RESEARCH ARTICLE

Actinobacteria Isolation from Forest Soils and Determination of Biological Activities

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Abstract: Actinobacteria bacterial group has one of the most populous population in microorganisms that extends very different and extensive habitats on earth especially the main character of the natural soil habitats. Since old times, bioactive metabolites of soil microorganisms have been studied and the results have provided that metabolites of these microorganisms have significant benefits to science, medicine, agriculture, and the pharmaceutical industry. In this study, isolation of Actinobacteria strains from forest soils, identification of morphological and molecular features, extraction of the bioactive metabolite of isolates and determination the antimicrobial, antibiofilm, and cytotoxic activities of bioactive extract were tested. The microbiological isolation methods for collected forest soil samples were used and after the determination of their morphological and molecular features, isolates were defined as *Brevibacterium spp.* that is a member of Actinobacteria. The antibiotic resistance of the isolates was determined by different methods and different concentrations of standard antibiotics. The chromium tolerance of isolates was also determined. The bioactive metabolites of isolates were produced in a modified medium and extracted. The antimicrobial, antibiofilm, cytotoxic activities of bioactive metabolites were determined against standard microorganisms and *Artemia salina* larvae were used as a test organism for cytotoxic tests. In the present study, results provided information about Actinobacteria that were isolated from forest soils. Isolates have antibiotic resistance and chromium tolerance abilities. Moreover, it has been shown that the Actinobacteria group is the largest bioactive metabolite producing group in terms of both antibacterial and antifungal activity and also contains a wide range of other compounds such as antibiofilm and cytotoxic compounds. The antimicrobial (MIC) concentrations of bioactive metabolites were detected 2500 µg/mL for standard bacteria cultures and 1250 µg/mL for yeasts. The antibiofilm (MBEC) value was determined at 1250 µg/mL. The 2500 µg/mL concentration of the extract was found to be the effective cytotoxic value. The results provide *Brevibacterium spp.* isolates have industrial and pharmaceutical potential and more detailed pharmaceutical researches are planned.

INTRODUCTION

Soil is a highly complex, heterogeneous, and nutrient-limited environment consisting of an organic matrix with liquid and gaseous pores possessing the highest microbial diversity on the Earth (1). Molecular phylogenetic analyses reveal that the soil and rhizosphere can contain thousands of unique bacterial species per gram (2). The phylum of Actinobacteria is well-known for its ability to produce a wealth of natural products with structural complexity and with diverse biological activities (3). Actinobacteria bacterial group has one of the most populous populations of microorganisms that extend very different and extensive habitats on earth. Actinobacteria and its members are the main characteristics of natural soil habitats. Especially, forest soils are eligible habitats for soil

microorganisms, it contents organic-inorganic materials, various organisms, and their wastes. Actinobacteria are *Gram*-positive, free-living, saprophytic bacteria, widely distributed in soil, water, and colonizing plants with a filamentous structure.

Bacteria produce a vast array of secondary metabolites, which have diverse and important ecological functions. It has become increasingly clear that secondary metabolite production often is triggered by intra- and inter-specific interactions between soil bacteria. Soil bacteria produce a large number of secondary metabolites that have many different physicochemical and biological properties (1). Bioactive metabolites of microorganisms especially soil microorganisms have studied for many years and the results have provided that metabolites of soil microorganisms have significant benefits to science, medicine, agriculture, and the pharmaceutical industry. Actinobacteria have also ability to synthesize antiviral (4), antibacterial, antifungal (5), antibiofilm, antitumoral (6), insecticidal (7), antioxidative (8), anti-inflammatory (9), anti-biofouling (10), immunosuppressive (11), anti-parasitic (7), plant-growth-promoting and herbicidal compounds (12), enzyme inhibitors (6) and industrially important enzymes. From the 22,500 biologically active compounds that have been obtained from microbes, 45% are produced by Actinobacteria, 38% by fungi, and 17% by unicellular bacteria (13).

The main focus of this study was to isolate, characterize and identification of Actinobacteria strains that were collected from forest soils in the Çanakkale-Gelibolu region in Turkey. The isolated Actinobacteria strain was growth in special media and extracted to obtain the bioactive metabolite in crude extract form. The collected crude extract was evaluated with its antibacterial, antifungal, antibiofilm, and cytotoxic features.

EXPERIMENTAL SECTION

Isolation and characterization

The soil samples were collected from 6 to 10 cm depth from forest regions in Çanakkale-Gelibolu, Turkey. For sampling, moist and fertile soils that are rich in organic matter were selected.

After collection, soil samples were serially diluted up to 10^{-5} and inoculated to special isolation modified media. The content of this medium was as follows: Dextrose (4 g/L), yeast extract (4 g/L), malt extract (10 g/L), $CaCO_3$ (calcium carbonate) (2 g/L) and agar (4 g/L). The cultures were incubated at 37 °C for 3-5 days (14). This culture-dependent isolation method was continued until the pure colonies shown on the agar surface typically resemble Actinobacteria. The morphological characterization of these colonies determined by using the *Gram* staining method.

Molecular identification

The selected pure cultures were identified by using their 16S rRNA (ribosomal RNA) sequence. After, DNA isolation PCR (Polymerase Chain Reaction) amplification methods were used. The 27F/1492R universal primers were used and 1450 base pairs of 16S rRNA were amplified by using PCR. Two primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used. Sequencing was performed by using the EURX Gene Matrix Bacterial Genomic DNA Purification Kit. The PCR protocol used was as follows: 95 °C for 5 minutes, 56 °C for 40 seconds, 72 °C for 60 seconds, 35 cycles of denaturation at 95 °C for 40 seconds, annealing at 56 °C for 40 seconds, and elongation at 72 °C for 45 seconds. Amplification was followed by a final extension at 72 °C for 5 minutes. After the PCR reaction was completed, 10 µL of the obtained PCR product was electrophoresed on a 1% 1× Tris-acetate-EDTA agarose gel containing ethidium bromide. PCR products were produced for bidirectional sequence analysis by mixing with their respective forward and reverse primers. The analyses were carried out in duplicate with the Sanger sequencing device and the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The 16S rRNA gene sequence was compared to a sequence in the public database using basic local alignment search tool (BLAST - Basic Local Alignment Search Tool) on the national center for biotechnology information (NCBI (National Center for Biotechnology Information)) website (www.ncbi.nlm.nih.gov). The homology of the 16S rRNA sequence of the isolate was analyzed by using BLAST software (15,16).

Determination of the antimicrobial resistance of isolates

Antibiotic resistance was determined by the disc diffusion method. The antibiotic resistance of Actinobacteria culture were determined both by disc diffusion and microbroth dilution methods against standard antibiotic discs; AmC-30 (amoxicilin+clavulanic Acid 30/10 µg), SAM-20 (ampicillin+sulbactam 10/10 µg), IPM-10 (imipenem 10 µg) B (bacitracin 0.004 µg), OFX-10 (ofloxacin 10 µg) and TEC-30 (teicoplanin 30 µg). 100 µL of Actinobacteria culture inoculated to MHA (Mueller Hinton Agar) and the standard antibiotic discs were put on the agar and incubated at 37°C, for 24 hours. After the incubation period, the inhibition zones around the discs were measured (17). The microbroth dilution method (minimum inhibitory concentration - MIC) was defined as the lowest concentrations necessary for the inhibition of growth. 6 different concentrations (156.25, 78.125, 39.062, 19.53, 9.75, and 4.87 µg/mL) of standard antibiotics chloramphenicol, amoxicillin and ampicillin were used. Overnight microorganism culture was adjusted to McFarland's 0.5 standard. 100 µL of each antibiotic concentrations and 100 µL of microorganism cultures were inoculated to the

well plates and incubated at 37°C, for 24 hours. After the incubation period, the wells were stained by 20 µL resazurin dye to observe the color difference between dead and living cells.

Screening of the chromium tolerance

Potassium chromate (K_2CrO_4) and chromium(III) chloride ($CrCl_3$) salts were used at 100, 75, 50, 25, and 12.5 mg/100 mL concentrations. The chromium powders were added to PYE (peptone, yeast extract) and MHA (Mueller Hinton Agar) at proper concentrations by using the agar dilution method (18). The 50 µL of Actinobacteria culture was inoculated to the medium and incubated at 37°C, 96 hours. Microbroth dilution method was also used at 25, 12.5 6.25, 3.125, and 1.562 mg/100 mL concentrations. 100 µL of K_2CrO_4 and $CrCl_3$ was added to *Brevibacterium* sp. that adjusted to McFarland 0.5 and incubated at 37°C, for 24 hours. Following the incubation period, a 150 µL aliquot was taken from each concentration wells and inoculated to MHA and incubated at 37°C, 96 hours. After the incubation, colonies on the plate were counted.

Fermentation and extraction of bioactive metabolites from *Brevibacterium*

For the fermentation step, a submerged culture method was used. The pure *Brevibacterium spp.* isolate was grown in the modified medium containing; 4 g/L of glucose, 10 g/L of malt extract, 4 g/L of yeast extract, 1 g/L of K_2HPO_4 (potassium hydrogen phosphate), $MgSO_4$ (magnesium sulfate), NaCl (sodium chloride), 0.001 g/L of $FeSO_4$ (iron(II) sulfate) and 2 g/L of $CaCO_3$ (calcium carbonate). The prepared cultures were incubated 37°C, for 7 days. After the incubation period, organic solvent extraction was performed. The broth was filtered through a Whatman No.1 filter and cultures were extracted three times with equal volume of ethyl acetate (EtOAc) at a 1:1 ratio. Then the EtOAc solution was evaporated for dryness by using a rotary evaporator at 30°C, 80 rpm, under 187 mbar vacuum and the crude extract was collected (19).

Determination of minimum inhibitory concentration (MIC) of bioactive metabolite

5 different concentrations crude extract were applied to *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 700603, *Bacillus subtilis* NRRL B478, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus pyogenes* ATCC 13615, *Listeria monocytogenes* ATCC 19111 and *Candida albicans* ATCC 90028 species by using 96 well plate assay. *Brevibacterium* crude extract was weighed and dissolved in %5 DMSO. Proper concentrations were

2500, 1250, 625, 312, and 156 µg/mL. Overnight microorganism cultures were adjusted to McFarland 0.5 standard. 100 µL of each extract concentrations and 100 µL of each microorganism cultures were inoculated to the well plates and incubated at 37 °C, for 24 hours. %5 DMSO, ketoconazole for fungi and chloramphenicol for bacteria species were also used as a control with 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, and 0.195 µg/mL values. After incubation period wells were stained by 20 µL resazurin dye to observe the color difference between dead and living cells (20, 21).

Biofilm eradication assay of bioactive metabolite

Microbial biofilms are communities of microorganisms embedded in a self-producing matrix, forming on living and nonliving surfaces. The inhibition effect of crude extract on biofilm formation was evaluated in 96-well plates. Briefly, overnight *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Candida albicans* ATCC 90028 cultures were adjusted to McFarland 0.5 standard. 200 µL of each bacteria were inoculated to the well plates and incubated at 37 °C, for 48 hours to form the biofilm at the bottom of the wells. After the incubation period, wells were gently washed twice with 100 µL of 0.9% NaCl (physiological saline). 100 µL of concentrations (1250, 625, 312, 156, and 78 µg/mL) of crude extract were added to each well and incubated at 37°C, 24 hours. After the incubation period, wells were stained by 20 µL resazurin dye. (22, 23, 24)

Brine shrimp lethality test of bioactive metabolite

A 24-h LC_{50} (lethal concentration) bioassay was performed in a 96 well plate using nauplii of the Brine shrimp *Artemia salina* (25). Commercially sold *Artemia* mix was used. 18 g of the *Artemia* mix was poured to the 500 mL of distilled water and incubated 48-52 hours at 30 °C. After larvae had been seen, they were taken and counted. Brine shrimp lethality bioassay was determined with crude extract concentrations; 2500, 1250, 625, 312, and 156 µg/mL with 10 *Artemia salina* larvae in each concentration wells. After 24 hours incubation period, the alive larvae were counted.

RESULTS AND DISCUSSION

Isolation, characterization and molecular identification of Actinobacteria

In the present study, 25 Actinobacteria strains were isolated from collected soil samples. *The Gram* staining method was applied to all of these isolates. Some of them are shown in Figures below 1. a, b, c.
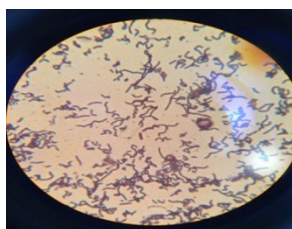
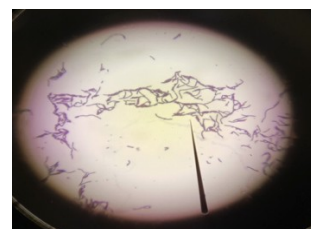**Figure 1.a**

Figure 1.b

Figure 1.c.

Actinobacteria isolates after *the Gram* staining method.

The selected pure cultures were identified by using their 16S rRNA sequence. The results showed that most of them identified as *Brevibacterium spp.* BLAST analysis of the isolate showed that it is 100% similar to *Brevibacterium spp.* called *Brevibacterium frigoritolerans* (Accession Numbers: MN062932.1 and MN845150.1) which are the many *Brevibacterium* species available in the Gen Bank. *Brevibacterium* is one of the soil microbial communities that uncommon members of the Actinobacteria class. *Brevibacterium* is a member of the Actinobacteria phylum and genus of the Brevibacteriaceae family that is aerobic, non-sporulating, *Gram*-positive rod to coccoid shape and grows in a saline environment.

Determination of the antibiotic resistance of isolates

The antibiotic resistance of these isolates was determined by using standard antibiotic discs; AmC-30 (amoxicilin+clavulanic acid 30/10 μg), SAM-20 (ampicillin+sulbactam 10/10 μg), IPM-10 (imipenem 10 μg), B (bacitracin 0.004 μg), OFX-10 (ofloxacin 10 μg) and TEC-30 (teicoplanin 30 μg). It showed the most sensible reaction to IPM-10 (imipenem 10 μg). Also, the other values are shown in Table 1. Additionally, the MIC values are 78.125 μg/mL for chloramphenicol and 4.87 μg/mL for amoxicillin and ampicillin.

Table 1. Antibiotic resistance results from the disc diffusion method in terms of millimeters (mm).

Standard Antibiotic Discs	*Brevibacterium spp.* inhibition zone diameters (mm)
AmC-30	19
SAM-20	16
IPM-10	20
TEC-30	8
OFX-10	12
B-0.04	0

Screening of the chromium tolerance

While 150-160 colonies were counted in chromium(III) chloride ($CrCl_3$) plate at 50 mg/100 mL concentration, 25 mg/100mL concentration plate formed 200-300 colonies. In 50 mg/100 mL potassium chromate (K_2CrO_4) plate 50-100 colonies and in 25 mg/100 mL plate 100-150 colonies were counted. Additionally, at the results of microbroth dilution for chromium(III) chloride ($CrCl_3$) and potassium chromate (K_2CrO_4), there was no formation of colonies. The test will be repeated using different concentrations. Chromium is one of the poisonous polluting metal ions that is found in soils. Several studies have demonstrated *Brevibacterium spp.* has chromium tolerance and chromium degradation abilities. Hence, chromium tolerance of isolates was tested by the agar dilution method. It showed significant tolerance to potassium chromate (K_2CrO_4) and chromium(III) chloride ($CrCl_3$) salts.

Determination of minimum inhibitory concentration (MIC) of bioactive metabolite

The MIC value of bioactive metabolite extract was tested at 2500, 1250, 625, 312, 156 μg/mL concentrations. The results are shown in Tables 2 and 3. The yeast species are more sensitive than bacteria species.

Table 2. MIC values of metabolite and chloramphenicol against bacteria species.

Microorganisms	MIC Values (µg/mL)	
	Bioactive Metabolites	Chloramphenicol
Escherichia coli ATCC 35218	2500	78.125
Staphylococcus aureus ATCC29213	2500	4.882
Listeria monocytogenes ATCC 19111	2500	9.765
Klebsiella pneumonia ATCC 700603	2500	19.531
Bacillus subtilis NRRL B478	2500	2.441
Staphylococcus epidermidis ATCC 14990	2500	9.765
Streptococcus pyogenes ATCC 13615	2500	39.0625

Table 3. MIC values of metabolites and ketoconazole against yeast species.

Microorganisms	MIC Values (µg/mL)	
	Bioactive Metabolites	Ketoconazole
Candida albicans ATCC 90028	1250	19.531
Candida krusei ATCC 6258	1250	39.0925

Several studies have demonstrated that secondary metabolites produced by soil bacteria can serve as weapons in microbial warfare, providing an advantage to producer strains when competing against other microbial competitors in the same ecological niche (26).

Biofilm eradication assay of bioactive metabolite
Antibiofilm studies demonstrated a dose-dependent activity. The antibiofilm activity of bioactive metabolite extract was examined by minimum biofilm eradication concentration (MBEC) assay. The antibiofilm value of bioactive metabolite extract was determined 1250 µg/mL for *Staphylococcus aureus*, 1250 µg/mL for *Staphylococcus epidermidis* and 1250 µg/mL for *Candida albicans*. It means, the extract showed the same efficiency to selected microorganisms and eradicate their biofilm structure at the same dose. 1250 µg/mL of bioactive metabolite extract is a proper concentration for eradicating the biofilm formation of tested microorganisms.

Brine shrimp lethality test of bioactive metabolite
The brine shrimp lethality test was determined at 2500, 1250, 625, 312, and 156 µg/mL concentrations. The lethality values of *Artemia salina* larvae are shown in Table 4. 2500 and 1250 µg/mL concentrations are the most effective doses on larvae. The larvae are counted out of 10 larvae.

Table 4. Results of the Brine shrimp lethality test.

Bioactive Metabolite Concentrations (µg/mL)	Amount of Alive *Artemia salina* Larvae (out of 10)	Percentage (%) Effect of Bioactive Metabolite
2500	0	100%
1250	2	80%
625	3 ± 1	60-70%
312	3 ± 1	60-70%
156	4 ± 1	50-60%

The cytotoxic efficacy of the bioactive metabolite extract is significantly observed at different concentrations. It can be used as an anticancer drug in cell culture studies. The studies will be continued to understand all of the efficacy mechanisms with different tests.

CONCLUSION

The search for novel microorganism species from soils has gained momentum in recent years. Nature acts as a prominent reservoir for isolating the microorganisms that have various biological activities. The results from in vitro experiments, a *Brevibacterium spp.* called *Brevibacterium frigoritolerans* was isolated. In this study, many biological activities of this isolate have been tested and proved. The isolated novel bacterial strain was characterized by morphological, molecular, antimicrobial resistance, and chromium tolerance features and activities. For the determination of *Brevibacterium spp.* isolates antibiotic resistance, AmC-30 (amoxicilin+clavulanic acid 30/10 µg), SAM-20 (ampicillin+sulbactam 10/10 µg), IPM-10 (imipenem 10 µg), B (bacitracin 0.004 µg), OFX-10 (ofloxacin 10 µg) and TEC-30 (teicoplanin 30 µg) standard antibiotic discs were used. IPM-10 (imipenem 10 µg) was the most effective and B (bacitracin 0.004 µg) is not effective at all. Chromium is a heavy metal and causes environmental pollution. It accumulates in soil and is a very carcinogenic substance. For this reason, the chromium tolerance of *Brevibacterium spp.* isolates were determined. Das and Mishra showed that *Brevibacterium spp.* tolerates to potassium chromate (K_2CrO_4) (27). This research proved that

Brevibacterium spp. isolates tolerate both potassium chromate (K_2CrO_4) and chromium(III) chloride ($CrCl_3$) salts. The crude extract was checked for antibacterial, antibiofilm, and cytotoxic activities to identify the mechanisms of bioactive metabolite. *Brevibacterium spp*. showed different bioactive features, which highlighted its importance as potential pharmacological agents. Hence, there could be a probability of a new bioactive compound in the crude extract, which might provide a basis for further development of novel compounds from *Brevibacterium spp*. In Oskay's research, bioactive metabolites of Actinobacteria showed antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* microorganisms (28). In the present study, bioactive metabolites of *Brevibacterium spp*. isolates that member of Actinobacteria showed antimicrobial activity against *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC 35218, *Klebsiella pneumonia* ATCC 700603, *Bacillus subtilis* NRRL B478, *Staphylococcus epidermidis* ATCC 14990*, Streptococcus pyogenes* ATCC 13615 *Listeria monocytogenes* ATCC 19111, *Candida albicans* ATCC 90028*, Candida krusei* ATCC 6258 and antibiofilm activity against *Staphylococcus aureus* ATCC29213, *Staphylococcus epidermidis* ATCC 14990*, Candida albicans* ATCC 90028 at different concentrations of the crude extract. It has been previously shown that bioactive metabolites of Actinobacteria at 0.1-10 µg/mL concentrations showed a 100% cytotoxic activity on *Artemia salina* larvae (29). In the current study, 80-100% cytotoxic activities at 2500 and 1250 µg/mL concentrations were determined. There are limited data in the literature about the activities of *Brevibacterium* bioactive metabolites. This research also provided new insight into the development of good candidates for pharmaceutical and bioactive natural products. The development of new drugs can be a candidate for curing several types of diseases (30). The current attempt of using *Brevibacterium* isolates and its bioactive metabolites will be favorable for antimicrobial, antitumoral, antibiofilm and cytotoxic activity tests.

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RESEARCH ARTICLE

Bioremediation Potential of Immobilized *Corynebacterium kutsceri* in the Treatment of Tannery Industry Effluent from Challawa Industrial Estate, Kano State, Nigeria

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Abstract: In the present study, bioremediation potentials of indigenous bacteria (*Corynebacterium kutsceri*) in the treatment of tannery effluent was investigated. Industrial tannery effluent samples from Mamuda Tannery Industries in Challawa Industrial estate, Kano State, Nigeria were collected for a period of six months (August 2017 to January 2018) for the experiment. Bacteria were isolated from the effluents using serial dilution, immobilized on agar-agar and biochemical tests were carried out to identify the bacteria. Different masses (5 g, 10 g, 15 g, 20 g, and 25 g) of the identified bacteria were used in the treatment of 250 mL of the effluents. Temperature, pH, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Suspended Solid (SS), Total Dissolved Solids (TDS), sulfate, phosphate, nitrate, chloride, and some heavy metals (Cr, Fe, Mn, Ni, Pb, Zn, Cd, and Cu) were determined before and after treatment of the effluents with the bacteria. The pre-treatment analysis showed that the values, 29.50±4.68, and 5.35±1.57, were observed for temperature (^{o}C) and the pH respectively. The levels (mg/L), 26.17±9.49; 3106±2753; 562±482; 444±507; 97.20±146.80; 268.34±411.01; 8.82±34.71 and 22.59±19.64 were observed for BOD, COD, SS, TDS, nitrate, sulfates, phosphate, and chlorides, respectively. The concentration (mg/L) of the heavy metals, before treatment (with the bacteria) were as; Cr (7.528±4.530); Fe (1.263±0.502); Ni (0.023 ± 0.021); Mn (0.277 ± 0.03); Pb (0.304 ± 0.20); Zn (0.058± 0.05); Cd (0.068±0.02) and Cu (0.012 ±0.02). The bacteria were identified to be *Corynebacterium kutsceri*. The post-treatment analysis showed that the COD (1376±248−2681±867 mg/L) has the highest value while Cu (ND −0.007±0.00 mg/L) has the lowest value. The post-treatment analysis using the different masses of the *Corynebacterium kutsceri* indicates that there is a great decrease in the levels of the physicochemical parameters and the heavy metals as well when compared with the levels observed at the pre-treatment analysis. The decrease could be attributed to, not only due to the increase in the exact mass of the bacteria but also to the multiplicity in the mass of the *Corynebacterium kutsceri* which subsequently increases the surface area for the remediation. The average high percentage reduction (70% to 100%) of these parameters and heavy metals implies that the *Corynebacterium kutsceri* has a higher potential for the treatment of effluents from the textile industries.

INTRODUCTION

With rapid industrialization, water pollution has become a major problem. The characteristics of industrial effluent depend upon the type of industrial raw material and the output of the product (1). Industrial effluents account for several point sources of water pollution, while developed nations adopt

stringent water quality requirement to control river pollution from point and non-point sources, the situation is different in the most developing countries like Nigeria. Wastewater treatment in Nigeria is not given the necessary priority it deserves and therefore, industrial wastes are discharged into receiving water bodies without treatment. The consequences of this include among others, river pollution, loss of aquatic life, uptake of polluted water by plants, disease burden, and shorter life expectancy in developing countries (2).

Tannery industrial wastewater is a serious consequence of the pollution point of view for streams, freshwater, and land used for agriculture. The lack of awareness in the modern industrial practice has resulted in the discharge of tannery effluents which exhibit the very high value of Cr, Sulfide, and chloride, Total Dissolved Solid (TDS), Total Suspended Solid (TSS), Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) in the water stream or land (3). Tannery effluent refers to as wastewater from the process of converting skin and hides into leather. The process of tanning requires a large volume of water, which is used to either cleanse the hides and skins or as an interaction medium between the hides and skin. During the tanning process, a large volume of effluents is discharged into the surrounding soil as well as a water source. These effluents may contain a variety of chemicals such as sodium sulfate, chromium sulfate, and non- ionic wetting agents that are used in the tanning process and may accumulate in the immediate environment of the tannery(4). There are two types of tanning. The first is the vegetable tanning in which plant extracts are employed to produce heavy leather such as belts and soles of footwear. The second is the chrome tanning which involves the treatment of skins/hides with chromium salts obtained by reduction of sodium dichromate reducing agent produces lighter leathers such as gloves and upper part of shoes. During the tanning process, at least about 300 kg chemicals are added per tonne of hides (5). A tannery is one of the important industries causing water pollution (6). There are more than 6000 tanneries in Nigeria with an annual processing capacity of 700,000 tons of hides and skins (7)(8). It was reported that the total amount of waste produced per slaughtered animal is approximately 35% of its weight (9). Also, for every 1000 kg of carcass weight, a slaughtered beef produces 5.5 kg of manure (excluding rumen contents or stockyard manure) and 100 kg of paunch manure (undigested food)(10). wastewater in the range of 30-35 L kg^{-1} skin/hide processed with variable pH, BOD, COD, high concentrations of suspended solids, and tannins including chromium (11). A single tannery can cause the pollution of groundwater around the radius of 7-8 kilometers (7).

Tannery effluent, when discharged into water bodies, alters the physical, chemical, and biological characteristics of water and depletes the dissolved oxygen, increases alkalinity, suspended solids, and sulfides which are deleterious to fish and other aquatic lives (12). Many conventional processes such as oxidation, chemical, and biological processes were carried out to treat wastewater from tanneries (6). Biological processes have received more attention because of their cost-effectiveness, lower sludge production and environmental friendliness in contrast to chemical/physical methods which are invariably cost-intensive and cannot be conveniently employed in all industries, especially in developing and underdeveloped countries (12). Microbes in the environment play an important role in cycling and destroying pollutants through bio-degradation (11). In the course of the last two decades, a wide variety of technologies had been developed for clean-up operations of contaminated sites. Bioremediation has evolved as the most promising one because of its economical safety and environmental features since organic contaminants become transferred and some of them are fully mineralized. Bioremediation of tannery effluents is an attractive environment-friendly, safe and cost-effective alternative technology to conventional methods. Microbes in the environment play an important role in cycling and destroying them through bio-degradation (3).

Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. It is based on the idea that all organisms remove substances from the environment to carry out growth and metabolism. The principles of bioremediation are that the microbe feeds on contaminants, digest it, and this waste is transformed into water and harmless gases which are later expelled out of the microbes. The resultant metabolic wastes that they produce are generally safe and somehow recycled into other products. Although the microorganisms are present in contaminated water, they cannot necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be stimulated. Naturally occurring degrade the hazardous organic wastes including xenobiotic compounds, such as pesticides, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in due course of time. However, metallic residues cannot be degraded in composting but may be converted into organic combinations that have less bioavailability than mineral combinations of the heavy metals. Microbial transformation of metals serves various function generally, it occurs either by redox conversions of inorganic forms or conversions from inorganic to organic forms and vice versa. Bioremediation is amenable to a variety of organic and inorganic compounds and may be applied either *in situ* or *ex-situ*. In addition to this, it is easy to

implement and maintain, does not require the use of expensive equipment or highly specialized personnel and is environmentally friendly and aesthetically pleasing to the public (13).

Immobilized bacteria can withstand various temperatures, pH and substrate concentrations; consequently increasing the efficiency and the lifespan of the bacteria. Immobilized bacteria are widely applied in the treatment of wastewater and can be separated and recovered after the treatment with the same efficiency.

This study was carried out in Kano. Kano lies on Latitude 11°30′N and 8°30′N and Longitude 11°5′N and 8°5′E and 8°5′E in Northern Nigeria. It is one of the developed industrial cities in Nigeria. Tannery and textile-related activities are the dominating industries and this could be one of the reasons for her high population density(14). The climate is characterized by well-defined wet and dry seasons. The wet season spreads from May to October, August usually being the wettest whereas the dry season lasts from November to April. River Challawa originates from the Challawa Gorge dam in Challawa village and stretches to River Kano that empties into Lake Chad. The river receives waste from tanneries and textile industries, urban water storm, and agricultural runoffs from farming communities along its course. River Challawa serves as fishing, farming, and water supplies for the communities in the area. The domestic water supply for Challawa, Sharada, and Bompai industrial areas and their surroundings originates from the Challawa River (14). Several workers have studied bioremediation of tannery effluent using microorganisms. However, limited literature is available on the bioremediation of tannery effluent using immobilized bacterial cells in the Challawa industrial estate. This research work focused on the potential of the immobilized bacteria in the treatment of industry tannery effluent in the Challawa industrial estate.

MATERIAL AND METHODS

Sample Collection
Effluents were collected from the Mamuda Tannery Industry in Challawa Industrial Estates, Kano, Nigeria. The effluents were collected for a period of six months (August 2017 to January 2018). It was collected from the effluent reservoir in the industries in sterile 4-liter plastic containers with a unique identification number and was preserved using an ice-box that was transported to Microbiology Laboratory, Department of Microbiology, the Bayero University of Kano for analysis.

Sample Preparation and Analysis
Immediately after the collection of the effluent, pH, SS, TDS, COD, BOD, nitrate, sulfates, phosphates, chlorides, and heavy metals (Cr, Fe, Mn, Ni, Pb, Zn, Cd, and Cu) concentrations were determined.

pH was determined using Ecotests pH meter and TDS was determined using AQUALYTIC TDS-Salinometer. BOD was determined as described by the standard method (7). COD, SS, nitrate, sulfates, phosphates, and chlorides were determined using DR/2010 HACH portable data logging spectrophotometer (8). The heavy metals were determined using Atomic Absorption Spectrophotometer (BUCK Scientific ACCUSYS 211) according to the manufacturer's instructions.

Isolation and Identification of the Bacteria from the Tannery Effluents
The bacteria were isolated from the effluents using Serial Dilution according to the method described by APHA (15). The biochemical tests such as oxidase, catalase, coagulase, indole (from 1% tryptone broth), citrate (Simmons citrate agar), methyl red (5 drops of MR), nitrate reduction, Voges-Proskauer (VP), Starch Hydrolysis, Glucose, Maltose, and Lactose tests were carried out on the bacterial isolates to identify the bacteria through the bacteria biochemical characteristics according to Ajao *et. al.* (16).

Determination of Growth Rate of the Bacteria in Effluent Sample
The bacteria growth rates were determined by transferring 2 mL of the bacterial isolates from the tannery effluent in broth medium into 100 mL sterile effluents in conical flasks and kept in an incubator (Giffrin cool) for 10 days. Control was also set up by incubating another 100 mL each of the sterile effluents without the bacteria. The optical density of the content was determined at the wavelength of 600 nm on a daily interval and recorded.

Immobilization of Bacteria
Agar solution and inoculi were prepared separately. Fifty milliliters (50 mL) of nutrient broth each of the inoculi was prepared in a McCartney bottle and incubated for 24 hours. A solution of agar-agar was prepared by dissolving 10 g of the powder in distilled water and made up to 500 mL mark in an Erlenmeyer flask and was sterilized in an autoclave (280A) for 15 minutes and allowed to cool to 40-45 °C (16). Four milliliters (4 mL) of the bacterial isolates in the nutrient broth was mixed with 36 ml of the prepared agar-agar media in petri-dish plates and then allowed to solidify. This was kept in the refrigerator for bioremediation.

Bioremediation (Treatment) of the Effluents
The solidified agar block (immobilized bacteria) was cut into cubes using a sterile knife; 0.1 mL phosphate buffer (pH 7.0) was added and kept in the refrigerator for 1 hour for curing. The phosphate buffer was decanted after 1 hour and the cubes were washed with sterile distilled water 3-4 times before it was used. Five grams (5 g), 10 g, 15 g, 20 g and 25 g of the immobilized bacteria were then weighed (17).

Two liters (2 L) of the effluent was supplemented with the minimum basal medium in g/L: NaCl (0.8), MgSO$_4$.7H$_2$O (0.001), KH$_2$PO$_4$ (2), NaNO$_3$ (2), CaCl$_2$.2H$_2$O (0.5) and NaHPO$_4$.12H$_2$O (2) and sterilized in an autoclave at 121 °C for 15 minutes. Two hundred and fifty milliliters (250 mL) of the effluents were transferred into different 250 ml conical flasks. The content was covered with a cotton-wool ramped with foil paper to avoid contamination. Five grams (5 g) of the immobilized bacteria were quickly transferred into each of the effluents in the conical flasks in an inoculating chamber (18). The same procedures were carried out for the 10 g, 15 g, 20 g and 25 g of the immobilized bacteria in separate 250 mL effluents in conical flasks and agitated for ten days in a shaker incubator (Gallenkamp-OC-4364-L) at the temperature 30 °C and velocity of 60 rpm.

The treated effluent samples were taken on the tenth day and analyzed for the parameters pH, SS, TDS, COD, BOD, nitrate, sulfates, phosphates, chlorides, and heavy metal concentrations (Post-treatment determination) at the different grams of bacteria to evaluate and compare the bioremediation efficiency.

Statistical Analysis
The data were represented as Mean ± Standard deviation and analyzed statistically using one-way Analysis of Variance (ANOVA) and Tukey's HSD as Post Hoc Tests with the aid of SPSS 16.0. The correlation coefficient was also used to measure the strength of the relationship between the different masses of the bacteria and the parameters. All $p \leq$ 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Results of the Physico-Chemical Parameters and Heavy Metals in Industrial Effluents before the Bioremediation
Results of the physicochemical parameters in the industrial effluents before the bioremediation were shown in Table 1. The mean level of temperature (29.50±4.68 °C) was found below the World Health Organization (WHO) (35 °C) and NESREA (National Environmental Standards and Regulatory Enforcement Agency) (40 °C) recommended standard limits; the average value of temperature observed in this present study is less than that observed (56.23 to 74.54 °C) by another researcher. High temperature brings down the solubility of gases in water that ultimately expresses as high BOD and COD (19). pH (5.35±1.57) was below the WHO (7.0-8.5) and NESREA (6-9). Maheshwari et al. (2017) reported that the level of pH in the effluents from the tannery industry in Vaniyambadi, India was 6.5 which was higher than that observed in the present study (20).

BOD (26.17±9.49 mg/L) was below the NESREA (200 mg/L) but above the WHO (10 mg/L). Maheshwari et al. (2017) reported that the level of BOD in the effluents from the tannery industry in Vaniyambadi, India was 348 mg/L which was higher than that observed in the present study. BOD is a measure of the content of organic substances in the wastewater which are biologically degradable with consumption of oxygen usually indicated as 5-day BOD. This is the amount of oxygen in milligrams per liter (mg/L) that consumed by microorganisms in 5 days at 20 °C for the oxidation of the biologically degradable substances contained in the water. The high values of BOD recorded are indicative of the presence of total solids in the effluents which are known to be organic with high oxygen demands for their oxidation under required conditions of temperature and oxidants and time, as a result, will naturally lead to the depletion of dissolved oxygen in the aqueous body. The biochemical oxygen demand of the untreated textile wastewater can cause rapid depletion of dissolved oxygen if it is directly discharged into the surface water sources another researcher (21).

The Chemical Oxygen demand (COD) is the amount of oxygen, in mg/L, required for the degradation of the compound of wastewater to occur. COD (3106±2753 mg/L) above the WHO (40 mg/L) and NESREA (40 mg/L). The level of COD in the effluents from the tannery industry in Vaniyambadi, India was 860 mg/L (20) which was lower than that observed in the present study. The higher the COD value of wastewater, the more oxygen demand to discharge water bodies another researcher(22). Akan et al. (2009) reported that a high COD value shows that the effluents have high oxygen demanding wastes which cause the depletion of Dissolved Oxygen (DO) which is a fundamental requirement for aquatic life.

SS (562±482 mg/L) is above the WHO (30 mg/L) and NESREA (10 mg/L). These high TSS values observed in all the industries understudied might be possible due to the presence of fine leather particles, residues from various chemical discharges and reagents from different waste liquors from tanneries and textile industries (23). The composition of solids present in tannery effluent mainly depends upon the nature and quality of hides and skins processed in the tannery (24). The average value of SS observed in this present study is less than that observed (3422±122 to 3700±122 mg/L) by Maheshwari et al. (2017). It is observed that a suspended solid absorb heat from sunlight, causing an increase in water temperature and subsequently decreases the level of dissolved oxygen (25).

TDS (444±507 mg/L) was above the WHO (250 mg/L) but below the NESREA (500 mg/L). The composition of solids present in tannery effluent

mainly depends upon the nature and quality of hides and skins processed in the tannery (24).

Nitrate (97.20±146.80 mg/L) was found above the WHO (50 mg/L) and NESREA (40 mg/L). Maheshwari et al. (2017) reported that the level of nitrate in the effluents from the tannery industry in Vaniyambadi, India was 95 mg/L which was lower than that observed in the present study. The high level of nitrate in the tannery effluents in the present study might be due to the release of ammonia from the deliming process and the presence of nitrogen in proteinaceous materials from unhairing operations. Momodu et al. (2017) have reported the presence of nitrate in receiving water which can lead to extensive undesirable algal growth associated with eutrophication.

Sulfates (268.34±411.01 mg/L) were below the NESREA (500 mg/L) but above the WHO (100 mg/L). The high level of sulfate in the tannery effluents in the present study might be due to the use of sulfuric acid. The sulfate and high phosphate concentration detected in the effluents are also known to cause eutrophication and algal boom (26).

Phosphate (18.82±34.717 mg/L) was found far above WHO (0.1 mg/L) and NESREA (3.5 mg/L). The sulfate and high phosphate concentration detected in the effluents are also known to cause eutrophication and algal boom (26).

Chlorides (22.59±19.64 mg/L) were below the recommended limits of WHO (250 mg/L) and NESREA (350 mg/L). The presence of chloride in the tannery effluents in the present study might be due to the use of sodium chloride in hide and skin preservation. Chlorides inhibit the growth of plants, bacteria, and fish in surface waters growing. The presence of these physicochemical parameters in the tannery effluent of the present study might be due to the nature of the raw materials and processes used in the tannery industries at the time of sampling.

The results of the heavy metals in the industrial effluents before the bioremediation were shown in Table 2. The mean level of chromium (7.528±4.530 mg/L) was found above the WHO (0.05 mg/L) and the NESREA (0.5 mg/L) recommended standard limits. Maheshwari (20) reported that the level of chromium in the effluents from the tannery industry in Vaniyambadi, India was 435 mg/L which was higher than that observed in the present study. The high concentration of chromium might be due to the use of chromium salts for tanning processes. Chromium can cause allergic reactions in the skin, damage the lungs, and asthma attacks maximum concentration of 0.1 mg/L was set up (27).

Iron (1.263±0.502 mg/L) was found above WHO (0.5 mg/L) and the NESREA (1.0 mg/L). Higher iron content may produce undesirable effects such as astringent taste, coloration, turbidity, deposits, and growth of iron bacteria in pipes affecting the acceptability of water for domestic use. Iron is an essential element in Maheshwari nutrition, and the health effect of iron in drinking water may include warding off fatigue and anemia (28).

Manganese (0.277 ± 0.03 mg/L) was above the NESREA (0.01 mg/L) and the WHO (0.02 mg/L). In another study, 1.02 mg/L of manganese was reported Yusuff and Sonobare, 2004 (29). Depending upon the exposure route, manganese may be among the least toxic of the trace elements if ingested low IQ of children is attributed to high manganese intake and hence at high concentration lead to neurotoxins and harms the brain (30).

Nickel (0.023 ± 0.021 mg/L) was above the NESREA (0.01 mg/L) and the WHO (0.02 mg/L). The concentration of nickel in the effluents from textile industries in Kano ranged (3.11 to 3.4 mg/L) (31) which is the same order of magnitude reported in the study by Ali et al (32). The most obvious anthropogenic source of nickel is scrap metal waste, notably alloyed metals including stainless steel. Nickel is considered as an essential trace element at very low concentrations. It does bioaccumulate in aquatic systems, and as such elevations above normal concentrations can result in deleterious aquatic effects (25).

Lead (0.304 ± 0.20 mg/L) was above the WHO (0.05 mg/L) and NESREA (0.1 mg/L). Galadima et al. (2012) (30) reported that the concentration of lead in the effluents from textile industries in Kano ranged (2.45 to 2.46 mg/L) which is the same order of magnitude reported in the study by Ali et al (32). Excess quantities of lead may impact Maheshwari health, especially affecting small children (33). The value 0.646 mg/L and 0.289 mg/L were reported for lead in wastewater samples from the textile industry in Kaduna (33).

Zinc (0.058± 0.05 mg/L) was below the WHO (5 mg/L) and the NESREA (0.2 mg/L). The concentration of zinc in the effluents from textile industries in kano ranged from 2.52 to 2.45 mg/L (31), which is the same order of magnitude reported by Ali et al. (2009). Zinc is a trace element that is essential for Maheshwari health; the danger of which can be to an unborn child when mothers absorbed large concentration of zinc and other health problem such as stomach cramps, skin irritation, vomiting and anemia (34).

Cadmium (0.068±0.02 mg/L) was above the NESREA (0.01 mg/L) but below the WHO (0.5 mg/L). Galadima et al. (2012) reported that the concentration of cadmium in the effluents from textile industries in Kano ranged from 1.23 to 2.06 mg/L, which is the same order of magnitude

reported by Ali et al. (2009). Cadmium is a nonessential trace element that enters the environment via anthropogenic activities such as industrial effluent, sewage-sludge, fertilizers, and pesticides. Cadmium adsorbs strongly to sediments and organic matter (35). It has a range of negative physiological effects on the organism, such as decreased growth rates and negative effects on embryonic development and children are likely to be exposed to cadmium when is highly toxic and absorbed in the skin and can cause lung damage and irritation with shortness of breath dry throat, headache, vomiting, extreme restlessness or irritability, etc (36). Other potential long-term effects are pulmonary damage and fragile bones (37).

Copper (0.012 ±0.02 mg/L) was found below WHO (1.00 mg/L) but above NESREA (0.01 mg/L) the recommended limits. Galadima et al. (2012) reported that the concentration of copper in the effluents from textile industries in Kano ranged from 1.06 to 1.01 mg/L. Copper is a reddish metal in a color that occurs naturally in rocks, soil, water, industrial activities, and sediment and has some practical uses in our society and are found in pipes, electrical wiring, and coins. The level of copper reported from the textile industry in Lagos ranged from 4.0 mg/L to 5.14 mg/L (29). Copper is generally remobilized with acid-base ion exchange or oxidation mechanism (33).

The presence of these heavy metals may be due to the addition of some raw materials containing these ions during the production process. It has been reported that the major problem associated with industrial effluents is the presence of heavy metal ions, which arise from the material used in the production process or a considerably high amount, from metal-containing raw materials (37). Heavy metals present as impurities in dye effluents or chelated as part of dye molecules. In metal complex dyes, the metal is coordinated or forms a chemical bond with the organic dye molecules. Thus, it is an indispensable constituent of dye and governs the fastness in absorbing colors. The highest value of heavy metal ions in the effluents severely affects soil fertility and depletes the soil quality and its nutrients. Besides, the variations of heavy metals concentration in the wastewater sample were due to the different types of dyestuff used in a different production of the threads when the samples were taken. The concentration of heavy metals could be a serious environmental nuisance if a large volume of such effluents is released into the environment regularly without proper treatment (31). In line with the findings by Yusuff and Sonibare (2004), Dan Azumi and Bichi (14) analyzed heavy metal in Kano from Challawa industrial estate and found high concentrations of metal ions. The presence of these heavy metals in the tannery effluent of the present study might be due to the nature of the raw materials and processes used in the textile industries at the time of sampling.

Table 1: Mean values (mg/L) ± S.D of physicochemical parameters in effluents from the industries before and after treatment of the effluents with the different masses of the bacteria.

Parameter	Before	After				
		5 g	10 g	15 g	20 g	25 g
Temperature(ºC)	29.50±4.68					
pH	5.35±1.57					
COD	3106±2753	1376ab±248	1588a±489	2279b±924	2681b±867	1881ab±626
BOD	26.17±9.49	0.81c±0.67	0.80b±0.61	0.80b±0.61	0.58bc±0.45	0.73b±0.38
SS	562±482	118ab±136	230ab±252	78ad±89	196bd±302	273ab±375
TDS	444±507	27be±16	25bc±15	25bc±16	27bc±18	25bd±20
Nitrate	97.20±146.80	18.97a±15.93	14.43a±8.88	18.67a±16.94	26.52a±13.06	20.28a±7.85
Chloride	22.59±19.64	12.90a±18.39	3.95bc±4.78	8.87a±10.95	5.54ab±4.66	8.25a±10.68
Sulfate	268.34±411.01	18.12a±19.26	15.99a±17.37	5.95b±8.91	13.31b±18.55	8.59b±12.82
Phosphate	18.82±34.71	2.03a±3.11	1.90a±3.15	2.10a±3.00	1.76b±2.74	1.76b±2.64

Replicate= 6 (months), Within the columns, means with different letters are statistically different ($p < 0.05$).

Table 2: Mean values (mg/L) ± S.D of heavy metals in effluents from the industries before and after the treatment of the effluents with the different masses of the bacteria.

Parameter	Before	After				
		5 g	10 g	15 g	20 g	25 g
Cr	0.171a±0.12	0.049a±0.05	0.046a±0.07	0.013a±0.02	ND	0.170a±0.00
Fe	0.822±0.63	0.532c±0.11	0.488b±0.18	0.485b±0.36	0.202a±0.21	0.287ab±0.29
Mn	0.217a±0.48	0.002a±0.00	ND	ND	ND	ND
Ni	0.285±0.06	0.180a±0.07	0.180a±0.09	0.100a±0.10	0.070a±0.11	0.055a±0.11
Pb	0.273±0.20	0.118a±0.05	0.076a±0.04	0.073a±0.04	0.051a±0.05	0.043a±0.05
Zn	0.035±0.03	0.028a±0.02	0.028ac±0.03	0.027a±0.02	0.020ab±0.02	0.013a±0.02
Cd	0.068±0.02	0.058a±0.02	0.058a±0.03	0.050a±0.02	0.058a±0.03	0.063a±0.02
Cu	0.172±0.16	0.125b±0.12	0.068b±0.08	0.122b±0.13	0.100b±0.11	0.078b±0.08

Replicate= 6 (months), Within the columns, means with different alphabets are statistically different ($p < 0.05$).

Table 3. Preliminary identification of the bacterial isolates.

Gram staining	Shape	Spore formation
+	Rod	-

+ = Positive   - = Negative

Table 4. Biochemical characteristics of the bacterial isolates.

Citrate	Catalase	Coagulase	Starch	Glucose	Oxidase	Lactose	Mannitol	Maltose	MR	Nitrate Reduction
-	+	+	+	+	-	-	-	+	-	-

MR=Methyl Red
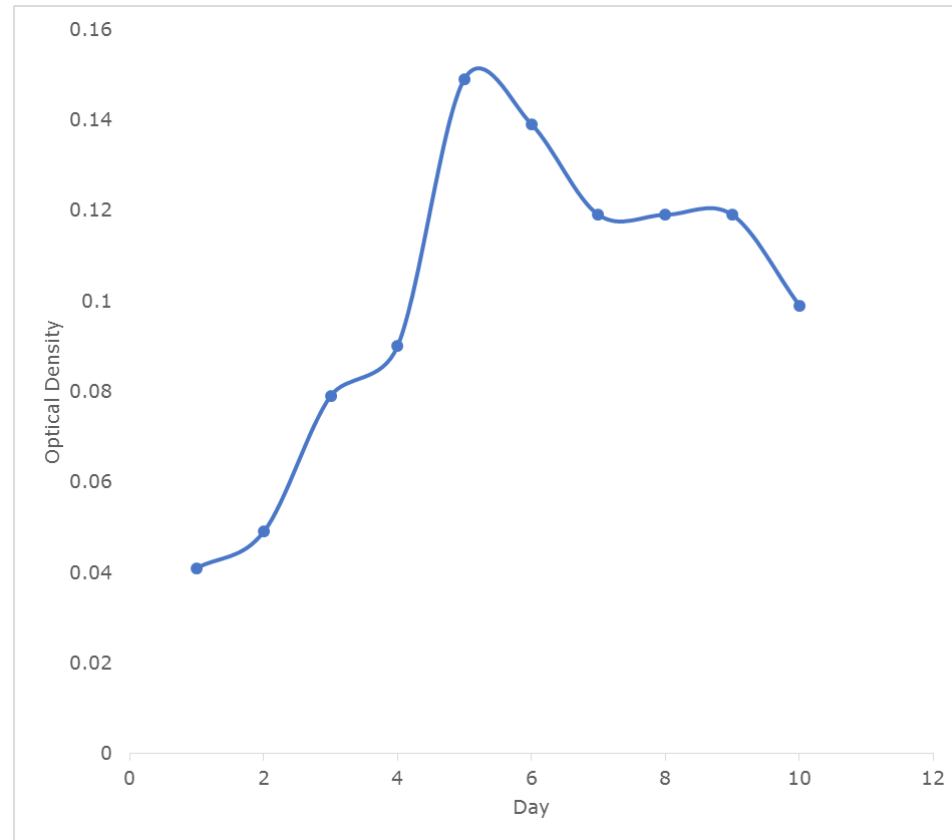
Figure 1: Growth rates (Cells/mL/day) of the *Corynebacterium kutsceri* in the effluent sample from the tannery industry.

Table 5: The correlation coefficient (r) between different masses of bacteria and physicochemical parameters.

Parameter	Correlation coefficient (r)	Percent dependence (rxrx100) (%)
SS	0.54*	30
TDS	-0.35	12
BOD	-0.62	39
COD	0.63*	40
NITRATE	0.53*	28
SULFATE	-0.68	46
PHOSPHATE	-0.69	48
CHLORIDE	-0.37	14

The correlation coefficient (r) with * is statistically significant ($p < 0.05$).
SS=Suspended Solid, TDS=Total Dissolved Solid, BOD=Biochemical Oxygen Demand and COD=Chemical Oxygen Demand

Table 6: The correlation coefficient (r) between different masses of bacteria and heavy metals.

Heavy metals	Correlation coefficient (r)	Percent dependence (rxrx100) (%)
Cr	-0.29	9
Fe	-0.85	72
Mn	-0.76	58
Ni	-0.99	97
Pb	-0.89	79
Zn	-1.00	99
Cd	0.33	11
Cu	-0.39	15

The correlation coefficient (r) with * is statistically significant ($p < 0.05$).

Table 7: Percentage reduction of the physicochemical parameters after-treatment of the effluents (250 mL) with the different masses (5 g, 10 g, 15 g, 20 g, and 25 g) of the *Corynebacterium kutsceri.*

Parameter (mg/L)	Percentage reduction (%)				
	5 g	10 g	15 g	20 g	25 g
COD	56	49	27	14	39
BOD	97	97	97	98	97
SS	79	59	86	65	51
TDS	94	94	94	94	94
Nitrate	80	85	81	73	79
Chloride	43	83	61	75	63
Sulfate	93	94	98	95	97
Phosphate	89	90	89	91	91

Table 8: Percentage reduction of the heavy after-treatment of the effluents (250 mL) with the different masses (5 g, 10 g, 15 g, 20 g, and 25 g) of the Corynebacterium kutsceri.

Parameter (mg/L)	Percentage reduction (%)				
	5 g	10 g	15 g	20 g	25 g
Cr	33	60	28	46	52
Fe	42	55	62	54	75
Mn	36	57	71	50	86
Ni	38	43	50	64	72
Pb	48	58	51	67	73
Zn	17	26	34	40	51
Cd	15	15	27	15	7
Cu	86	43	100	71	86

Results of the Preliminary Identification and Biochemical Characteristics of the Bacterial Isolates

Results of preliminary identification and biochemical characteristics of bacterial isolates were shown in Tables 3 and 4 respectively. After 48 hours of incubation, the nutrient agar media plates were checked for bacteria growth. The results showed that the bacterial isolate was found to be gram-positive, rod-shaped and recorded negative results for spore formation. The results of the biochemical test indicated that the bacteria were positive for catalase, maltose, glucose, starch hydrolysis, and coagulase tests. The bacteria showed negative results for citrate, oxidase, nitrate reduction, MR, lactose and mannitol tests. Base on the Preliminary Identification and biochemical test results, the bacteria isolated were identified to be *Corynebacterium kutsceri (C. kutsceri)*.

Mohammed *et al*., (2012) (3) investigated the enriched inoculums of *Corynebacterium sp* and *Pseudomonas putida,* separately inoculated into anthracene-contaminated water at the temperature of 28 °C under an optimum pH of 7.2 for 96 hours. Maheshwari et al., (2017) (20) isolated and immobilized *Pseudomonas putida* and *Bacillus cereus* isolated from the tannery effluents of Vaniyambadi, India.

Figure 1 shows the growth rates of *C. kutsceri* in the effluent sample from the tannery industry. The initial growth phase of the *C. kutsceri* occurred on the 3rd day of incubation as the growth rate increases up to the 5th-day incubation when the maximum growth was observed. Beyond the 5th day, the growth of the bacteria declined (which might be due to a shortage of nutrients in the effluents) until it reached its phase of death (which might be due to the unavailability of nutrients in the effluents). This implies that the isolate can bioremediate most in the effluent on 5th day.

Results of the Physicochemical Parameters and Heavy Metals in the Industrial Effluents after the Bioremediation

Table 1 shows the mean results of the physicochemical parameters in the industrial effluents before and after the bioremediation using the different masses (5 g, 10 g, 15 g, 20 g, and 25 g) of the *C. kutsceri*. Also, Table 5 shows the results of the correlation coefficient (r) between the different mean masses of bacteria and the physicochemical parameters*.*

The mean value (mg/L) of the SS after the bioremediation varies between 78±89 and 273±375. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the SS was in the order 15 g>5 g>20 g>10 g>25 g. A positive and significant correlation (0.54*) exists between the masses of the *C. kutsceri* and the SS.

There was a low correlation (30 %) between the masses of the *C. kutsceri* and the SS.

The mean value (mg/L) of the TDS after the bioremediation was between 25±15 and 27±16. A negative and no significant correlation (-0.35) exist between the masses of the *C. kutsceri* and the TDS. There was a low correlation (12%) between the masses of the *C. kutsceri* and the TDS.

The mean value (mg/l) of the BOD after the bioremediation varies between 0.58b±0.45 and 0.81±0.67. In the present study, the values of the BOD agree with the report Maheshwari *et al*. (2017) (20) in which the concentration of nitrate decreased in biologically (immobilized *Pseudomonas putida* and *Bacillus cereus*) treated tannery wastewater. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the BOD was in the order 20 g>25 g>15 g=10 g>5 g. A negative and no significant correlation (-0.62) exists between the masses of the *C. kutsceri* and the BOD. There was a low correlation (39%) between the masses of the *C. kutsceri* and the BOD.

The mean value (mg/L) of the COD after the bioremediation varies between 1376±248 and 2681±867. In the present study, the values of the COD agree with the report Maheshwari *et al*. (2017) (20) in which the concentration of nitrate decreased in biological (immobilized *Pseudomonas putida* and *Bacillus cereus*) treated tannery wastewater. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the COD was in the order 5 g>10 g>25 g>15 g>20 g. A positive and significant correlation (0.63*) exists between the masses of the *C. kutsceri* and the COD. There was a low correlation (40 %) between the masses of the *C. kutsceri* and the COD.

The mean value (mg/L) of the nitrate after the bioremediation varies between 14.43±8.88 and 26.52±13.06. In the present study, the values of the nitrate agree with the report Maheshwari *et al*. (2017) (20) in which the concentration of nitrate decreased in biological (immobilized *Pseudomonas putida* and *Bacillus cereus*) treated tannery wastewater. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the nitrate was in the order 10 g>5 g>15 g>25 g>20 g. A positive and significant correlation (0.53*) exists between the masses of the *C. kutsceri* and nitrate. There was a low correlation (28%) between the masses of the *C. kutsceri* and the nitrate.

The mean value (mg/L) of the sulfate after the bioremediation varies between 5.95±8.91 and 18.12±19.26. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the sulfate was in the order 15 g>25 g>20 g>10 g>5 g. A negative and no significant

correlation (-0.68) exist between the masses of the *C. kutsceri* and the sulfate. There was an average correlation (46 %) between the masses of *C. kutsceri* and the sulfate.

The mean value (mg/L) of the phosphate after the bioremediation varies between 1.76±2.64 and 2.10±3.00. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the phosphate was in the order 25 g>20 g>10 g>5 g>15 g. A negative and no significant correlation (-0.69) exist between the masses of *C. kutsceri* and the phosphate. There was an average correlation (48%) between the masses of *C. kutsceri* and the phosphate.

The mean value (mg/L) of the chloride after the bioremediation varies between 5.54±4.66 and 12.90±18.39. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the chloride was in the order 10 g>20 g>25 g>15 g>5 g. A negative and no significant correlation (-0.37) exist between the masses of *C. kutsceri* and the chloride. There was a very low correlation (14%) between the masses of *C. kutsceri* and the chloride. The decrease in the concentration of the physicochemical parameters after the bioremediation was not only due to the increase in the mass of *C. kutsceri* but might be also due to the increase in surface area of the different mass of *Corynebacterium Kutsceri.*

Table 2 shows the mean results of the heavy metals in the industrial effluents before and after the bioremediation using the different masses (5 g, 10 g, 15 g, 20 g, and 25 g) of the *C. kutsceri*. Also, Table 6 shows the results of the correlation coefficient (r) between the different mean masses of bacteria and heavy metals*.*

The mean value (mg/L) of the chromium after the bioremediation varies between 3.048±2.70 and 5.416±4.07. This is in agreement with the work of Maheshwari *et al*. (2017) (22) in which the concentration of chromium decreased in biologically (immobilized *Pseudomonas putida* and *Bacillus cereus*) treated tannery wastewater. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the chromium was in the order 10 g>25 g>20 g>5 g>15 g. A negative and no significant correlation (-0.29) exists between the masses of the *C. kutsceri* and the chromium. There was a very low correlation (9%) between the masses of the *C. kutsceri* and the chromium.

The mean value (mg/L) of the iron after the bioremediation varies between 0.310±0.20 and 0.732±0.11. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the iron was in the order 25 g>15 g>10 g>20 g>5 g. A negative and no significant correlation (-0.85) exist between the masses of the

C. kutsceri and the iron. There was a high correlation (72%) between the masses of the *C. kutsceri* and the iron.

The mean value (mg/L) of the manganese after the bioremediation varies between 0.003±0.01 and 0.015±0.01. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the manganese was in the order 25 g>15 g>10 g>20 g>5 g. A negative and no significant correlation (-0.76) exists between the masses of the *C. kutsceri* and the manganese. There was an average correlation (58 %) between the masses of the *C. kutsceri* and the manganese.

The mean value (mg/L) of the nickel after the bioremediation varies between 0.077±0.06 and 0.172±0.05. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the nickel was in the order 20 g>25 g>15 g>10 g>5 g. A negative and no significant correlation (-0.99) exists between the masses of *C. kutsceri* and the nickel. There was a very high correlation (97%) between the masses of the *C. kutsceri* and the nickel.

The mean value (mg/L) of the lead after the bioremediation varies between 0.082±0.11 and 0.158±0.20. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the lead was in the order 25 g>20 g>10 g>15 g>5 g. A negative and no significant correlation (-0.89) exists between the masses of the *C. kutsceri* and the lead. There was a high correlation (79%) between the masses of the *C. kutsceri* and the lead.

The mean value (mg/L) of the zinc after the bioremediation varies between 0.028±0.03 and 0.048±0.04. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the zinc was in the order 20 g>25 g>15 g>10 g>5 g. A negative and no significant correlation (-1.00) exist between the masses of the *C. kutsceri* and the zinc. There was a very high correlation (99%) between the masses of the *C. kutsceri* and the zinc.

The mean value (mg/L) of the cadmium after the bioremediation varies between 0.050±0.02 and 0.063±0.02. The relative potential or efficiency of the different masses of the *C. kutsceri* in remediating the cadmium was in the order 15 g>5 g>20 g=1 g>25 g. A positive and no significant correlation (0.33) exists between the masses of the *C. kutsceri* and the cadmium. There was a very low correlation (11%) between the masses of the *C. kutsceri* and the cadmium.

The mean value (mg/L) of the copper after the bioremediation varies between not detected and 0.007±0.00. The relative potential or efficiency of

the different masses of the *C. kutsceri* in remediating the copper was in the order 15 g>25 g>20 g>5 g>10 g. A negative and not significant correlation (-0.39) exists between the masses of the *C. kutsceri* and the copper. There was a low correlation (15%) between the masses of the *C. kutsceri* and the copper. There was a reduction in the concentration of all the heavy metals after the bioremediation process when compared with the concentration of the raw samples before the bioremediation. This is in agreement with the work of Maheshwari *et al*. (2017) in which the concentration of chromium decreased in biologically (immobilized *Pseudomonas putida* and *Bacillus cereus*) treated tannery wastewater. This is also in line with the report made by Ajao et al. (2011) and Galadima et al. (2012). Also, Table 6 shows the results of the correlation coefficient (r) between the different mean masses of bacteria and heavy metals*.* In the present study, the decrease in the concentration of the heavy metals after the bioremediation was not only due to the increase in the mass of the bacteria but might be also due to the increase in surface area of the different mass of the bacteria.

Percentage reduction of the physicochemical parameters and the heavy metals by the *Corynebacterium Kutsceri*
Table 7 shows the percentage reduction of the physicochemical parameters after the treatment of the effluents (250 mL) by the different masses (5 g, 10 g, 15 g 20 g, and 25 g) of the *C. kutsceri.* The percentage reduction (%) of SS ranged (51-86); TDS (94); BOD (97-99); COD (14-56); nitrate (73-85); chloride (43-83); sulfate (93-98); phosphate (89-92).

Table 8 shows the percentage reduction of the heavy metals after-treatment of the effluents (250 mL) with the different masses (5 g, 10 g, 15 g 20 g, and 25 g) of the *C. kutsceri.* The percentage reduction (%) of Cr ranged (28-60); Fe (42-75); Mn (36-86); Ni (38-72); Pb (48-73); Zn (17-51); Cd (7-27) and Cu (43-100). Maheshwari *et al*. (2017) studied the bioremediation by free and immobilized bacteria isolated from tannery effluent. The percentage reduction of nitrate and nitrite was ranged from 45 to 75% when treated with *P. putida*, whereas they were ranged from 60 to 79% on the 15th day of the treatment. The level of BOD was reduced drastically after the 15th day of exposure by the free cells of both bacteria. The maximum percentage of reduction (83%) was shown by immobilized *B. cereus.* The percent reduction of COD ranged from 16% to 65% in both bacterial strains. The percentage of removal of Cr(VI) was 86% and 91% for free and immobilized cells, respectively. In *B. cereus* treated sample, it reached 84% and 89% on the 15th day respectively. It was concluded that both *P. putida* and *B. cereus* investigated in this study are highly recommended

RESEARCH ARTICLE

for beneficial bioremediation applications for *in-situ* and off-site removal of pollutants.

Bioremediation of textile industrial effluent using the mixed culture of *Pseudomonas aeruginosa* and *Bacillus subtilis* immobilized on agar-agar in a bioreactor was carried out by Ajao et al. (2011). The result indicates overall % reduction in COD, BOD, nitrate, sulfate, phosphates as 83%, 97%, 61.3%, 62.8%, 61.2% respectively. Heavy metals were also bio-sorbed. It was concluded that immobilized cells represent a promising application in the bioremediation of textile industrial effluent and possible reusability of the cells for its commercial application can be achieved.

In general, immobilization makes the enzyme more resistant to temperature, pH, and substrate concentration swings giving it a longer lifetime and higher productivity per active unit (35). Although several workers described microbial degradation of tannery effluent, limited literature is available on the bioremediation of tannery effluent using immobilized bacterial cells in the present study area.

CONCLUSION

The tannery industrial effluent samples contained variable levels of the physicochemical parameter and heavy metals, in which some of them were higher than the recommended standard limits. The presence of these physicochemical parameters and heavy metals might be due to the nature of the raw materials and processes used in the tannery industries at the time of sampling. Based on the preliminary identification and biochemical test results, the bacterial isolate was identified to be *C. kutsceri*. There was an overall decrease in the concentration of the physicochemical parameters and the heavy metals after the bioremediation using the different masses of the immobilized *C. kutsceri*. However, the decrease was not only due to the increase in the mass of the *C. kutsceri* but might be also due to the increase in surface area of the different mass of the immobilized *C. kutsceri*. The average high percentage reduction (70% to 100%) of these parameters and heavy metals implies that the immobilized *C. kutsceri* is having a higher potential for the treatment of the tannery industrial effluents.

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Comparative DFT Study of a Ruthenium Complex

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Abstract: A comparative density functional theory (DFT) study of the Ruthenium complex (pentamethylcyclopentadienyl)(diisopropylmethylphosphine)(chloro)(trichlorosilyl)ruthenium hydride is reported. The molecule contains a ruthenium (Ru) atom, which, like other transition metals, is computationally difficult to handle due to the near degeneracy of their electronic states. Calculations were carried out in the gas phase using GAMESS software (the General Atomic and Molecular Electronic Structure System), which is an ab-initio quantum chemistry package. Five different basis sets were used, namely: Sapporo non-relativistic SPK DZP, SBKJ, 3-21G, STO3G, and STO6G. The molecule was optimized in quintuplicate with each of the basis sets. The computational results were compared with real X-ray data to assess how well the basis sets worked for a molecule containing a transition metal such as ruthenium. As the most computationally expensive basis set, the Sapporo non-relativistic SPK DZP was expected to give the most accurate results. However, unexpectedly, 3-21G, a computationally cheaper basis set, exhibited the best performance.

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INTRODUCTION

Density functional theory (DFT), as the most common computational method for molecular calculations, has been used for decades to investigate the properties of molecules and molecular systems by ab-initio calculations. (1–6) It has been applied to a wide range of real atomic systems, including transition metal (TM) complexes. (3,7–11) However, it is well known that TM complexes are computationally difficult as they have unique properties, such as near degenerate electronic states, which lead to less accurate computational results than for other atomic types. (8–11) Nonetheless, DFT is still the most used computational approach as there is no better alternative (with a lower computational cost and better accuracy) available. (6)

Owing to the errors that arise in quantum chemical calculations of TM complexes, it is necessary to investigate suitable computational methods to minimize calculation errors. One of the most effective ways of determining the effectiveness of computational methods is to compare their results with real experimental data. By doing so, we aim to contribute to search for better parameter sets that give better results for ab-initio quantum chemical calculations of TM complexes, and also to contribute to the improvement of computational approaches by adding some new data to the existing literature.

Scientists need to know how much improvement they can expect from using a more computationally expensive model (for instance, when computation time is doubled). Therefore, expected improvements in accuracy with increased computational resources need to be investigated in order to decide if a particular model is worthwhile. The most effective way of doing this is to carry out comparative studies with new models and real data and contribute to the available literature, as with the current study. Moreover, these types of studies should include models that are both computationally cheap and expensive for comparison and accuracy. Hence, the

main aim of the current work is to determine the effectiveness of the computationally expensive Sapporo non-relativistic SPK DZP and SBKJ basis sets by comparison to the computationally cheaper STO3G, STO6G, and 3-21G.

The CAM-B3LYP functional, which was recently introduced, solves the long-range self-interaction error problem with its range-separated structure. (12,13) Furthermore, it has been used for transition metal complexes in previous studies.(14–17) Hence, it was also selected for investigation in our current study. As our results showed, it is an efficient functional for optimization of the ruthenium complex since it gave tolerable bond length errors according to the comparison of the calculated results with the real X-ray data, even for the computationally cheaper basis sets.

Here, five different basis sets were investigated for the optimization of the structure of (pentamethylcyclopentadienyl) (diisopropylmethylphosphine)(chloro) (trichlorosilyl)ruthenium hydride, and the calculated results were compared against real X-ray data for the molecule.(18)

RESULTS AND DISCUSSION

The optimized geometry of the molecule is given in Figure 1. As can be seen in Figure 1, Ru is bonded

MATERIALS AND METHODS

All calculations were carried out using GAMESS software (the General Atomic and Molecular Electronic Structure System), which is an ab-initio quantum chemistry package.(6,19) The hybrid Becke three-parameter Lee–Yang–Parr, modified by the Coulomb-attenuating method (CAM-B3LYP) was selected for this work as exchange correlation functional.(12) Five different basis sets were used to optimize the structure of the molecule: SBKJ (Stevens, Bash, Krauss, and Jasien) valence, Sapporo non-relativistic SPK DZP, 3-21G, STO3G, and STO6G.(20–24) All calculations were performed in the gas phase and all of the optimizations started from the same molecular configuration. All of the computational parameters were kept the same for all calculations, except for the basis sets. After the optimizations, frequency calculations were also carried out with the same levels of theory to confirm that were no imaginary frequencies, hence the optimized conformations are confirmed as real minima on the potential energy surface.

to five ring carbons (C1, C2, C3, C6, C48) and four other atoms (Si27, P26, Cl32, and H31) in half-sandwich form.

Figure 1: Optimized geometry of the molecule, as determined by the Sapporo non-relativistic SPK DZP basis set.

The calculated bond lengths of the molecule are given in Table 1, and the percentage of calculation errors for each bond and each method are presented in Table 2. As can be seen from Tables 1 and 2, the overall performance of the basis sets varies greatly, while the performance of individual

basis sets also varies with the type of bonded atom. For instance, according to Table 1, the STO-6G basis set gives better performance than the STO-3G basis set for all of the bonds. Besides, STO-6G gives more errors for Ru bonds (with near 25%) than the C-C bonds (with near 4%).

Table 1: Bonded atoms and bond lengths calculated using basis sets and determined experimentally with X-ray data.

Atom 1	Atom 2	X-ray (Å)	SBKJ (Å)	Sapporo SPK DZP (Å)	3-21G (Å)	STO-3G (Å)	STO-6G (Å)
C1	C2	1.414	1.425	1.419	1.407	1.508	1.356
C1	C9	1.496	1.512	1.495	1.495	1.527	1.514
C2	C4	1.484	1.513	1.496	1.499	1.517	1.517
C3	C2	1.441	1.449	1.427	1.447	1.351	1.519
C3	C5	1.497	1.516	1.499	1.500	1.519	1.524
C3	C6	1.442	1.453	1.439	1.454	2.102	1.474
C33	C38	1.530	1.548	1.534	1.544	1.557	1.554
C33	C39	1.532	1.546	1.529	1.539	1.552	1.553
C34	C36	1.525	1.546	1.531	1.540	1.554	1.554
C34	C37	1.526	1.545	1.527	1.540	1.555	1.551
C48	C1	1.447	1.464	1.443	1.465	1.467	1.565
C48	C8	1.496	1.510	1.493	1.495	1.487	1.522
C6	C48	1.415	1.428	1.414	1.417	1.768	1.454
C6	C7	1.500	1.514	1.496	1.499	1.466	1.502
P26	C33	1.861	1.875	1.861	1.852	1.884	1.913
P26	C34	1.848	1.880	1.868	1.857	1.889	1.905
P26	C46	1.822	1.841	1.820	1.821	1.857	1.859
Ru25	C1	2.316	2.335	2.252	2.365	1.638	2.507
Ru25	C2	2.345	2.365	2.309	2.400	2.310	2.548
Ru25	C3	2.229	2.256	2.233	2.234	1.591	1.679
Ru25	C48	2.241	2.280	2.240	2.281	1.578	1.638
Ru25	C6	2.240	2.284	2.262	2.270	1.458	1.654
Ru25	Cl32	2.413	2.449	2.430	2.427	1.679	1.752
Ru25	P26	2.369	2.440	2.410	2.377	4.890	1.785
Ru25	Si27	2.315	2.374	2.341	2.307	1.767	1.833
Si27	Cl28	2.099	2.134	2.127	2.102	2.040	2.054
Si27	Cl29	2.089	2.105	2.093	2.066	2.044	2.043
Si27	Cl30	2.092	2.110	2.089	2.075	2.055	2.049

Table 2: Percentage calculation errors for each bond by basis set.

Atom 1	Atom 2	Error % SBKJ	Error % Sapporo SPK DZP	Error % 3-21G	Error % STO-3G	Error % STO-6G
C1	C2	0.78	0.35	-0.50	6.65	-4.10
C1	C9	1.07	-0.07	-0.07	2.07	1.20
C2	C4	1.95	0.81	1.01	2.22	2.22
C3	C2	0.56	-0.97	0.42	-6.25	5.41
C3	C5	1.27	0.13	0.20	1.47	1.80
C3	C6	0.76	-0.21	0.83	45.77	2.22
C33	C38	1.18	0.26	0.92	1.76	1.57
C33	C39	0.91	-0.20	0.46	1.31	1.37
C34	C36	1.38	0.39	0.98	1.90	1.90
C34	C37	1.25	0.07	0.92	1.90	1.64
C48	C1	1.17	-0.28	1.24	1.38	8.15
C48	C8	0.94	-0.20	-0.07	-0.60	1.74
C6	C48	0.92	-0.07	0.14	24.95	2.76
C6	C7	0.93	-0.27	-0.07	-2.27	0.13
P26	C33	0.75	0.00	-0.48	1.24	2.79
P26	C34	1.73	1.08	0.49	2.22	3.08
P26	C46	1.04	-0.11	-0.05	1.92	2.03
Ru25	C1	0.82	-2.76	2.12	-29.27	8.25

Ru25	C2	0.85	-1.54	2.35	-1.49	8.66
Ru25	C3	1.21	0.18	0.22	-28.62	-24.67
Ru25	C48	1.74	-0.06	1.78	-29.59	-26.91
Ru25	C6	1.96	0.98	1.34	-34.91	-26.16
Ru25	Cl32	1.51	0.73	0.60	-30.40	-27.38
Ru25	P26	2.98	1.72	0.32	106.39	-24.66
Ru25	Si27	2.54	1.12	-0.35	-23.68	-20.82
Si27	Cl28	1.67	1.33	0.14	-2.81	-2.14
Si27	Cl29	0.77	0.19	-1.10	-2.15	-2.20
Si27	Cl30	0.86	-0.14	-0.81	-1.77	-2.06

Figure 2 shows a comparison of the calculated C−C bond lengths with those obtained from the X-ray data, and Figure 3 shows the errors in the calculated C−C bond lengths. As shown in Figures 2 and 3, the Sapporo non-relativistic SPK DZP basis set gives the best approximation of the C−C bond lengths, while STO-3G gives the worst approximation. The most significant error for the C−C bond lengths was in relation to the C6 position calculated using the STO-3G basis set; 45% error was seen for the C3−C6 bond and 24% for the C6−C48 bond. On the other hand, when we look at the overall picture in Figure 2, it is clear that all of the approximations are in tolerable agreement with the C−C bond lengths determined via X-ray data, except for those obtained using the STO-3G basis set.
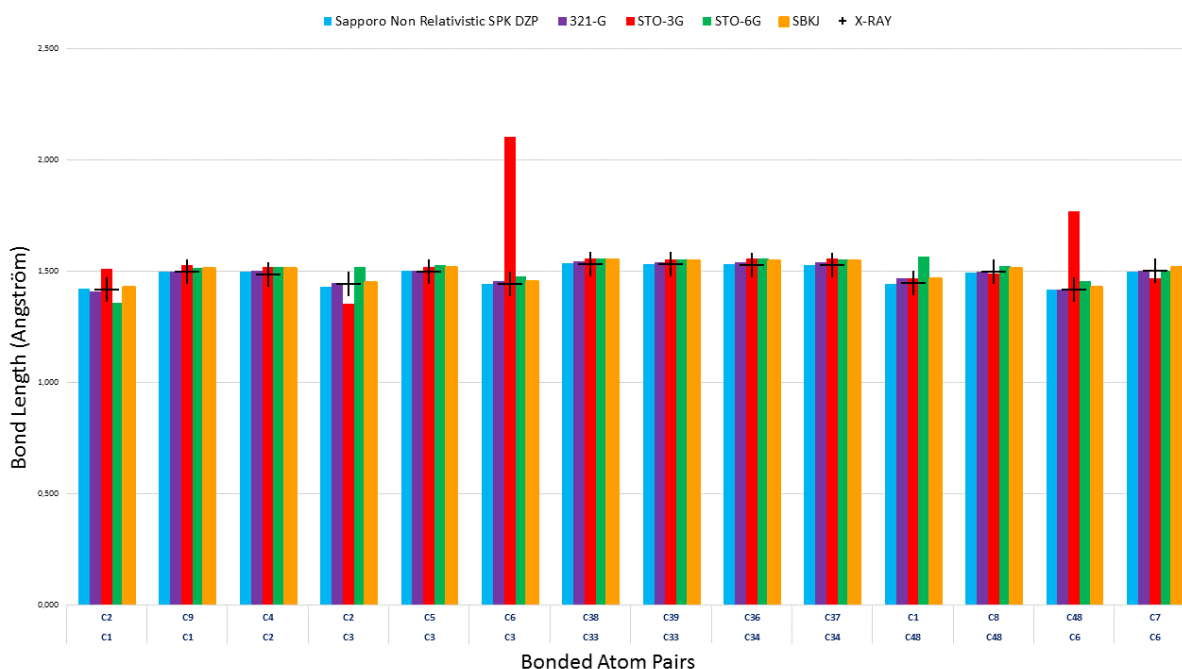
Figure 2: Comparison of calculated C−C bond lengths with those obtained from X-ray data.

Figure 3: Percentage differences between calculate and experimentally determined C−C bond lengths.

Figure 4: Comparison of calculated bond lengths for Ru, Si, Cl, and P atoms with those obtained from X-ray data.
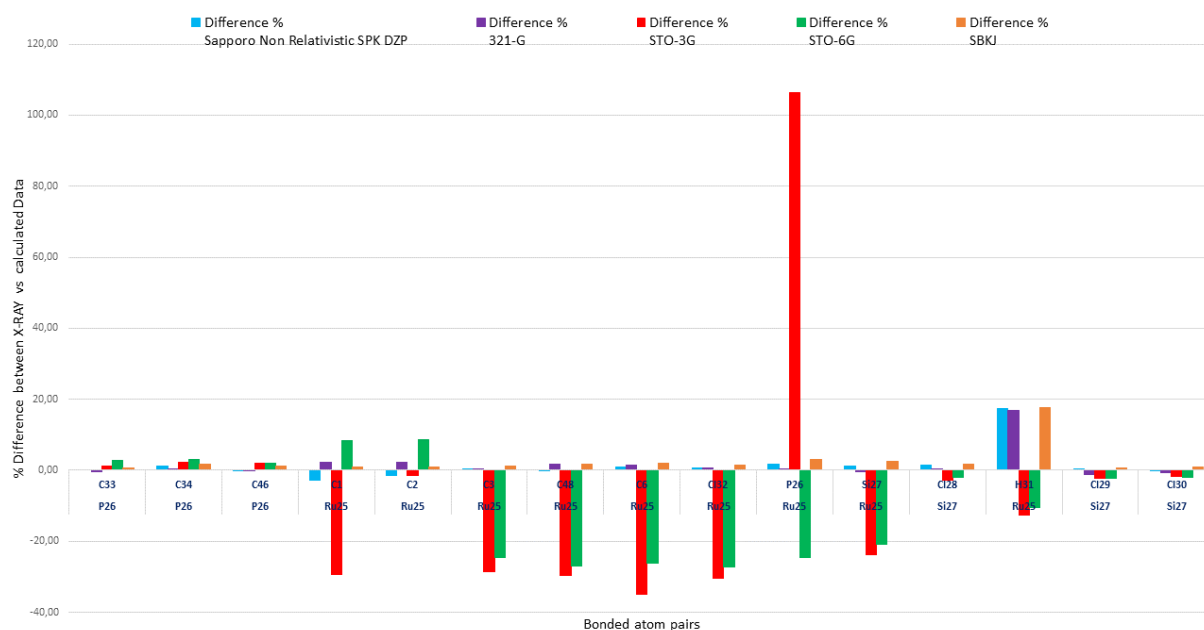
Figure 5: Percentage differences between calculate and experimentally determined bond lengths for Ru, Si, Cl, and P atoms.

Figure 4 shows a comparison of the calculated and experimentally obtained bond lengths for Ru, Si, Cl, and P atoms, while Figure 5 shows the percentage errors for these calculated bond lengths. It can be clearly seen that the performance of the basis sets differs significantly for Ru bonds, while closer agreement was observed for P–C and Si–Cl bonds. Clearly, neither of the Slater-type orbital basis sets (STO-3G nor STO-6G) give a good fit for Ru bonds. Although the STO-6G basis set shows better performance than the STO-3G, as expected, both approximations give intolerable errors for Ru bonds. Thus, this type of basis set is not recommended for quantum chemical calculations for complexes with

transition metals such as Ru. The best performance for Ru bonds surprisingly came from 3-21G, a computationally cheaper method, rather than from the more computationally expensive Sapporo non-relativistic SPK DZP or SBKJ basis sets.

Figure 6 shows a comparison of the calculated and experimentally determined C−H bond lengths, and Figure 7 shows the percentage errors for the calculated C−H bond lengths. The results show that each of the five basis sets gave larger errors for H atoms than for any other atoms. All of the basis sets gave longer bond lengths than those obtained from the X-ray data for all H bonds.

Figure 6: Comparison of calculated C−H bond lengths with those obtained from X-ray data.
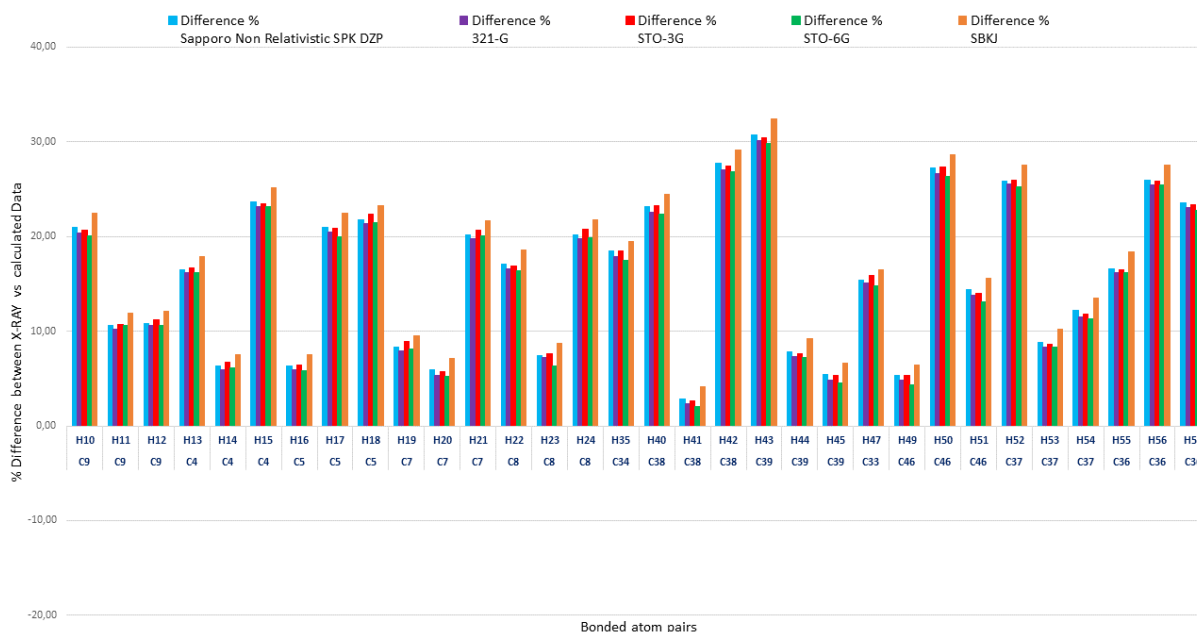
Figure 7: Percentage errors for calculated C−H bond lengths.

The calculation times and RMS (Root Mean Square) errors for specific bond types are presented in Table 3. The table shows that the fastest method is STO-3G with 604 minutes; hence, it is the cheapest method in a computational sense. However, STO-3G is the worst method when considering the percentage errors for all bond types.

On the other hand, the Sapporo non-relativistic SPK DZP basis set is the slowest one at 5290.2 minutes,

requiring an eight-fold longer calculation time than STO-3G. The Sapporo non-relativistic SPK DZP basis is the most computationally expensive method used in this work. Although it gave the most accurate results for C−C bond lengths, as expected, this was unexpectedly not the case for Ru bonds or C−H bonds.

Surprisingly, 3-21G, the second-cheapest method in computational terms with 640.7 minutes, gave the

most accurate results for Ru bonds with a 4.501% RMS error, and the second-most accurate results for C–C and C–H bonds with results very close to those obtained using the best-performing basis set.

Hence, when all of the bonds are considered with total RMS errors, 3-21G exhibits the best performance, being superior to the other more computationally expensive basis sets.

Table 3: The RMS error percentages for bonded atom groups and the calculation times.

Basis Set	Total calculation time (min)	Total RMS error	C–C bonds RMS error	Ru bonds RMS error	C–H bonds RMS error
Sapporo SPK DZP	5290.2	13.057	0.401	4.636	17.735
3-21G	640.7	12.722	0.687	4.501	17.275
STO-3G	604.0	22.059	14.221	33.428	17.623
STO-6G	1517.7	14.974	3.256	16.530	17.005
SBKJ	1300.9	13.983	1.124	4.795	18.968

The performance of each of the basis sets is shown graphically in Figure 8, which shows all the calculation times and the RMS error percentages for all bond types and all basis sets. Thus, we can compare both the calculation times and the errors simultaneously to compare the performances of all

basis sets. Clearly, the Sapporo non-relativistic SPK DZP and the 3-21G basis sets have similar error rates for all types of bonds, while there is a large gap between their calculation times (88.17 hour vs. 10.68 hour).
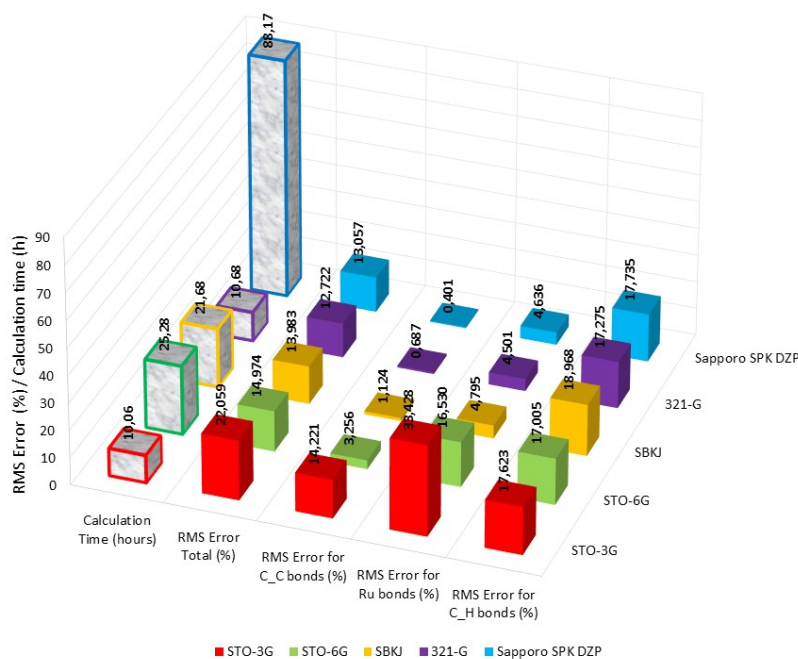
Figure 8: 3D graph comparing the performance of the basis sets, showing the RMS error percentages and calculation times for each basis set and each bond type.

CONCLUSIONS

Considering the overall picture in Figure 8, the following conclusions can be drawn:

a)   Slater-type orbital (STO   -*G) basis sets are not recommended for complexes involving Ru and similar transition metals.

b)   All of the tested methods exhibited some difficulties in calculating H bonds, but the Slater-type orbital basis sets (STO-6G and even STO-3G) performed better than the other tested basis sets.

STO-6G gives the smallest RMS error for H bonds, as seen in Figure 8.

c)   The Sapporo non-relativistic SPK DZP, 3-21G, and SBKJ valence basis sets give similar error rates for C–C and Ru bonds, but with radically different calculation times of 5290.2 min, 640.7 min, and 1300.9 min, respectively.

d)   Hence, among those tested in this work, the 3-21G basis set shows the best performance with a short computation time and high accuracy, and it is recommended for use for complexes with Ru and similar transition metals.

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RESEARCH ARTICLE

Catalytic performance of polyvinylidene fluoride (PVDF) supported TiO₂ additive at microwave conditions

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Abstract: TiO_2 is a widely used additive material to improve the properties of filtration membranes. As far as its contribution to filtration performance, TiO_2 acts as a catalytic agent. In this study, the catalytic activity of TiO_2 added PVDF flat sheets (membranes) prepared by the phase inversion method, and we tested them on the oxidation of benzyl alcohol at microwave irradiation. We investigated the structure, thermal behaviors, and morphology of samples by FTIR, TG-DTA, and SEM analysis, respectively. Contact angle measurements determined the hydrophilicity, and we observed the interaction between TiO_2 and PVDF chain. TiO_2-added PVDF membranes had improved filtration and rejection performance. 32.4% BSA rejection was obtained with composites while it was 7.5% for the pristine membrane. We obtained 93.86% conversion and 56% selectivity with composites in a microwave-assisted batch reactor. The decomposition of the polymer increased with a 6% additive due to the catalytic effect of TiO_2 then, and it decreased with further loading. We obtained detailed evidence by activation energies (E_a) of decomposition calculated by Kissinger's method. The microwave-assisted catalytic effect of TiO_2 inside the polymeric matrix promise to obtain fast, high yielded, and disposable membrane materials for catalytic reactors to in-situ production of fine chemicals.

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INTRODUCTION

Filtration and separation membranes caused great interest in obtaining more improved performance, such as high purity, fast, and easy yields. One of the most effective routes to obtain highly efficient filtration membranes was the preparation of composites, which are a combination of two or more components. Zeolite, metal oxides, carbon-based molecular sieves, and clays were commonly used organic/inorganic filler materials into the polymer matrix (1). Researchers preferred TiO₂ as additive materials for filtration membranes due to their multi-functional properties such as providing thermal and physical resistance, tunable pore, and channels in addition to its photocatalytic activity (2). Degradation of organic compounds were

conducted by using TiO₂ at the form of suspended in solution or immobilized to support by NIPS, TIPS and spinning processes or grafting to membrane surface (3–9). Among those, suspended TiO₂ provided high activity, but separation from the solution and regeneration became an environmental issue, so it restricts the usage of suspension. Researchers adopted the application of the TiO₂-immobilized polymer matrix, and they used composite membranes, mainly separation of particles, degradation of organic contaminants, production of fine chemicals, and bacterial removal. Researchers used Ag particles combined with TiO₂-Perlite for the degradation of methylene blue removal under UV light radiation (10). Hydroxyl groups that emerged on the surface of composites turned to hydroxyl radicals when UV

radiation exposed to them, and these induced to the decomposition of organic dye, waste, and bacteria.

Some authors also prepared TiO_2 dispersed filtration membranes and investigated in terms of additive size, additive ratio, as far as they met the composition, and coagulation conditions (11,12). An extended summary was reported by Mendret et al. about TiO_2-deposited polymers, particularly for the combination of TiO_2 and PVDF (13). TiO_2 provided excellent hydrophilicity and permeation to filtration membranes. Mainly, embedded membranes having nanosized particles performed better antifouling properties compared with their pristine forms (14,15). Self-cleaning ability is related to the photocatalytic activity of TiO_2 immobilized on the membranes.

Some authors prepared additive contained polymeric or ceramic matrixes to achieve instant reaction of substrates during filtration as catalytic membrane reactors. The common advantages of these systems are easy reaction and separation with low cost and reusability. Some researchers conducted phenol hydroxylation and p-aminophenol production by hydrogenation of p-nitrophenol on ceramic membrane reactors with high yield (16,17). For the oxidation of phenol with peroxide, the authors used a packed bed reactor consisting of FeZSM-5 zeolite immobilized on silicon carbide (SiC) foam catalyst. At 60 ⊕C and 1 mL min^{-1} flow, the researchers obtained 3.4 kJ mol^{-1} activation energy of phenol degradation with 95 h^{-1} turnover frequency. These kinds of systems require polymeric materials that have high physical and chemical resistance. PVDF is known for its high chemical, physical, and thermal resistance, and we see its use for chemical reactors and applications using UV light irradiation (18).

On the other hand, TiO_2 can be used to dispose of polluting polymers, organics, and polymeric materials economically, for instance, the application of polyethylene degradation by PAM grafted TiO_2 (19). A group of researchers investigated the photocatalytic degradation of the polystyrene matrix by the immobilized TiO_2, and they recorded a decrease at the molecular weight of the polymer (20). Thermal behaviors are also essential indicators about the resistance capacity of polymer, and those provide information about the interaction of additive with a polymer matrix (21).

In general, under appropriate rates, TiO_2 contributed better thermal stability to the polymer matrix. Polymer coated by nucleated additives induces crystalline formation on polymer during the phase separation (22–24). According to literature overviewed, TiO_2 immobilized/embedded/

coated polymeric composite membranes have better thermal resistance in addition to improved flux, rejection, and other filtration properties compared with pristine forms.

In this study, we aimed to determine the catalytic and thermal oxidative degradation properties and their filtration performance of TiO_2-blended PVDF membranes prepared at different mass ratios. In distinct energy supply, we conducted the oxidation experiments of benzyl alcohol in a microwave radiated batch reactor cooled with a condenser. We also investigated the polymer decomposition behaviors of TiO_2 (inside the structure) by detailed thermal analysis and decomposition enthalpies calculated by the Kissinger's method. We evaluated whether these are usable or not as a catalytic membrane at microwave conditions to get a fast and environmentally friendly design.

We tested only 0.5-4% TiO_2 contained filtration membranes in terms of filtration performances due to the inconvenience of the filtration ability of high loading composites. We also investigated the effect of additive ratio and dispersion of particles on the membrane properties on the heat resistance of composite membrane and disposal method for polymeric waste by the obscured thermal-oxidative activity of TiO_2 in addition to its well-known photocatalytic activity.

EXPERIMENTAL SECTION

Materials
Polyvinylidene fluoride, PVDF (Solef 6010), was used as the polymer material without any purification with N, N-dimethylformamide (DMF; Sigma Aldrich) as a solvent and distilled water. For the synthesis of TiO_2, titanium(IV) isopropoxide, $Ti[OCH(CH_3)_2]_4$ (284.22 g/mol, 97% w/w, 0.960 g/mL), sodium chloride, NaCl (58.44 g/mol), ethanol, C_2H_5OH (46.06 g/mol, 0.789 g/mL) were purchased from Sigma-Aldrich. We used a model protein-solution with bovine serum albumin, BSA (MW: 66000 Da, ≥98% purity, Sigma Aldrich) with boric acid, H_3BO_3 (MW: 61.83 g/mol, Sigma-Aldrich), ortho-phosphoric acid, H_3PO_4 (MW: 98 g/mol, Sigma-Aldrich) and acetic acid, CH_3COOH (MW:60.05 g/mol, 99.7%, Sigma-Aldrich) as acidity adjustment. We procured benzyl alcohol and hydrogen peroxide (30% w/v) from Panreac.

Preparation of TiO_2 and TiO_2-PVDF composite membranes
We prepared the TiO_2-blended PVDF membranes by wet-phase inversion technique determined in a report (25). To synthesize TiO_2 particles, titanium(IV) isopropoxide (0.11 mol/L) and NaCl solution (%1 w/w) were mixed in a round-bottomed flask and stirred with a magnetic stirrer for ten minutes. We then left the obtained slurry to

rest for approximately 12 hours. It was then filtered and washed with hot ethanol and water. The precipitate was dried at 60 ºC and ground to powder at the size of 5-100 μm. An appropriate amount of dry solid was weighed and dissolved in DMF by ultrasonication. We added it to the polymer solution obtained by dissolving PVDF (1.6 g) in DMF (10 mL) as a 14% mass ratio in terms of polymer to solvent. The mixture of PVDF/DMF/TiO_2 was stirred at 250 rpm at 65 ºC for 12 hours, and we kept it in the ultrasonication bath for 10 minutes to obtain a homogeneous dispersion. The mixture was waited for a time to prevent air

bubbles and cast on to a flat surface (15 cm x 15 cm) uniformly by a 300 μm casting knife at 25 ºC. After exposure to air for 10 seconds, we quickly immersed the glass plate into the coagulation bath. Figure 1 represents the steps of preparation. Membranes were dried and used for characterization and alcohol oxidation. We stored the filtration membranes in distilled water for filtration experiments. Membranes contain TiO_2 at the rate of 0, 0.5, 1, 2, 4, 6, and 8% according to the total mass of combination were determined as PVDF, T05-P, T1-P, T2-P, T4-P, T6-P, and T8-P respectively.
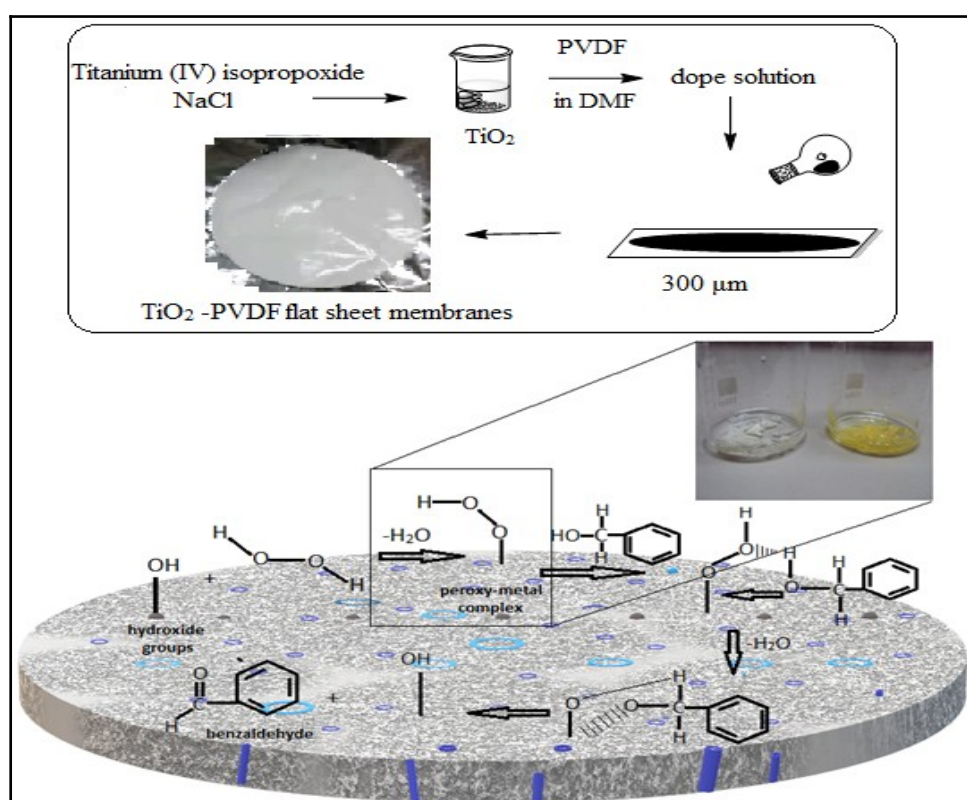
Figure 1: Illustration for the preparation of TiO_2-PVDF and peroxy metal complex.

Characterization of TiO_2 and TiO_2-PVDF composite membranes

We recorded the FTIR spectra of TiO_2 and membranes in the region between 4000 and 650 cm^{-1} by a Perkin-Elmer FTIR spectrometer. Thermal stability and content of inorganic matter of samples were investigated by the thermogravimetric analyzer (TG-DTA, Exstar 7020) under the aerial atmosphere. We have adjusted typical conditions as about 5-7 mg of sample weight and 20 ºC/min heating rate. We evaluated the thermal decomposition effect of TiO_2 to polymer, and we heated the samples in 50-1000 ºC temperature range by 5, 10, 15, 20 and 30 ºC/minute heating rates. The hydrophilicity of membranes was analyzed by a KSV Attention contact angle analyzer (Finland) at 25 ºC temperature. The morphology of membranes was

examined by Carl Zeiss ULTRA Plus SEM device (Germany) at 10 kV. We photographed the cross-section images of samples broken in liquid nitrogen. As a model contaminant, we used BSA to determine membrane performance. We have analyzed the absorbance of the contaminant with a UV-visible spectrophotometer (PG Instruments, T80) at 280 nm wavelength (24).

We determined the water uptake capacity (WU) of membranes stored in water by measuring the weight of wet (W_w) and dried (W_d) membranes dried at 40 ºC in a vacuum oven for 2 hours. We used Equation 1 to calculate.

$$WU(\%) = \frac{W_w - W_d}{W_w} x100$$

(1)

Pore percentage called as membrane porosity (PO) was calculated by following eq. (2):

$$PO(\%) = \frac{W_w - W_d}{dA\delta} \, x100 \tag{2}$$

Where d is the density of deionized water at 25 °C (the temperature of studies conducted), A is the area of the membrane (cm^2) and δ is the thickness of wet membrane (cm).

Water flux, rejection and antifouling performances of composite membranes

We measured the pure water flux (PWF) performances of membranes by collecting the permeated water (L/m^{2}h) at ultrafiltration cross-flow membrane cell. We adjusted the transmembrane pressures (TMP) as 100, 200, and 300 kPa after pre-conditioning of membranes for 1-3 h (26-27). We calculated WF by Equation (3) (28).

$$WF = \frac{V}{At} \tag{3}$$

where V is the volume of water-permeated (L), A is the surface of the membrane (1.7x10^{-3} m^2), and t represents the sampling time (h). Compaction factor (CF) means the physical resistance of membranes, and we calculated that by dividing the initial WF$_i$ value by constant WF$_c$ value. We prepared the 0.5 g/L BSA solution with phosphate buffer (0.01 mol/L, pH:7.4) and filtered it at 200 kPa. We determined the concentration of BSA in collected permeate with a UV-Vis spectrophotometer and calculated the rejection performance with Equation (4).

$$R(\%) = \left(1 - \frac{C_p}{C_f}\right) x100 \tag{4}$$

Where C_p and C_f stand for the concentration of initial and final solutions of BSA, respectively.

Catalytic activity experiments

A previous study investigated the catalytic activities on benzyl alcohol conversion of TiO$_2$-PVDF samples in a batch reactor (29) We put 0.02 g of TiO$_2$-PVDF membrane pieces (1.5x1.5 cm) kept in benzyl alcohol for the diffusion of the substrate into a 50 mL reaction cup along with 1 mmol benzyl alcohol and 1 mmol H$_2$O$_2$. We then applied 500 W microwave radiation in a cooled microwave oven for 8 minutes. We analyzed a sample with GC, taken from the reaction mixture with a syringe (without any filtration). We washed the pieces of the membrane with acetone and used these samples three times. We performed the calculation of conversion and benzaldehyde selectivity of samples according to Equations 6 and 7. We used powder TiO$_2$ as a catalyst with the same reaction conditions. We filtered the sample taken from the powder catalyst with a syringe filter before injection to the column.

$$Conversion\% \, (C\%) = \frac{C_{alcohol(initial)} - C_{alcohol(final)}}{C_{alcohol\,(initial)}} x100$$

(6)

$$Selectivity\% \, (S\%) = \frac{Amount\ of\ benzaldhyde}{Amount\ of\ all\ products} x100 \tag{7}$$

RESULTS AND DISCUSSION

FTIR analysis of TiO$_2$ and TiO$_2$-PVDF membranes

We carried out FTIR analyses to understand the interaction of TiO$_2$ and the polymer chain. We have observed satisfactory evidence proving the blend of TiO$_2$ with a chain structure of PVDF in Figure 2.
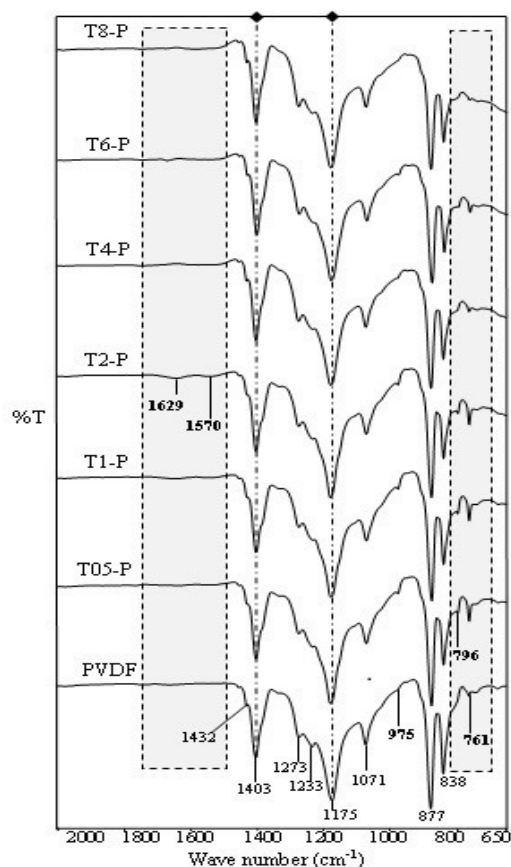
Figure 2: FT-IR spectra of TiO_2-PVDF samples.

α-phases of PVDF ascribed with 761, 796, and 975 cm^{-1} bands disappeared with increasing TiO_2 amount. This outcome indicated the formation of an amorphous structure with increased TiO_2 loading. We could see the superimposed FTIR bands of pristine and composites at the high wavenumber, but deviations emerged at the bands of 761, 976, and 1233 cm^{-1} due to increasing TiO_2. We observed characteristic CH_2 and CF_2 stretching vibrations at 1403, and 877-1175 cm^{-1}, respectively, and CF stretching bands of the PVDF chain at 1071 cm^{-1}. We obtained an essential indication for TiO_2 and PVDF interaction with the band at ca. 1570 cm^{-1} indicating the presence of OH groups on TiO_2 bonding with the PVDF chain (30-32). The band at 1629 cm^{-1} emerged due to the presence of the Ti-O-Ti structure and indicated possible hydrogen bonding between the oxygen of TiO_2 and fluorides of PVDF (23–25, 33). Figure 3 represents the possible bonding.
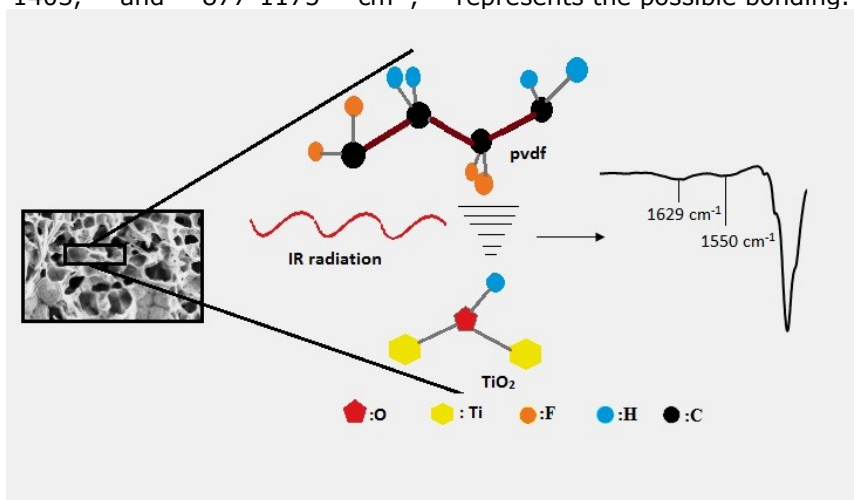
Figure 3: Estimated bonding of Ti-O-Ti with PVDF chain.

Morphology of composites

Figure 4 presents the cross-section SEM images of the composites to understand better the effect of TiO_2 addition on the structure of the membrane.
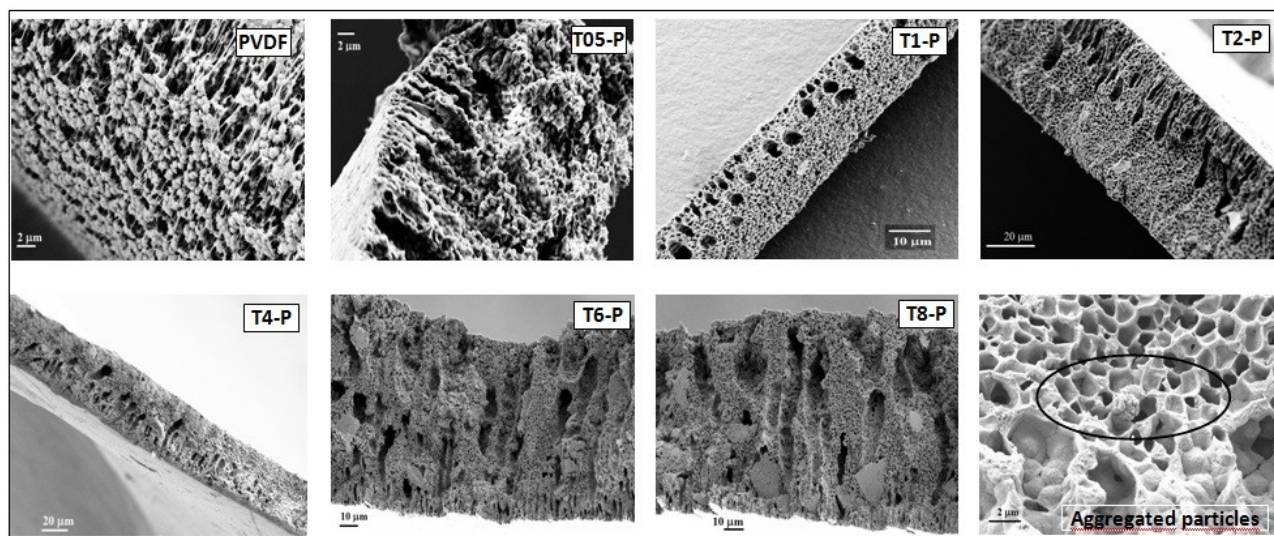
Figure 4: SEM images of TiO_2-PVDF samples.

Bare PVDF and additive contained samples showed different morphology as mainly symmetric and asymmetric forms. PVDF membrane structure, having large holes among the polymer clusters, became sponge-like as could be seen at the image of T1-P with macroscopic voids. We observed that, for T2-P and T4-P, the uniform membrane structure consisting of finger-like pores connected to the sponge side. In general, we obtained appropriately dispersed particles for the samples which have finger-like pores, despite the aggregated TiO_2 remnants available among the pores. We also observed a severe deviation from uniformity for samples containing additive amounts at 6% and further.

Furthermore, a high amount of additive pressed the micro-pores and channels, which determine the permeation and rejection performances of membranes. Additive, along with the solvent type, has a prominent effect on the formation of membrane structure such as a sponge, macro, and finger-like properties according to the affinity of water (11). Researchers reported the crystalline form of TiO_2 and DMF as a solvent to result in a thicker structure of hollow fiber membranes (34-35).

High water affinity of TiO_2 accelerated to mixing of coagulation water with polymer solvent (DMF) during phase inversion. Thus, the solvent separation occurred faster than additive-free samples. Due to the hydrophobic nature of PVDF, polymeric lumps formed containing large holes at the bare PVDF membrane. Structural changes observed by SEM images were compatible with the permeation results presented in Figure 6. With the further amount of TiO_2 deposition, convenient finger-like structures of membrane began to turn on dense sponge-like with large agglomeration. 4% TiO_2 amount was concluded as a maximum ratio to obtain sufficient filtration behavior.

Thermal analysis and thermal catalytic activity of composites

The thermal stability of samples was analyzed by TG and DTA to understand the effect of additive on to polymeric structure at the temperature ranges of 30-1000 ºC (Figure 5). Ash contents obtained for the PVDF and TiO_2 contained PVDF samples were different from the additive ratios.
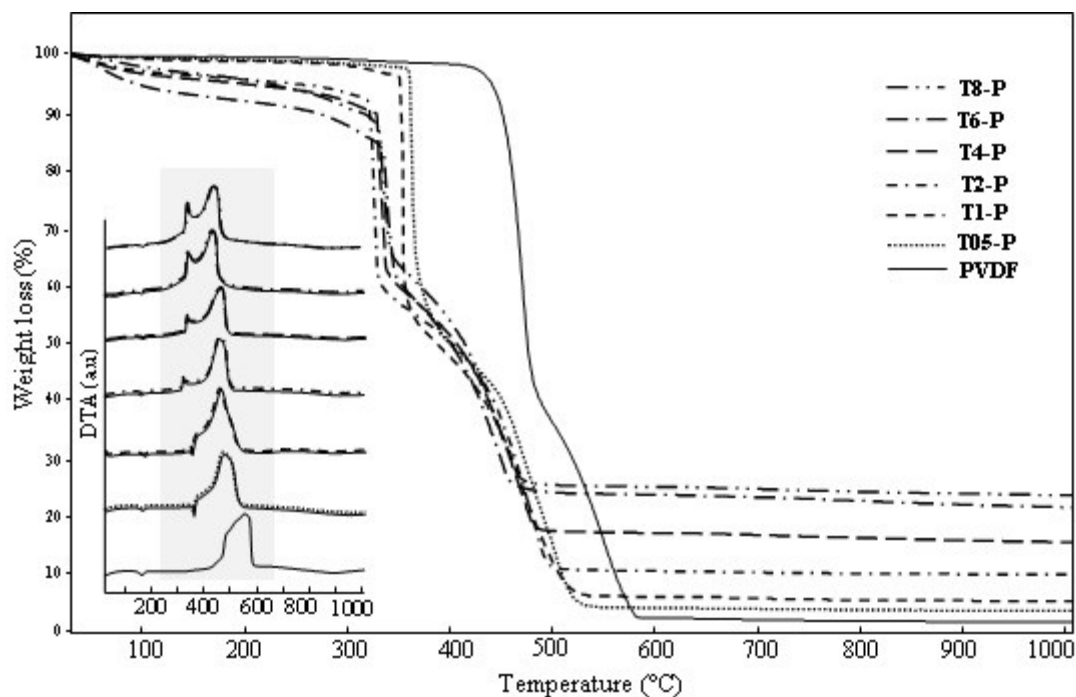
Figure 5: TG and DTA curves of TiO_2-PVDF samples.

During the calculation of the dope solution, only polymer and additives burned along with a small amount of solvent in the thermal analyzer. After the exposure of samples to heat, the remaining weight indicated to TiO_2 inside the membranes. The process burned all of the organic contents of the polymer. In Figure 5, we recorded that the degradation of neat PVDF started at 470 ºC, whereas the degradation temperatures of composites were between 348-339 ºC, which was a lower temperature than neat PVDF. We know that TiO_2 deposition onto polymeric support has

two opposite effects for the thermal behaviors of the samples. (1) TiO_2 may cover the surface of the polymer and improve the thermal resistance of the composite by protecting the polymer from direct exposing of oxygen or (2) thermal catalytic oxidation of TiO_2 may be dominant, and it may increase the thermal degradation of the organic structure due to thermal catalytic effect of TiO_2 at high temperatures (36). Patra and coworkers explained the increasing thermal resistance for the additive containing samples due to the escape of the radicals (20).

Table 1: Thermal behaviors of TiO_2-PVDF samples.

Sample	t_{onset} (ºC)	t_{max} (ºC)	t_{endset} (ºC)	$t_{\%10}$ (ºC)
PVDF	374	470	593	450
T05-P	351	362	546	361
T1-P	328	348	565	347
T2-P	328	348	541	347
T4-P	300	326	523	321
T6-P	312	332	503	320
T8-P	305	339	494	288

Rather than shielding, the catalytic thermal decomposition effect of additives dominated, and it decreased the on-set and max temperatures of TiO_2 contained samples (Table 1). Thermal degradation of samples accelerated, and end-set temperatures were recorded between 494-565 ºC, whereas it was 593 ºC for neat PVDF. Reduced degradation temperature values of additive containing samples are clear indicators for the thermal decomposition effect of TiO_2. Differential

thermal analysis (DTA) curves confirmed the results of the thermal degradation of the polymeric structure (Figure 5). Exothermic peaks of PVDF at 440-560 ºC emerged after the burning of organic components was observed at lower temperatures with increasing TiO_2 amounts. Besides, we observed for T4-P, T6-P, and T8-P one more exothermic peak, which represents degradation, and these were different from PVDF. An endothermic peak at around 170 ºC may

correspond to the melting point of polymer (37). Increasing of additive resulted in a decrease at the crystallinity of polymer, and TiO₂ clusters started to agglomerate differently. A disordered structure meant weakened binding of OH groups on TiO₂ with a PVDF chain pointed by FTIR results (see the section about FTIR). That induced the degradation effect of TiO₂ at high temperatures. We conducted the thermal analysis of samples to demonstrate the degradation effect of the additive at high heating rates: 5, 10, 15, 20, 30 ⁰C/minute, and activation energy of samples were calculated by Kissinger's method (38) according to TG results obtained under air. We calculated the activation

energies of degradation by linearization of β / T_{max}^2 versus $1/T_{max}$ according to Equation 1.

$$\left(\frac{\beta}{T_{max}^2} \right) = \left(\frac{AR}{E_a} \right) - \frac{E_a}{RT_{max}} \qquad (1)$$

Where β is the heating rate, A is a pre-exponential factor, E_a is the calculated activation energy for degradation of samples, and R is the universal gas constant. By using the maximum degradation occurred, we calculated the activation energy values. We presented T_{max} and E_a values calculated by the mentioned method in Table 2.

Table 2: T_{max} and degradation energy values of TiO₂-PVDF samples.

TiO₂content	β(°C/min)					
(Wt. %)	5	10	15	20	30	E_a (kJ/mol)
-	447	470	444	460	482	193.5
0.5	342	362	334	345	353	135.8
1	340	348	355	365	378	138.5
2	335	347	348	355	374	136.9
4	314	326	337	347	366	95.1
6	329	332	336	343	351	217.4
8	334	339	339	344	351	305.5

The E_a of PVDF was 193.5 kJ/mol, and it was observed as 136.9 and 95.1 kJ/mol for T2-P and T4-P respectively under the air atmosphere. Thermal properties of composites given in Table 1 and Figure 5 are compatible with the activation energy values. In general, activation energy decreased with an increased amount of additive, relatively with changes of T_{onset} and T_{max} values. E_a values provided clear information about how the additive rate affected the degradation process. To bare TG results, very little increase was observed for max decomposition as can be assumed negligible. But 217.4 and 305.5 kJ/mol E_a values recorded for T6-P and T8-P respectively indicated that further loading of additive prevented the

degradation of the polymer due to the shielding effect of agglomerated spherical TiO₂ clusters. The thermal stability of PVDF decreased because of the thermal oxidative effect of TiO₂ until the 4% TiO₂, which is the final sufficient composition as a filtration membrane. After that activation energy increased, the shielding effect became dominant when the 6% and further additive loadings. We have observed competition between shielding and thermal decomposition effects until the 6% additive.

Filtration Performances of composites
We tested the water flux (WF) of membranes cross-flow filtration system (see Figure 6).
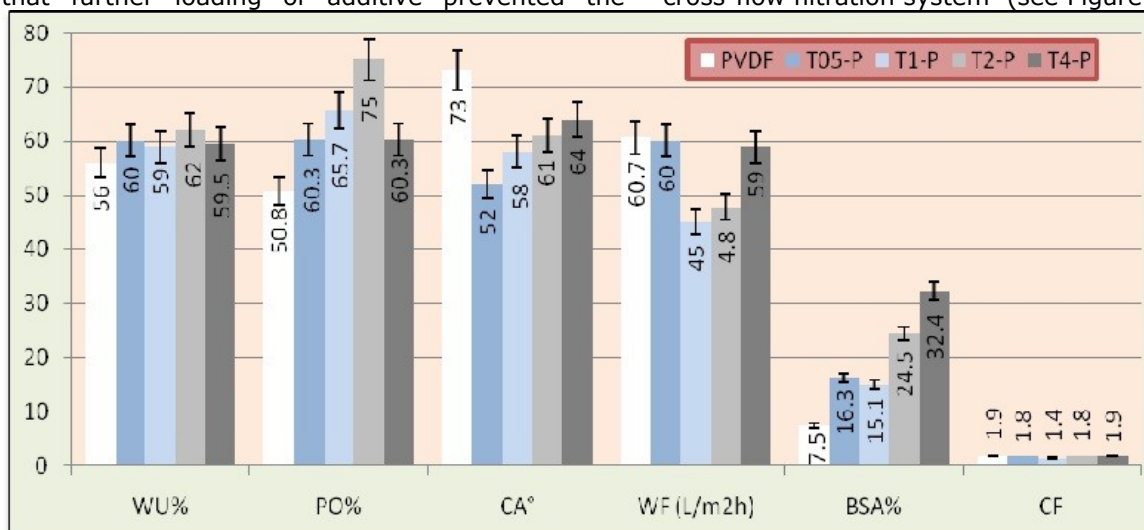
Figure 6: Filtration performances of PVDF and TiO₂-PVDF samples.

WF values decreased with increasing hydrophilic TiO_2 additive, which is controversial with the phenomena of the more hydrophilic, the more improved in water flux (39). Water permeation is commonly not only depending on hydrophilicity but also porosity, thickness, and water uptake (11). T2-P showed the highest porosity and water uptake for T2-P, which were 75% and 62%, respectively. Porosity and water uptake values of composite membranes were higher than that of the pristine PVDF membrane. As a controversy, water permeation of composites was lower than the PVDF membrane. In that case, we understood that the thickness and pore size determined the permeation performances of membranes. We saw the formation of a large number of small pores during phase separation. That was result of accelerated process induced by hydrophilic additives with increasing porosity. A relative change was observed among the porosity and water uptake values. Porosity and water uptake of PVDF were 50.8% and 56% respectively. High porosity with low water uptake of composites was also another proof for the existence of small pores. Effect of hydrophilic additive was confirmed by contact angle measurements. It is known that the smaller contact angle means the better hydrophilicity (40). Lower contact angle values were obtained for all samples compared with contact angles of PVDF (73º). Roughness of membrane surface suppressed the hydrophilic character of additive with further TiO_2 loading ratios. Due to agglomerated particles contact angles values were began to rise. Thick skin layer had a respectable effect on filtration performance and physical properties of membranes. Formation

of a thicker and stuck layer was induced by increasing viscosity of dope solution. That was confirmed by lower compaction values of membranes. Denser composition of composite membranes performed lower compaction values. We have concluded that the type and amount of additive have a considerable effect on the pores, voids, and channels of all structures with physical effects. BSA rejection performances were investigated to interpret structural characteristics of membranes. Dense structure of membrane provided an advantage as high rejection performance. 32.4% BSA rejection values was obtained for T4-P sample while it was only 7.5% for PVDF. Permeable polymeric lumps determined in SEM images of PVDF (Figure 4), provided low rejection although moderate flux. New structures formed by TiO_2 addition presented tightened skin which was beneficial for filtration and higher rejection. Generally it was known that particle accumulation on the surface occurs by electrostatic interaction between surface and particles (38). Flux recovery values a little reduced due to the interaction of BSA and surface groups. The roughness of the membrane promoted to an accumulation of rejected particles on the surface of the membrane.

The catalytic activity of composites
We have used a round-bottomed flask to test the catalytic activity of TiO_2-PVDF flat sheets on benzyl alcohol oxidation. We adjusted the alcohol and peroxide ratio to 1:1 with 0.06 g catalyst without solvent. We put the reaction flask cooled with a condenser into the microwave furnace. Table 3 shows the results of optimization experiments.

Table 3: Conversion and benzaldehyde selectivity of TiO_2-PVDF composite samples.

Entry	Catalyst (amount, g)	Temperature (ºC)	Reaction Time (min.)	Conversion-selectivity (%)
1	-	R.T.	240	-
2	-	80	240	-
3	-	80*	8	-
4	TiO_2 (0.06)	R.T.	240	-
5	TiO_2 (0.06)	80	240	26.2-33.5
6	TiO_2 (0.06)	80*	8	86.0-4.4
7	T2-P (0.06)	R.T.	240	-
8	T2-P (0.06)	80	240	25.5 - 45
9	T05-P (0.06)	80*	8	-
10	T1-P (0.06)	80*	8	53.15 -21.4
11	T2-P (0.06)	80*	8	67 - 29
12	T4-P (0.06)	80*	8	62.9-45.4
13	T6-P (0.06)	80*	8	66.6-16.2
14	T8-P (0.06)	80*	8	27-26
15	T4-P (0.02)	80*	8	70.7 - 36
16	T4-P (0.04)	80*	8	76.9 - 5.9
17	T4-P (0.08)	80*	8	54 - 20
18**0	T4-P (0.06)	80*	8	48.46 - 4.35
19**0.2	T4-P (0.06)	80*	8	59.4 - 34.3
20**0.4	T4-P (0.06)	80*	8	62.5 - 48

21**0.6	T4-P (0.06)	80*	8	56.37- 56
22**0.8	T4-P (0.06)	80*	8	56.5 - 56
23**1	T4-P (0.06)	80*	8	73 - 38.7
24**1.5	T4-P (0.06)	80*	8	93.86 - 5.84
25-300W	T4-P (0.06)	80*	8	76 - 17
26-700W	T4-P (0.06)	80*	8	70 - 36
2. reuse	T4-P (0.06)	80*	8	63.4 - 74
3. reuse	T4-P (0.06)	80*	8	80 - 37
4. reuse	T4-P (0.06)	80*	8	59 - 35

We carried out the reaction in a 50 mL flask with a condenser in a 500 W microwave, with 1 mmol H_2O_2 and 1 mmol benzyl alcohol ratio unless otherwise stated. We introduced the mixture to an HP-5 column for GC analysis.
*: in microwave radiation.
**: H_2O_2: Benzyl alcohol ratio (mol)

We investigated the effects of various parameters on the reaction yield. A high conversion with high selectivity was a considerable result. A satisfactory result was obtained with 0.06 g T4-P sample. That was directly related to easy transferring of substrate to active sites of catalyst. Structural properties such as pore distribution, pore radius, cross-section thickness, and the additive were essential determiners of substrate flow and, consequently, the reaction yield. Moderate pores provided easy transferring of substrate conversely, pressed structure complicated transportation. As could be seen, T4-P has a more hydrophobic character inferred from its high contact angle and low water uptake capacities. That makes it more attractive to organic substrate. The more organic substrate diffused inside the active sites give the more selectivity. On the other hand, selectivity performance of T4-P may due to its prevention effect of over peroxide transferring due to its hydrophobicity. Experiments conducted to understand peroxide ratio at the reaction yield supported this estimation. High conversion but low selectivity was obtained with increased peroxide ratio. That meant that benzyl alcohol was converted into other oxidation products. Changes of peroxide alcohol ratio may resulted in different products (39). 0.4 (H_2O_2: benzyl alcohol molar ratio) was considered as optimum peroxide amount. The results of 0 and 0.2 ratios specified stoichiometric peroxide shortage. Reason for low selectivity, although increased peroxide ratio may be decomposition or transferring problem of peroxide (29). The catalyst amount had a direct effect on the product type. Highest conversion was obtained when the TiO_2-PVDF was 0.04 g but low selectivity. It decreased when it was further than 0.06 g. Increase at the conversion was related with high amount of catalysis. However, the exact reason may be over-oxidation of benzaldehyde or direct conversion of benzoic acid to its by-products (see Figure 7) (41).
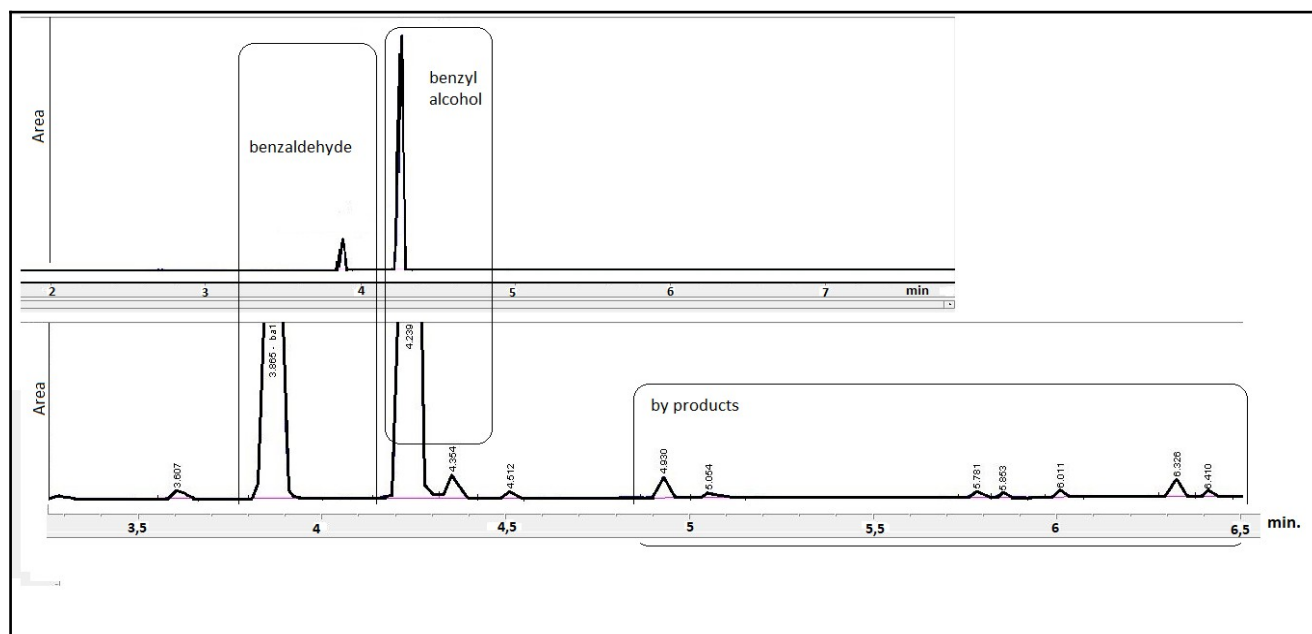
Figure 7: GC peaks of benzaldehyde and other products.

In this study, we saw that complex peroxy-metal structures formed as a result of the interaction of hydrogen peroxide and metals on the catalyst (Figure 1). During the oxidation, peroxide decomposed quickly in the presence of a high amount of catalyst to give active hydroxyl radicals. Even though a high number of hydroxyl radicals desired to the conversion of a substrate, the formation of active reactants quickly may cause over oxidation of reactant, consequently low selectivity. We did not observe notable results with different microwave radiation. (see Table 3). We separated TiO_2-PVDF pieces from the mixture and washed with acetone, then reused three times. Washing with organic solvent wiped off all organic residues and opened the channels. However, the structure gets shrunk with further reuse due to microwave radiation. Benzyl alcohol conversion and benzaldehyde selectivity decreased gradually, with ongoing usage. TiO_2 embedded PVDF flat sheets performed moderate yield in addition to easy usage, low cost, and fast results at solvent-free area. The advantages of easy separation from the reaction mixture without solvent and reusability were considered remarkable output for oxidation reactions.

CONCLUSION

We combined the well-known advantages of TiO_2 additive on PVDF membranes to obtain sufficient, fast, and environmentally friendly chemical production systems with microwave radiation.TiO_2 provided improvement filtration and better BSA rejection by increasing hydrophilicity of the polymeric matrix for almost all membranes. 4% TiO_2 contained samples presented 32.4% BSA rejection while it was only 7.5% for PVDF. TiO_2 interaction with PVDF ascribed by the FTIR bands of Ti-OH and Ti-O-Ti vibrations.

We found a clear insight by the thermal behaviors of TiO_2 on the membrane structure, which are not available in the primary thermal analysis results. Activation energies belong to polymeric decomposition were recorded at different heating rates. Unlike preventing effect, TiO_2 induced thermal decomposition of the polymer due to its catalytic activity, especially at high temperatures.

TiO_2-PVDF membrane pieces were used as a microwave-induced catalyst to obtain benzaldehyde from benzyl alcohol in a batch reactor. 62.9% conversion and 45.4% selectivity with the T4-P sample. We investigated the possibility of faster and cleaner in-situ production of fine chemicals on the TiO_2-catalyzed filtration system combined with microwave radiation. TiO_2-added PVDF self-recyclable membranes have a high potential to the production of chemicals on

filtration systems with microwave energy, besides their well known hydrophilic, antifouling, and high rejection properties.

Availability of the data

The processed data required to reproduce these findings are available to download from [https://data.mendeley.com/datasets/w2678cnzsg/1].

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RESEARCH ARTICLE

Poly(o-aminobenzyl alcohol) Films with and without Organic Compound on AISI 316 Surface; Synthesis and the Corrosion Performances

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Abstract: In this work, we obtained the anticorrosive properties of polymer films (SS/PABA and SS/PABA-ORG) synthesized on stainless steel surface by adding an organic substance to aniline derived *o*-aminobenzyl alcohol monomer synthesis medium. Firstly, we prepared the polymer coating bath by dissolving 0.15 M *o*-aminobenzyl alcohol monomer in the electrolyte solvent containing acetonitrile and 0.15 M $LiClO_4$. From this bath, poly (*o*-aminobenzyl alcohol) (PABA) film was synthesized in 30 segments by cyclic voltammetry (CV) technique at a scanning rate of 50 mV/s at a potential range of -0.20/1.80 V on the AISI 316 (SS) working electrode in contrast to the platinum electrode. For the synthesis of organic structure doped polymer film (PABA-ORG), we repeated the same synthesis process by dissolving $C_{21}H_{27}NO_{2(k)}$ (ORG) at low concentration in the same bath. We investigated the corrosion performances of bare SS, SS/PABA, and SS/PABA-ORG substrates, using open circuit potential – time, anodic polarization, and AC impedance techniques in the solution of corrosion study. As a result, we observed that the organic additive added to the synthesis medium caused changes in the synthesis behavior of PABA. Corrosion performance tests showed that PABA and PABA-ORG films increased the corrosion protection performance of the SS electrode, and we observed a reduction of the corrosion rate of the SS electrode.

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INTRODUCTION

Corrosion is the physical and chemical erosion of metals and a momentous event that can cause serious harm to humanity despite today's developing technology. This phenomenon is because metals generally undergo electrochemical and chemical reactions within the atmosphere due to environmental factors. So the materials might receive severe damage. To prevent these damages and losses, researchers have been developing various methods and materials for years. Stainless steel, which is one of these materials, is resistant to many corrosive environments with its quality and

stable oxide layers formed by chromium and nickel metals in its compositions. However, it can corrode faster than expected in environments containing chloride and sulfate (1-3). AISI 316 series stainless sheets of steel, which are frequently preferred by the industry due to their cheapness, easy availability, and machinability, also have the disadvantage mentioned in such environments. Although there are many methods used to prevent this and increase the protective effect of stainless steel, conductive polymer coatings have received much attention in the literature recently (4-10). In particular, polyaniline and its derivatives are conductive polymers that are frequently studied and

give fruitful results. The main characteristics of conductive polymers are their high conductivity, corrosion-resistant, and non-corrosive enough to be brought to the metal level.

Furthermore, their electrochemical coating on technical metals results in meager costs, under constant potential, constant current, and cyclic voltammetry. Besides, one of the commonly applied techniques in recent years is cyclic voltammetry. In this technique, after the monomer oxidation, the polymer film is converted into induction and oxidation forms during synthesis. After the monomer oxidation, there is a conversion of the polymer film into induction and oxidation forms during synthesis. As known, once most inorganic inhibitors are harmful to the environment, organic inhibitors containing amine groups are useful to prevent corrosion. Amine-based inhibitors compose a thin adsorbed layer on the substrate, and this layer has a polymeric structure and is extremely protective against corrosion (11-13). The research made in the literature shows that the organic structure can add to the matrix of conductive polymer films (14). This study aim is to investigate the anti-corrosive properties of poly (o-aminobenzyl alcohol) synthesized from an aniline derived monomer o-aminobenzyl alcohol on AISI 316 stainless steel. Also, we synthesized the polymer coating (PABA-ORG) obtained in a mixture solution including o-aminobenzyl alcohol (ABA) and 2,6-di-*tert*-butyl-4-{(*E*)-[(4-hydroxyphenyl)imino]methyl}phenol (M$_w$: 325.45) (ORG) compound having varying degree of hydrophobic chain length and the steric environment on AISI 316 stainless steel (SS) in LiClO$_4$ + acetonitrile medium. We explored the

corrosion performances of PABA coatings synthesized without and with 0.50 mM ORG compound in a 3.5 % NaCl solution. We evaluated the electrochemical measurement techniques of PABA polymer films with and without ORG compound obtained on SS (SS/PABA and SS/PABA-ORG) and bare SS.

MATERIALS AND METHODS

All electrochemical experiments were carried out with three-electrode in single-cell technique on CH Instruments electrochemical workstation. In the experiment cells, Ag/AgCl (3M KCl) and square platinum plate with a surface area of 0.25 cm^2 electrodes were reference and counter electrodes, respectively. AISI 316 electrode with a 0.50 cm^2 surface was the working electrode. Shortly before use, the SS electrode surface was carefully polished with 400 and 1200 grid abrasive paper, respectively, and washed with acetone+ethanol (1:1) mixture and bi-distilled water in this order. For the poly (o-aminobenzyl alcohol) (PABA) coating, we dissolved 0.15 M o-aminobenzyl alcohol monomer in acetonitrile (ACN) containing 0.15 M lithium perchlorate. Electropolymerization was carried out by cyclic voltammetry (CV) technique in 30 segments between -0.20 and 1.80 V at a scan rate of 50 mV s^{-1}. The fitting curves in the Nyquist and Bode diagrams in Figure 5c were analyzed using the ZView2 software. The structure of the organic compound named 0.50 mM 2,6-di-*tert*-butyl-4-{(*E*)-[(4-hydroxyphenyl)imino]methyl}phenol (M$_w$: 325.45) joined to the PABA polymer film is given in Figure 1. The electropolymerization of PABA polymer film containing an organic compound (PABA-ORG) we applied the same process as that of PABA polymer film.
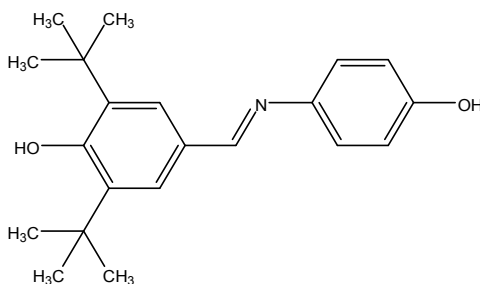
Figure 1: Chemical structure of the organic compound.

RESULTS AND DISCUSSION

Synthesis of the Polymeric Film

In this study, we synthesized PABA film in 0.15 M o-aminobenzyl alcohol (ABA) monomer dissolved in the solution of LiClO$_4$ in ACN by CV technique in one step. We coated Poly (o-aminobenzyl alcohol) (PABA) homopolymer films with and without organic compound (ORG) of 0.50 mM concentration on the

surface of the SS electrode using cyclic voltammetry at a scan rate of 50 mV/s. Figure 2 shows the film growth curves observed for SS in 0.15 M ABA (a) and 0.15 M ABA + 0.50 mM organic compound (ABA-ORG) (b) containing 0.15 M LiClO$_4$ + ACN solution. Film growth curves of electrodes obtained in both ORG compound-containing and ORG compound-free PABA medium, which are referred to here as SS/PABA-ORG and SS/PABA respectively,

showed in Figure 2. We obtained the works at a scan rate of 50 mV.s^{-1}.

We carried out 30 segments for the synthesis of PABA or PABA-ORG coating on the SS electrode. Both coated electrodes exhibited entirely different electrochemical behavior in CV curves. The anodic current values increased after the potential value of 0.89 V during the first cycle in ABA monomer containing LiClO$_4$ + ACN solution, while we observed them after the potential value of 0.88 V for LiClO$_4$ + ACN + ABA monomer mixture with the organic compound. The 8.84x10^{-4}, a peak current value obtained for the ABA monomer medium, was related to the monomer oxidation of o-aminobenzyl alcohol on the SS electrode. The monomer oxidation process in ABA and ORG compound mixture moved to previous potential value, while its current intensities were incredibly high as 1.12x10^{-3} A than that the presence of only ABA. This case clarifies itself for being a better conductive medium in the presence of ORG compound concerning only the presence of ABA. It showed that the addition of ORG

compound to ABA solution provided an increase in the electropolymerization rate.

Furthermore, because it was not observed an additional oxidation peak in cyclic voltammogram (Figure 2b), we concluded that the organic compound is of high purity. Also, we did not observe the dissolution of the SS electrode for both coated electrodes. On the other hand, the peak current increases obtained for monomer oxidation of coated SS electrodes were significantly high in the first anodic cycles, while their values decreased with increasing cycle numbers. These behaviors of SS electrode in LiClO$_4$ and ACN medium containing ABA with and without ORG compound could be explained by the lower conductive surface of synthesized PABA film, for the SS electrode surface. Moreover, monomer oxidation peak intensities recorded for ABA + ORG compound mixture were comparatively higher when compared with that of only the ABA medium. After the growth of the polymer film, the process covered the SS electrode surface, a green uniform film for both o-aminobenzyl alcohol conditions with and without ORG compound.
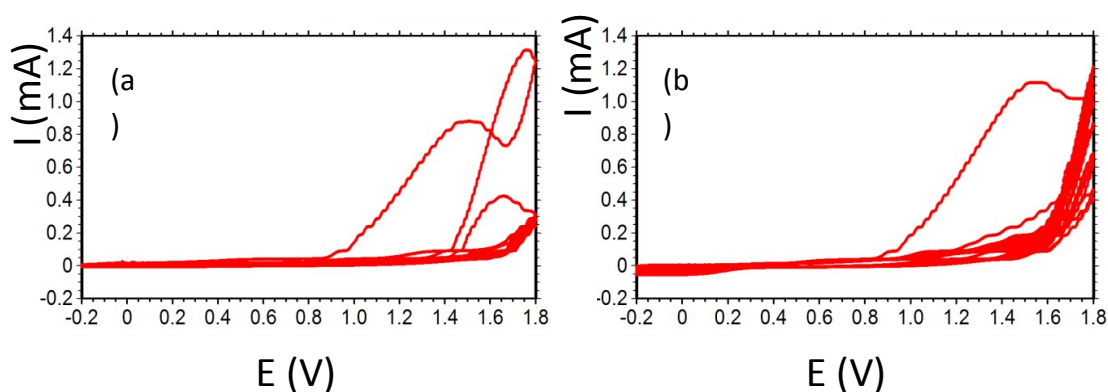
Figure 2: CVs of SS/PABA (a) and SS/PABA-ORG (b) homopolymer film synthesis.

Corrosion protection abilities of coated electrodes
The anticorrosive properties of the PABA and PABA-ORG coated SS electrodes in 3.5 % NaCl were evaluated with EIS, E$_{ocp}$-time plots, and anodic polarization plot.

We plotted the E$_{ocp}$ values of SS, SS/PABA, and SS/PABA-ORG electrodes against the exposure time at the first half-hour and different exposure times (see Figure 3). Initially, the E$_{ocp}$ measurement of bare SS metal was -0.234 V, while these values recorded for SS/PABA and SS/PABA-ORG electrodes in 3.5 % NaCl electrolyte solution were 0.140 V and 0.070 V, respectively. The E$_{ocp}$ values obtained for uncoated and deposited electrodes shifted to an anodic direction in time. Yet, the E$_{ocp}$ measurements of the SS/PABA-ORG electrode raced to the cathodic direction for a short time after 1654 s of exposure

time. We reported these values of SS/PABA to shift to the positive domain to 0.165 V. Afterwards, according to our observations, the E$_{ocp}$ values of SS/PABA-ORG electrode shifted to the anodic domain from 0.053 to 0.206 V, between 2 and 168 h of exposure time (see Figure 3b). These values recorded for SS/PABA electrode remained constant close to the same potential value for 168 min.

Consequently, we noticed that the SS/PABA-ORG substrate had significantly positive potential region comparison with the only PABA deposited SS metal. We attributed these positive measurements obtained for the SS/PABA-ORG electrode to the formation of, especially nickel and chromium oxide layers, which built up with an extended immersion period, at metal/polymer interface. On the other hand, the E$_{ocp}$ measurements of bare SS metal were carried to the cathodic region from -0.076 to -0.124

V, between 2 and 168 h of immersion period. This case indicated that chloride ions increased the active dissolution of SS the underlying chromium

and nickel oxide layer utilizing its aggressive property and small ion diameter.
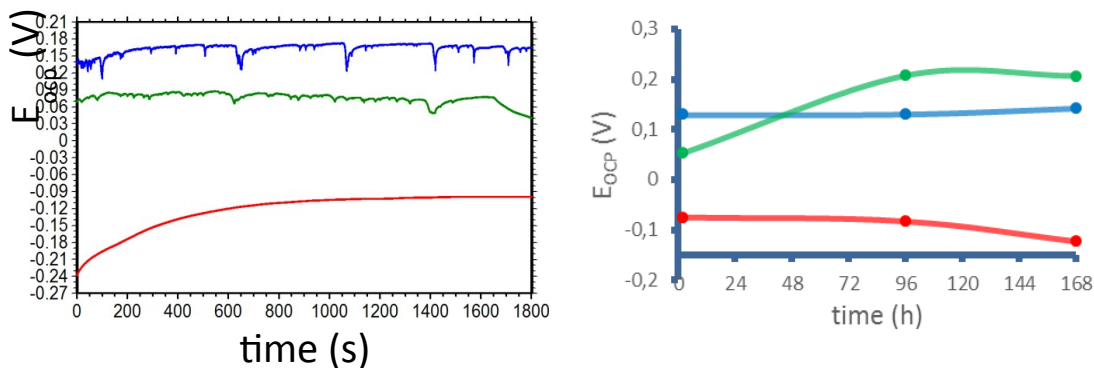
Figure 3: Open circuit potential-time plots of **SS**, **SS/PABA,** and **SS/PABA-ORG** electrodes at first half-hour (a) and different immersion times (b).

Figure 4 compares the anodic polarization plots of SS, SS/PABA, and SS/PABA-ORG substrates after 168 h of exposure period in the solution of corrosion. In the presence of bare SS, we determined the corrosion potential value (E_{corr}) as – 0.130 V. Besides, the current values obtained for bare SS were highest when compared with those of coated SS electrodes. This case showed active dissolved of stainless steel in the wider potential region, while current values of the SS electrode remained almost constant in the high potential domain, owing to the formation of chromium and nickel oxide layers. On the other hand, the corrosion potential values of SS/PABA and SS/PABA-ORG electrodes were anodic direction when compared with that of the bare SS electrode. Therefore, this result implied conversion of SS electrode from

anodic dissolution to chromium and nickel oxide layers under the catalyzing effect of polymer film after the corrosive types reached to the SS. The E_{corr} values of polymer film coated electrodes were high due to oxide layers formation and reduction of PABA film. As a result, stabilities of PABA and PABA-ORG coatings were high while permeability was low in aggressive medium and ensured important anodic protection on SS in extended time. Also, the current values of SS/PABA and SS/PABA-ORG electrodes were significantly lower than that of the bare SS electrode. This case showed the effective barrier properties of polymer coatings on the SS electrode. The lowest current values recorded for PABA-ORG coating indicated that ORG compound addition to poly(o-aminobenzyl alcohol) structure exhibited an excellent barrier property on the SS surface.
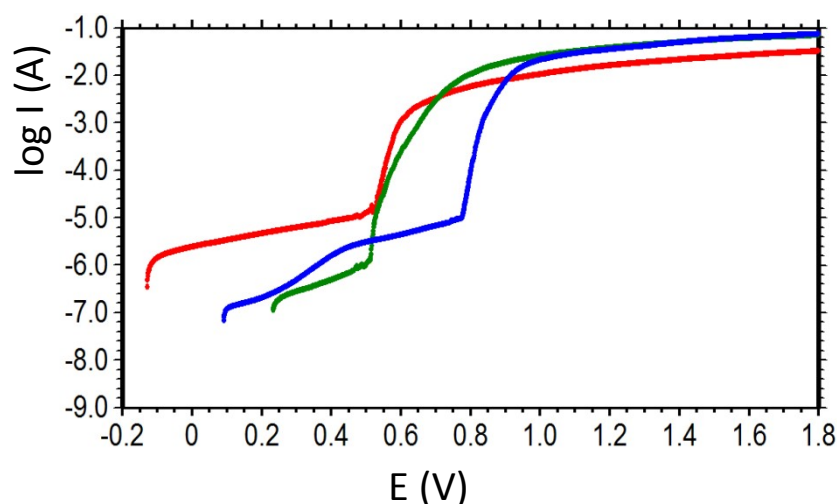
Figure 4: Anodic polarization plots of **SS**, **SS/PABA**, **SS/PABA-ORG** electrodes, after 168h of exposure time.

The Nyquist diagrams obtained for uncoated SS and PABA coated SS (SS/PABA), and PABA-ORG coated

SS are present in Figure 5 for 2, 96, and 168 h of immersion times in the solution of corrosion. There

was a depressed semicircle for metal corrosion at the high-frequency region for bare and deposited electrodes. In Nyquist curves, a one-time constant defined as depressed semicircle at the high-frequency region gave the polarization resistance (R_p). The R_p value obtained for the bare SS electrode was the sum of the resistance of oxide layers (R_o) and the charge transfer resistance (R_{ct}) corresponding to the metal/solution interface formed at the bottom of the pores. In contrast, the fact that almost all curves obtained for coated electrodes were composed of a depressed semicircle gives the R_p value, including the R_o, the R_{ct}, and the polymer film resistance (R_{pf}). Besides, the linear region at the low-frequency side of the Nyquist plots recorded for all of them denoted the presence of Warburg impedance (Z_w) showing a noticeable formation of layers, after 2 h of immersion time in Figure 5a. Warburg impedance assumes the diffusion resistance consisting of corrosion types of the substrate because of being at approx. 45° of angle, in phase angle, ($^-$)-log f plots (14-18). The observation of the linear portion at the low-frequency district showed that the coating had active barrier property.

Consequently, we observed a typical charge transfer process occurring under diffusion control as a linear part. As a result, ion diffusion processes taking place through the pores of the coating and oxide layers towards the underlying metal surface at a metal/solution interface denoted the presence of Warburg impedance. We observed that the bare SS electrode had important anti-corrosion properties due to the formation of stable chromium and nickel oxide layers, after 2 h of the exposure period. In the case of uncoated SS and coated electrodes, there was still a linear portion defined as the Warburg impedance at the lowest frequency domain, which indicated the active protective properties of the oxide and polymer films on surfaces, after 96 h. Nevertheless, we noticed on phase angle-logf plot that the $^-$ values of coated electrodes obtained a maximal value at approx. 60° (Figure 5b). Higher $^-$ values of PABA and PABA-ORG polymer film coated SS electrodes attributed to limited corrosive products diffusion process taking place through the

pores of the coating and the formation of new oxide films towards the underlying SS surface at metal/solution interface (14-18). In Figure 5c, the incremental R_p value of depressed semicircle obtained polymer-coated both electrodes at the high frequency indicated the growth of the passive films consisted of nickel and chromium oxides, metal/polymer interface. In the presence of SS/PABA and SS/PABA-ORG electrodes, the one-time constant at high frequency composed of the total resistance of R_{ct} value corresponding to the dissolution of metal at the bottom of the pores, R_{pf} value and R_o value. The linear portion at the low-frequency region of the Nyquist curves denoted the presence of Warburg impedance, pointing out an unusual formation of films (14-18). It is also well known that Warburg coefficient (σ) values give an idea about the diffusability of ion through the polymer film and oxide layers or only the former. We investigated the diffusive properties of polymer films deposited on the SS surface and calculated the Warburg coefficients with details given in the literature (17-18) and (see Table 1). The σ value of SS/PABA and SS/PABA-ORG electrodes was comparatively higher when compared with that of uncoated SS substrate, after 168 h of immersion period (Table 1). We obtained this highest σ value obtained for SS/PABA-ORG electrode by investigation of the $^-$-logf plot. In $^-$-log f plot, the peak representing polarization resistance expanding between mid and low frequencies in the curve of SS/PABA-ORG electrode indicated the highest phase angle than those of SS/PABA and bare SS electrodes (Figure 5B(c)). So, it was evident that on SS electrode coated with PABA polymer in the organic compound medium had lower porosity structure and number (or only the former), when compared with that of only PABA polymer. As a result, these low porosity properties allowed the limited ion diffusion from the pores of the coating on the metal surface. So, organic compound situated in polymer film structure reduced more the porosity structure of SS electrode by more catalyzing the formation of oxide layers on the SS surface with time. Consequently, the more formation of nickel and chromium oxide layers on the SS surface inhibited mainly the dissolution of stainless steel metal.

Table 1: Corrosion performance values of coated and uncoated SS electrodes, after 168 h period of immersion.

Electrode	E_{OCP} (mV)	R_P (kΩ)	σ (Ω.s$^{-\frac{1}{2}}$)
SS	-124	5.08	2200.79
SS/PABA	148	30.80	10484.84
SS/PABA-ORG	206	31.16	12576.56

CONCLUSIONS

In this study, we have synthesized PABA film with and without organic compound on the SS surface by cycling the potential in $LiClO_4$ + acetonitrile solution.

The cyclic voltammetric curves demonstrated that PABA coatings synthesized with and without organic compound medium had different morphologic structure and different potential values of monomer oxidation. We investigated the corrosion

performances of SS/PABA and SS/PABA-ORG electrodes in a 3.5 % NaCl solution. It was evaluated with E_{ocp}-time curves, anodic polarization curves, and the AC impedance diagrams and of PABA polymer films synthesized with and without ORG compound obtained on SS surface. This study clearly showed that all the PABA-ORG coating provided valuable protective property against corrosive types such as aggressive chloride ions.

The Nyquist diagrams revealed that PABA-ORG film exhibited significant catalytic efficiency on the formation of protective oxide layers on the SS surface when compared with only PABA film. Consequently, PABA-ORG coating was low permeable and high stability under aggressive chloride medium and ensured exceptional anodic protection on SS over a longer time.
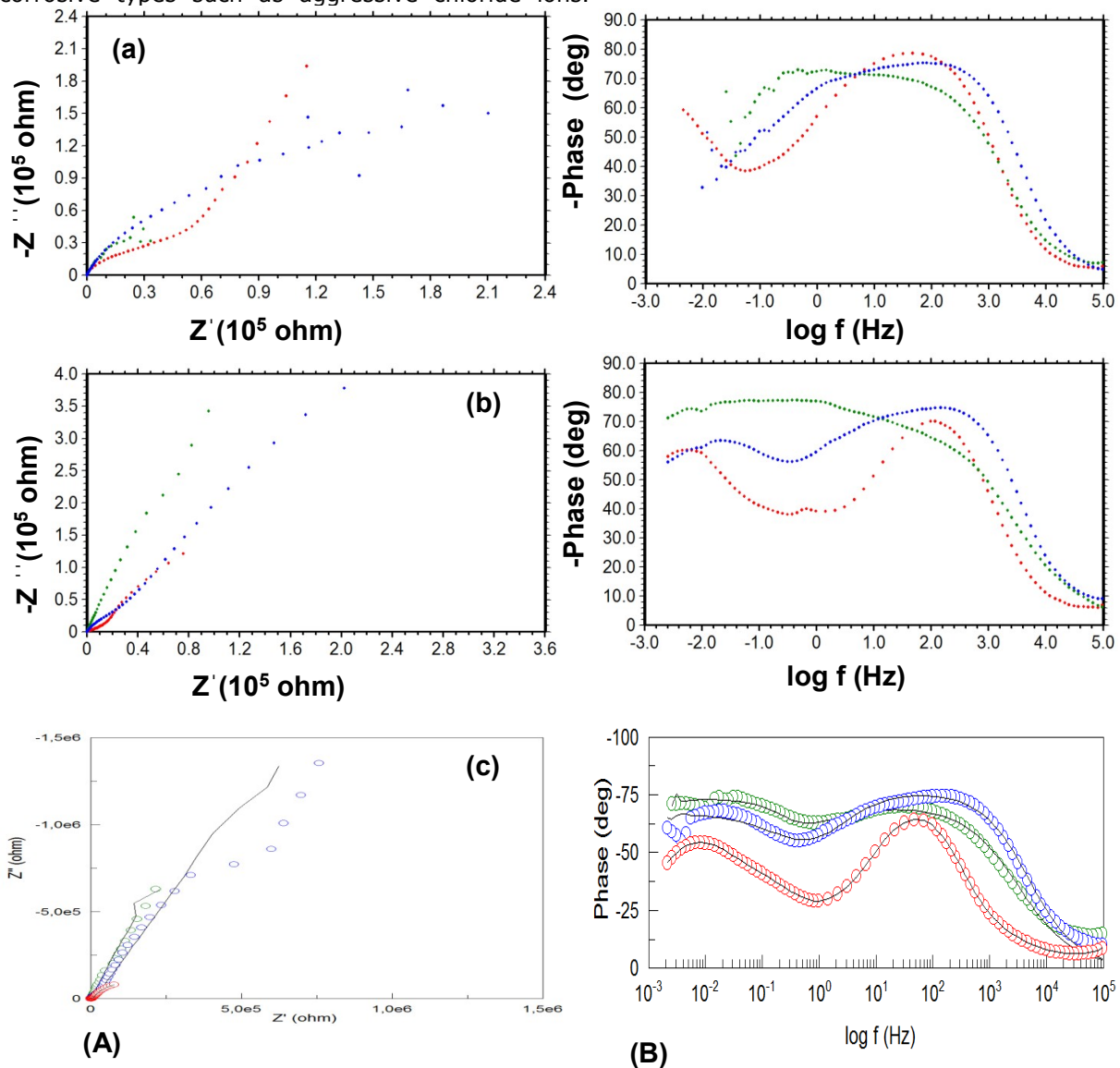
Figure 5: Nyquist (A) and Bode (B) curves recorded for **SS**, **SS/PABA** and **SS/PABA-ORG** electrodes after 2 (a), 96 (b) and 168 (c) h of exposure time in 3.5 % NaCl solution.

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Identifying the Novel Pyrimidine-Based CDK2 Inhibitors as Anticancer Agents Using Text-Mining and Combined Molecular Modeling Approaches

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Abstract: The cycline-dependent kinase (CDK) protein is a vital target used in anti-cancer drug designing studies. Many FDA-approved drugs, including anti-cancer drugs, use pyrimidine as a crucial fragment. In the current study, a small molecule database (Specs SC) that text mining studies include more than 210.000 compounds, and we filtered 6668 molecules that carry "pyrimidine" fragments. We then screened these compounds at the binding pocket of CDK-2 target using molecular docking and molecular dynamics (MD) simulations approaches. Besides, we compared the binding free energies of screened compounds with pyrimidine-based FDA-approved anti-cancer drug Abemaciclib which targets CDK. Based on the comparison of docking scores of screened compounds, we used top-7 hits in 100 ns MD simulations. We also applied the same MD simulations protocol (100 ns) to the Abemaciclib-bound CDK-2 complex structure. We then calculated the average Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) energies. Our MM/GBSA results showed that 6 out of 7 compounds have better MM/GBSA scores than FDA-approved compound Abemaciclib. Thus, together with a combination of text mining and integrated molecular modeling approaches, we identified novel pyrimidine-based hits against CDK.

INTRODUCTION

Cycline-dependent kinases (CDKs) are the catalytic subunits of a large family of serine/threonine protein kinases, having crucial roles in the regulation of cell cycle progression. There are two main groups in the family: Cell cycle CDKs and transcriptional CDKs (1). We often observe the over-expression of CDKs in many cancer types; thus, their inhibition may lead to cell cycle arrest or apoptosis (2). Although the human genome encodes 21 CDKs, researchers have shown that only seven (CDK1-

4, 6, 10, 11) of them indeed play a direct role in the progression of the cell cycle. Thus, targeting the CDK enzyme function is a rational approach to prevent unregulated cancer cell proliferation, and the authors considered these targets in many anti-cancer drug design studies (3, 4). Pyrimidine derivatives have attracted considerable interest in recent years in diverse biological problems (5, 6). Experts use pyrimidine derivatives in many different biological applications, including anti-tumor (7), antiviral (7, 8), antioxidant (9), antifungal (10), and hepatoprotective (11) roles. FDA-approved drug Brigatinib (marketed as Alunbrig) is a pyrimidine-based small-molecule used in targeted cancer therapy (12). Another FDA-approved drug Abemaciclib (commercial terms Verzenio and Verzenios) specifically targets CDK. Physicians mainly use them in advanced or metastatic breast cancer treatment. However, it has some adverse side effects such as diarrhea, nausea, vomiting, leukopenia (i.e., low white blood cell count) including neutropenia, anemia, thrombocytopenia (i.e., low platelet count), stomach pain, fatigue, decreased appetite, and headache (13, 14). Therefore, in the current study, pyrimidine-based new lead compounds targeting CDK were investigated using integrated text-mining and molecular modeling approaches. For this aim, we used MarvinSketch software (15) in 210,596 molecules obtained from Specs-SC drug-like small molecule database in an IUPAC text file format. We then screened this text file by a Python-based text mining in-house script and identified 6668 molecules as compounds that include the "pyrimidine" phrase in their text files. Moreover, we used these filtered compounds at the active site of the CDK-2 enzyme, and screened compounds were ranked based on their interaction energy scores. We used top-7 compounds that show better docking scores than FDA-approved drug Abemaciclib in molecular dynamics (MD) simulations. Trajectory frames collected throughout the MD simulations were used in Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) calculations and based on average MM/GBSA scores, we identified novel 6 pyrimidine-based small molecules that have better MM/GBSA scores than Abemaciclib (Table 1).

MATERIALS AND METHODS

Data Collection

Text mining helps find significant molecules by quickly screening them from large databases using keywords (16). In this study, 210,596 molecules obtained from Specs-SC were prepared in the IUPAC text file format using MarvinSketch software. We then screened this text file to find pyrimidine-based molecules using a Python-based text mining and obtained 6,668 molecules.

Ligand Preparation

Ligand preparation consists of the analysis of a 3D structure of a ligand, generation, and optimization steps. We have prepared 6,668 compounds using the OPLS-2005 forcefield (17) LigPrep module (18) of the Maestro Molecular Modeling Suit. A problem to be addressed is the ionization of the ligand in physiological conditions. We have used the Epik module (18) at the physiological pH of 7.4 for potential ionization states. We also produced all possible stereoisomers and tautomers and considered 32 structures per ligand.

Protein Preparation

We obtained the CDK-2 target from the protein data bank (PDB), and a 2XMY PDB-coded protein structure was used (19). We fixed the missing side chains, backbone atoms, and loops with Prime (20). For protonation states, structural optimization and minimization, the PROPKA and OPLS-2005 force field were used, respectively (21). We considered water molecules around 5.0 Å of the co-crystallized ligand in the docking simulations.

Molecular Docking Simulations

Molecular docking studies investigate interactions occurring in protein-protein or ligand-protein complexes and rank candidate poses according to predicted affinity scoring functions (22). Docking processes predict ligands to bind with the most appropriate conformation in the binding pocket of the target protein using different algorithms. We used Glide to carry out the molecular docking between the investigated ligands and CDK-2 protein to obtain the binding poses as well as docking scores of the studied ligands (23). The conformations obtained during the docking were ranked using the Glide score function (24). Residues set the binding pocket within 10 Å vicinity of a co-crystallized ligand. All ligands were initially docked into the binding pocket of CDK using a grid-based docking program Glide standard precision (SP) of Maestro Molecular Modeling pocket (20, 23) and requested 10 docking poses for each ligand (25, 26).

Molecular Dynamics (MD) Simulations

There is evidence that some systems require MD simulations to discover proper binding fit (27). Long MD simulations can find more and energetically favorable configurations. MD simulations were implemented up to 100 ns using Desmond V.4.9 to investigate the conformational stability of the complexes of selected hit molecules with CDK-2 (28). We solvated the complex structures in the orthorhombic simple point charge (SPC) water model (29).

Moreover, we neutralized the systems with counter ions (0.15 M NaCl solution). We set the system as Lennard-Jones interactions cutoff of 10 Å on periodic boundary conditions (23). We used a 2.0 fs time step in the integration steps.

We have used Nose-Hoover thermostat (30) and Martyna-Tobias Klein protocols (31) to control the temperature and pressure of the systems at 310 K and 1 bar, respectively.

Molecular Mechanics/Generalized Born Surface Area (MM/GBSA)

Protein-ligand complexes were also analyzed by MM/GBSA to estimate the free binding energies of studied ligands. The MM/GBSA calculations were applied to complex structures using Schrödinger's Prime module (20). The software extracted the frames of ligand-protein complexes from the MD trajectory of each complex at every 10 ps. For protein flexibility, we used the VSGB solvation model (32), which is a realistic parameterization of the solvation and OPLS-2005 force field.

Therapeutic Activity Prediction

Binary QSAR models in MetaCore/MetaDrug underwent therapeutic activity prediction, pharmacokinetic predictions, and ADME/toxicity profile for the compounds under investigation. It is an advanced data analysis program based on protein-protein interactions, protein-DNA interactions, disease, and toxicity. In Metacore/Metadrug, the software normalized the predicted therapeutic activity values to 0 and 1 (i.e., 0 meaning inactive, 1 meaning active molecules). Therapeutic activity results of selected 7 hit compounds, and the FDA-approved two drugs (Abemaciclib and Ribociclib) are in Table 1. Also, we carried out the therapeutic activity calculations for specific CDK2 inhibitors (A-674563 and MK-8776). Activity values of A-674563 and MK-8776 are 0.44 and 0.79, respectively.

RESULTS AND DISCUSSION

The current study aims to identify novel pyrimidine-based small compounds against CDK-2 from a small molecule library using combined text mining and molecular modeling approaches. For this aim, we used the Specs SC database, which includes 210,596 drug-like compounds. Figure 1 summarizes the flowchart of all applied procedures in the current study. All 210,596 compounds imported from the Specs-SC database in .sdf file format were converted to .name IUPAC text file format with MarvinSketch program (MarvinSketch, 2018) and identified 6,668 compounds that contain the phrase "pyrimidine." We then converted these compounds to .sdf file format and prepared 2D molecular structures of selected pyrimidine-derivatives to energetically optimized 3D structures using the LigPrep module of Maestro molecular modeling Suite. Afterward, we used these prepared compounds in molecular docking simulations. These 6,668 pyrimidine derivative compounds were screened at the active site of the CDK-2 (PDB ID: 2XMY) using a grid-based docking program Glide/SP (standard precision). Docking scores of these compounds were measured, and selected top-7 compounds for MD simulations to better understand their structural and dynamical profiles at the active site of the enzyme. Trajectory frames of each complex system were collected and analyzed throughout the MD simulations. Figure 2 shows MM/GBSA free energy plots of each selected hits, as well as FDA-approved drugs Abemaciclib and Ribociclib. Table 1 summarizes the top-docking scores and average MM/GBSA binding free energies of selected hit compounds as well as FDA-approved compounds. Docking scores of Glide/SP for the selected hit compounds are similar. However, a few selected compounds (i.e., compounds 2523, 2657, and 6447) have in a different range of MM/GBSA scores. There are a few reasons. (i) Some of the hit compounds (i.e., compound 2523) do not maintain their initial docking poses (Figure S13); (ii) Used docking scoring functions and MM/GBSA scoring functions are different; thus they may not necessarily produce the same ranking. For this reason, we also performed docking simulations using another docking program GOLD. As well as, instead of a prolonged (100-ns) MD simulations, we also repeated simulations with three times with short (10-ns) MD simulations for selected hit compounds and FDA-approved drugs (Abemaciclib, Ribociclib) and specific CDK-2 inhibitors (A-674563 and MK-8776), (Table S1). GOLD docking scores showed that selected hit compounds 6447 and 2657 also have high docking scores. Besides, Figures S1, S2, and S3 give the protein RMSD plot of compounds 2657, 5925, and 6447 during 10 ns simulations,

respectively. The results show that the average ΔG values obtained as a result of 10 ns simulations are very close to each other.

We provided, in Figure 3, the protein (Cα) RMSD plots of selected 7 hit compounds and FDA-approved drug Abemaciclib. RMSD plots of all studied systems show that all the systems reach the equilibrium after the 20-ns time scale. The deviations from the initial conformations are not significant (<4.0 Å). Within the studied systems, the Mol-236 lead compound had higher structural fluctuations compared to other systems.

Together with protein-RMSDs for the studied systems, we further measured the ligand-RMSDs. We plotted two different ligand RMSD graphs: LigFitLig and LigFitProt. While we considered the rotational motion in LigFitLig RMSDs, throughout the MD simulations, we adopted the translational motion of the ligand in LigFitProt RMSDs. Thus, in LigFitLig, heavy atoms of the ligand align themselves, and we recorded the deviations from the initial conformation throughout the simulation. However, in LigFitProt RMSDs, heavy atoms of ligand aligned to the backbone atoms of protein, and we measured the deviations from the initial positions as RMSDs. Ligand RMSD shows how stable the ligand is regarding the protein and its binding pocket. We showed the LigFitProt's RMSD of a ligand when we first matched the protein-ligand complex with the reference protein backbone, and then the RMSD of the ligand heavy atoms is determined. If the measured values are significantly larger than the protein's RMSD, then the ligand is likely to have diffused away from its original binding position. LigFitLig displays a ligand's RMSD that is aligned and measured just according to its reference conformation. This value for RMSD measures the internal fluctuations in the ligand atoms.

Figure 4 shows the LigFitProt RMSD plots of studied ligands. As it can be seen clearly from the Figure, most of the studied ligands do not have significant translational changes throughout the MD simulations. We observed the maximum deviations for FDA-approved drug Abemaciclib, Mol-2655 and Mol-2657, which have around 5.0 Å throughout the simulations. Results show that studied systems tightly bound to the binding pocket of the target structure; thus, they do not diffuse from their initial positions.

Figure 5 shows the LigFitLig RMSD plots of studied ligands. As we can see from the RMSD plots, all the ligands have less than 3.0 Å average RMSD values that represent the structural stability of studied compounds at the active site of the target protein. We also investigated the root-mean-square fluctuation (RMSF) values of studied compounds to check the effect of each ligand to the target protein structure. (Figure 6) Residue numbers around

40, 70, and 150 have higher fluctuations compared to other regions when the studied compounds bind.

We showed the 2D ligand interaction diagrams of hit compound Mol-2657 in Figure 7. The compound constructs stable non-bonded chemical interactions with mainly following residues throughout the MD simulations: Lys20, Lys129, Asn132, and Asp145.

Figure 8 displays a time-line representation of the interactions and contacts (H-bonds, Hydrophobic, Ionic, Water Bridges). The top panel shows the total number of specific contacts produced throughout the MD simulation period by the protein with the ligand. The bottom panel displays which residues interact with the ligand in each trajectory frame. Many residues allow more than one direct interaction with the ligand, defined by darker orange color, depending on the scale to the right of the map. Figure 8 displays that Mol-2657 constructs critical chemical interactions with residues Lys129, Asn132, and Asp 145, which are stable throughout the simulation time.

We screened the therapeutic activity values of selected 7 hit compounds against cancer using binary quantitative structure-activity relationship (QSAR) models in Metacore/Metadrug. Therapeutic activity values of all selected compounds greater than 0.5 (Table 1).
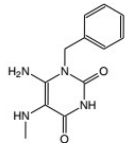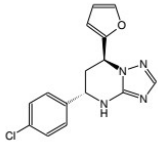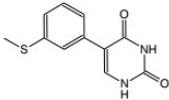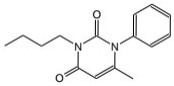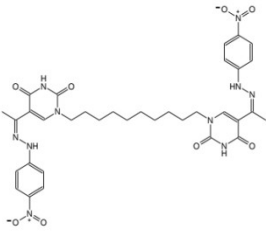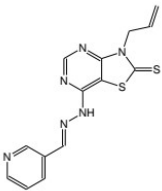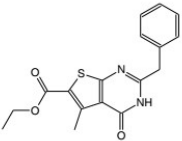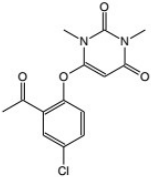
CONCLUSION

In this study, we carried out advanced integrated text mining, virtual screening, and hybrid molecular modeling strategies to identify the pyrimidine-based inhibitors targeted to the binding site of CDK-2. Thus, we have selected 6,668 pyrimidine derivatives from the small molecule database of Specs-SC for this aim. We screened these identified compounds against the CDK-2 target using molecular docking and used the top-docking poses of selected 7 hits in long MD simulations (100 ns). MM/GBSA binding free energy calculations have been conducted for all ligands. We also performed the same protocol (100 ns molecular dynamics simulation) for the FDA-approved drug Abemaciclib. The therapeutic activities of the selected 8 compounds were screened in Metacore/Metadrug using binary QSAR models. We evaluated the docking scores and MM/GBSA scores in Table 1. We observed that the compound 2523 has a high docking score, however its corresponding MM/GBSA score was low compared to MM/GBSA scores of other compounds. When we visualize the trajectory
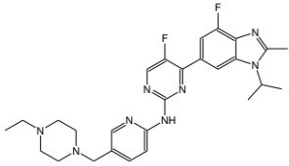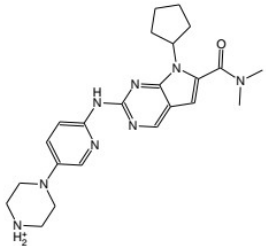
frames throughout the simulations, we observed that this compound does not stay at the binding pocket throughout the simulations and loses its initial docking pose. (see Figure S11 Thus, we have not evaluated the binding energy of compound 2523. Here, we showed that integrated text-mining and molecular modeling approaches leading to 7 novel pyrimidine-based CDK-2 inhibitors. Thus, such new compounds may open new paths for developing small inhibitors against CDK-2.

Table 1. 2D structures, top-docking scores in Glide/SP and Gold, and average MM/GBSA scores of selected 7 hits and FDA-approved 2 CDK inhibitors.

Compounds	2D Structure	Glide Docking Score (kcal/mol)	Gold Docking Score (kcal/mol)	MM/GBSA (kcal/mol)	Predicted Therapeutic activity
Mol-30		-9.359	-4.956	-48.91±3.29	0.53
Mol-236		-9.385	-6.453	-46.52±3.95	0.75
Mol-2623		-9.382	-6.882	-52.85±4.64	0.52
Mol-2655		-9.298	-6.963	-47.32±3.58	0.53

Compounds	2D Structure	Glide Docking Score (kcal/mol)	Gold Docking Score (kcal/mol)	MM/GBSA (kcal/mol)	Predicted Therapeutic activity
Mol-2657		-9.573	-7.533	-74.38±7.91	0.69
Mol-5925		-9.746	-7.00	-51.90±3.02	0.74
Mol-6447		-9.317	-7.675	-62.14±3.20	0.56
Mol-2523		-9.373	-5.969	NA	0.30

Compounds	2D Structure	Glide Docking Score (kcal/mol)	Gold Docking Score (kcal/mol)	MM/GBSA (kcal/mol)	Predicted Therapeutic activity
Abemaciclib		-6.818	-9.170	-45.49±5.90	0.78
Ribociclib		-5.187	-7.568	-47.40±3.16	0.68
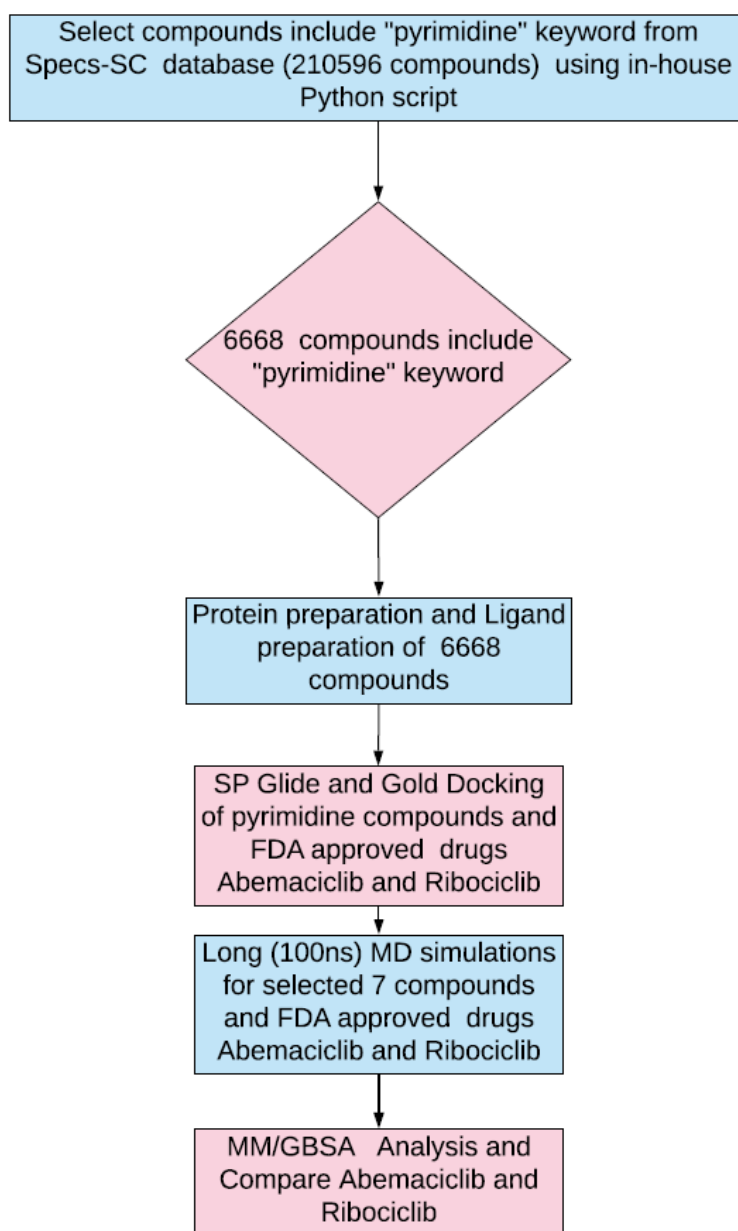
Figure 1: Applied virtual screening workflow at the current study.
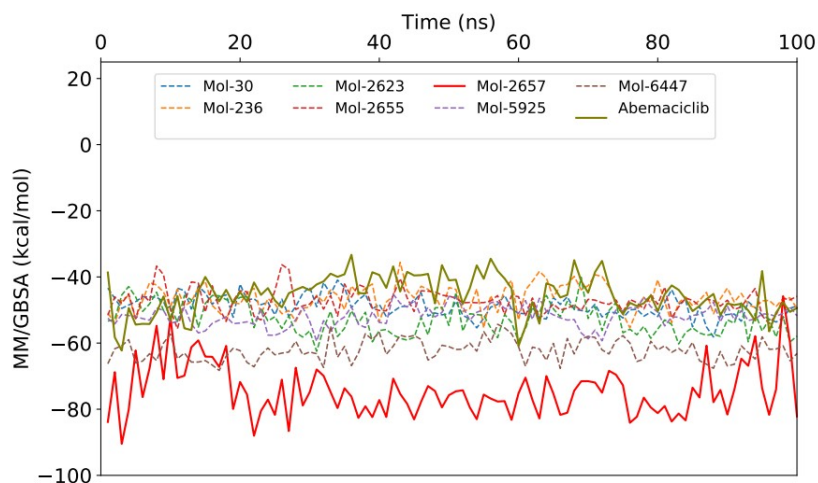
Figure 2: MM/GBSA plots of selected 7 compounds and FDA-approved drug Abemaciclib during the MD simulations.
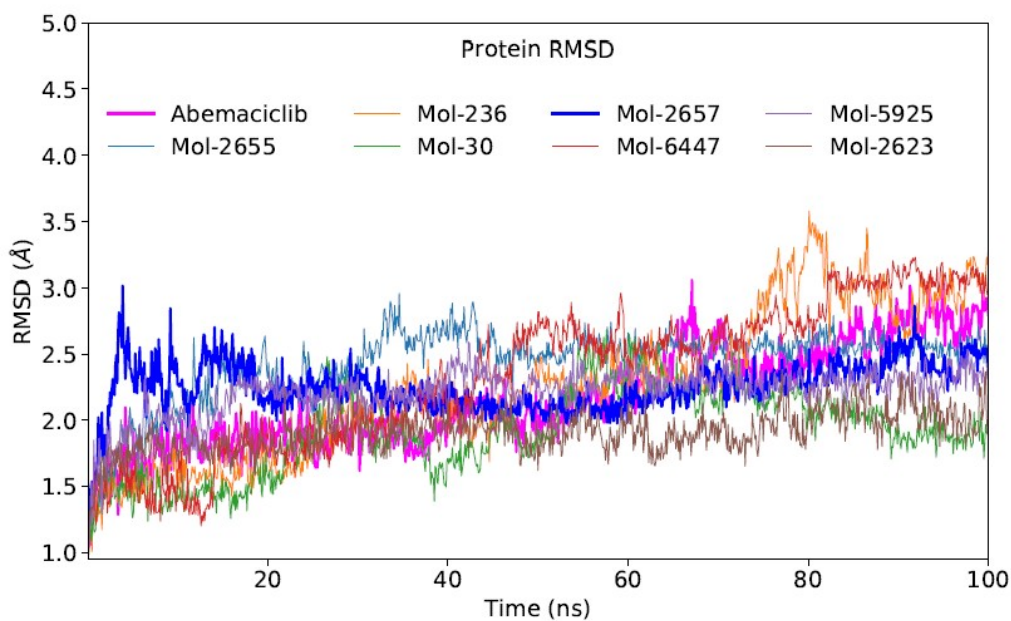
Figure 3: Protein-RMSD plots of selected 7 compounds and FDA-approved drug Abemaciclib throughout the MD simulations.
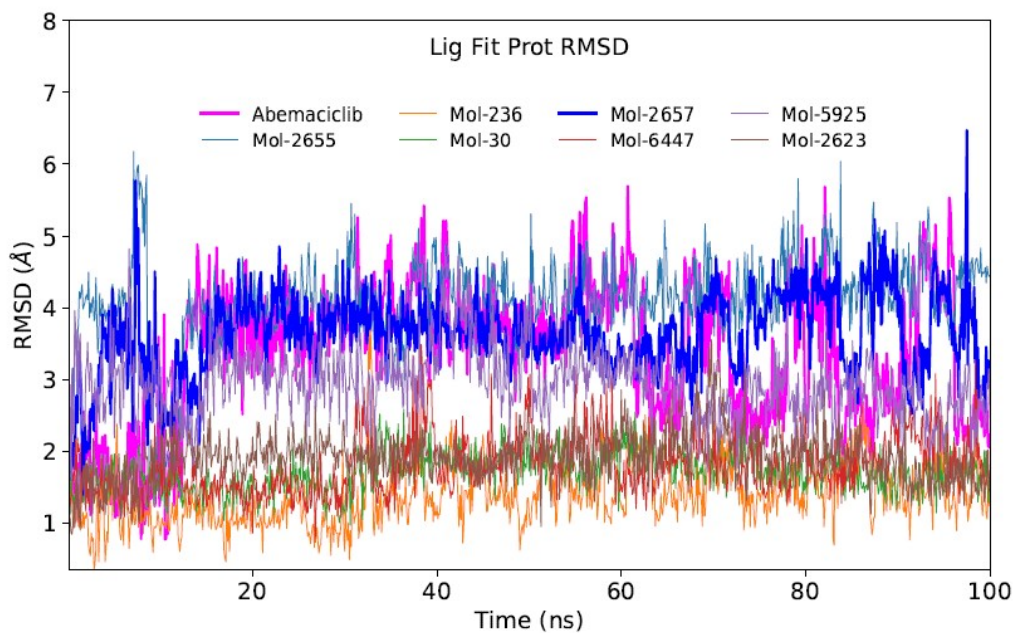
Figure 4: LigFitProt RMSD graph for selected 7 compounds and FDA-approved drug Abemaciclib.
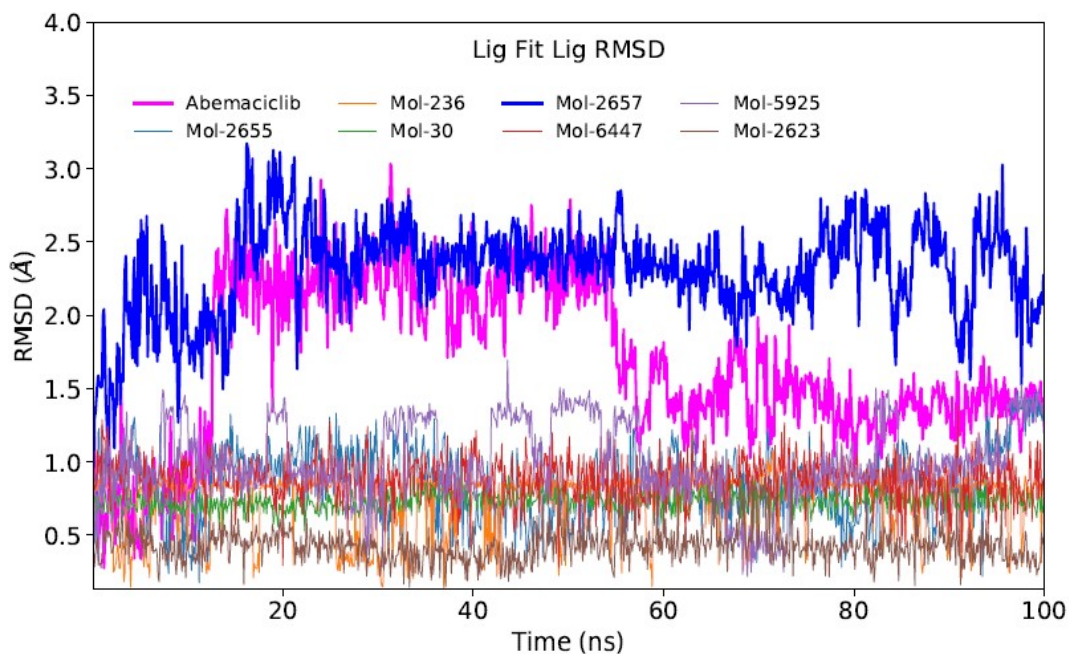
Figure 5: LigFitLig RMSD graph for selected 7 compounds and FDA-approved drug Abemaciclib.
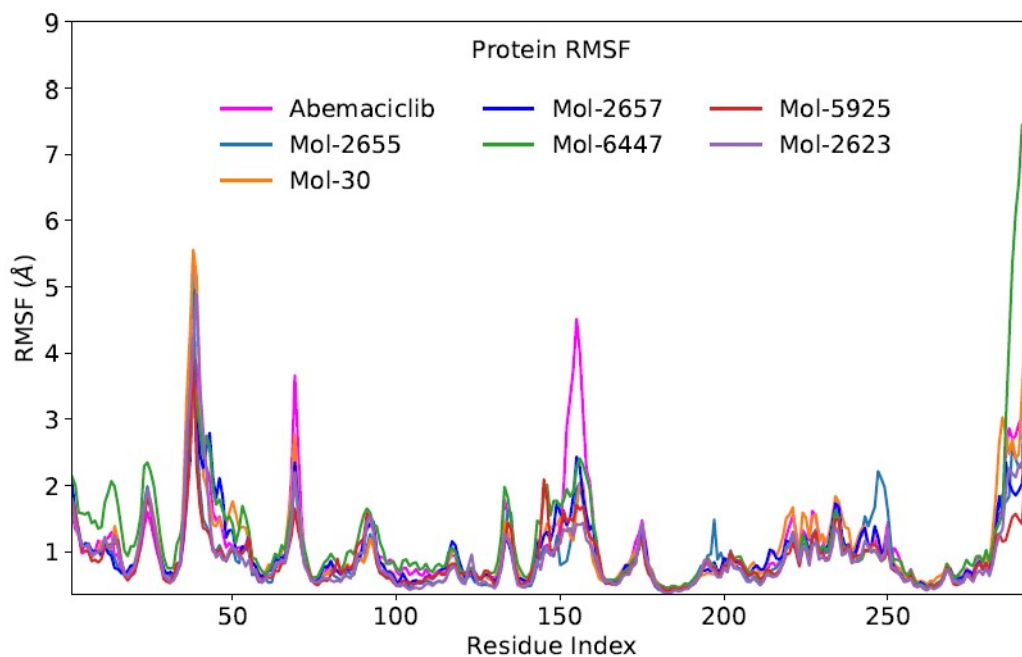
Figure 6: RMSF plots for selected 7 hit compounds and FDA-approved drug Abemaciclib.
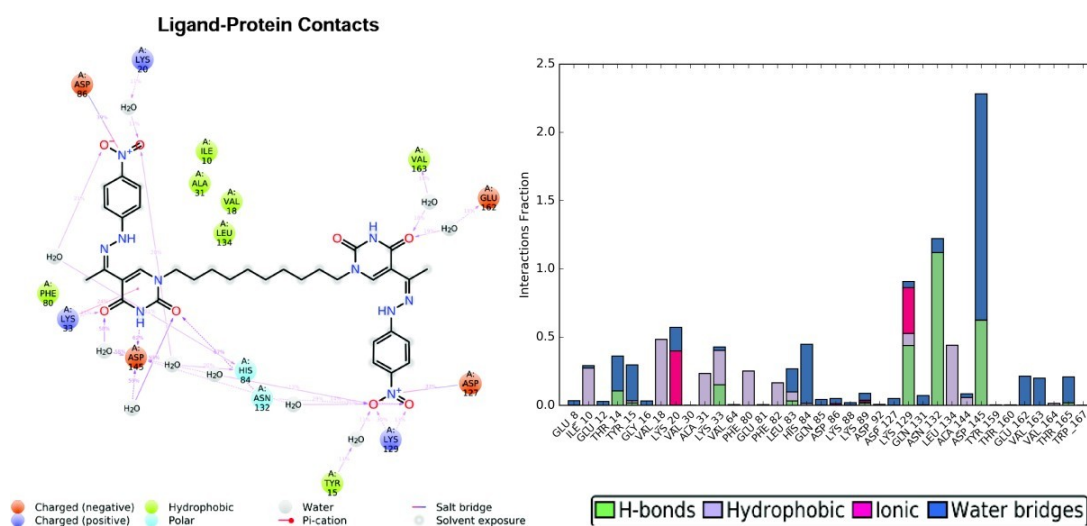
Figure 7: Mol-2657's 2D ligand interaction graph. The figure also shows interaction fractions throughout the simulations.
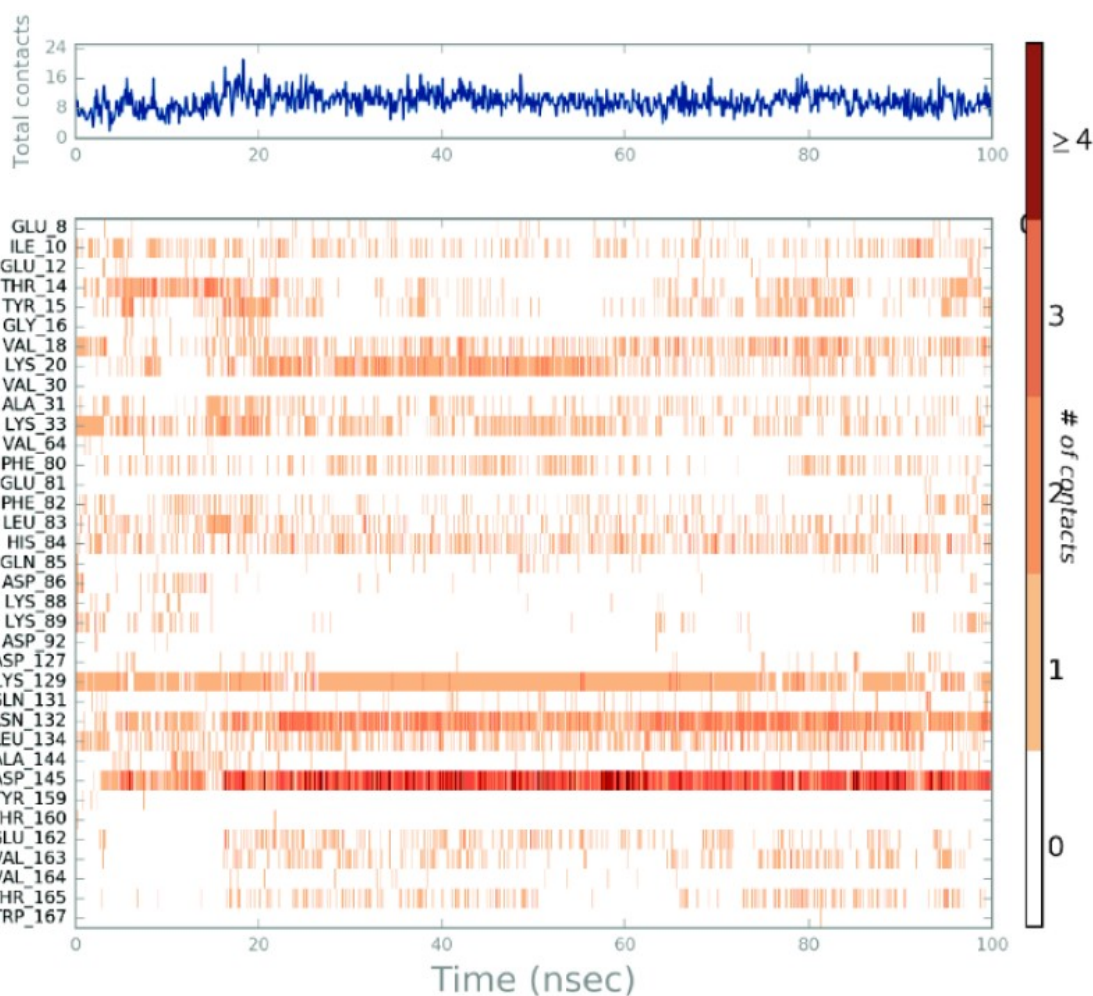
Figure 8: Protein-ligand interactions throughout the simulations. While top-panel shows total contacts with the ligand, the bottom-panel represents the formed and broken interactions between the protein and ligand during the simulation.

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SUPPLEMENTARY INFORMATION

Table S1. Molecular dynamics simulations results of selected 7 hit compound, FDA-approved two drugs (Abemaciclib and Ribociclib) and two selective CDK2 inhibitors (A-674563 and MK-8776)

Compounds	Gold Docking Scores (kcal/mol)	MD_1 (10 ns) (ΔG/kcal/mol)	MD_2 (10 ns) (ΔG/kcal/mol)	MD_3 (10 ns) (ΔG/kcal/mol)	Average (ΔG/kcal/mol)
30	-4.956	-46.13	-46.15	-47.59	-46.62
236	-6.453	-47.37	-46.99	-42.95	-45.77
2657	-7.533	-75.19	-82.62	-75.62	-77.81
5925	-7.000	-51.46	-53.85	-55.04	-53.45
6447	-7.675	-60.63	-56.98	-60.32	-59.31
2523	-5.969	-43.47	-40.95	-39.53	-41.31
2655	-6.963	-45.13	-45.08	-45.99	-45.40
Abemaciclib	-9.170	-41.75	-39.04	-40.05	-40.28
Ribociclib	-7.568	-49.40	-52.64	-50.99	-51.01
A-674563	-9.685	-46.98	-47.75	-47.24	-47.32
MK-8776	-7.529	-42.12	-44.35	-44.90	-43.79
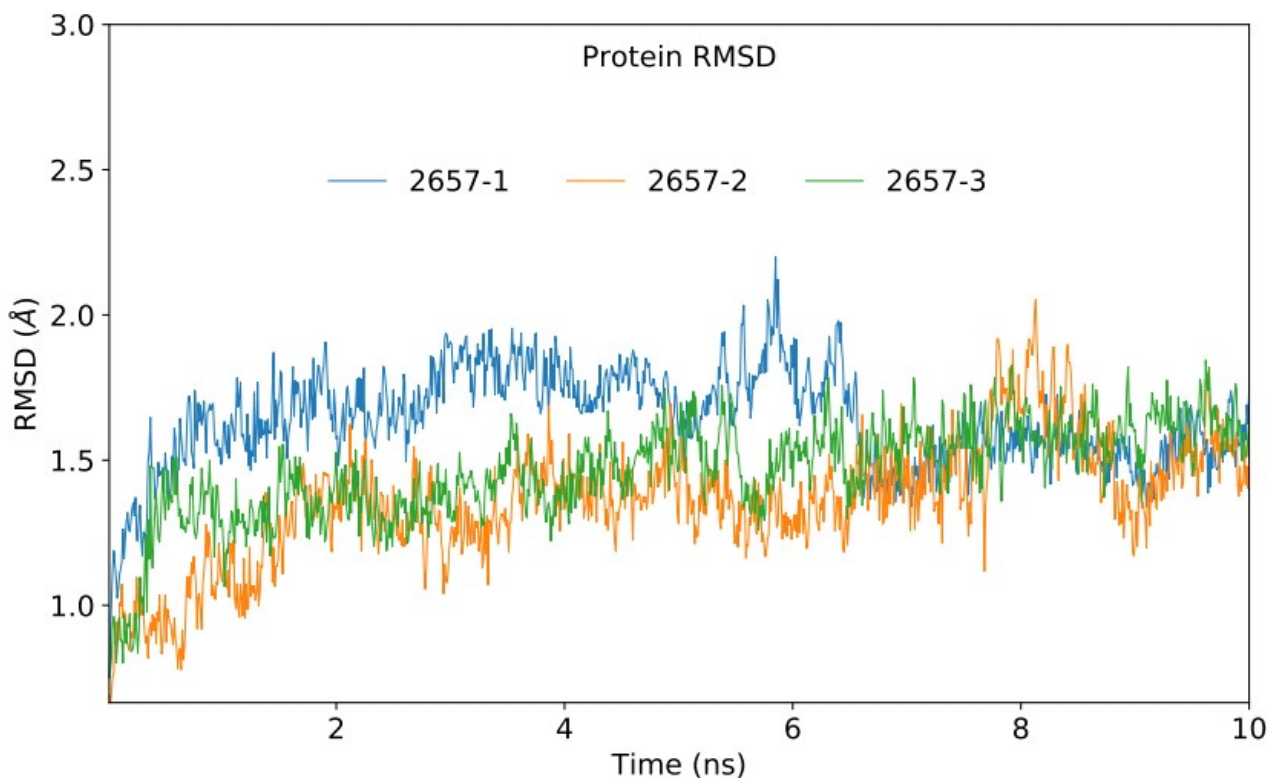
Figure S1: Protein RMSD plot of compound 2657 as a result of 3 separate 10 ns simulations.
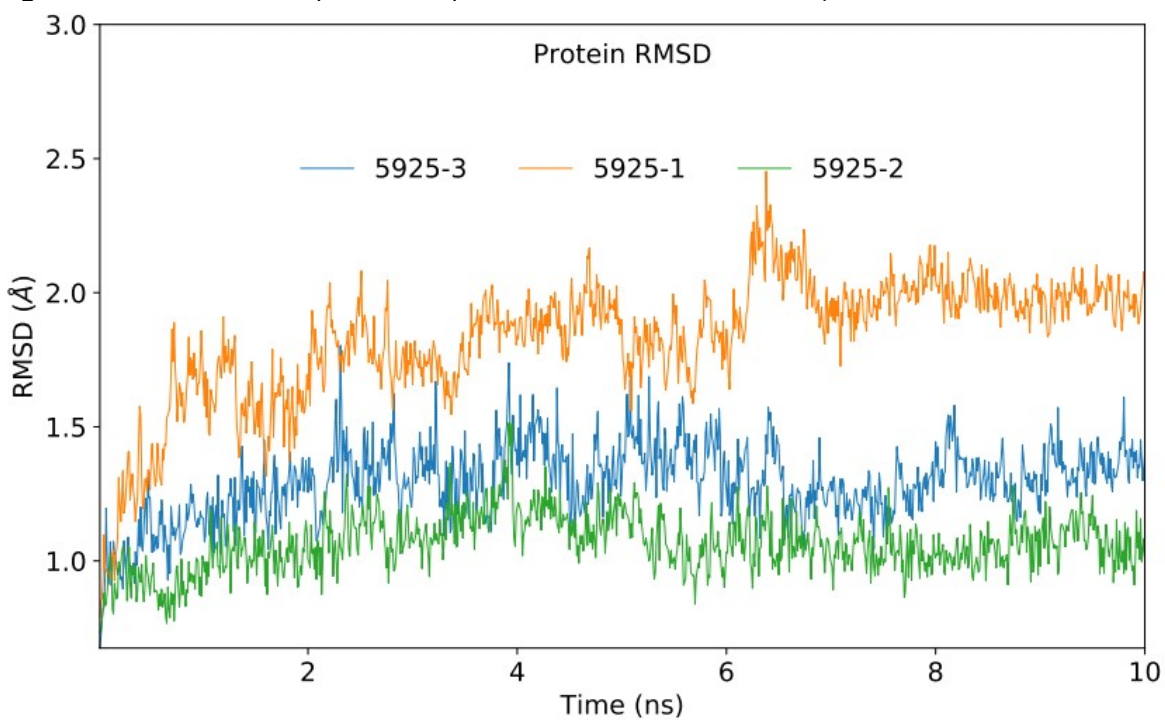
Figure S2: Protein RMSD plot of compound 5925 as a result of 3 separate 10 ns simulations.
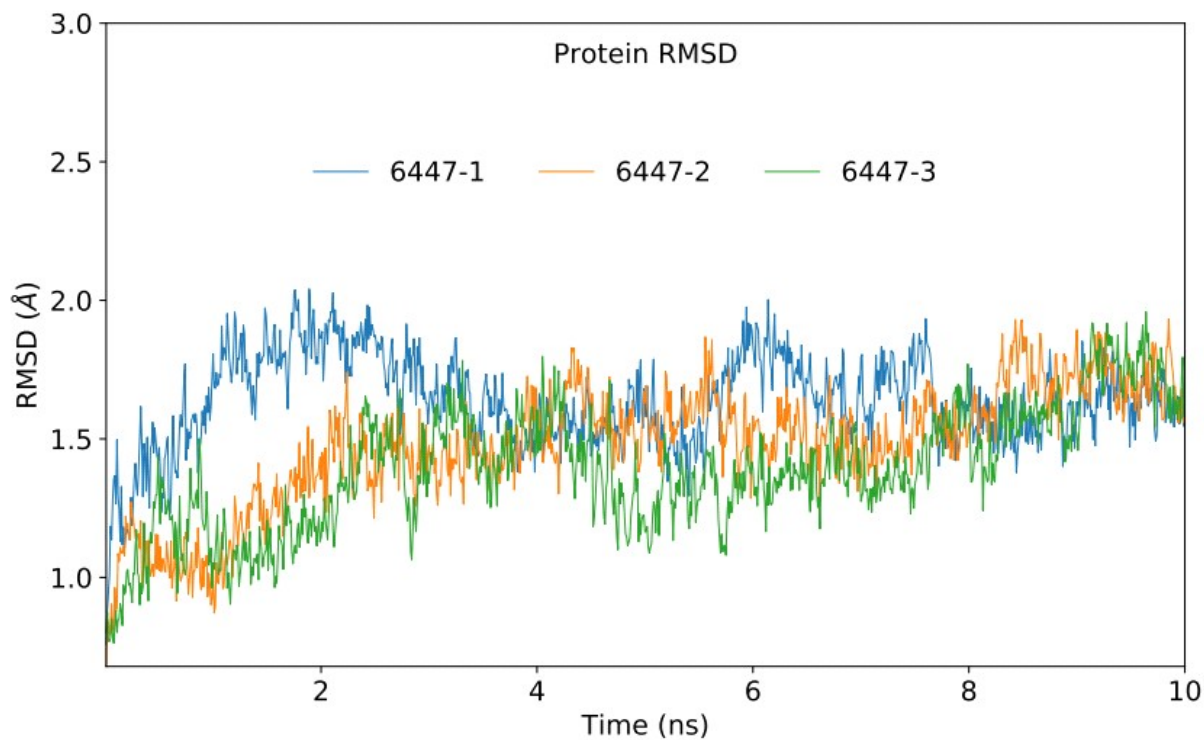
Figure S3: Protein RMSD plot of compound 6447 as a result of 3 separate 10 ns simulations.
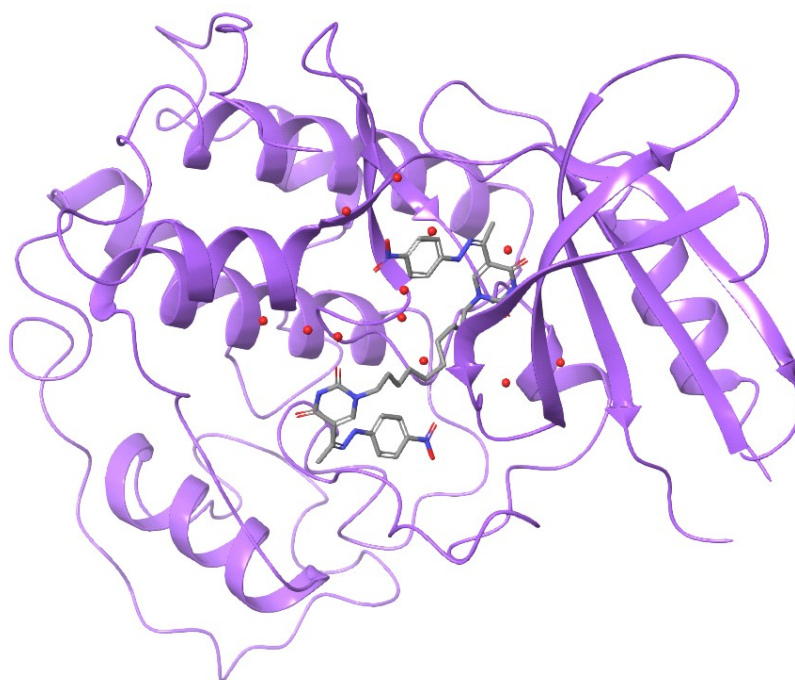
Figure S4: 2XMY-Compound 2657 complex structure.
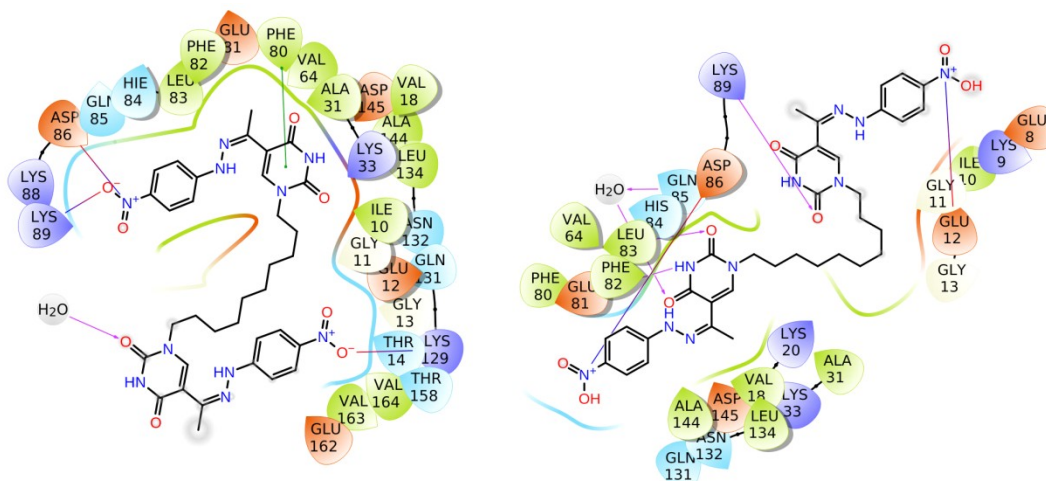
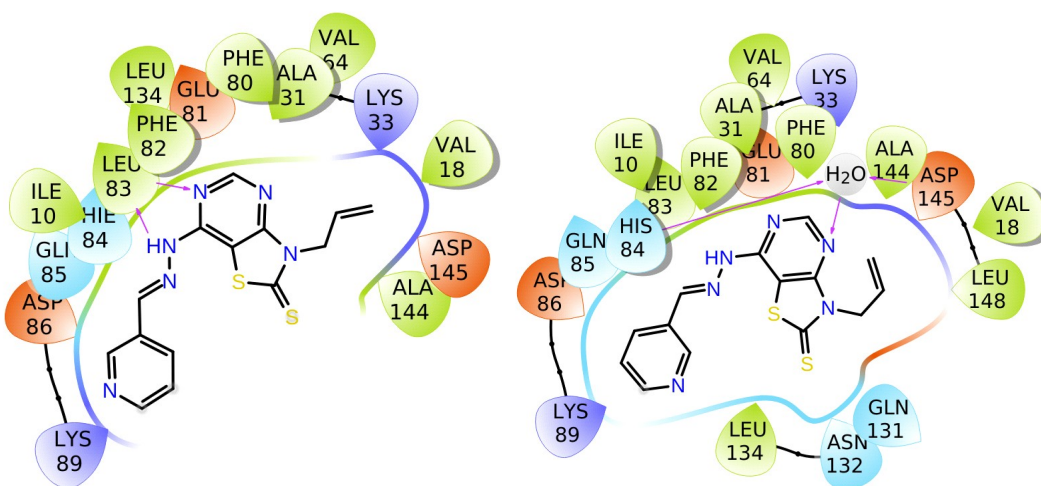**Figure S5:** Ligand interactions diagram of compound 2657 (left Glide docking and right Gold docking)

Figure S6: Ligand interactions diagram of compound 5925 (left Glide docking and right Gold docking)
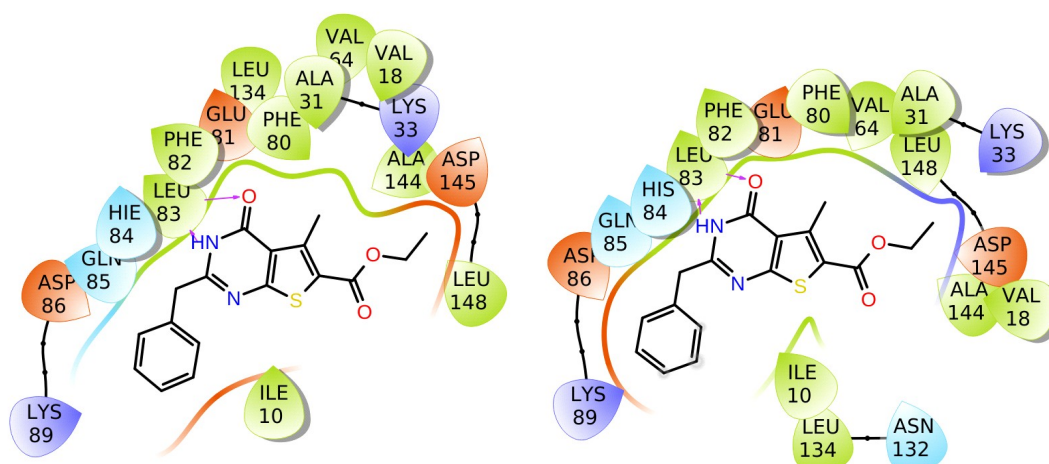
Figure S7: Ligand interactions diagram of compound 6447 (left Glide docking and right Gold docking)
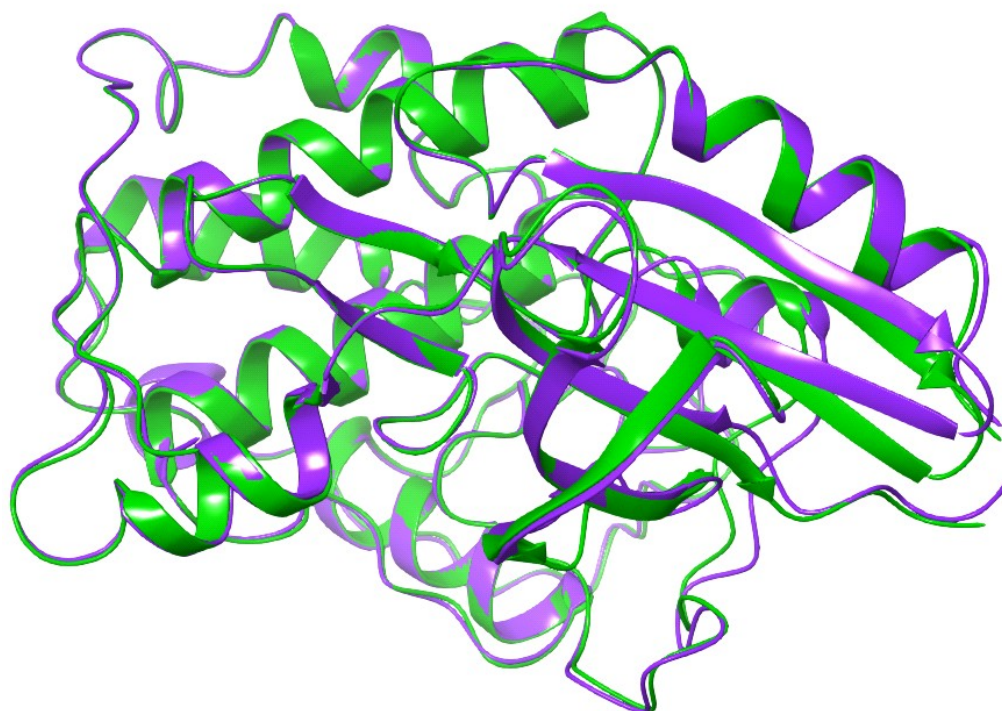
Figure S8: Alignment of 2XMY and 2XNB (Alignment Score: 0.014 ; RMSD: 0.583 A)
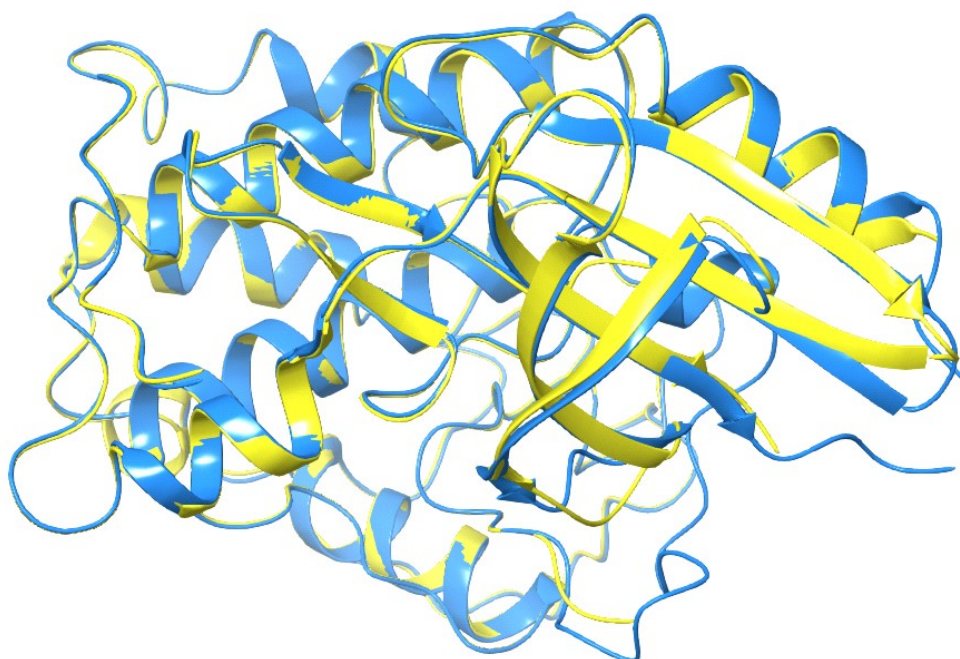

Figure S9: Alignment of 2XMY and 2W05 (Alignment Score: 0.016; RMSD: 0.629 A)
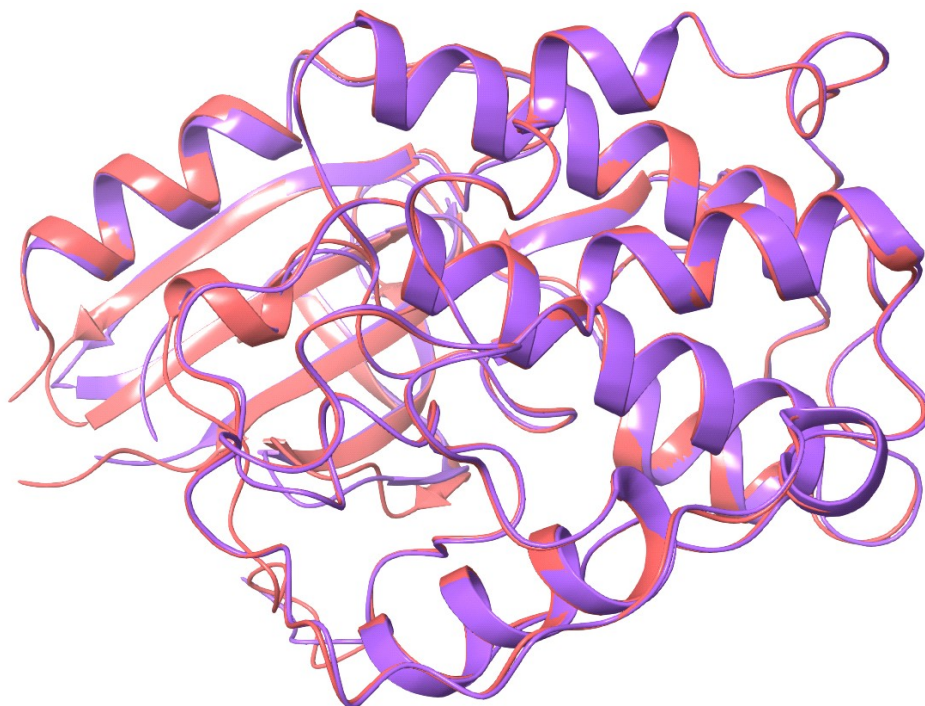
Figure S10: Alignment of 2XMY and 1Y91 (Alignment Score: 0.018; RMSD: 0.670 A)

Table S2: A method development-validation1.

Title	Stars	t Q	Job Name	docking score
glide-dock_SP_validation_se…				
2XMY - minimized	☆☆☆		glide-dock_SP_validat…	
2m	☆☆☆	0	glide-dock_SP_validat…	-8.629
2c	☆☆☆	0	glide-dock_SP_validat…	-7.510
2c	☆☆☆	0	glide-dock_SP_validat…	-7.510
2d	☆☆☆	0	glide-dock_SP_validat…	-7.428
2n	☆☆☆	0	glide-dock_SP_validat…	-7.077
2h	☆☆☆	0	glide-dock_SP_validat…	-6.729
glide-dock_SP_validation_se…				
2XMY - minimized	☆☆☆		glide-dock_SP_validat…	
2i	☆☆☆	0	glide-dock_SP_validat…	-7.499
2l	☆☆☆	-1	glide-dock_SP_validat…	-7.490
2j	☆☆☆	0	glide-dock_SP_validat…	-6.880
2k	☆☆☆	-1	glide-dock_SP_validat…	-4.472

Table S3. A method development-validation2.

Title	tomer probability	docking score
▼ glide-dock_SP_validation_set3_inactive_…		
⊢ 2XMY - minimized		
⊢ 12	1.000	-8.839
⊢ 17	1.000	-8.174
⊢ 16	1.000	-6.361
▼ glide-dock_SP_validation_set3_active_pv…		
⊢ 2XMY - minimized		
⊢ 11e	1.000	-9.554
⊢ 11a	1.000	-9.273
⊢ 11h	1.000	-7.286
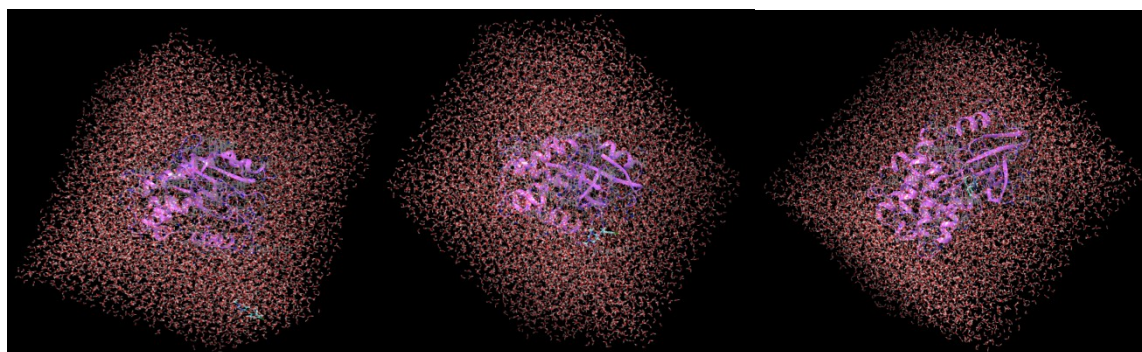
FigureS11: Initial (right), after 60 ns (center) and last (left) poses of compound 2523 throughout 100 ns molecular dynamics simülations.

Assessment of Elemental Content, Antioxidant Activity, and Total Phenolic Content of Vitis sylvestris Gmelin

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Abstract: In this study, we determined the Cu, Fe, Mn, and Zn contents of Vitis sylvestris Gmelin using flame atomic absorption spectrometry (FAAS), and examined the antioxidant properties of Vitis sylvestris Gmelin using radical scavenging capacities and total phenolic content tests. We found the average elemental contents; Cu, Fe, Mn, and Zn as 1.506 ± 0.042 mg/kg, 0.796 ± 0.020 mg/kg, 2.333 ± 0.033 mg/kg, and 3.191 ± 0.262 mg/kg, respectively. When we examined the antioxidant activity tests applied to different extracts, we determined the highest extraction yield with the methanol extract. DPPH radical scavenging activity, ABTS radical scavenging activity, and total phenolic content values in methanol extract were determined to be respectively 3.957 ± 0.146 mg TEAC/g fw, 9.062 ± 0.273 mg TEAC/g fw, and 2.365 ± 0.028 mg GAE/g fw. When we evaluated the antioxidant activity and total phenolic content results for all extracts statistically, we determined that there was generally a statistically significant difference between each extract ($p < 0.01$). Vitis sylvestris Gmelin has high antioxidant content when considering the data obtained. Also, we determined that it is an effective candidate in the protection against reactive oxygen species.

INTRODUCTION

Copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) are essential elements for human health and should be taken into the body to maintain many important metabolic activities in the human body (1, 2). These elements play an essential role in the growth, development, and health of the human body (2-5). We can find Cu in the structure of many enzymes; similarly, Zn is defined as a co-factor in some enzymes and is involved in various metabolic activities. For example, if we do not take copper and zinc into the human body in sufficient quantities, then the bodily functions may be adversely affected. Consuming various vegetables, fruits, and food products supply the essential elements needed by the human body.

Antioxidants protect our body against diseases by reacting with almost all classes of compounds in the structure of living organisms, by taking a role in the reduction of reactive oxygen species (ROS), which cause significant damage. Antioxidant compounds have an essential role against diseases such as cancer, diabetes, gastrointestinal diseases, neurodegenerative diseases, and aging caused by excessive reactive oxygen species. Many synthetic antioxidant compounds are available, but it is clear that these compounds have toxic and mutagenic effects. In recent years, we started to prefer

natural antioxidant compounds rather than synthetic ones, and we can take these compounds from various sources, including vegetables and fruits. Fruits and vegetables are rich antioxidant sources concerning phenolic compounds, anthocyanins, vitamins, and mineral elements in the literature (6, 7). Authors state that the adverse effects of free radicals reduce by consuming foods, including antioxidants, because of their positive effects on health (6, 8-10).

Turkey, a country rich in fruit and vegetable varieties, is a potential source of phenolic compounds, and one of them is wild grapes and grape varieties (11, 12). In the literature, there are studies about antioxidant activity, phenolic, vitamin, and elemental content of wild grapes grown in different regions (13-16). As a result of biodiversity, which is emerging due to physical geography characteristics, climate differences, and abundant water resources, it is determined that there is no data about wild edible Vitis sylvestris Gmelin reported (17) to naturally grown in Pertek-Tunceli district (Turkey). In this context, we investigated the element content, antioxidant activity, and total phenolic content of Vitis sylvestris Gmelin.

MATERIAL AND METHOD

Reagents and standards
All chemicals and solvents used in sample and standard solution preparation were of analytically pure grade and obtained from Sigma-Aldrich and Merck. We used ultrapure water (Milli-Q, Millipore 18.2 μΩ cm-1) in the experimental studies. We drew calibration graphs by standard solutions prepared in different concentration levels and used these graphs to evaluate the obtained data.

Element contents were determined using Perkin Elmer AAnalyst 800 flame atomic absorption spectrometer (FAAS) (Perkin Elmer, Inc., Shelton, CT, USA). The operation conditions are as follows: We set the wavelength and slit width for Cu, Fe, Mn, Zn respectively 324.8-07 nm, 248.3-0.2 nm, 279.5-0.2 nm, 213.9-0.7 nm, and acetylene and air flow rate was 2.0 L/min and 17.0 L/min. We used a Shimadzu 1800 UV-Vis spectrophotometer for antioxidant activity measurements.

Sample preparation
Vitis sylvestris Gmelin (wild grape) naturally grown in Pertek-Tunceli, Turkey was gathered manually on the season, and then samples were mixed, washed with tap water, lastly passed through deionized water. We then stored them in the freezer until the analysis time.

Wet Digestion procedure for elemental analyses: We separated Vitis sylvestris Gmelin samples into small pieces, transferred approximately a 1-g sample into the beakers, and dissolved with 2 mL of concentrated 1:1 HNO3: H2O2 mixture. We evaporated the samples near dryness with occasional stirring and repeated the same procedure once again. We completed the final volumes to 5 mL with 1.0 M HNO3 and centrifuged to obtain homogeneous solutions. We carried out elemental analyses in the solutions using FAAS. Blank samples and standard reference materials (0.25 g) (NIST-1547 peach leaves) were also prepared using the same procedure and analyzed. We expressed the results as mean values ± standard deviation of three independent analyses based on fresh weight.

Extraction procedure for antioxidant activity: We separated Vitis sylvestris Gmelin samples into small pieces, approximately 5 g samples transferred into the beakers, and acidified 10 mL of extraction solvents such as water, acetonitrile, and methanol with 0.1% HCl to the samples. We then mixed the samples with extraction solvents for 60 min at room temperature and then centrifuged (10 min at 5000 rpm). We passed the clear solutions through a 0.45 μm injection filter. For DPPH and ABTS radical scavenging activities, we applied total phenolic content tests to different sample extracts. We expressed the results as mean values ± standard deviation of three independent analyses based on fresh weight.

Antioxidant Activity Tests
DPPH radical scavenging activities in the extracts were determined using the method applied by Brand-Williams et al. (18). We made the volumes of extracts taken in a certain amount to 2.5 mL with DPPH solution and then incubated for 30 min at room temperature. We used a UV-Vis spectrophotometer for the absorbance measurements of the solutions at 517 nm. Trolox was the standard and stated the results as Trolox-equivalent on fresh weight (mg TEAC/g fw).

We used the method proposed by Re et al. (19) to measure ABTS radical scavenging activity of the sample extracts. The extracts were made up to 2.5 mL with ABTS solution and incubated at room temperature for 30 minutes in the dark. At the end of the incubation time, we measured the absorbance values with a UV-Vis spectrophotometer at 734 nm. Trolox was the standard, and we stated the results as Trolox equivalent on fresh weight (mg TEAC/g fw).

Determination of Total Phenolic Content
We determined the total phenolic contents by measuring the absorbance value of the color formed by adding the Folin-Ciocalteu reactant and 2% sodium carbonate solution to the extracts (20). We determined the absorbance values by

measuring with a UV-Vis spectrophotometer at 755 nm after 30 minutes of incubation at room temperature. The standard was gallic acid and we expressed the experimental data in terms of gallic acid equivalent on fresh weight (mg GAE/g fw).

We used GraphPad Software (version 5.01 for Windows, GraphPad Software, USA) for the statistical analyses for antioxidant activity and total phenolic content. We established the significance between the groups using the one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. We considered the differences statistically significant when p < 0.01.

RESULTS AND DISCUSSION

We used FAAS to determine Cu, Fe, Mn, and Zn contents in Vitis sylvestris Gmelin. We found the Cu, Fe, Mn, and Zn contents were 1.506 ± 0.042 mg/kg, 0.796 ± 0.020 mg/kg, 2.333 ± 0.033 mg/kg, and 3.191 ± 0.262 mg/kg, respectively. We tested the accuracy of the method with standard reference material (NIST-1547 peach leaves). We determined the certified values for this standard reference material to be 3.75 ± 0.37 mg/kg for Cu, 219.8 ± 6.8 mg/kg for Fe, 97.8 ± 1.8 mg/kg for Mn, and 17.94 ± 0.53 mg/kg for Zn. In the present study, we found the Cu, Fe, Mn, and Zn contents obtained with the used method to be 3.48 ± 0.25 mg/kg, 198.7 ± 7.0 mg/kg, 90.1 ± 2.1 mg/kg, and 17.08 ± 0.48 mg/kg, respectively. We found the recovery values for these elements to be 93% for Cu, 90 % for Fe, 92% for Mn, and 95% for Zn.

A literature study has reported that Cu, Fe, Mn, and Zn concentrations in grapes found from 0.4±0.1 to 2.4±0.1 µg/g, 6.6 ± 0.1 to 14.8 ± 0.3 µg/g, 3.1±0.2 to 41.8±0.2 µg/g, and 2.1±0.1 to 20.4±1.0 µg/g in a study, respectively (21). In another study, researchers determined the element contents in the grape as 2.6±0.1 µg/g for Cu, 3.8±0.2 µg/g for Fe, 3.4±0.2 µg/g for Mn, and 2.2±0.1 µg/g for Zn (22). Researchers examined the elemental contents of some fruits and vegetables in a study. They found Cu and Zn concentrations in the grape to be 3.5 mg/kg and 6.1 mg/kg, respectively (23). In the present study, the data concerning elemental contents were compatible, found with the literature data.

We determined the antioxidant activity of Vitis sylvestris Gmelin using DPPH and ABTS radical scavenging activities and total phenolic content tests, applying these tests to the extracts of the samples processed with water, acetonitrile and methanol, and evaluated the results statistically. There is a mention in a literature report that antioxidant activity results changed significantly depending on the solvent type (24-27). In the current study, we applied the antioxidant activity and total phenolic content test applied to different extracts; we obtained the highest results for all tests with methanolic extracts. As seen in Figure 1, average DPPH radical scavenging activity results were determined to be 0.537 ± 0.060 mg TEAC/g fw for aqueous extracts, 3.785 ± 0.077 mg TEAC/g fw for acetonitrile extracts, 3.570 ± 0.08 mg TEAC/g fw for ethanolic extracts, and 3.957 ± 0.146 mg TEAC/g fw for methanolic extracts. We evaluated the results statistically. We found out that there were statistically significant differences between acetonitrile, ethanolic and methanolic extracts of aqueous extracts, and also between ethanolic and methanolic extracts. On the other hand, we determined that there were no statistically significant differences between ethanol and methanol extracts of acetonitrile extracts (p < 0.01).

We found the average ABTS radical scavenging activity results for water, acetonitrile, ethanol, and methanol extracts as 2.285 ± 0.120 mg TEAC/g fw, 7.984 ± 0.355 mg TEAC/g fw, 8.471 ± 0.642 mg TEAC/g fw, and 9.062 ± 0.273 mg TEAC/g fw, respectively (Figure 2). When we examined the data statistically, we determined that there were statistically significant differences between acetonitrile, ethanolic, and methanolic extracts against aqueous extracts, and also between acetonitrile and methanolic extracts. On the other hand, we found that there were no statistically significant differences between acetonitrile and methanol extracts of ethanol extracts (p < 0.01). While we found the highest total phenolic content in methanolic extracts (2.365 ± 0.028 mg GAE/g fw), the lowest total phenolic content was in aqueous extracts (0.592 ± 0.045 mg GAE/g fw) (Figure 3). When we statistically compared the data for total phenolic contents, we found that there was a statistically significant difference between every two extracts except for acetonitrile and ethanolic extracts (p < 0.01).
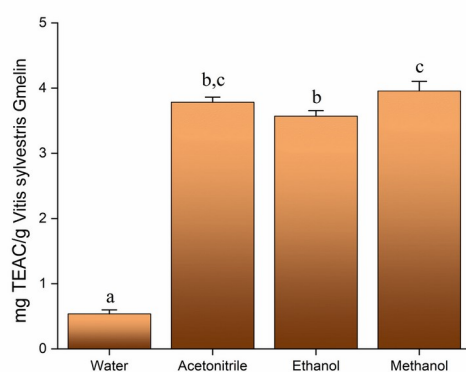
Figure 1: DPPH radical scavenging activity of Vitis sylvestris Gmelin.
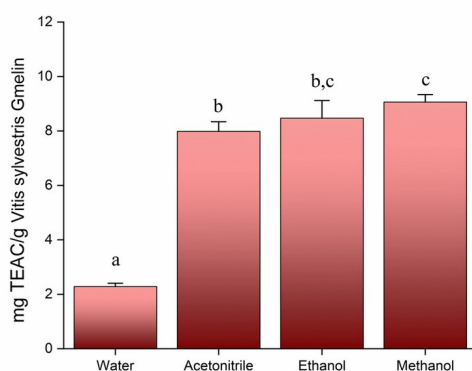

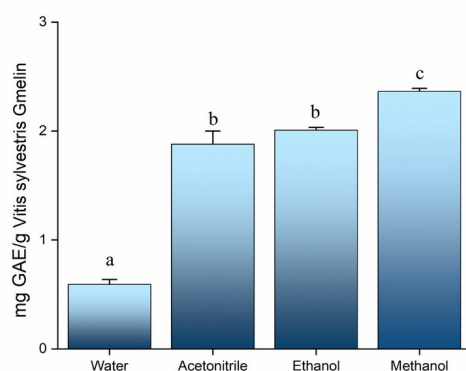**Figure 2:** ABTS radical scavenging activity of Vitis sylvestris Gmelin.

Figure 3: Total phenolic content of Vitis sylvestris Gmelin.

In a study, researchers examined phenolic profile and antioxidant activity of different grape varieties and reported total phenolic content results in a range from 0.44 ± 0.02 to 7.94 ± 0.19 mg gallic acid equivalents/g grape sample (28). Antioxidant activity of black grape obtained applying different drying methods determined with DPPH and ABTS assay, total phenolic content tests. Literature reports that antioxidant activity results changed depending on different drying methods. The highest value for total phenolic content found to be 20.21 mg/g DW in fresh grapes, but this value in the freeze-, oven- and sun-dried samples continued to decrease. The values of DPPH and ABTS antioxidant activities in fresh grapes determined as 66.07 mmol TE/kg DW and 137.65 mmol TE/kg DW (29). Total phenolic compounds of red grape varieties examined by Correia and

Jordão found to be average 1341.0 mg/L, and antioxidant capacities determined varying from 3.96 to 32.96 mm/L Fe(II) (30). The results in the presented study were compatible with those found in the literature. Researchers determined that the data changed importantly depending on different conditions such as drying method, extraction method, and solvent type, as stated in the literature (24-27, 30).

CONCLUSIONS

In this study, we investigated the elemental content and antioxidant activity of Vitis sylvestris Gmelin. Elemental analysis results carried out as applied to the wet digestion method were determined to be 1.506 ± 0.042 mg/kg for Cu, 0.796 ± 0.020 mg/kg for Fe, 2.333 ± 0.033 mg/kg for Mn, and 3.191 ± 0.262 mg/kg for Zn, respectively. We have found that DPPH and ABTS radical scavenging activity, total phenolic content results tested with different polar solvents are 3.957 ± 0.146 mg TEAC/g fw, 9.062 ± 0.273 mg TEAC/g fw, 2.365 ± 0.028 mg GAE/g fw for methanol extracts determined as the best solvents. Vitis sylvestris Gmelin naturally grown in Pertek, Tunceli; according to the obtained results can be evaluated as an antioxidant food, especially, we will add new data with this study about the fruit to the literature.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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Graphene Oxide-Ionic Liquid Used as Solid-Phase Microextraction Coating for Polyphenolic Compounds' Extraction and Determination with GC-MS After On-Fiber Derivatization in Wine

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Abstract: We describe the use of a home-made fiber coated by graphene oxide modified by an ionic liquid having methylimidazolium cation with an amino-functional group for the extraction of polyphenolic compounds (P.C.s). We then performed the determination by gas chromatography coupled with mass spectrometry after on-fiber derivatization. The authors optimized the main parameters influencing the extraction and derivatization processes. The on-fiber derivatization was employed within 15 min at 60 $^{\circ}$C using 20 µL of trimethylsilyl reagents. Under the optimized conditions, the calibration curves for 12 P.C.s were linear from 0.1 to 1000 µg/L, and the detection limits were between 0.02 and 0.1 µg/L. We determined the single fiber repeatability obtained for all calibration points and the fiber to fiber reproducibility for 100 µg/L to be < 14.82% and < 5.87%, respectively. The extraction efficacy of the home-made fiber due to high intermolecular and electrostatic attractions was much better than the commercial fibers. We successfully applied the method to the analysis of P.C.s in wine samples with the recoveries from 72.8 to 99.9%.

INTRODUCTION

Polyphenolic compounds (P.C.s) are involved in color formation in plants, and plants can also synthesize some new polyphenolic compounds to protect against pathogens in stress situations (1). Polyphenolic compounds are essential in terms of their positive effects on human health. Their antimutagenic, anticarcinogenic, antioxidant, anti-inflammatory, antiallergic, and antipathogenic properties are reported (2, 3). Wine is a significant natural antioxidant source when compared to other alcoholic beverages (4).

Because of the complexity of the sample matrix, literature mentions of sample pretreatment techniques, including liquid-liquid extraction (LLE), solid-phase extraction (SPE), stir bar sorptive extraction (SBSE), and solid-phase microextraction (SPME) before their chromatographic detection for extraction and preconcentration of polyphenolic compounds in wine samples (5-8). SPME, an equilibrium-based sample pretreatment technique, provides a single-step analysis by integrating sampling, extraction, preconcentration, and sample introduction for the target analytes. The main advantages of SPME are fast mass transfer, its solvent-free, and simple nature, and lower sample volume (9). Enrichment of organic compounds from

different kinds of sample matrices is essential, and so new coatings must be developed on fibers for SPME. Literature suggests the orientation of the preparation of new coating materials to the selectivity performance to target analytes as well as the development of more durable, firmer, and highly consistent supports (10).

Graphene oxide (G.O.) obtained by oxidation of epoxy (C-O-C), hydroxyl (C-OH), carbonyl (C=O) and carboxylic acid (COOH) groups from basal and surface corners of a single graphite layer has excellent properties such as large surface area, excellent structure, and good chemical stability (11). Recently, graphene oxide-based materials have been progressed and used as coating materials in the sample preparation technique such as SPME, SPE because of its facile functionalization (12, 13). Ionic liquids (I.L.s), being potential environmentally friendly solvents, have a great attraction in separation science due to low volatility, high viscosity, excellent thermal stability, and high polarities (14). Besides, its use as stationary phase in liquid chromatography, gas chromatography, and capillary chromatography, and extraction material in micro liquid extraction techniques, I.L.s have been preferred in SPE and SPME as sorbent and coating material both alone and grafted on different supports such as graphene oxide and carbon nanotubes to improve extraction efficiency and stability (15-18).

So far, liquid chromatography-diode array detector (LC-DAD) and liquid chromatography-mass spectrometry (LC-MS) techniques have been reliable solutions for the determination of P.C.s. However, rarely has gas chromatography-mass spectrometry (GC-MS) been preferred due to the need for derivatization for polar P.C.s. In the literature, direct methods (19,20), solid supported-LLE (SS-LLE) (5), SPE method using molecularly imprinted polymers (6), and SPME method using commercial polyalcoholic fiber (P.A.) (8) are available. Along with them, application of carbowax-templated resin (CW/TPR) (21), polystyrene-divinylbenzene-polyacrylonitrile (PS-DVB-PAN) coated fiber (22), and poly(ionic liquid)-based molecularly imprinted polymer (PIL-MIP)-coated fiber (23) before L.C. to wine, fruit juices, and beer samples. Many extraction techniques including LLE (24), liquid-liquid microextraction (LLME) (25), SPE using Oasis MAX cartridges (26), SPSE using polydimethylsiloxane (PDMS) coated stir bar (27), SPME with P.A. fiber (28- 30) are present for the determination of P.C.s in wine, fruit juice, and medicinal plant samples before GC-MS/FID or multidimensional GC-MS techniques. Despite the most probable preference of L.C. methods, G.C. can also be a choice for the determination of P.C.s in a complex sample matrix. GC-MS technique has some advantages such as better chromatographic separation using a capillary column, improved detectability, lower matrix effects, more accurate result, cost-effectiveness, and a more straightforward interface concerning LC-MS.

References concerning the SPME-GC-MS method using only commercial fibers are available for some polyphenolic compounds such as resveratrol, gallic acid, caffeic acid, p-coumaric acid, ferulic acid, syringic acid, and protocatechuic acid (28-30). SPME fibers coated with graphene oxide modified with ionic liquids having vinyl- and benzylimidazolium by sol-gel technique have been performed and used for extraction and determination of phthalate esters and polyaromatic hydrocarbons in water and coffee samples in our previous studies (18, 32). In this work, the extraction performance of coating possessing graphene oxide modified with amino-terminated methylimidazolium cation was evaluated for the analysis of twelve P.C.s (syringic acid, protocatechuic acid, cinnamic acid, p-coumaric acid, sinapinic acid, ferulic acid, caffeic acid, quercetin, kaempferol, chlorogenic acid, resveratrol, and gallic acid) in wine using direct immersion-SPME coupled with GC-MS. The graphene oxide-(1-(3-aminopropyl)-3-methylimidazolium bis(trifluoromethylsulfonyl)imide) (GO-[APMIM][NTf$_2$]) coated fiber was prepared with layer by layer coating technique onto the surface of stainless steel wire using a crosslinker agent. We performed the derivatization of P.C.s using trimethylsilyl (TMS) reagents on the fiber. The authors compared the efficiency of GO-[APMIM][NTf$_2$] coated fiber with commercial fibers and G.O. coated fiber. Finally, we applied the optimized SPME method for the determination of P.C.s in real white, red, and fruit wine samples.

MATERIALS AND METHODS

Chemicals and Materials
We procured the P.C. standards (caffeic acid, gallic acid, quercetin, kaempferol, chlorogenic acid, resveratrol, syringic acid, protocatechuic acid, cinnamic acid, p-coumaric acid, sinapinic acid, and ferulic acid) from Alfa Aesar (Karlsruhe, Germany). We also purchased ethyltrimethylsilane (ETMS), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), and trimethylchlorosilane (TMCS) as derivatization compounds from the same vendor. 1-Methylimidazole, 3-bromopropylamine hydrobromide, bis(trifluoromethane) sulfonimide lithium (LiNTf2), and graphene oxide (2 mg/ mL, dispersion in water) were obtained from Sigma-Aldrich (St. Louis,USA). We purchased the modified dihydroxyethylene urea as a cross-linking from Hunstman (Utah, USA). All other used reagents were of analytical reagent grade. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, USA). We procured a manual SPME holder, amber glass vials (20 mL) with screw caps and polytetrafluoroethylene/ silicone septa, and polyacrylate (PA, 85 μm), divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm),

carboxen/polydimethylsiloxane (CAR/PDMS, 85 μm) and carbowax/ polyethylene glycol (CW/PEG, 60 μm) fibers from Supelco (Bellefonte, USA). Using a 5 μL microsyringe from Hamilton (Reno, USA), we produced the SMPE fibers and obtained a stainless steel wire having O.D. 150 μm from a local market (Istanbul, Turkey).

Stock standard solution of 1000 mg/L of each P.C.s was prepared using methanol and stored at -18 ºC. The intermediate solution of the mixture standard of P.C.s was prepared at 200 mg/L in methanol and stored at 4 ºC. We prepared the pH 8 buffer solution using 1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl).

The analyzed samples, including red wine, white wine, and fruit wines, were purchased from local supermarkets in Izmir. The alcohol content was in the range of 12%-13.8% for the studied wines. The bottles of wine were stored at 4 ºC and protected from light before analysis. The authors prepared synthetic wine solutions including 12% (v/v) of ethanol with pH adjusted to 3.5 by tartaric acid for optimization and performance evaluation of the DI-SPME method.

Instrumentation
We performed the G.C. analysis on a Trace 1300 gas chromatograph QP2010 equipped with an ISQ QD single quadrupole mass spectrometer and split/splitless injector (Thermo Scientific, USA). A TG-5MS fused silica capillary column (30.0 m × 0.25 mm I.D., 0.25 μm film thickness) supplied from Thermo Scientific (West Palm Beach, FL, USA) was used. High purity helium was employed as the carrier gas, at a flow rate of 1.2 mL min-1. We employed the following separation temperature program in G.C. for TMS derivatives: initially oven temperature held at 80 ºC for 3 min, then programmed at 10 ◦C min-1 to 220 ◦C (held for 4 min), finally increased to 280 ◦C at 20 ºC min-1 (held for 2 min). The injection port temperature was at 250 °C for GO-[APMIM][NTf2] coated SPME fiber. We carried out all injections on the splitless mode for 5 min. After each analysis, we heated all fibers at a desorption temperature for 5 min in the extra G.C. injection port to prevent carry-over effects. The authors operated the MS in the electron impact (E.I.) at 70 eV under selected ion monitoring mode (SIM) by monitoring two relevant m/z fragments for TMS derivatives given in Table S1. The GC-MS ion source and interface were set at 250 ºC and 280 ºC, respectively. Figure 1 gives the chromatogram of P.C.s and their M.S. spectra.

The Fourier transform infrared (FTIR) spectra of G.O. and GO-[APMIM][NTf2] were obtained by using a Thermo Fisher Scientific Nicolet iS10 model FTIR Spectrometer. We employed thermogravimetric analysis (TGA) for investigation of their thermal properties. The TGA was performed between 30 and 450 °C at a rate of 10°C/min under a nitrogen atmosphere with a Perkin–Elmer Diamond TG/DTA instrument. X-ray Diffractometer measurements were made by a Philips X'Pert PROBE model with a monochromatic Cu-Kα X-ray source at 2θ = 10-60°. The authors evaluated the surface morphology GO-[APMIM][NTf2] coated fiber with scanning electron microscopy (SEM, Carl Zeiss 300VP, Jena, Germany).

Derivatization Procedure
By on-fiber derivatization process, the fibers after SPME firstly were inserted into a flask, dried under nitrogen atmosphere for 5 min and then transferred into the headspace of a 1.5 mL glass vial containing 20 μL of BSTFA: TMCS (9:1, v:v) solution and held at 60 °C for 15 min.

Preparation of the Fiber
After etching of stainless steel wire as previously described (18), the tip of wire was dipped into dihydroxyethylene urea cross-linking reagent for 1 h, then inserted into G.O. dispersion in water for 30 min. These steps were repeated for three times for efficient coating. We dipped the G.O. coated fiber vertically into the 0.02 g [APMIM][NTf2], which was synthesized according to the previous methods given in the literature (18, 33), in 5 mL of methanol for 2 h at room temperature. This step was repeated in sextuplicate to increase the thickness of the fiber coating. Then, the home-made fiber prepared was dried at room temperature overnight and conditioned in sextuplicate in the injection port of G.C. at 250 °C for 5 min under nitrogen atmosphere.
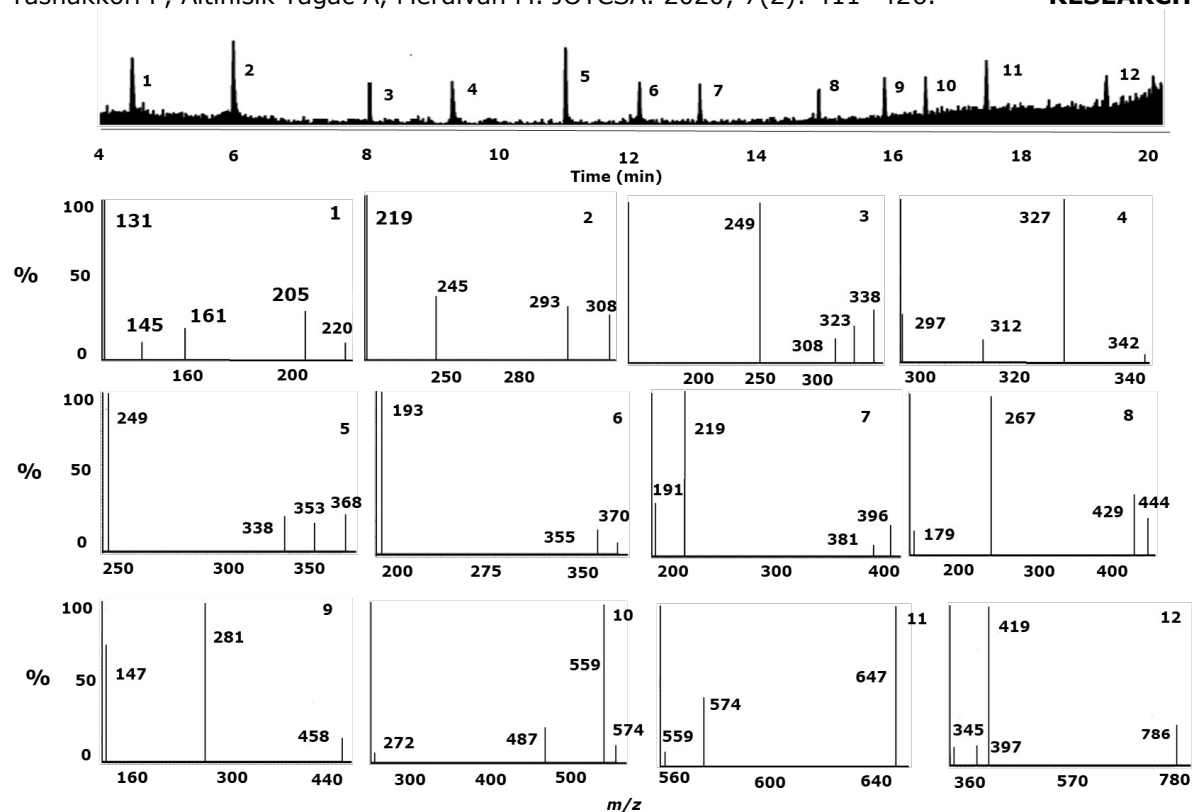
Figure 1. Chromatogram of P.C.s standards and M.S. spectra of P.C.s by the proposed method. Peak identification: (1) cinnamic acid, (2) p-coumaric acid, (3) ferulic acid, (4) syringic acid, (5) sinapinic acid, (6) protocatechuic acid, (7) caffeic acid, (8) resveratrol, (9) gallic acid, (10) kaempferol, (11) quercetin, (12) chlorogenic acid.

The characterization results of GO-[APMIM][NTf2] coating material by FTIR, XRD and T.G. were shown in Figure S1. FTIR (cm-1): 2914, 1847, 1578, and 1456. XRD (2θ) = 23.67°, 38.17° and 40.51°. TG (25-450°C): from 100% to 70% weight loss.

DI-SPME Procedure for Analysis of P.C.s

Firstly, synthetic wine or wine sample solution was prepared by adjusting pH to 8 and placed into a 20-mL amber glass vial. The vial was placed in a metallic block on a magnetic heater. After equilibration for 5 min, the 1 cm tip of the GO-[APMIM][NTf2] coated SPME fiber was exposed to the test solution at adjusted temperature for a particular time while stirring at 400 rpm using a stirring bar. Extractions were performed using a metallic block and a heater with a magnetic stirrer. After extraction, the fiber was dried under a nitrogen stream for 5 min, derivatized by BSTFA:TMCS mixture, and inserted into GC-MS for analyzing P.C.s. Because of the complex composition of wine, we have used two home-made fibers prepared in the extraction experiments. Using one prepared GO-[APMIM][NTf2] coated fiber, we were successful to perform 150 injections with no remarkable decrease in recovery and repeatability.

RESULTS AND DISCUSSION

Optimization of the Derivatization Process

G.C. analysis needs a derivatization procedure for hydroxyl functional groups. The most commonly used derivatization reagents are TMS reagents containing N-methyl-N-(trimethylsilyl)trifluoroacetamide), N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide, BSTFA and TMCS for derivatization of polyphenolic compounds and their metabolites (25, 28, 34, 35). TMS reagents are hydrolyzed in aqueous solutions. Because of this, the SPME fiber after extraction was dried under nitrogen atmosphere. Therefore, drying time for fiber after extraction, suitable derivatizing reagent and volume, and time and temperature of derivatization were investigated. The optimal conditions to get the highest signal came to an agreement at 60 °C for 15 min using 20 µL of BSTFA: TMCS (9:1) after drying the fiber for 5 min under nitrogen gas for all P.C.s studied following the extraction.

Optimization of the SPME Conditions

SPME experimental conditions such as sample pH, ionic strength, extraction temperature, and extraction time were investigated to obtain reproducible results with high extraction performance of the GO-[APMIM][NTf2] coated SPME

fiber for P.C.s by using DI-SPME method. We carried out all the experiments in triplicate using synthetic wine sample solutions containing 100 µg/L P.C.s standard and reported the mean values.

Sample pH and Ionic Strength
The pH of a sample solution affects the retention of analytes on the surface of the coating material of SPME fiber. It is possible to observe an interaction between the analyte and the sorbent when the polarities of them are close to each other. The pH of the sample is adjusted to obtain reproducible extraction efficiency, taking into account the pKa of analytes. Depending on the pKa values of P.C.s, we performed the extraction experiments to control the effect of pH at pH 4 and 8. The authors saw that the extraction efficiencies of P.C.s studied were higher at pH 8 except syringic acid and gallic acid (Figure 2A). The reason is that the P.C.s studied were ionized mainly at pH 8 and the sorption between the ionized P.C.s and imidazolium cation took place by electrostatic interaction. Besides, the aromatic ring and –O.H., -OCH3 and -COOH groups in P.C.s caused dipole-dipole and π-π interactions during the sorption to the surface of GO-[APMIM][NTf2] coated fiber. The time of analysis is optimal at pH 8 and selected for further studies because the peak areas of most P.C.s studied were high at pH 8.

In HS-SPME and rarely in DI-SPME methods, increasing the ionic strength of the aqueous solution improved the peak areas, favoring the extraction of analytes into the fiber and solubility of the extracted compounds. Thus, we investigated the impact of ionic strength on the uptake of P.C.s after exposure to the fiber at three different NaCl concentrations (0%, 10%, and 25%, w/v). As seen in Figure 2B, the addition of NaCl caused a slight increase in the peak areas of P.C.s (10%, w/v) initially and then led to a more decrease in the extraction efficiency. The presence of a high amount of salt may cause an increase in the viscosity of the solution, which decreases the mass transfer rate and affects the peak areas of P.C.s negatively. So, we used 10% NaCl solution as a test for further experiments.

Extraction Temperature and Extraction Time
Extraction temperature is an essential factor for the extraction efficiency. It influences the mass transfer and affects the extraction time in the SPME method. Therefore, we investigated the effect of extraction temperature on the extraction efficiency of P.C.s by exposing the fiber to the sample solution at 30 and 70 °C for 30 min. Figure 2C shows the temperature profiles obtained. The authors achieved the highest extraction efficiency at 30 °C for all P.C.s. Increasing temperature caused a decline in the peak areas of P.C.s. This event may be explained by that the high temperature causes a rapid motion of neutral or ionized P.C.s but decrease the diffusion of P.C.s on fiber coating due to the exothermic extraction process (36). The researchers performed subsequent experiments at 30 °C for the home-made fiber because of the maximum extraction efficiency at this temperature.

The extraction time deals with the interaction of analytes in solution and fiber coating in SPME. The extraction time was investigated from 15 to 90 min at 30 °C by stirring at 400 rpm to acquire the adsorption equilibrium for P.C.s on the fiber surface. As shown in Figure 2D, the peak areas of all P.C.s reached the maxima in 30 min. Thus, considering the extraction efficacy and the analytical time, 30 min was chosen as the optimized fiber exposure time.
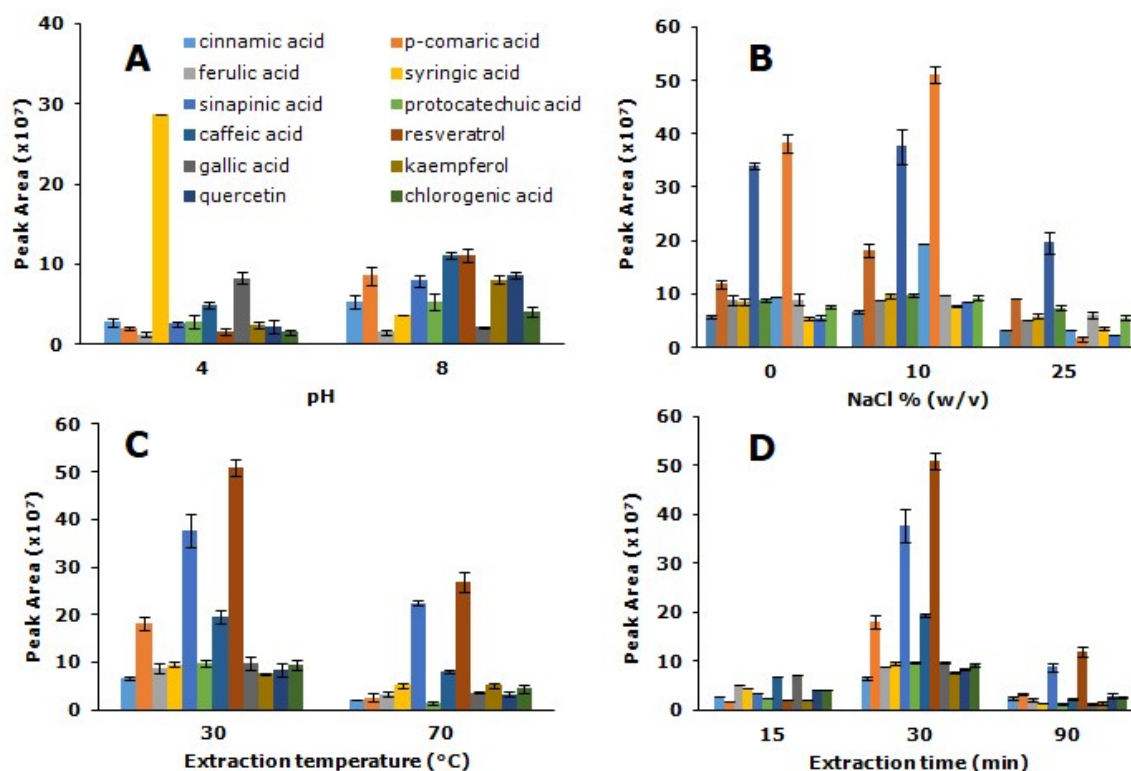
Figure 2. Effects of pH of sample solution (A), ionic strength (B), extraction temperature (C), and extraction time (D) of GO-[APMIM][NTf2] fiber on the DI-SPME method.

Comparison of Extraction Efficiency of GO-[APMIM][NTf₂] Coated Fiber with Commercial Fibers and G.O. Coated Fiber

DI-SPME method was also optimized for P.A., DVB/CAR/PDMS, CAR/PDMS, and CW/ PEG commercial SPME fibers to compare their extraction efficiency with the GO-[APMIM][NTf₂] coated fiber. These fibers were conditioned on the injection port of G.C. before the extraction experiments at 280 °C, 270, 300, and 240 °C for P.A., DVB/CAR/PDMS, CAR/PDMS, and CW/ PEG for 30 min according to the manufacturer recommendation, respectively. We carried out the SPME extraction experiments in the range of 15- 60 min with 100 μg/L P.C.s solution at pH 8 and 400 rpm to obtain the optimum extraction time. The authors obtained the highest extraction efficiency as 30 min for all commercial SPME fibers.

We studied the extraction temperature at 30 and 70 °C at the optimized extraction time. The experimental results indicated that the optimum extraction temperature was 30 °C for all commercial fibers. Although the optimal extraction parameters of commercial fibers are the same as the GO-[APMIM][NTf₂] coated fiber, the extraction efficiency of the home-made coated fiber was much higher than those commercial fibers (Figure 3). Within the commercial SPME fibers, the peak areas of cinnamic acid and kaempferol with P.A. fiber and the peak area of cinnamic acid with CW/PEG fiber were only higher than that of the home-made fiber. As seen in Figure 2, the presence of [APMIM][NTf₂] caused much increase in the extraction efficacy of P.C.s with respect to only G.O. coated fiber.
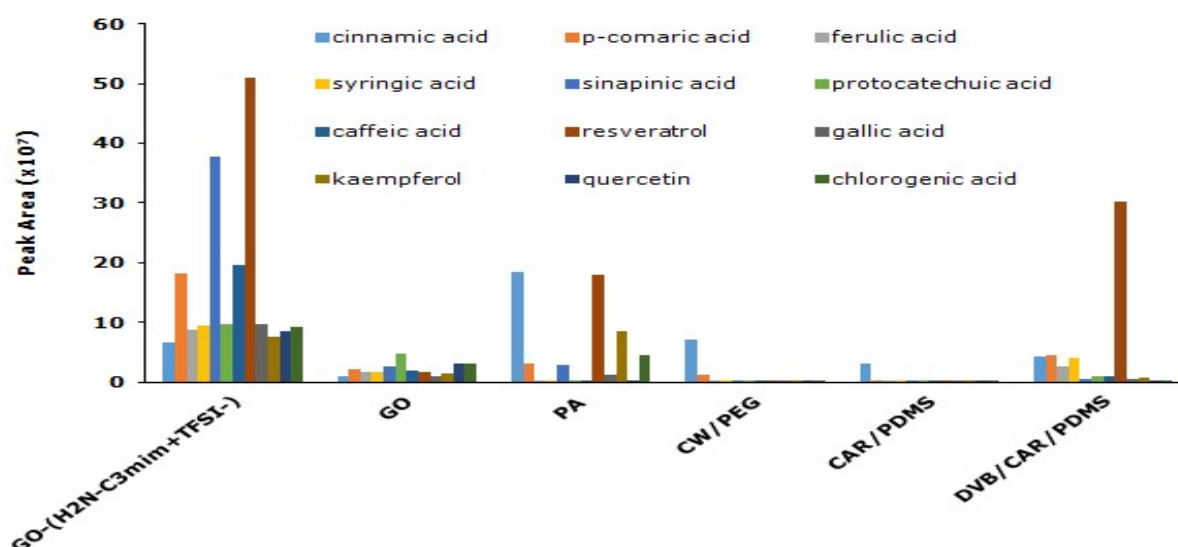
Figure 3. Comparison of the extraction efficiencies of GO-[APMIM][NTf$_2$] coated fiber with G.O. coated and P.A., CAR/PDMS, DVB/CAR/PDMS, and CW/ PEG commercial fibers. Conditions: C$_{PCs}$ = 100 µg/L; sample pH = 8; stirring rate = 400 rpm; extraction temperature = 30 °C; extraction time = 30 min.

Table 1. Analytical figures of merit for GO-[APMIM][NTf2] coated fiber in DI-SPME-GC-MS method.

PCs	Linear range (µg/L)	R2	LOD (µg/L)	Precision (RSD, %)	Fiber-to-Fiber (RSD, %)
Cinnamic acid	0.1-1000	0.998	0.05	2.48-11.37	2.39
p-Coumaric acid	0.25-1000	0.998	0.1	2.22-9.23	2.79
Ferulic acid	0.25-1000	0.998	0.1	2.57-9.68	2.18
Syringic acid	0.1-1000	0.999	0.02	1.32-14.82	4.68
Sinapinic acid	0.1-1000	0.997	0.02	0.71-13.29	3.04
Protocatechuic acid	0.1-1000	0.997	0.02	0.92-8.42	5.71
Caffeic acid	0.25-1000	0.997	0.05	3.48-14.74	1.64
Resveratrol	0.1-1000	0.997	0.02	0.93-10.53	4.79
Gallic acid	0.25-1000	0.997	0.02	0.90-9.85	2.33
Kaempferol	0.1-1000	0.997	0.02	0.78-9.85	5.87
Quercetin	0.1-1000	0.998	0.02	3.14-9.24	4.06
Chlorogenic acid	0.1-1000	0.997	0.02	2.73-11.92	2.25

Table 2. Comparison of the proposed method with previous SPME and other extraction methods for determination of P.C.s studied.

	Coating Material/ Sorbent	Extraction type	Sample	PCs	LR (µg/L)	LOD (µg/L)	RSD (%)	Recovery (%)	Refs
LC-DAD	MIP	SPE	wine	GA PrA	10-70* 0.1-4.5	0.4* 0.1*	6.4-8.0 7.0-8.1	89.1-98.3 95-100	(6)
GC-MS		LLME	plasma	Phenolic acids	10-5000	0.5-16.9	3.8-18.4	80-110	(25)
LC-FLD	PDMS CW/TPR	SBSE SPME	Wine, must, fruit juice	trans-res trans-res/ cis-res	0.5-50 5-150/ 2-150	0.1 2/0.5	6.9 5.3/4.8	82-105	(21)
LC-DAD	PA	SPME	Wine, spirit, grape juice	res	0.1-500	0.4	6.5-12.6	92.2-99.4	(8)
GC-MS	PDMS stir bar	SBSE	Wine	res, picetannol	0.2-1	0.004-0.015	5-9	79-109	(27)
GC-MS	PA	SPME	Wine and grape	res	1-150	0.09	2.4	85-116	(28)
LC-DAD	PIL/MIP	SPME	Fruit juice and beer	CA FA	0.1-200 0.05-200	0.019-0.024 0.011-0.042	2.3-8.2 4.6-8.0	80.1-111 72.1-109	(23)
LC-MS/MS	PS-DVB-PAN	SPME	Wine, berry, grape	CA res	1.5-500 5-500	0.5 1.5	5 4	82 77	(22)
Multidimensional GC-MS	PA	SPME	Wine	res	10-5000	7.08	3.0-9.2	72.7-94.7	(29)
GC-MS	Oasis MAX	SPE	Wine	trans-res	up to 2500	0.24	8	92.5-108.2	(26)
GC-FID	PA	SPME	Synthetic solution	pCuA, SyA, PrA, FA, CA, GA	2.2-354.4	0.01-1.77	9.78-17.89	-	(30)
GC-MS	GO-[APMIM][NTf2]	SPME	Wine	CnA, SyA, SnA, PrA, Res, Kfl, Qcn, ChA pCuA, FA, CA, GA	0.1-1000 0.25-1000	0.02-0.05 0.02-0.1	0.78-11.92 0.90-14.74	72.8-99.9 80.7-99.8	This study

LC-FLD: Liquid chromatography-fluorescence detector MEPS: Microextraction packed sorbent,
CnA: Cinnamic acid, SyA: Syringic acid, SnA: Sinapinic acid, PrA: Protocatechuic acid, Res: resveratrol, Kfl: Kaempferol, Qcn: Quercetin, ChA: Choloregenic acid, pCuA: p-Coumaric acid, F.A.: Ferulic acid, CA: Caffeic acid, GA: Gallic acid. *** µg/mL**

Surface Morphology of GO-[APMIM][NTf2] Coated Fiber

We characterized the surface morphology of the G.O. and GO-[APMIM][NTf2] material by SEM (Figure 4). In Figure 4a, it is evident G.O. sheets seem the sheet-like structure with a smooth surface and wrinkled edge. After a combination with I.L. (Figure 4b), the coating material had a rougher surface, which pointed out that I.L. stacks assembled on the surface of the G.O. layers. The GO-[APMIM][NTf2] is porous with a much rougher surface, which indicates that the coating material has a large surface area. Figure 4c-d shows that the coating possessed a homogeneous and porous structure. The porous structure of the coating could have increased the available surface area of the fiber, as well as its extraction ability. From the SEM images in Figure 4c-d, the coating thickness was determined as 27.5 µm for the GO-[APMIM][NTf2] coating material.
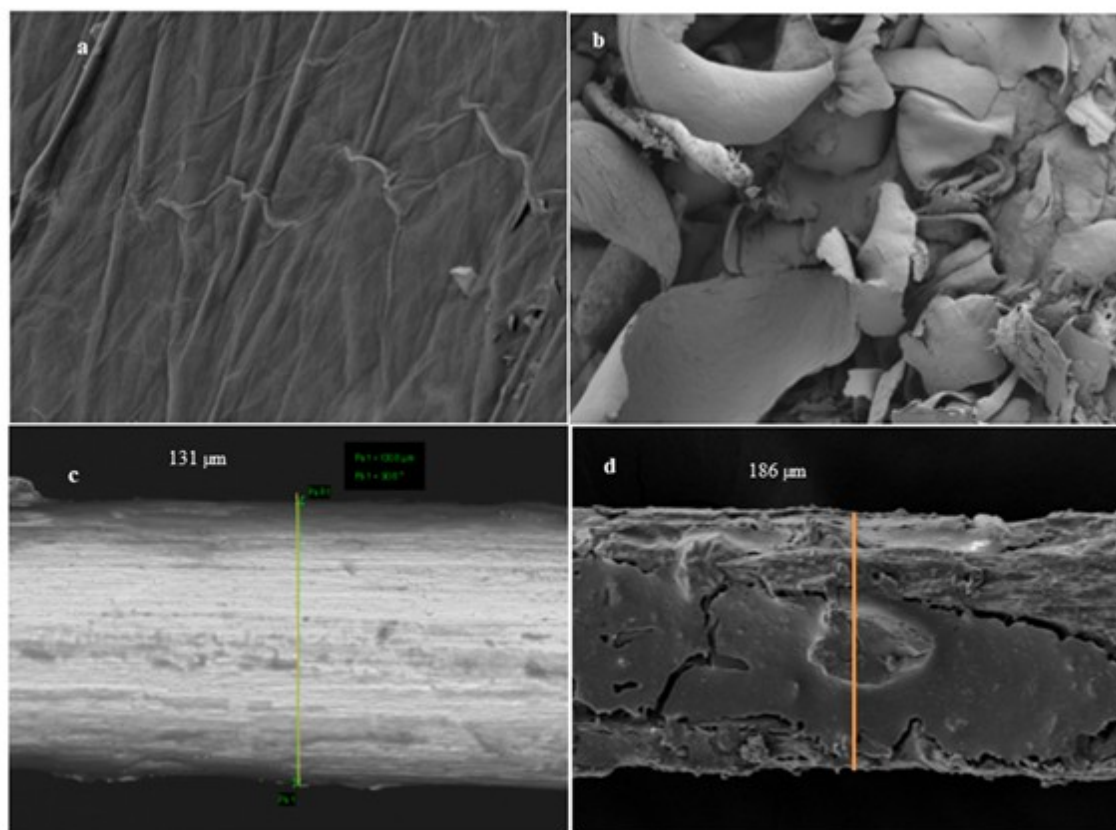

Figure 4. SEM images of GO, 2 µm, 10000 X (a); GO-[APMIM][NTf2] 20 µm, 500 X (b); etched stainless steel fiber, 20 µm, 1000 X (c); GO-[APMIM][NTf2] fiber, 20 µm, 1000 X (d).

Application to Real Sample Analysis

The proposed GO-[APMIM][NTf2] coated fiber was used for the analysis of twelve P.C.s in red wine, white wine, and fruit wine samples by DI-SPME-GC-MS method (Table 3). By SPME, known as a non-exhaustive method, the amount of analytes found in the sample represents the free concentration of analytes (20). We examined the recoveries of 12 P.C.s by spiking 5 and 200 µg L-1 concentration of P.C.s to the wine samples to evaluate the accuracy of the proposed DI-SPME-GC-MS method with the home-made coated fiber. As shown in Table 3, we acquired the recoveries of P.C.s in the range of 75.4- 99.8 % for red wine, 75.2- 99.9 % for white wine, and 71.2- 99.7 % for fruit wines with the RSDs less than 13.71% depending on the P.C.s and samples. Figure 5 shows the typical chromatograms of fruit wines, white wine, and red wine samples as blank and red wine spiked of P.C.s standard.
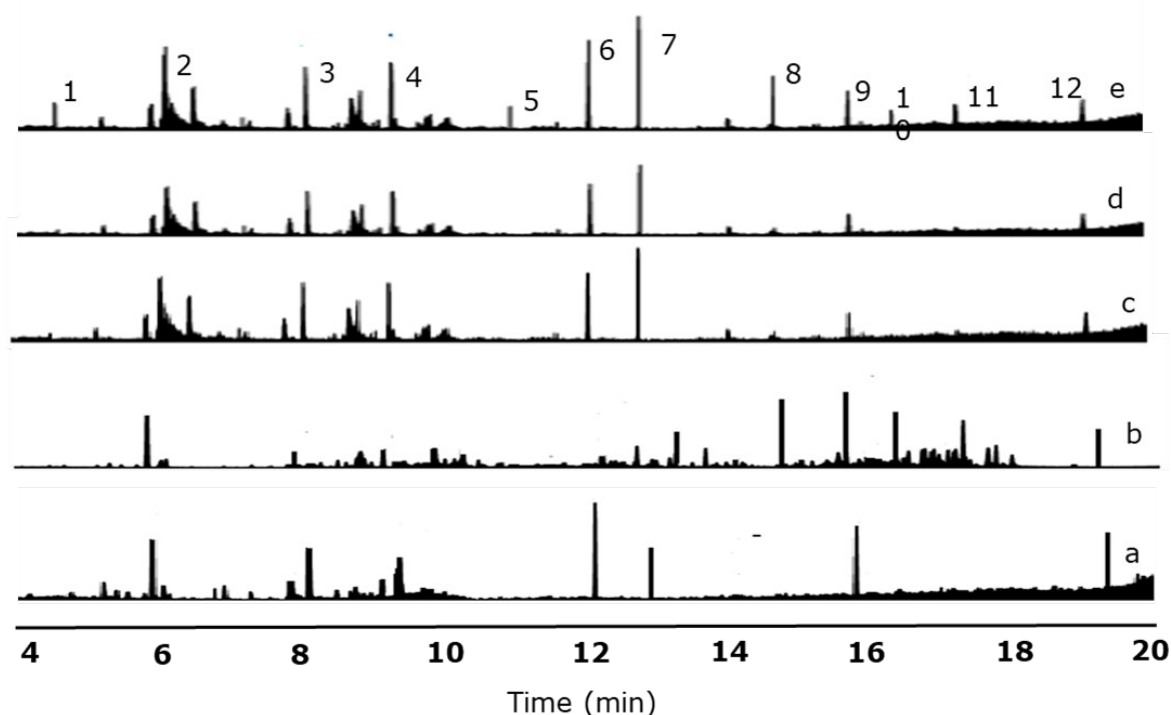
Figure 5. Chromatograms of P.C.s for wine samples by the proposed method. (a) blank melon wine, (b) blank blueberry wine, (c) blank white wine, (d) blank red wine, and (e) blank red wine spiked with P.C.s at 20 µg L-1. Peak identification: (1) cinnamic acid, (2) p-coumaric acid, (3) ferulic acid, (4) syringic acid, (5) sinapinic acid, (6) protocatechuic acid, (7) caffeic acid, (8) resveratrol, (9) gallic acid, (10) kaempferol, (11) quercetin, (12) chlorogenic acid.

CONCLUSION

The GO-[APMIM][NTf2] coated fiber was prepared and successfully applied for the determination of P.C.s in wine samples by the proposed DI-SPME under optimized conditions (30 °C, 30 min, 5 min desorption) and on-fiber derivatization (20 min) combined to GC-MS method. The developed home-made fiber exhibits high durability, excellent thermal behavior, high fiber-to-fiber reproducibility, and long term stability without a reduction in the extraction performance after more than 150 extraction cycles. Also, the extraction efficiency of the GO-[APMIM][NTf2] coated fiber was much better than the studied commercial fibers (P.A.,

CAR/PDMS, DVB/CAR/PDMS, and CW/PEG). The prepared fiber presents a wide linear range, low LODs, and excellent repeatability and reproducibility in the determination of the P.C.s in different kinds of wine samples. Besides, by using the home-made SPME fiber, good recoveries for the analysis of P.C.s were succeeded in the wine samples.

The performance of the present coating material could be due to electrostatic interactions between imidazolium cation and the P.C.s, as well as the п-п and dipole-dipole interactions with G.O. and I.L., and the P.C.s. Thus, GO-[APMIM][NTf2] coated fiber can be taken into account as SPME fiber for the extraction of P.C.s in various kinds of food samples.

Table 3. Analytical results of P.C.s* in wine samples (n= 3).

Wine samples	Added (µg/L)		CnA	pCuA	FA	SnA	CA	ChA	SyA	PrA	GA	Kfl	Qcn	Res
Red	0	Found	58.3±7.1	255.7±0.3	382.0±0.3	ND	2.8±0.2	108.0±0.6	270.0±0.6	1.4±0.8	194.0±3.2	233.012.3	103.7±0.9	81.5±0.6
	5	RR,RSD(%)	96.7±6.13	97.6±9.7	97.5±8.4	75.4±7.2	84.0±8.9	98.9±2.4	99.7±3.9	91.6±7.1	99.8±6.3	99.5±10.6	99.2±6.8	98.9±0.2
	200	RR,RSD(%)	95.2±5.2	96.8±7.2	96.4±3.3	79.2±4.9	87.5±1.5	89.9±2.5	96.8±1.0	92.5±6.6	90.1±5.6	98.1±6.2	94.3±5.4	97.6±1.0
White	0	Found	2.9±0.7	69.6±0.9	85.1±0.4	ND	308.0±1.4	7.8±2.7	54.8±0.2	496.0±0.1	491.0±4.9	ND	ND	ND
	5	RR,RSD(%)	89.4±5.3	95.18±.3	99.0±12.4	75.22±.8	99.6±1.8	86.0±9.4	98.1±6.6	99.9±4.0	99.9±7.8	83.5±10.1	80.5±8.3	80.0±4.9
	200	RR,RSD(%)	91.8±2.5	93.1±5.8	91.9±1.5	78.4±2.4	97.4±3.8	92.9±6.5	93.4±1.3	97.3±0.9	97.3±4.0	88.9±8.1	88.7±5.8	84.7±0.6
Black mulberry	0	Found	ND	238.0±0.1	301.0±0.2	ND	415.5±2.7	447.5±0.1	280±0.5	1.6±0.4	407.0±0.8	ND	338.0±1.8	ND
	5	RR,RSD(%)	86.4±,5.0	99.4±6.8	99.6±3.1	76.6±9.2	96.5±5.6	99.6±5.7	99.5±6.6	81.6±5.4	99.6±2.4	78.2±4.4	99.5±1.1	86.2±10.2
	200	RR,RSD(%)	92.60±0.8	93.6±0.2	93.8±3.5	80.9±0.9	93.3±3.6	95.1±1.2	93.4±0.3	83.7±3.2	92.9±2.9	89.9±2.1	94.7±0.3	88.8±5.4
Blueberry	0	Found	ND	107.0±0.8	ND	ND	415.5±4.5	3.7±0.5	ND	1.7±0.2	416.0±2.1	448.5±7.9	469.0±6.7	482.0±0.2
	5	RR,RSD(%)	82.2±8.3	88.7±8.3	80.7±8.1	77.6±3.1	99.5±5.2	82.2±6.3	80.0±10.5	79.6±8.4	99.6±8.2	99.6±6.9	99.7±7.8	99.7±4.5
	200	RR,RSD(%)	88.5±4.6	86.1±5.1	82.4±4.1	85.1±2.3	96.8±6.5	91.0±4.7	83.8±4.3	83.2±5.2	86.1±0.9	86.2±5.0	87.1±1.9	83.0±3.5
Melon	0	Found	ND	371.0±1.8	311.0±2.0	ND	453.0±0.1	294.0±0.2	272.0±4.5	441.0±7.9	312.0±0.8	ND	ND	ND
	5	RR,RSD(%)	81.8±4.63	99.7±1.3	99.8±1.5	73.4±7.7	99.8±0.9	99.6±1.8	99.6±3.9	99.9±0.6	99.8±1.8	84.2±4.8	84.4±5.1	81.6±1.4
	200	RR,RSD(%)	93.7±1.3	95.7±0.5	95.9±0.8	89.9±0.1	96.4±0.7	95.1±0.8	92.4±0.7	96.0±0.6	91.4±0.9	93.5±1.1	88.5±1.2	96.5±2.8
Red plum	0	Found	ND	448.0±8.5	364.0±4.2	ND	957.0±6.6	314.0±4.2	ND	ND	ND	398.0±8.5	975.0±10.6	ND
	5	RR%,RSD	84.6±4.8	99.7±1.3	99.5±6.0	75.2±1.4	99.8±6.8	99.4±3.96	78.8±8.4	72.8±7.2	77±.64.1	99.±51.9	99±.89.2	76.2±6.6
	200	RR%,RSD	91.0±4.4	98.3±1.4	93.1±3.5	85.1±4.4	95.1±0.8	97.4±3.0	89.5±7.9	83.0 ±3.2	81.7±2.7	96.6±2.2	97.0±0.5	93.3±4.3

[a]Values are mean ± standard deviation

[b]RR : Relative recovery; ND: not detected

* P.C.s; CnA: Cinnamic acid, SyA: Syringic acid, SnA: Sinapinic acid, PrA: Protocatechuic acid, Res: t-resveratrol, Kfl: Kaempferol, Qcn: Quercetin, ChA: Chlorogenic acid, pCuA: p-Coumaric acid, F.A.: Ferulic acid, CA: Caffeic acid, GA: Gallic acid

SUPPORTING INFORMATION SUMMARY

Details of parameters of SIM mode for P.C.s after derivatization and characterization studies of GO-[APMIM][NTf2] coating material are given in the supporting information.

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Graphene Oxide-Ionic Liquid Used as Solid-Phase Microextraction Coating for Polyphenolic Compounds' Extraction and Determination with GC-MS After On-Fiber Derivatization in Wine

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Supporting Information

Table S1. Parameters of SIM mode for PCs after derivatization.

Compound	Molecular weight	TMS groups	TMS derivatized molecular weight	t_R (min)	Characteristic fragments[a]
Cinnamic acid	148	1	220	4.48	<u>131</u>, 161, 205, 145, 220
p-Coumaric acid	164	2	308	6.01	<u>219</u>,293,245,308
Ferulic acid	194	2	338	8.07	<u>249</u>,323,338,219
Syringic acid	198	2	342	9.30	<u>327</u>,342,312,297
Sinapinic acid	224	2	368	11.01	<u>353</u>,368,338,249
Protocatechuic acid	154	3	370	12.13	<u>193</u>,370,355,73
Caffeic acid	180	3	396	13.04	<u>291</u>,396,381,73
Resveratrol	228	3	444	14.71	<u>267</u>,179,429,444
Gallic acid	170	4	458	15.82	<u>281</u>,147,179,458
Kaempferol	286	4	574	16.44	<u>487</u>,574,559,272
Quercetin	302	5	662	17.35	<u>647</u>,574,559,662
Chlorogenic acid	354	6	786	19.16	<u>419</u>,786,397,345

[a] Quantitations are underlined.

Characterization of GO-[APMIM][NTf $_2$] coating material

In the FTIR study (Figure S1(A)), as well as the main peaks of GO, the presence of IL was verified by the peaks at 2914, 2847, 1578 and 1456 cm^{-1} corresponding to the stretching vibrations of C-H in imidazole ring, aliphatic groups, C-N and C=N groups in imidazole ring, respectively. In thermal gravimetric analysis curves (Figure S1(B)), GO-[APMIM][NTf$_2$] coating with fewer thermally labile oxygen functional groups has a mass loss in the range of 200-450°C at the low slope (from 95% to 70% weight loss) beside the moisture loss at 150 °C. In XRD powder patterns (Figure S1(C)), the appearance of new peaks at 2θ = 23.67°, 38.17° and 40.51° explain the exfoliation of GO due to the removing of water molecules and the oxide groups and interaction with IL.
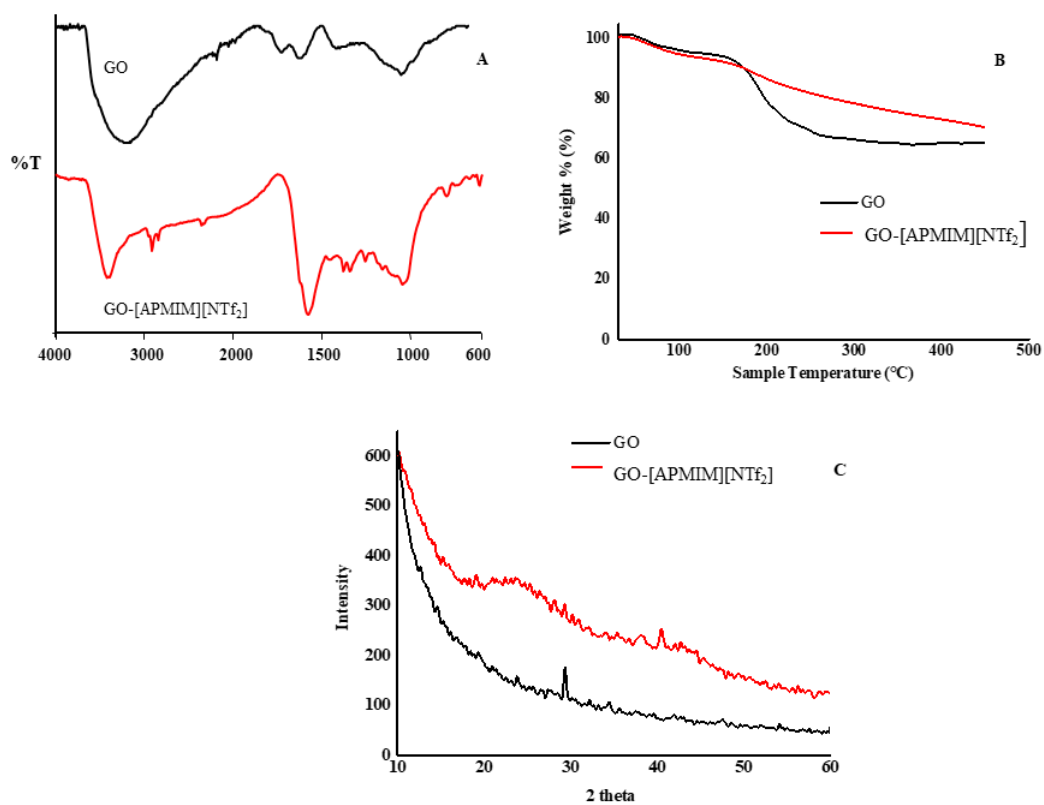
Figure S1: FTIR spectra (A), thermogravimetric analysis (B), and XRD patterns of GO and GO-[APMIM][NTf$_2$] (C).

Eco-friendly Synthesis of Quinoxaline Derivatives Using Mineral Fertilizers as Heterogeneous Catalysts

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Abstract: The synthesis of quinoxaline derivatives were heterogeneously catalyzed by phosphate-based catalyst fertilizers, MAP, DAP, or TSP. The reaction affords the desired products in excellent yields at ambient temperature. A series of studies were conducted to investigate the effect of solvent reaction, its volume, and catalyst amount. A study of the recyclability of catalysts removed from the reaction mixture by simple filtration was also carried out to find that the three phosphate-based catalysts retain their catalytic activities up to six cycles.

INTRODUCTION

Since the US Environmental Protection Agency (EPA) had developed the concept of "green chemistry" to protect human health and environment (1), enormous research efforts have been devoted to designing clean and eco-friendly chemical processes (2-4). In this area, considerable interests have focused on heterogeneous catalysis (5-7) due to its advantages to reduce energy consumption but to recover and recycle the used catalyst. Thereby, a large variety of heterogeneous catalysts have been developed and widely used to carry out numerous organic syntheses (8). Among the greener catalysts developed, phosphate-based minerals appear as the obvious choice for Moroccan researchers as they represent the first mining wealth of their country. For this reason, they contribute to the valorization of these minerals as promising catalysts. S. Sebti and co-workers were the first team to introduce natural (9), doped (10) or modified (11) phosphate not only as

heterogeneous catalysts for organic reactions but also as the support of metal catalysts (12-13).

Motivated by the desire to introduce a new generation of phosphate-based heterogeneous catalysts, our laboratory was particularly interested in the catalytic performance evaluation of some phosphate fertilizers for Knoevenagel condensation and 1-(benzothiazolylamino)-methyl-1-2-naphthol derivatives (14-15).

In continuation of our ongoing effort to extend the application of these novel fertilizer phosphates as green and reusable heterogeneous catalysts, we develop in the present paper a straightforward and efficient process to synthesize quinoxaline derivatives utilizing phosphate-based catalysts, mono-ammonium phosphate (MAP), di-ammonium phosphate (DAP), or triple superphosphate (TSP).

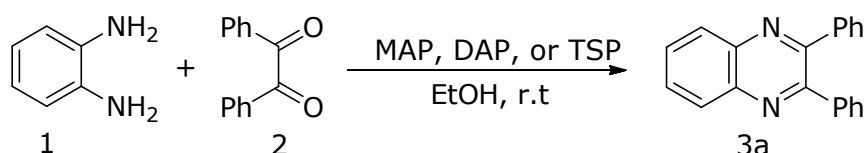The quinoxaline derivatives are a class of nitrogen-containing fused heterocyclic compounds, which

attracted considerable interest since their basic skeleton involved as a core structure of several drug molecules such as quinacillin (15), brimonidine (16), varenicline (17), clofazimine (18), echinomycin (19-20) and actinomycin (21). Some other quinoxaline-containing compounds have shown many biological properties (22), such as antimicrobial (23), antidiabetic (24), anti-inflammatory (25), antioxidant (25), anticancer (26), antileishmanial and antitrypanosomal (27) activities. Additionally, quinoxaline derivatives act as inhibitors of the aldose reductase enzyme (28) and the kinase protein (29). Furthermore, the quinoxaline structural nucleus has recently identified to possess inhibitory effects on the corrosion of some metals (30-32).

In light of their importance, numerous methods have been designed to synthesize quinoxaline derivatives. Most broadly, they involve 1,4-addition of 1,2-diamines to diazenylbutenes (33), oxidative cyclization of α-haloketones and 1,2-diamines (34), oxidative coupling of epoxides with ene-1,2-diamines (35), and condensation of 1,2-diamines with 1,2-dicarbonyl compounds (36-37). The latter method was chosen as the reaction model.

EXPERIMENTAL

Chemicals and instruments
Chemicals were purchased from Fluka or Aldrich companies. The spectral data were compared with those of the known compounds reported in the literature. Melting points were recorded on a Wagner & Munz HEIZBANK Kofler bench. Thin layer chromatography (TLC) on silica gel SIL G/UV 254 plates were used to monitor the progress of the reactions.

Synthesis of quinoxaline derivatives: General procedure
In a typical reaction, to a solution of 1,2-diamine (1 mmol) and 1,2-dicarbonyl (1 mmol) in EtOH (2 mL) was added phosphate-based catalyst MAP, DAP or TSP (0.006 g). The resulting mixture stirred at 800 rpm at ambient temperature for appropriate reaction time as indicated by TLC. The solid product formed was dissolved in hot EtOH and filtered to separate the catalyst, and the resulting filtrate was placed in ice-bath to give a pure product as crystals. All prepared products were identified as based on comparison of their melting points and their ^{1}H NMR and ^{13}C NMR data with authentic samples reported in the literature. Supplementary material includes the NMR data is joined to this paper.

Recyclability of MAP, DAP and TSP
The recyclability of the catalyst was examined using the model reaction under optimized reaction conditions. The catalyst is recovered by simple filtration at the end of the reaction, washed with hot EtOH, dried at oven at 80 °C for 6 hours, and then reused.

RESULTS AND DISCUSSION

In a preliminary study, the condensation of 1,2-diamines **1** with 1,2-dicarbonyl **2** (Scheme 1) chosen as a test reaction for the catalytic activity of catalysts MAP, DAP, and TSP. Table 1 gathers the results and noticeably displays that in the presence of catalysts, the reaction led to the expected product in higher yields (94-99%) within shorter reaction times (Table 1, entries 2-4).

Scheme 1. Condensation of 1,2-diamines with 1,2-dicarbonyl compounds.

Table 1. Results and physical characteristics of 2,3-diphenylquinoxaline **3a.**

Entry	Catalyst	Time[a]	Yield[b] (%)
1	-	60	23
2	MAP	4	94
3	DAP	2	99
4	TSP	2	96

[a] Time reported in min monitored by TLC. [b] Isolated yields.

These encouraging results prompted us to investigate the solvent effect on the reaction. The results displayed in Table 2 show that the reagents react efficiently in the different solvents. However, the yields decreased smoothly when the reaction is carrying out in cyclohexane and THF. The best yields are obtained in EtOH. Consequently, we chose it as an optimal solvent for this reaction to avoid the use of the toxic solvent and thus to contribute to the protection of the environment.
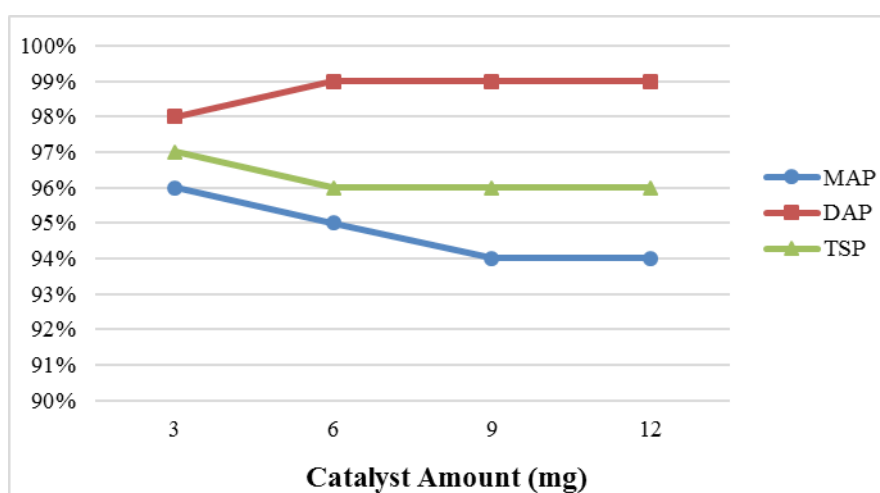
Table 2. Solvent effect on the quinoxaline **3a** synthesis [a].

Entry	Solvent	Catalyst	Time (min)	Yield[b] (%)
1		MAP	4	94
2	EtOH	DAP	2	99
3		TSP	2	96
4		MAP	4	95
5	MeOH	DAP	2	96
6		TSP	2	90
7		MAP	4	98
8	Isopropanol	DAP	2	96
9		TSP	2	89
10		MAP	4	92
11	CH_3CN	DAP	2	95
12		TSP	2	94
13		MAP	4	92
14	AcOEt	DAP	2	94
15		TSP	2	94
16		MAP	4	94
17	Dioxane	DAP	2	93
18		TSP	2	92
19		MAP	4	92
20	$CHCl_3$	DAP	2	93
21		TSP	2	90
22		MAP	4	87
23	Cyclohexane	DAP	2	86
24		TSP	2	82
25		MAP	4	73
26	THF	DAP	2	78
27		TSP	2	74

[a] Reaction conditions: 1,2-diamine (1 mmol) and 1,2-dicarbonyl (1 mmol), Solvent (2 mL), 0.006 g of catalyst, r.t; [b] Isolated yields.

Further experiments were conducted to determine the optimum catalyst loading. As revealed in Figure 1, yields increase smoothly with about 1% when DAP amount increases, and decrease smoothly with 2% or 1% when MAP and TSP amounts increase, this result allows us to deduce that the amount of the optimal catalyst is 0.003 g, thus this quantity is widely sufficient to give good yields at 96% for MAP, at 98% for DAP and at 97% for TSP. One can also notice that the reaction times are concise 4, 2 and 2 min in the presence of MAP, DAP, and TSP catalysts, respectively.

Figure 1. Effect of the catalyst amount on the quinoxaline **3a** synthesis.

In order to explore the reliability of the protocol developed to synthesize the quinoxaline derivatives, various aryl 1,2-diamines were condensed with various 1,2-dicarbonyl compounds (Scheme 2) under the optimal reaction conditions. Table 3 shows that the heterogeneous catalysts MAP, DAP,

and TSP afford the products in high to excellent   yields 89-99% in short reaction times 2-4 min.
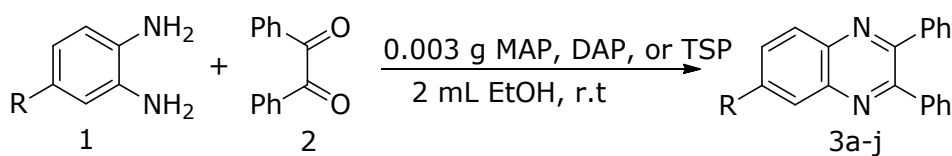
Scheme 2. Condensation of aryl 1,2-diamines with 1,2-dicarbonyl compound.

Table 4 collects the yields and reaction times of the condensation of 1,2-diamines with 1,2-dicarbonyl catalyzed by MAP, DAP, and TSP and those brought out in the literature. As can be seen, phosphate fertilizers MAP, DAP, and TSP underwent the reaction more rapidly than Fe_3O_4–SiO_2–imid–PMA^n, $VOSO_4$, and MIL-101-Cr-NH-RSO$_3$H, though the yields were comparable.

Table 4. Comparison between the present results and those given in the literature.

Entry	Catalyst	Condition	Time (min)	Yield (%)	Ref
1	Fe_3O_4@SiO_2–imid–PMA^n (0,5 mol%)	EtOH, r.t.	10	97	(38)
2	$VOSO_4$ (3 mol%)	EtOH, r.t.	20	96	(39)
3	MIL-101-Cr-NH-RSO$_3$H (3.9 mol%-SO$_3$H)	MeOH, 45 °C	12	93	(40)
4	MAP (0.003 g)	EtOH, r.t.	4	96	This work
5	DAP (0.003 g)	EtOH, r.t.	2	98	This work
6	TSP (0.003 g)	EtOH, r.t.	2	97	This work

The catalytic efficiency of the catalyst is determined not only by its capacity to lead to the formation of the desired product with a good yield and short reaction time but also by its recyclability and its natural regeneration. Finally, it seemed necessary to check the reusability of the three catalysts (MAP, DAP, and TSP) in the optimum reaction conditions.
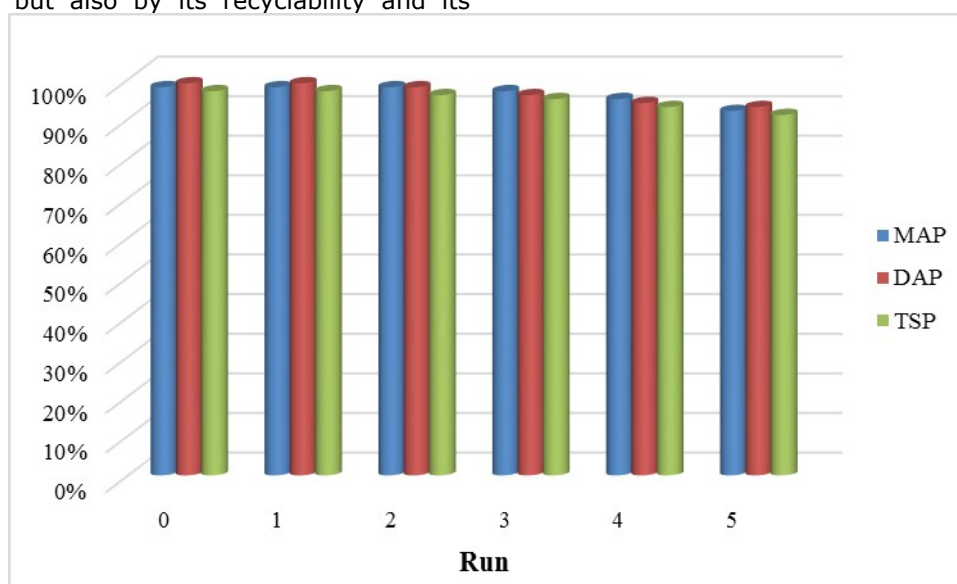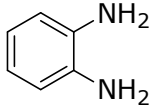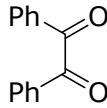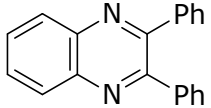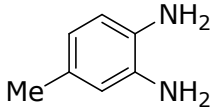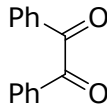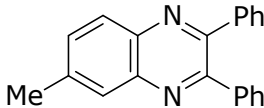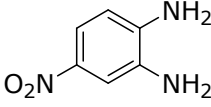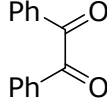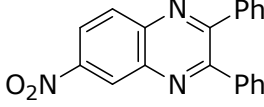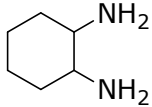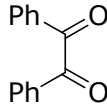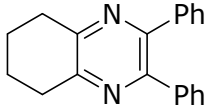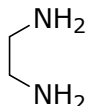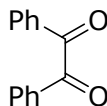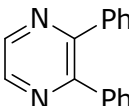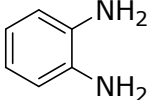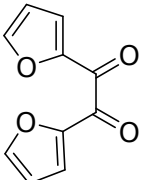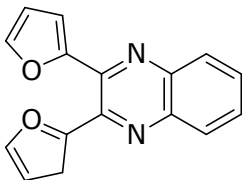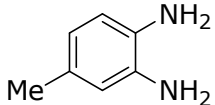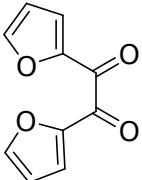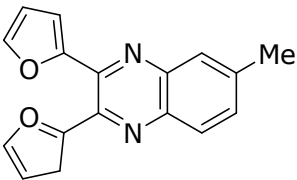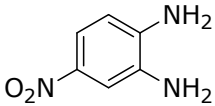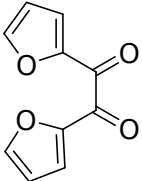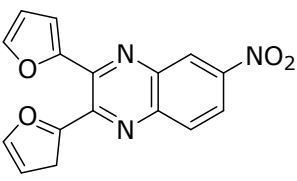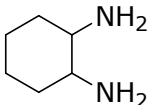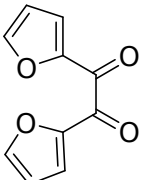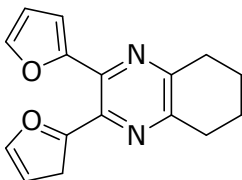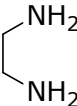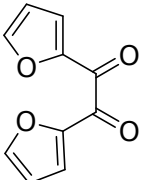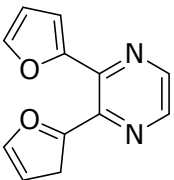

Figure 2. Recyclability of MAP, DAP, and TSP (0.003 g) in the condensation of 1,2-diamines with 1,2-dicarbonyl compounds at optimal conditions.

The catalysts remain functional throughout the six runs with a slight loss of their catalytic capacity that can be estimated by about 5% in yield.

Table 3. Quinoxaline derivatives synthesis catalyzed by MAP, DAP, and TSP at room temperature [a].
[a] Reaction conditions: 1,2-diamine (1 mmol) and 1,2-dicarbonyl (1 mmol), EtOH (2 mL), 0.003 g of catalyst, r.t. [b] Isolated yields.

Entry	aryl 1,2-diamines	1,2-dicarbonyl	Product	Molecular weight (g/mol)	Catalyst	Time (min)	Yield[b] (%)	TON	TOF (h^{-1})
1(3a)				282.3	MAP	4	96	36.67	547.31
					DAP	2	98	43.17	1308.18
					TSP	2	97	75.78	2296.36
2(3b)				296.4	MAP	4	97	37.16	554.63
					DAP	2	98	43.17	1308.18
					TSP	2	99	77.34	2343.64
3(3c)				327.3	MAP	4	95	36.39	543.13
					DAP	2	94	41.41	1254.85
					TSP	2	97	75.78	2296.36
4(3d)				314.4	MAP	4	88	33.71	503.13
					DAP	2	90	39.65	1201.51
					TSP	2	90	70.31	2130.60
5(3e)				232.28	MAP	4	90	34.48	514.63
					DAP	2	88	38.77	1174.85
					TSP	2	91	71.09	2154.24

No.	Amine	Diketone	Product	MW	Method	Time	Yield		
6(3f)	(benzene-1,2-diamine, NH$_2$/NH$_2$)	(furil)	(2,3-di(furan-2-yl)quinoxaline)	262.26	MAP	4	90	34.48	514.63
					DAP	2	93	40.97	1241.51
					TSP	2	91	71.09	2154.24
7(3g)	(4-methylbenzene-1,2-diamine, Me, NH$_2$/NH$_2$)	(furil)	(2,3-di(furan-2-yl)-6-methylquinoxaline, Me)	276.29	MAP	4	94	36.02	537.61
					DAP	2	92	40.53	1228.18
					TSP	2	97	75.78	2296.36
8(3h)	(4-nitrobenzene-1,2-diamine, O$_2$N, NH$_2$/NH$_2$)	(furil)	(2,3-di(furan-2-yl)-6-nitroquinoxaline, NO$_2$)	307.26	MAP	4	91	34.86	520.29
					DAP	2	89	39.21	1188.18
					TSP	2	92	71.87	2177.88
9(3i)	(cyclohexane-1,2-diamine, NH$_2$/NH$_2$)	(furil)	(2,3-di(furan-2-yl)-5,6,7,8-tetrahydroquinoxaline)	266.29	MAP	4	86	32.95	491.79
					DAP	2	89	39.21	1188.18
					TSP	2	88	68.75	2083.33
10(3j)	(ethylenediamine, NH$_2$/NH$_2$)	(furil)	(2,3-di(furan-2-yl)pyrazine)	212.2	MAP	4	88	33.72	503.28
					DAP	2	86	37.88	1147.88
					TSP	2	90	70.31	2130.61

CONCLUSION

To sum up the present study, phosphate-based fertilizers MAP, DAP, and TSP act as active heterogeneous catalysts for the synthesis of quinoxalines from various 1,2-diamines and 1,2-dicarbonyl compounds at ambient temperature. The optimum reaction conditions were not determined only by choice of the "greener" solvent and its volume, but also by the determination of the most appropriate weight of the catalyst, in order to present an environmentally benign protocol for this synthesis. Moreover, the study of the recyclability results shows that the catalysts can be quickly recovered and reused for at least six runs.

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Eco-friendly Synthesis of Quinoxaline Derivatives Using Mineral Fertilizers as Heterogeneous Catalysts

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Supplementary Information

*2,3-diphenylquinoxaline (**3a**, Table 3, Entry 1).* M.p.: 127-128°C (126-127°C [36-37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 7.32-7.36 (m, 6H); 7.42-7.46 (m, 4H); 7.86-7.88 (m, 2H); 8.14-8.16 (m, 2H). ^{13}C NMR (300 MHz, DMSO-d6): δ(ppm) : 128.50; 129.22; 129.26; 130.16; 130.84; 139.23; 140.93; 153.49.

*6-methyl-2,3-diphenylquinoxaline (**3b**, Table 3, Entry 2)*. M.p.: 121-122°C (120-122°C [36-37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 2.47 (s, 3H); 7.30-7.47 (m, 6H); 7.68-7.71 (m, 4H); 7.92 (m, 1H); 8.01 (m, 1H); 8.04 (m, 1H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 21.83; 127.96; 128.82; 129.15; 130.13; 133.02; 139.42; 141.00; 152.56; 153.30.

*6-nitro-2,3-diphenylquinoxaline (**3c**, Table 3, Entry 3).* M.p.: 141-142°C (140-142°C [37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 7.36-7.46 (m, 6H); 7.47-7.54 (m, 4H); 8.38 (m, 1H); 8.58 (m, 1H); 8.93 (m, 1H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 124.05; 129.87; 130.29; 138.45; 139.69; 143.46; 148.11; 155.86; 156.51.

*2,3-Diphenyl-5,6,7,8-tetrahydroquinoxaline (**3d**, Table 3, Entry 4).* M.p.: 153–154°C (152–155°C [37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 1.99–2.03 (m, 4H), 3.11 (m, 4H), 7.30–7.33 (m, 6H), 7.44–7.49 (m, 4H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 22.9, 32.0, 128.2, 128.4, 129.9, 139.2, 149.8, 151.1.

*2,3-Diphenylpyrazine (**3e**, Table 3, Entry 5)*. M.p.: 119–120°C (121–122°C [37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 7.30–7.37 (m, 6H), 7.43–7.48 (m, 4H), 8.62 (s, 2H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 128.6, 128.9, 129.8, 139.0, 142.4, 153.1.

*2,3-di(furan-2-yl)quinoxaline (**3f**, Table 3, Entry 6).* M.p.: 133–135°C (134–136°C [36-37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 6.59 (m, 2H), 6.68 (m, 2H), 7.65 (m, 2H), 7.79 (m, 2H), 8.17 (m, 2H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 112.00, 112.93, 129.06, 130.67, 140.71, 142.85, 144.22.

*2,3-di(furan-2-yl)-6-methylquinoxaline (**3g**, Table 3, Entry 7).* M.p.: 124–125°C (122–124°C [36-37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 6.71 (m, 4H), 7.68 (m, 3H), 7.99 (s, 1H), 7.99 (m, 1H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 21.46, 111.42, 111.97, 112.40, 127.30, 127.88, 132.21, 138.53, 139.98, 140.65, 141.12, 141.92, 143.35, 143.52, 150.40.

*2,3-di(furan-2-yl)-6-nitroquinoxaline (**3h**, Table 3, Entry 8).* M.p.: 154–156°C (152–154°C [36-37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 6.62-6.65 (m, 2H), 6.97 (m, 2H), 7.68 (m, 2H), 8.32 (m, 1H), 8.59 (m, 1H), 9.04 (m, 1H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 111.38, 111.52, 113.55, 114.38, 122.49, 123.98, 129.47, 137.98, 141.88, 142.98, 143.45, 143.95, 144.41, 146.64, 148.89, 148.98.

*2,3-Di(2-furyl)-5,6,7,8-tetrahydroquinoxaline (**3i**, Table 3, Entry 9)*. M.p.: 150°C (150–151°C [37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 1.91–1.97 (m, 4H), 2.99–3.04 (m, 4H), 6.45–6.51 (m, 4H), 7.50 7.51 (m, 2H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 22.65, 31.87, 111.34, 111.63, 139.22, 143.35, 150.77, 150.81.

*2,3-Di(2-furyl)pyrazine (**3j**, Table 3, Entry 10).* M.p.: 77–78°C (78–80°C [37]); ^{1}H NMR (300 MHz, DMSO-d6): δ (ppm): 6.53–6.72 (m, 4H), 7.56–7.57 (m, 2H), 8.53 (m, 2H). ^{13}C NMR (300 MHz, DMSO-d6): δ (ppm): 111.82, 112.15, 112.33, 141.76, 143.84, 150.40.

¹H and ¹³C NMR spectra of Quinoxalines:

¹H NMR spectrum of 2,3-diphenylquinoxaline (DMSO-d6).

¹³C NMR spectrum of 2,3-diphenylquinoxaline (DMSO-d6).
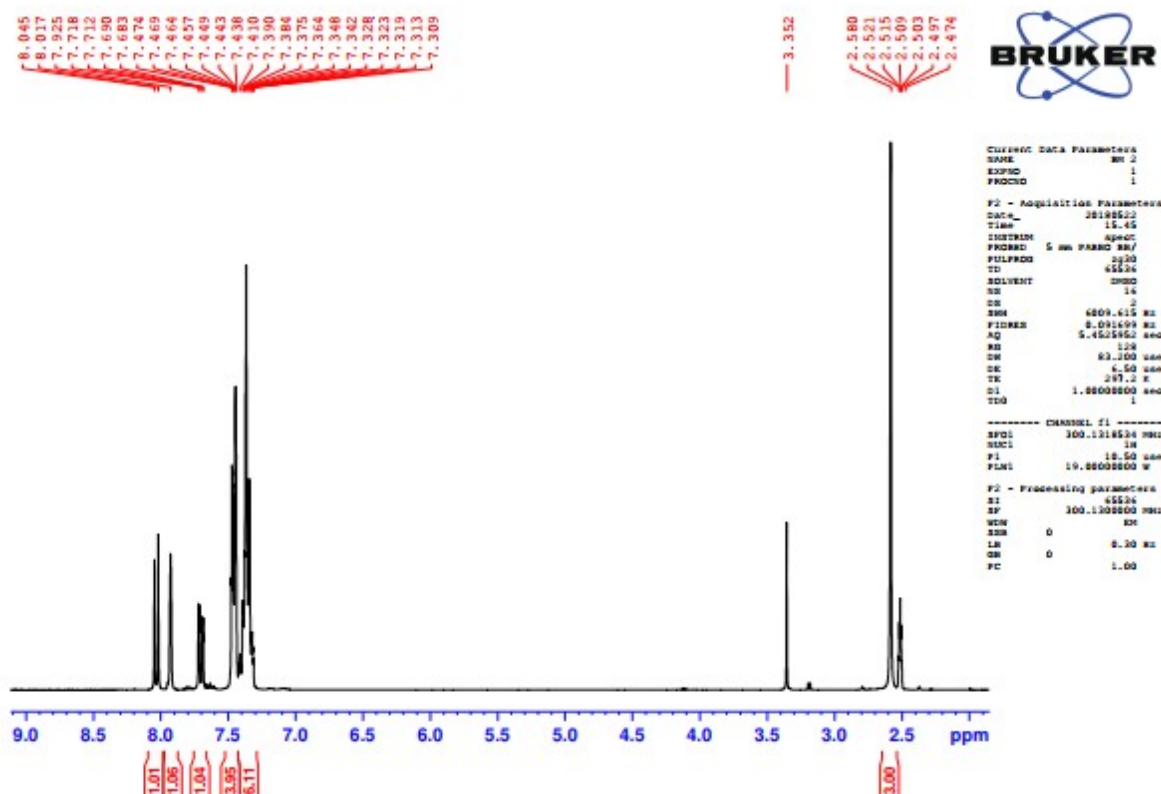
¹H NMR spectrum of 6-methyl-2,3-diphenylquinoxaline (DMSO-d6).
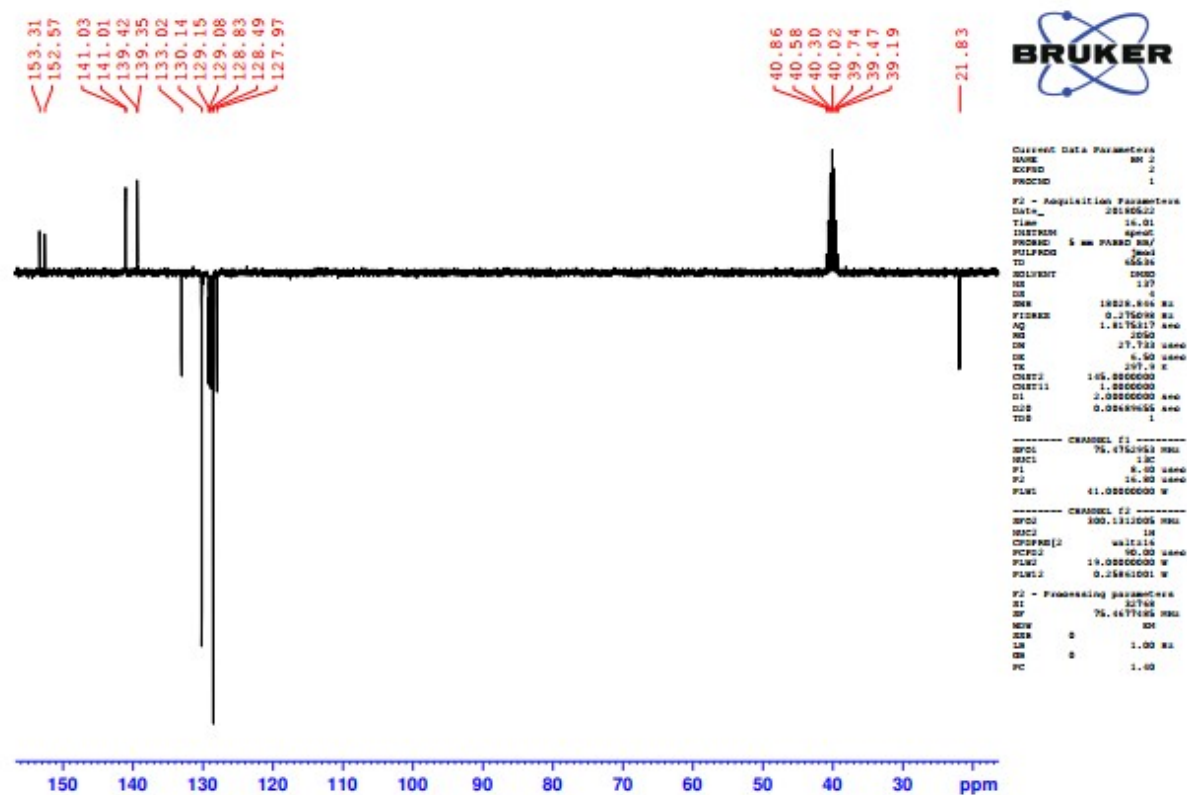
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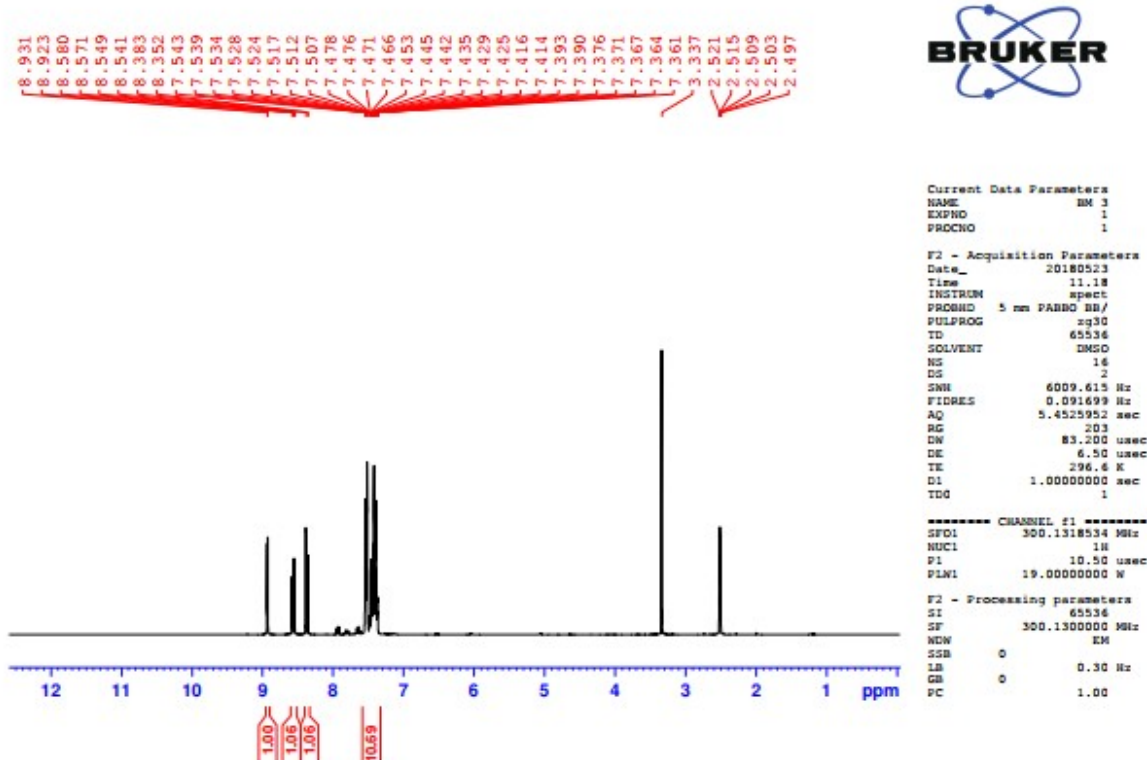
¹H NMR spectrum of 6-nitro-2,3-diphenylquinoxaline (DMSO-d6).
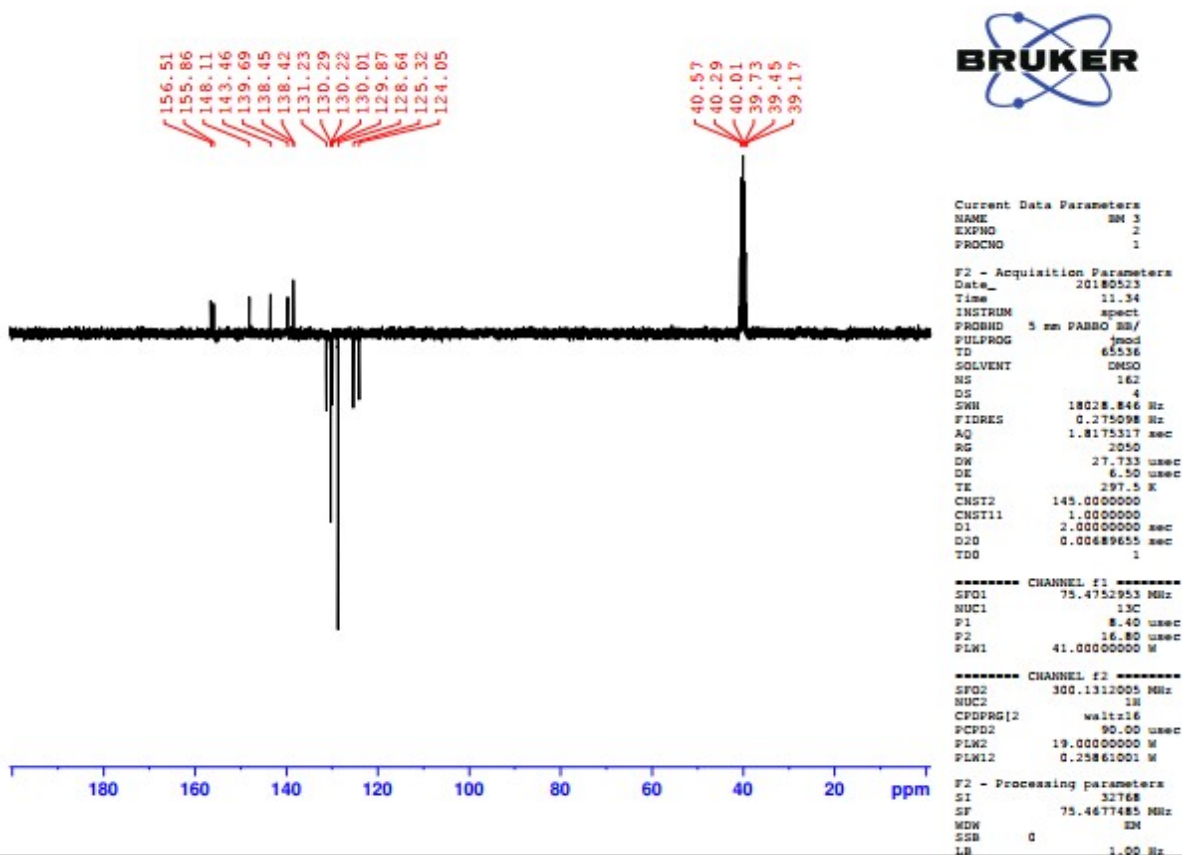
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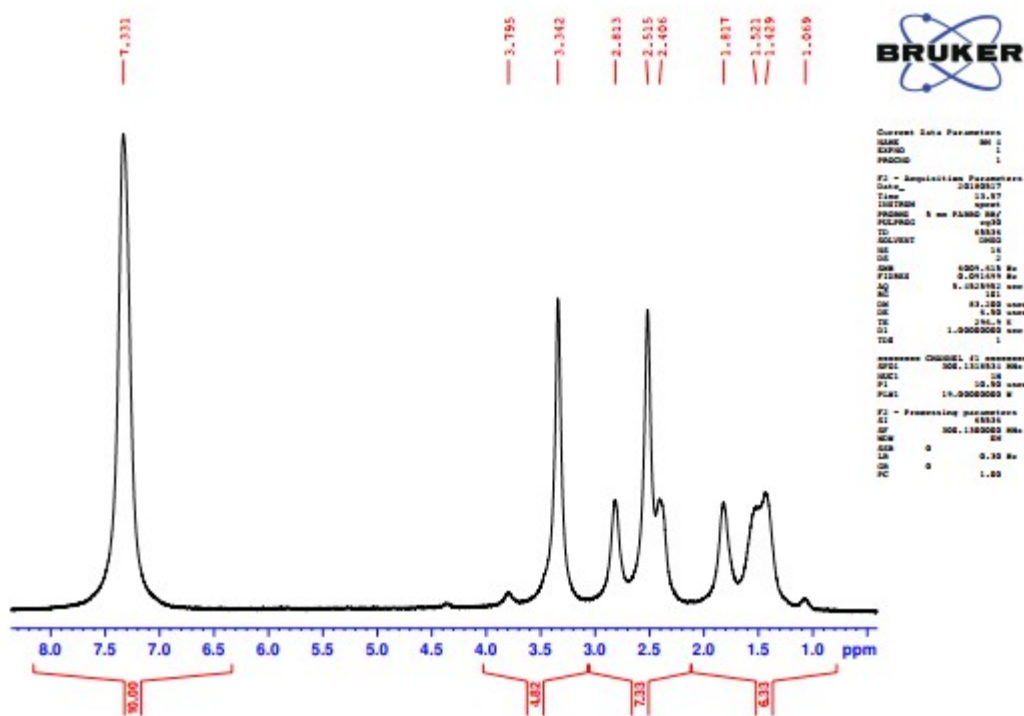
¹H NMR spectrum of 2,3-Diphenyl-5,6,7,8-tetrahydroquinoxaline (DMSO-d6).
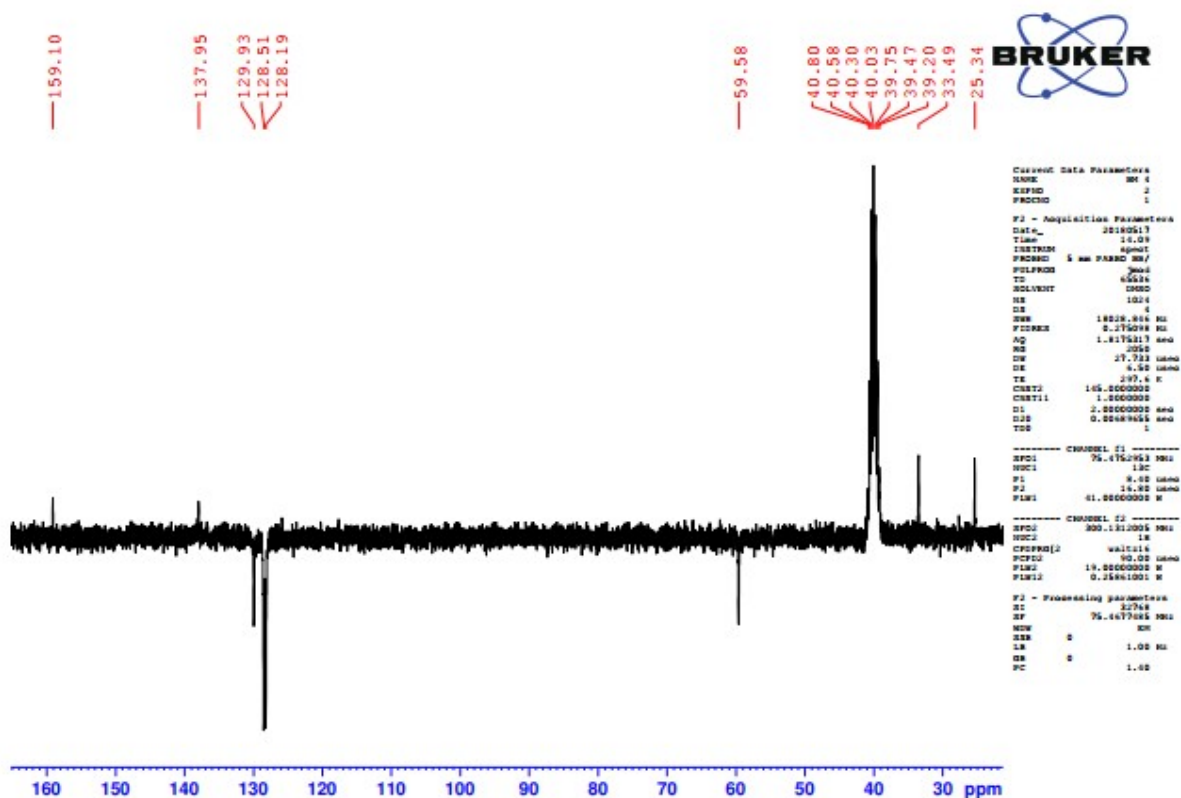
¹³C NMR spectrum of 2,3-Diphenyl-5,6,7,8-tetrahydroquinoxaline (DMSO-d6).

¹H NMR spectrum of 2,3-Diphenylpyrazine (DMSO-d6).

¹³C NMR spectrum of 2,3-Diphenylpyrazine (DMSO-d6).

The Evaluation of Syngas Loss from Disposable Syringe Sampling System: A Case Study

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Abstract: The main advantage of standard gas sampling methods is their capability to keep a consistent gas composition from sampling to the analysis. On the other hand, the disadvantages include the price and possible fragility of the sampling equipment and the speed of sampling. Thus, a new tool was required with low cost and fast sampling during fieldwork. Disposable plastic syringes fulfill all these requirements with the additional advantage of applicability for multiple samplings. With the utilization of 3-way stopcocks, a filter media, and a butterfly needle, a simple but efficient sampling system was prepared. Even though the holding time of specific components is not as high as it is for a standard sample holder, the precise analysis of the gas samples is possible. After 3 hours of holding time, the concentration decrease was 0.4% CO, 1.6% CO_2, 8.0% H_2, 0.2% CH_4, 0.5% C_2H_4, 0.8% C_2H_6, 3.5% H_2S (3-part syringe) and 2.5% H_2S (2-part syringe) In the article, equations are proposed for the examined components so that the measured concentrations could be adjusted based on the elapsed time from sampling. Syringes have been successfully utilized in the field of applied pyrolysis, for small and pilot-scale experiments, and can even be used in an industrial environment.

INTRODUCTION

As the energy demand of humanity is getting higher, the available energy resources must be used with the best possible efficiency. The most common utilization of solid fuels is energy production via thermochemical processes, which include, for example, combustion, gasification, and pyrolysis. Combustion is the most frequently used thermochemical process; however, the role of pyrolysis and gasification is predicted to increase in the future in both energy production and further branches of industry. During combustion, carbon monoxide, water, and heat are produced from the solid matter at high temperature, in the presence of oxygen to achieve complete combustion (1-3). In the case of gasification, the solid base material is

turned into gaseous fuel – syngas – at high temperature and during partial oxidation. The main constituents of syngas are carbon monoxide and hydrogen (4). As a result, syngas can be widely used as a base material in the chemical industry, for energy or synthetic natural gas production as well (5). Pyrolysis can be carried out at a lower temperature than gasification, and usually in the absence of air. The final products, beside syngas, include significant amounts of liquid hydrocarbons such as pyrolysis oil and tar, as well as char that can be further utilized via combustion or gasification.

To adequately control both pyrolysis and gasification processes, the composition of the produced gas should be known. The compositions of the gases can

vary greatly depending on the base material, the type of technology used, and the parameters of the experiment. The quantity of the following components should be monitored:
-        carbon monoxide (CO): the main component; the highest possible quantity should be in the syngas;
-        hydrogen (H_2): the main component; the highest possible quantity should be in the syngas;
-        oxygen (O_2): produced if there is a leakage in the system, so its determination is essential;
-        hydrocarbons (C_xH_y): fundamentally, the generation of methane is expected, but a high amount of ethane and ethene can be produced if the holding time in the reactor is short; their production is mostly a characteristic of pyrolysis, but their presence during gasification is disadvantageous, implying that the technology should be changed;
-        carbon dioxide (CO_2): due to the addition of an oxidizing medium, partial oxidation occurs during gasification and CO_2 is produced; the regulation of the quantity of the oxidizing medium can lead to the maximization of CO and the minimization of CO_2 production;
-        hydrogen sulfide (H_2S): produced from base materials with high sulfur content in the sub-stoichiometric environment; essential to know its quantity for environmental considerations and the utilization of the gas.

Gasification and pyrolysis experiments of various base materials – coals, biomasses, wastes – had been conducted with small-scale and pilot-sized (Figure 1.) systems for many years to optimize syngas production. In the case of the lab-scale system, the tests were fast, so frequent sampling was necessary to determine if any parameters of the experiments had to be changed. On the other hand, the use of the pilot-sized system required a more significant number of samplings from several gas outlets to monitor the various technological steps.

Thus, the preparation of a sampling technique appropriate for both systems was required with the following criteria:
-        sampling should be fast, to monitor sudden changes in the gas composition;
-        the sampling system could be used not only in laboratory conditions but for in situ samplings;
-        it should be cheap, as a high number of samples are needed in case of further analyses;
-        the internal volume should be suitable to take samples of varying quantities (e.g., the produced gas quantity is small in the lab-scale system);
-        the gas composition should stay nearly constant in the sample holder until the analysis.

1 – Perfusor pump
2 – Plastic syringe
3 – Insulation
4 – Water heater pipe
5 – Base material
6 – Tube furnace
7 – Heat resistant steel tube
8 – Wadded up filter
9 – Liquid collector
10 – Ice water
11 – Glass Allihn reflux condenser
12 – Hoffmann clamp
13 – Rotameter
14 – Gas sampling point
15 – Flare
16 – Extractor

1 – Feeder
2 – Reactor
3 – Base material
4 – Tube furnace
5 – Temperature controller
6 – Char transporter screw
7 – Char collector
8 – Venturi scrubber
9 – Steam generator
10 – Cyclonic separator
11 – Filter
12 – Gas meter
13 – Liquid collector
14 – Flare
15 – Gas sampling point

Figure 1: Lab-scale pyrolysis system (left) and pilot size gasification system (right).

The most frequent gas sampling methods (canister, sample cylinder, sampling bag, different types of sampling bulbs and gas-tight syringes) focus on holding the samples for a long time, e.g., bags for 2 days, canisters for 30 days (6). Thereby, the same gas composition is assured for the analysis as the composition during sampling. The volume of the samplers is at least 100-500 cm^3, which makes the flushing and filling take several minutes, especially if the sampler is rigid. These properties of standard tools can be disadvantageous in case of experiments of smaller-scale. Glass gas-tight syringes provide fast sampling with low volume. However, they are fundamentally used for normal laboratory conditions and are not suitable for fieldwork in a dusty or contaminated environment. Furthermore, such

syringes are fragile and easily blocked, rendering them single-use samplers if the immediate analysis is not possible – as it is the case for our systems.
Thus, disposable plastic 3-part syringes were tested as samplers. Some of the main advantages of syringes, in contrast with the current sampling methods, are the speed of sampling, which is less than 0.5 minute, including the assembly of the sampler system and the cost, which can be a fraction of the price of the other samplers. Some examples of the approximate average retail prices of certain samplers in 2019 were as follows:
–   1 L multi-layer foil gas sampling bag with valve and septum: 17 EUR / 19 USD;
–   125 mL glass gas sampling bulb with stopcock and septum: 233 EUR / 269 USD;

− 250 mL polypropylene gas sampling bulb with stopcock: 100 EUR / 111 USD;
− 250 µL gas-tight syringe: 78 EUR / 86 USD;

I contrast, the proposed sampling system from disposable syringes would cost 1.1 EUR / 1.2 USD, with 0.6 EUR / 0.7 USD per sample using one butterfly needle and one flushing syringe.

Syringes are mainly used in the medical field. Their significance is rather low in the case of chemical analysis, where they are almost solely used as liquid feeders (7, 8). Furthermore, the application of disposable syringes as gas samplers has been examined in a few publications. During their experiments to analyze greenhouse gas fluxes between the soil and the atmosphere, Collier et al. (9) prepared a static chamber and collected gas samples from the chamber headspace using disposable syringes. The samples were immediately injected into vials, so there was no change in the gas composition during sampling. De Quirós et al. (10) used disposable insulin syringes to sample bubbles in the cardiovascular system of stranded cetaceans. After sampling, the contents were injected into a vacutainer. The reason to use disposable syringes was the inapplicability of gas-tight syringes (fragile and easily clogged) during fieldwork.

The gas holding capability of plastic syringes is scarcely tested. Cho et al. studied the air contamination of CO_2-filled empty syringes with time both mathematically and experimentally. However, as the syringes were not closed off in any way, the results were not useful for the current experiment (11). On the other hand, plastic syringes filled with sulfur hexafluoride and perfluoropropane, sealed off with a plastic cap, and a 3-way stopcock were used by Ohji et al. (12), and the concentration decrease of these was examined concerning time for 24 hours. The concentration in their experiments decreased by 1-1.5 % for a 3-way stopcock and by 60-63 % for the plastic cap of the syringe. According to these results, the two

examined components can be stored with minimal concentration decrease in a syringe with a stopcock for one day. The investigation of the effects of syringe material (plastic and glass), storage time (0 and 30 min) and storage temperature (0-4 and 22 °C) on normal arterialized blood gas values (12 vol % O_2 and 5 vol% CO_2) was carried out by Knowles et al. (13). It was concluded that samples from plastic syringes should be instantly analyzed, while glass syringes should be used to store the samples.

Syringes are adequate for few-second-long medical processes. However, they are subjected to higher and longer stress during gas sampling, holding, and injection. Furthermore, these otherwise disposable tools could be reusable in case of gas sampling. After sampling, the storability of the gas is dependent on the material of the syringe and the stopcock. The barrel of the 3-part syringe is made from polypropylene, the base material of the plunger is isopropylene, the body of the stopcock is polycarbonate, and the handle is polyethylene. Among these polymers, polypropylene and polyethylene are not considered gas-tight materials, so the gas sample loss had to be examined. Moreover, if H_2S is present in the gas, it can be bound by the rubber plunger. There are some commercially available 2-part syringes, but these are difficult to obtain in large size (50 mL). On the other hand, 3-part syringes with rubber plunger are better sealed and provide easier plunger sliding, compared to the 2-part ones. Thus, 3-part syringes sealed with 3-way stopcocks were used for the experiments to test their performance as syngas samplers and sample holders. With high gas quantities kept in the samplers, the changes in gas composition were tested at various time intervals.

MATERIALS AND METHOD

Materials

Calibration gas from Linde was used to model raw syngas. The exact gas composition is summarized in Table 1.

Table 1: The gas mixture used to test the method.

Gases, gas mixtures	Composition, vol%
Hydrogen	50
Carbon monoxide	25
Carbon dioxide	12
Methane	6
Ethane	3
Ethylene	2
Hydrogen sulfide	2

Romed type 2-part and 3-part disposable plastic syringes (50 mL), B.Braun type 3-way stopcocks, household cotton wool, and B.Braun type 23G scalp vein butterfly needle were used to prepare the

sampling system (23G = 0.023" outside diameter of the needle). The assembled gas sampling system is illustrated in Figure 2.
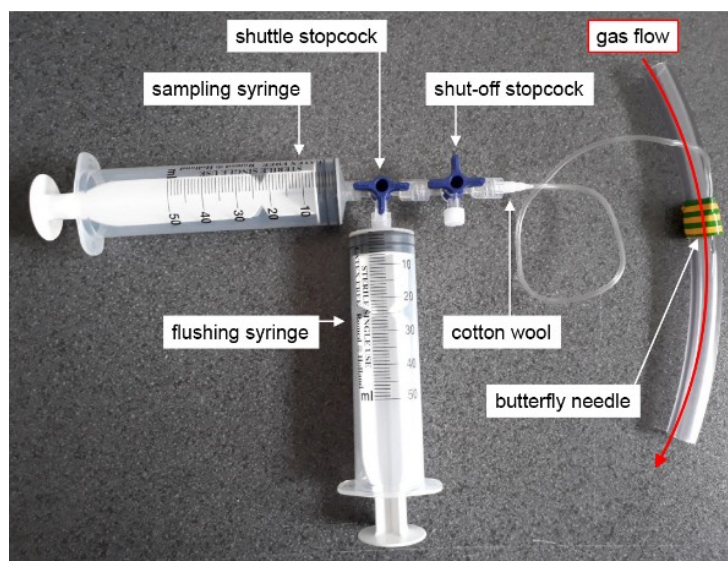
Figure 2: The gas sampling system.

Methods

The sampling method was prepared with the usage of simple medical infusion tools. Disposable syringes were used for both gas sampling and injecting the sample to a gas chromatograph for analysis. Plastic single-use syringes are available with standard conical Luer connectors (14). The two most widespread types are Luer-Slip and Luer-Lock connectors. In the case of Luer-Slip connectors, the connecting item – for example, needle, stopcock, injector, etc. – can be simply slipped in or on the cone-shaped connector. The connectors are kept together by frictional force. As the connectors can be easily disconnected, the proper connection should always be monitored. Considering that the Luer-Lock type connectors require the two connecting items to be screwed together, making disconnection significantly harder, Luer-Lock connectors were chosen.

A piece of cotton wool is used as a gas filter because of its high specific surface. It is supposed to separate materials that are either solid or able to condense and were not separated by the gas cleaning equipment. Thus, the gas chromatograph can further be protected.

Additionally, a tap or inlet is necessary where a plastic, rubber, or silicone tube can be connected to the sampler. Various sized needles were tested to connect the tube with the sampler. The trial of various types of needles revealed that using a relatively thin needle can cause many problems: the needle can bend, slip out of its plastic socket and cause injuries, get clogged and make the sampling longer because of the small inside diameter. The use of a thick needle can result in a giant hole on the tube, and, in the case of rigid tubes, gases can escape, and most of the produced components during syngas production are toxic and flammable. Thus, a 23G-25G size butterfly needle was chosen

for the experiments. The tube can be pierced slantwise with the butterfly needle, just like it is done in case of blood tests or infusions, with the wings taped to the tube to keep it in position.

After assembling the sampling system, the steps of the sampling are the following (Figure 3.):

- Put the shuttle stopcock in a position that allows gas to flow through all three passages (Figure 3/a.). The shut-off stopcock should be mirroring the shuttle stopcock, the passages parallel to the gas flow opened, and the perpendicular passage closed. This way, both syringes can be filled with the gas.

- The tube of the butterfly needle should be flushed with the sample gas, by using one of them as a flushing syringe (Figure 3/b.). As the sample gas enters the tube, the residing air is sucked to the flushing syringe. The total inner volume of the stopcock, the butterfly needle, and its tube is around 1.3 mL, so approximately 10-15 mL gas is necessary for complete flushing.

- Turning the shuttle stopcock closes the passage to the flushing syringe (Figure 3/c.), so the clean sample can only flow to the sampling syringe (Figure 3/d.).

- When sampling is finished, the sampling syringe can be closed off by further turning the shuttle stopcock, opening the passage to the flushing syringe (Figure 3/e.). The gas from the flushing syringe can be injected back to the original gas flow (Figure 3/f.). Therefore it enables the user to protect themselves from the toxic components that could be released after the sampling process.

- The final step is to disconnect the empty flushing syringe and the shuttle and sampling stopcocks after the shut-off stopcock is closed (Figure 3/g.). The sample can be held in the sealed syringe that can be connected to the sampling loop of the gas chromatograph (Figure 3/h.).
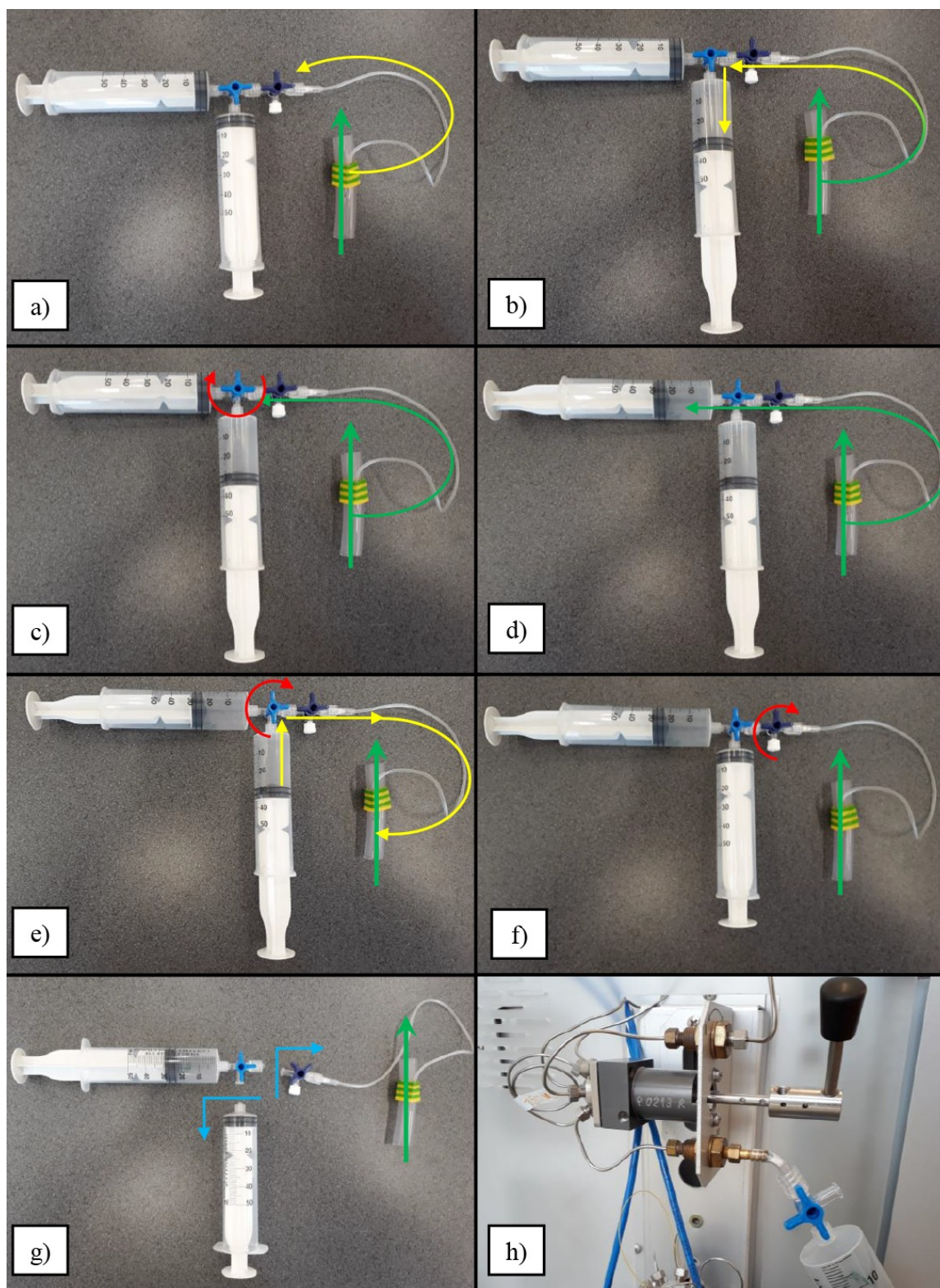
Figure 3: The operation of the sampling system.
(a – connecting the sampler to the sampling point; b – filling the flushing syringe with air/previous sample;
c – closing the flushing syringe; d – sampling; e – stopping the gas sample flow; f – closing off the sampling
point; g – removing the sampling syringe; h – attaching the sampling syringe to the gas chromatograph)

A Dani Master gas chromatograph with a TCD detector was used to test the gas holding capability of the syringes. The gas chromatograph operating conditions can be seen in Table 2.

Table 2: GC operation conditions.

Detector	Thermal Conductivity Detector (TCD)
Temperature	110 °C
Voltage	4 V
Signal range	1
Sample injection	Loop, 200 μL
Injector	S/SL
Mode	Splitless
Split purge	10 mL/min
Temperature	40 °C
Pressure control	400 kPa
Carrier gas	Helium 6.0
The first column (Q-Bond 1)	Restek RT-Q-Bond (Fused Silica PLOT)
Length x Inner diameter x Film thickness	30 m x 0.32 mm x 10 μm
The second column (Q-Bond 2)	Restek RT-Q-bond (Fused Silica PLOT)
Length x Inner diameter x Film thickness	15 m x 0.53 mm x 20 μm
The third column	Restek RT-Msieve 5A (Fused Silica PLOT)
Length x Inner diameter x Film thickness	30 m x 0.53 mm x 50 μm
Configuration of the column with software*	
Length x Inner diameter x Film thickness	45 m x 0.32 mm x 20 μm
Oven temperature	36 °C
Method events	Times of switching the valve
flow in all three columns	0 – 1.80 min
flow in only the Q-Bond column	1.80 – 5.70 min
flow in all three columns	5.70 – 12.5 min
Aux gas	Nitrogen 5.0
Aux gas pressure	270 kPa
Cycle time	12 min
Evaluation software	Clarity 3.0.6.589

*The software can recognize only one column, so column parameters that could substitute for all three columns and result in realistic carrier gas flow values had to be entered.

Samples were analyzed immediately, and 3, 6, 9, 12, 18, 24, 36, 48, 59, 70, 95, and 120 hours after sampling. The calibration of the instrument was checked after 9, 18, and 24 hours and corrected if necessary. Furthermore, the start-up of the instrument was always followed by calibration (before the 36-, 48-, 95- and 120-hour examination).

RESULTS AND DISCUSSION

As some experiments require much more samplings as the laboratory system can analyze at once, the period during which the samples can be used with relatively low concentration change had to be tested, because these syringes were designed to hold liquids, the composition of the gas inside changes over time as air enters the syringe. For these tests, various gas mixtures were prepared from the gases available and sealed inside the syringe with the stopcock. The gas mixtures were kept on room temperature and were not exposed to direct sunlight. The composition was analyzed at given time intervals. The signals given by the original mixture were considered 100%, and the changes related to this are shown in Figure 4. The decrease in the quantity of the components can be described with polynomials in two or three variables. Moreover, exponentials are also indicated in the figure with the coefficient of determination for each equation.

The yellow zones in Figure 4. demonstrate the range of the standard deviations of the measurements. It can be observed for all examined components, that the standard deviation of the measurements increased as the elapsed time became longer between the sampling and the measurement. Thus, the shorter the elapsed time is before measurement, the better the proposed equations are to describe the changes in composition.

In the case of CO, CO_2, CH_4, C_2H_4, and C_2H_6, a similar trend was experienced in the concentration changes. The decrease in H_2 and H_2S amount (in a 2-part syringe) showed a correlation, however, due to the better sealing ability of the syringes with rubber plunger, no further investigations were carried out about the composition changes in 2-part syringes. The evolution of the H_2S content (in a 3-part syringe) shows significantly distinct characteristics. The comparison of the diagrams reveals that the decrease of hydrogen content is the most significant for approximately the first 13 hours, then the decrease in the concentration of hydrogen sulfide becomes drastically higher (in a 3-part syringe).

The reason for this phenomenon can be explained by the examination of the used syringe. Both polypropylene (barrel) and polyethylene (handle) are non-polar molecules. Hydrogen is also non-polar and has the smallest size among the examined components, so it is expected to escape at the fastest rate from the syringe (15). However, the plunger is made from isoprene, and sulfur is used for crosslinking. The crosslinking of sulfur between the double-bonds of the isoprene chains is called vulcanization (16). The quantity of sulfur affects the characteristics of the product; for example, increasing sulfur content results in higher rigidity. As the plunger is soft and able to deform, many unsaturated bonds can be found between the

isoprene chains. Thus, instead of escaping to the atmosphere, hydrogen sulfide diffuses into the plunger material at a faster rate than hydrogen could escape through the wall of the syringe. The characteristic odor of hydrogen sulfide can be smelled on the plunger for several weeks after the analysis of hydrogen sulfide-containing samples, and this can, therefore, be further proven. The plunger also becomes gradually more rigid after 30-50 samplings, and sliding it in the barrel also becomes difficult, as the plunger is further vulcanized due to the diffusion of sulfur. This issue can be avoided with the use of a 2-part syringe in case of hydrogen sulphide containing samples.

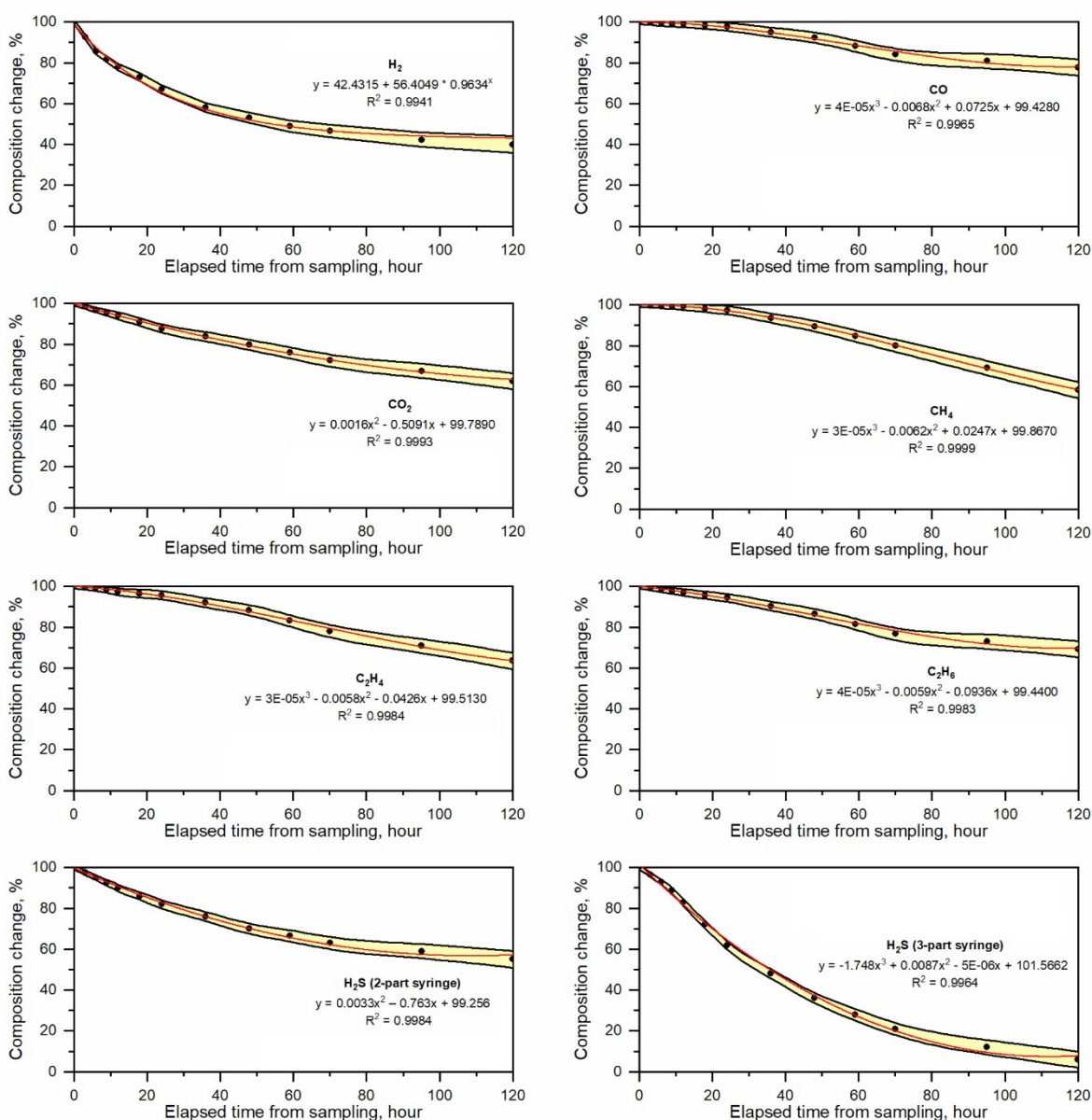**Figure 4:** The changes in the composition of the calibration gas in the syringe in relation to time.

Ideally, the measurement of the stored gases should be carried out immediately after sampling as the component concentration decreases over time.

In case of the first measured time (3 hours), the concentration decrease was 0.4% CO, 1.6% CO_2, 8.0% H_2, 0.2% CH_4, 0.5% C_2H_4, 0.8% C_2H_6, 3.5%

H₂S (3-part syringe) and 2.5% H_2S (2-part syringe). However, the initial concentration can be calculated using the equations given in Figure 4. Since the standard deviation for each component increases over time, the measurements should be carried out as soon as possible.

CONCLUSIONS

A new gas sampling technique for systems of various sizes was developed, using disposable plastic syringes with stopcocks and a butterfly needle, and the syngas holding capability of the syringes after sampling was examined. The hydrogen content decreases exponentially, making it one of the earliest components to leave the sample holder, along with H_2S in the 3-part syringe. Equations describing the expected gas composition related to the holding time were also proposed, making it possible to calculate the original composition and concentration of the examined gas samples. As the standard deviations of the equations also increase over time, the accuracy of the calculations decreases. Thus, the initial measurement of the samples is suggested.

ACKNOWLEDGEMENT

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RESEARCH ARTICLE

Spectral, DFT, molecular docking and antibacterial activity studies of Schiff base derived from furan-2-carbaldehyde and their metal(II) complexes

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Abstract: Metal(II) complexes of cobalt(II) (1), copper(II) (2), nickel(II) (3), and zinc(II) (4) with Schiff base ligand derived from furan-2-carbaldehyde were synthesized and elucidated by IR, electronic, mass, ^{1}H NMR, and magnetic susceptibility measurements. Using DFT-based optimization of structures, bond length, bond angle, HOMO-LUMO energy gaps, and molecular electrostatic potential maps (MEP) of ligand and complexes 1-3 were theoretically calculated at the B3LYP/LANL2DZ level of theory. HOMO-LUMO energy gap was calculated which allowed the calculation of comparable properties like chemical hardness, chemical inertness, and chemical potential. The Ni(II) and Cu(II) complexes showed potent inhibition against all the bacterial strains. In comparison with antibacterial activity, molecular docking studies were carried out with protein receptor SEC2 (PDB: 1STE) in Staphylococcus aureus.

INTRODUCTION

The Schiff base ligands with nitrogen and oxygen donor atoms act as good chelating agents for the transition and non-transition metal ions. The azomethine (-CH=N-) linkage present in Schiff base ligands and it's metal (II) complexes show a wide range of biocidal activities such as antibacterial (1) antifungal (2) anti-inflammatory

(3) anticancer, anti-diabetic (4), and antitumor activities. Furthermore, the interactions of these complexes with DNA have gained much attention due to their possible applications as new therapeutic agents (5-7). The high affinity for the chelation of the Schiff bases towards the transition metal ions is utilized in preparing their complexes in the solid form (8-10). The tractability of Schiff base ligands can be

enhanced by hydrogenation of (C=N) bands, and they should thus coordinate metal ions more easily. For these explanations, condensed Schiff bases have newly expanded significant consideration (11). Herein we report the synthesis and characterization of furan-2-ylmethylidene-hydrazinylidene-ethyl-phenol **[AF],** which is derived from 2-[(1E)-1-hydrazinylidene-ethyl] phenol and 2-furancarboxaldehyde and it's metal(II) complexes.

EXPERIMENTAL

All the chemicals used were of analytical grade and were as procured. The reagent grade chemicals 2-hydroxyacetophenone, hydrazine hydrate, 2-furancarboxaldehyde, and metal(II) salts were purchased from Sigma-Aldrich and used without further purification. The melting point had been recorded on an electro-thermal melting factor apparatus and are uncorrected. 1H-NMR spectra were recorded on a Bruker 400 MHz spectrometer at IISc, Bangalore, and Karnataka, INDIA. The chemical shifts have been proven in d values (ppm) with tetramethylsilane

(TMS) as an internal standard. LC-MS changed into acquired the usage of a C-18 column on Shimadzu, LCMS 2010A, Japan. The FT-IR spectra of the compounds were taken as KBr pellets (100 mg), the usage of Shimadzu FT-IR spectrometer. Magnetic susceptibility has been measured at 35 °C through the Gouy technique.

Synthesis of 2-{(1E)-1-[(2E)-(furan-2-ylmethylidene) hydrazinylidene] ethyl} phenol [AF]

The Schiff base ligand was prepared by refluxing 2-hydroxyacetophenone (4.02 g, 0.02 mol) and hydrazine hydrate, each dissolved in 30 mL ethanol with continuous stirring. The stirring was continued for 3 h and to obtain the white product. A solution of the isolated 2-[(1E)-1hydrazinylidene ethyl]phenol (2.7g 0.02 mol) in 10 mL ethanol was then refluxed with an ethanolic solution of furan-2-carbaldehyde (1.8 g, 0.02 mol) for 5 h after adding 1 mL of glacial acetic acid (Scheme 1). The obtained solid product was filtered off, washed several times with methanol and recrystallized from hot methanol, and dried over anhydrous $CaCl_2$.
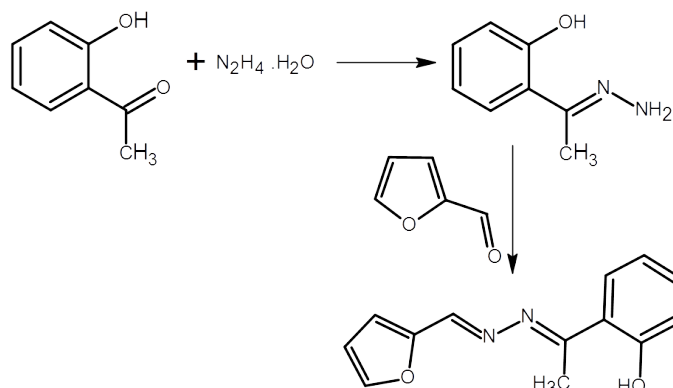
Scheme 1: Synthesis of Schiff base ligand AF **.**

Yield: 78%: M.p.: 178–180 °C, Anal. Calc. for $C_{13}H_{12}N_2O_2$ = C(68.41%) H(5.30%) and N(12.27%), Found: C(67.11%) H(5.68%) and N(11.94%), LC-MS: m/z 228 [M+1].

Synthesis of metal complexes
Ethanolic solution of metal(II) chlorides [Co(II), Ni(II), Cu(II) and Zn(II)] (2 mmol) added in drops to an ethanolic solution of the ligand (AF) (4 mmol) and the mixture was refluxed on a water bath for 3-4 h. The solid complexes obtained was filtered, washed with hot ethanol and dried in vacuum over anhydrous calcium chloride.

Co(II) Complex [1]:- Pale yellow solid, yield: 61%. Mp: > 300 °C. Anal. C(53.44%), H(3.79%), Co(10.09%), N(9.59%), Cl(12.13%), Found: C(53.62%), H(4.03%), Co(10.44%), N(10.21%), Cl(11.82%), 23 λm cm^{-2} Ω^{-1} mol^{-1}.

Ni(II) Complex [2]:- Light green solid, yield: 53%. Mp: > 300 °C Anal. C(52.37%), H(3.61%), Ni(10.21%), N(8.89%), Cl(12.05%), Found: C(52.62%), H(3.91%), Ni(10.44%), N(9.22%), Cl(12.63%),21 λm cm^{-2} Ω^{-1} mol^{-1}.

Cu(II) Complex [3]:- Dark brown solid, yield: 55%. Mp: > 300 °C Anal. C(55.12%), H(4.19%), Cu(12.11%), N(8.66%), Cl(13.22%), Found: C(55.41%), H(4.51%), Cu(11.81%), N(8.05%), Cl(13.87%), 28 λm cm^{-2} Ω^{-1} mol^{-1}

Zn(II) Complex [4]:- Light cream solid, yield: 62%. Mp: > 300 °C Anal. C(51.43%), H(4.16%), N(8.12%), Zn(9.12%), Cl(10.18%), Found: C(51.92%), H(3.83%), Zn(8.78%), N(8.73%), Cl(10.51%), 21 λm cm^{-2} Ω^{-1} mol^{-1}.

Biological studies
Antibacterial assay
The obtained compounds were screened *In-vitro* against S*. aureus, B. Subtilis,* and *S. Pneumonia* (gram-positive) *P. aeruginosa and K. pneumonia* (gram-negative) bacterial strains by using disc diffusion method (12, 13) . Ciprofloxacin was used as a standard for the current study with a concentration of about 10 µg/disc. Sterile molten Muller and Hynton agar cooled to 45 °C was injected with different strains. Fresh inocula containing ~100 cells mL^{-1} were used. Homogeneously, the inoculums were transferred on to a molten agar media with sterile cotton swabs by disease-free technique. A Whatman, no 2 filter paper disc of 6 mm diameter containing 200 µL/disc of sample, was placed over the inoculated medium. The plates were left uninterrupted at room temperature for two hours and incubated at 37 °C for 24 h. Zone inhibition was measured with the zone reader scale.

***In-silico* docking studies**
HEX 8.0 docking software in support with the discovery visualizer studios were used to perform interaction studies with protein receptor SEC2 (PDB: 1STE) in Staphylococcus aureus obtained from RCSB protein data bank (14).

RESULTS AND DISCUSSION

The metal (II) complexes are soluble in DMSO, DMF, and are sparingly soluble in chloroform. The complexes are stable at room temperature. The elemental analysis data of the ligand [AF] and its metal complexes [Co(II), Ni(II), Cu(II) and Zn(II)] indicate that the metal to ligand ratio is 1:2.

^{1}H NMR and Mass spectra
To identify the structure of free ligand AF, the resonance signals at δ 12.12, δ 7.11–7.75, δ 3.52-3.81, and δ 2.75-2.95 ppm are due to OH, Ar-protons, -CH$_2$, and -CH$_3$, respectively. The

sharp peak at 8.08 ppm corresponding to (-CH=N-) proton is shifted towards the upper field, which proves the formation of coordination bond between the metal and imine nitrogen. The deprotonation of OH proton has been observed in the spectrum of Zn(II) complex, which supports the phenolic oxygen atom was coordinated with metal ions and ^{1}H NMR spectra of ligand AF and Zn(II) complex provided in the supplementary file.

The mass spectrum of ligand (AF) shows (Fig. 3) a well-defined molecular ion peak at m/z 288, which is equivalent with (M+ 1), was proposed molecular formula weight of ligand. The molecular ion peaks of the Cu(II), Co(II) Ni(II) and Zn(II) complexes were appeared at m/z of 588.93, 584.31, 584.07 and 590.79 respectively, which are equivalent to the stoichiometric ratio of 1:2 (M:L) type. Further, the observed molecular mass in all the spectra of the prepared complexes are in consistency with their proposed molecular structures.

IR spectra
The ligand AF showed the characteristic band at 3334 cm^{-1} due to stretching vibrations of OH group and the absorption bands of 2920, 1590, 1298, 916 and 1357 cm^{-1} are due to Ar-CH, C=N, C-O, N=N and −CH$_3$ respectively (15). The OH stretching vibrations have disappeared with complexation in the metal (II) complexes, indicating coordination through the OH group. Additional support by the v(C−O) stretching shifted to the lower frequency at 1245-1253 cm^{-1}. The C=N band also shifted lower frequency by 1512–1589 cm^{-1} indicating coordination of azomethine nitrogen (16, 17). The new IR bands witnessed in the range at 570–522 cm^{-1} in the spectra of the metal complexes suggest the presence of M−O bonding in their structure, and M−N bands are tentatively assigned to absorptions in the regions 443–499 cm^{-1} (18).

Electronic spectra
The electronic spectra of the ligand AF and their metal complexes are set in Table 1. The uncoordinated ligand showed two absorptions bands at 250, 290 nm due the π→π*, n→ π*, for (C=N) groups respectively (19, 20), and for the metal complexes, the bands appeared clearly in the range of 460 to 785 nm due to the π→ d_x^2 $_{-y}^2$and /or (π σ) → $d_x^2$$_{-y}^2$ LMCT transition in all complexes (21-23).

Table 1: UV-Visible Bands of the AF and their metal complexes.

Compound	Solvent	λ_{max} in (cm^{-1})	Transition	Magnetic moment (BM)	Geometry
Co(II)-AF	DMF	16,150 20,270	$^4T1g(F) \rightarrow A2g(F)$ $^4T_{1g}(F) \rightarrow {}^4T1_g(P)$	4.81	Octahedral
Ni(II)-AF	DMF	10,354 17,556 23,712	$^3A_{2g}(F) \rightarrow {}^3T_{2g}(F)$ $^3A_{2g}(F) \rightarrow 3T_{1g}(F)$ $^3A_2g(F) \rightarrow {}^3T_{1g}(P)$	3.11	Octahedral
Cu(II)-AF	DMF	13,478-14,331	$^2E_g \rightarrow {}^2T_{2g}$	1.92	Distorted Octahedral
Zn(II)-AF	-	-	-	-	Diamagnetic complex

Computational studies

The calculations, optimized geometry of ligand, Co(II), and Ni(II) complexes are carried out with Gaussian 09 software using B3LYP/6-311, + +G(d,p)(5D, 7F) basic set in the gas phase (24).

Frontier molecular orbital analysis (FMOs) and Molecular electrostatic potential (MEP)

In FMOs, the HOMO act as donor and LUMO as electron acceptor between the occupied and unoccupied molecular orbitals are shown in Figures 1 and 4. The energy gap ΔE of HOMO-LUMO for the ligand is 0.917 eV, in which HOMO orbital is delocalized on the nitrogen atom and CH$_3$ group while the LUMO orbital is occupied on phenyl and thiophene rings (25). The energy gaps ΔE of Co(II) and Ni(II) complexes are 2.311 and 2.778 eV. In HOMO orbitals for Co(II) and Ni(II) complexes are mainly delocalized on corresponding metal ions and thiophene and phenyl rings. Whereas in LUMO, electrons are concentrated on nitrogen, methyl group, and some part of metal ions. These tell us the chemical reactivity and stability of the ligand and their corresponding metal (II) complexes (26).
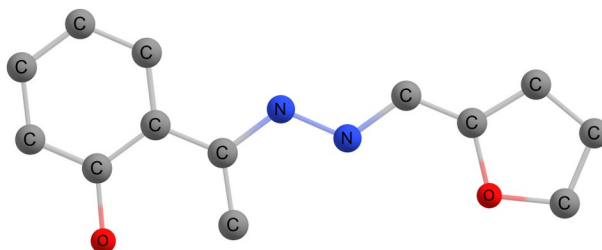
Figure 1: Optimized geometry of schiff base AF.
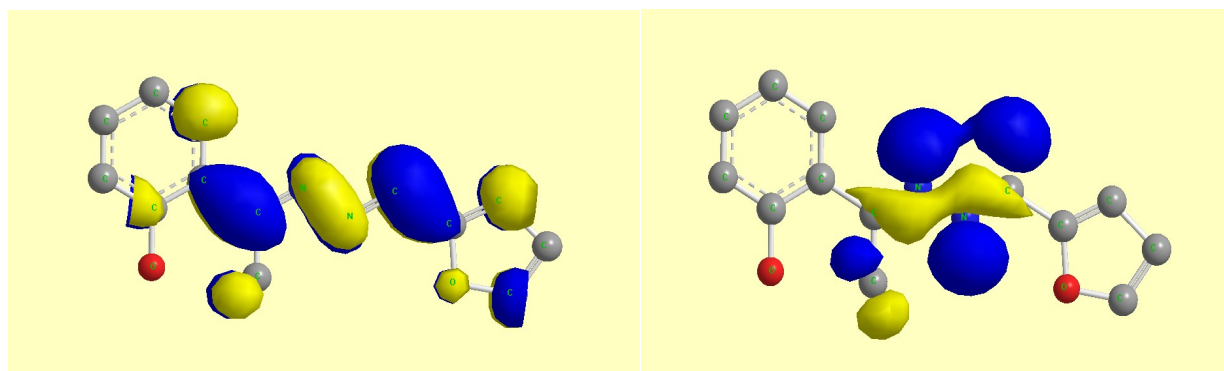
Figure 2: E_{LUMO}-E_{HOMO} [-4.820 eV and -3.903 eV] molecular Schiff base AF.

Molecular electrostatic potential (MEP)

MEP relates to the chemical reactivity and electronegativity by the electron and proton of the molecules (Figure 5). Red color shows the negative electrostatic potential and mostly contains on the nitrogen and methyl moiety,

whereas the blue color represents the positive electrostatic potential contains on the phenyl and OH group of the molecule. The potential shows the oxygen is nucleophilic center nitrogen acts as an electrophilic center and gives molecular interactions in the compounds (27).
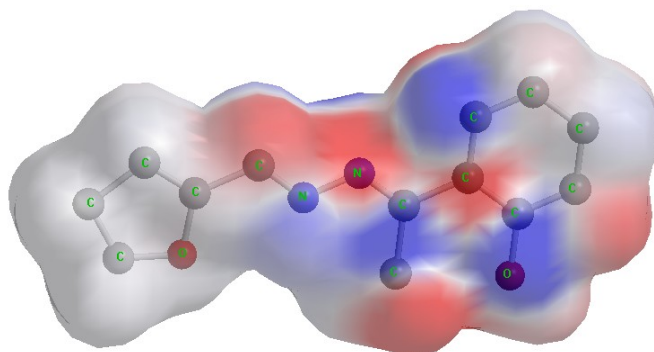
Figure 3: Molecular electrostatic potential (MEP) of ligand

Table 2: Selected parameters of Schiff base AF.

Bond	Bond length (Å)	Angle	(°)	Dihedral angle	(°)
C(8)-C(10)	1.540	N(9)-C(8)-C(10)	119.999	N(9)-C(8)-C(10)	119.999
N(9)-N(11)	1.352	C(8)-N(9)-N(11)	120.000	C(8)-N(9)-N(11)	120.000
N(11)-C(17)	1.244	N(9)-N(11)-C(17)	120.000	N(9)-N(11)-C(17)	120.000
C(12)-O(13)	1.410	O(13)-C(12)-C(14)	110.999	O(13)-C(12)-C(14)	110.999
C(12)-C(14)	1.324	O(13)-C(12)-C(17)	124.499	O(13)-C(12)-C(17)	124.499
C(12)-C(17)	1.540	C(14)-C(12)-C(17)	124.500	C(14)-C(12)-C(17)	124.500
O(13)-C(15)	1.410	C(12)-O(13)-C(15)	104.000	C(12)-O(13)-C(15)	104.000

The energy gaps ΔE of Co(II) and Ni(II) complexes are 2.311 and 2.778 eV. In HOMO orbitals for Co(II) and Ni(II) complexes are mainly delocalized on the corresponding metal ions and thiophene and phenyl rings. Whereas in LUMO, electrons are concentrated on nitrogen, the methyl group, and some part of metal ions. These tell us the chemical reactivity and stability of the ligand and their corresponding metal Co(II) and Ni(II) complexes and represented in Figures 5 and 6, and for Cu(II) and Zn(II) complexes were represented in supplementary file (28).
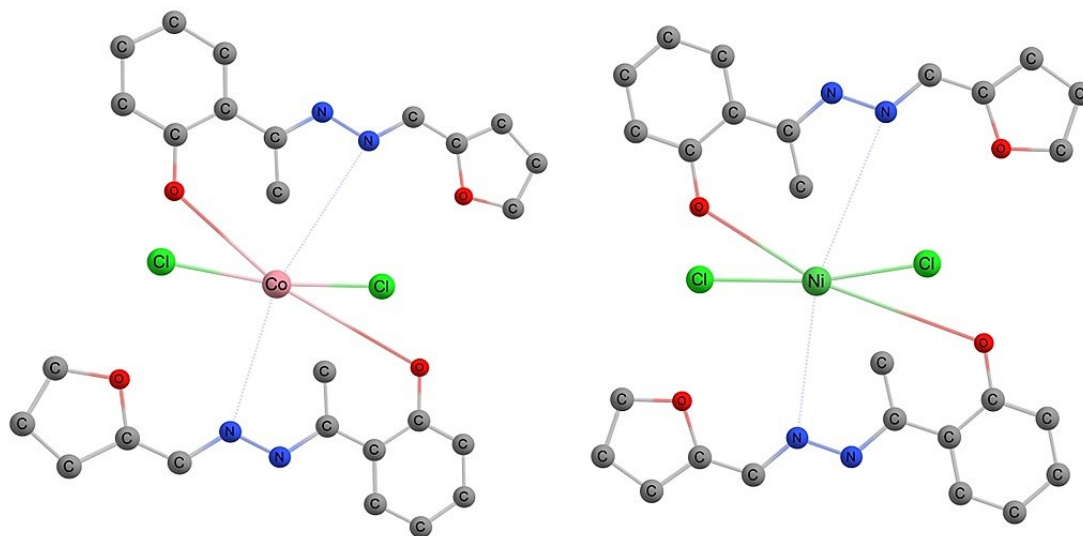
Figure 4: Optimized geometry of Co(II) and Ni(II) complexes.
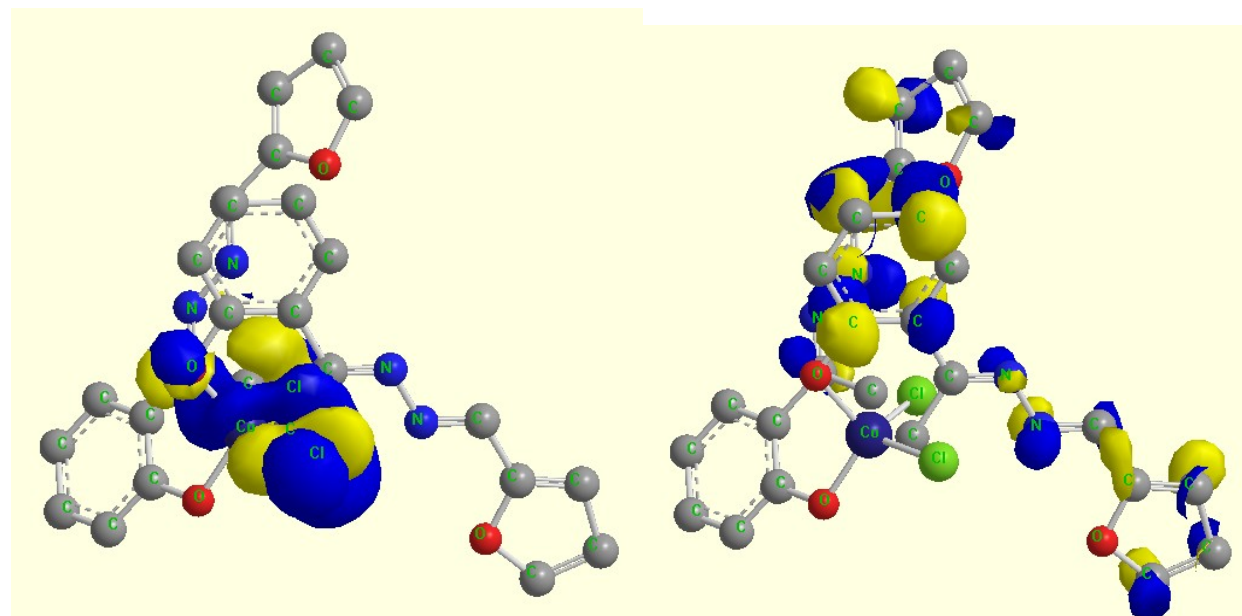
Figure 5: E_{LUMO}-E_{HOMO} [-4.951 eV and -2.631eV] molecular orbitals of Co(II) complexes.
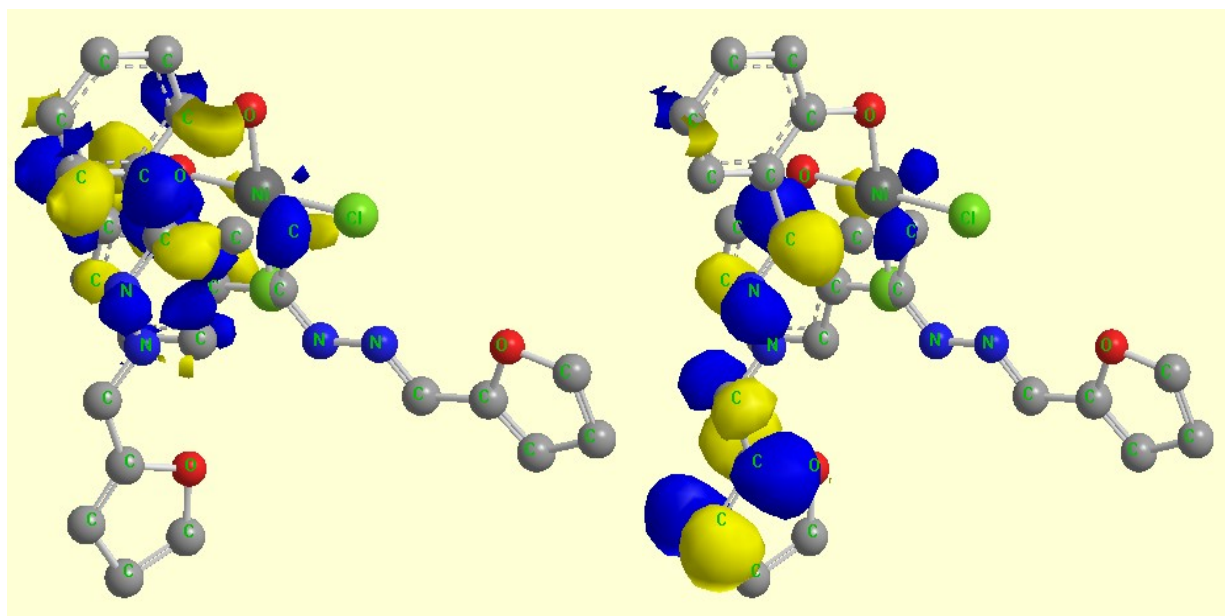
Figure 6: E_{LUMO}-E_{HOMO} [-8.811eV and -6.033 eV] molecular orbitals of Ni(II) complexes.

Biological studies
Antibacterial activity
The obtained results of the antibacterial assay of synthesized compounds have given in Table 3. The Ni(II) and Cu(II) complexes showed potent inhibition against all the bacterial strains, in which B. subtilis and K. pneumonia showed the

highest activity towards these cell lines. On the other hand, Co(II) complex showed promising activity towards gram-negative bacterial strains of P. aeruginosa and B. subtilis. While the ligand AF and Zn(II) showed the least activity towards all the bacterial strains (29, 30).

Table 3: Antibacterial data of AF and their metal complexes.

Compounds	*P.aeruginosa*	K. pneumonia	B. subtilis	S. pneumonia	S. aureus
AF	6	8	9	8	7
Co(II)	12	13	15	13	13
Ni(II)	15	18	19	15	12
Cu(II)	15	18	20	17	16
Zn(II)	12	13	15	11	13
Ciprofloxacin	16	19	22	18	17

Molecular docking
In accordance with antibacterial activity, it is significant to carry out *in silico* docking studies. The protein receptor SEC2 (PDB: 1STE) in Staphylococcus aureus revealed that the excellent docking interactions with different amino acids and E-total score (31). The Ni(II) complex showed excellent binding affinity towards enzyme receptor 1STE of -287.16

kcal/mol, while Co(II) and Cu(II) complexes also exhibit good binding interactions docking score of -257.11 and -222.99 kcal/mol. But the ligand, AF, showed the least binding interactions of -155.73 kcal/mol. The ligand and the receptor interactions involve binding with different amino acids like His6, Phe20, Thr136, Ser165, Phe167, Glu198, Phe200, Val236, Thr237, Leu250, and Leu253.

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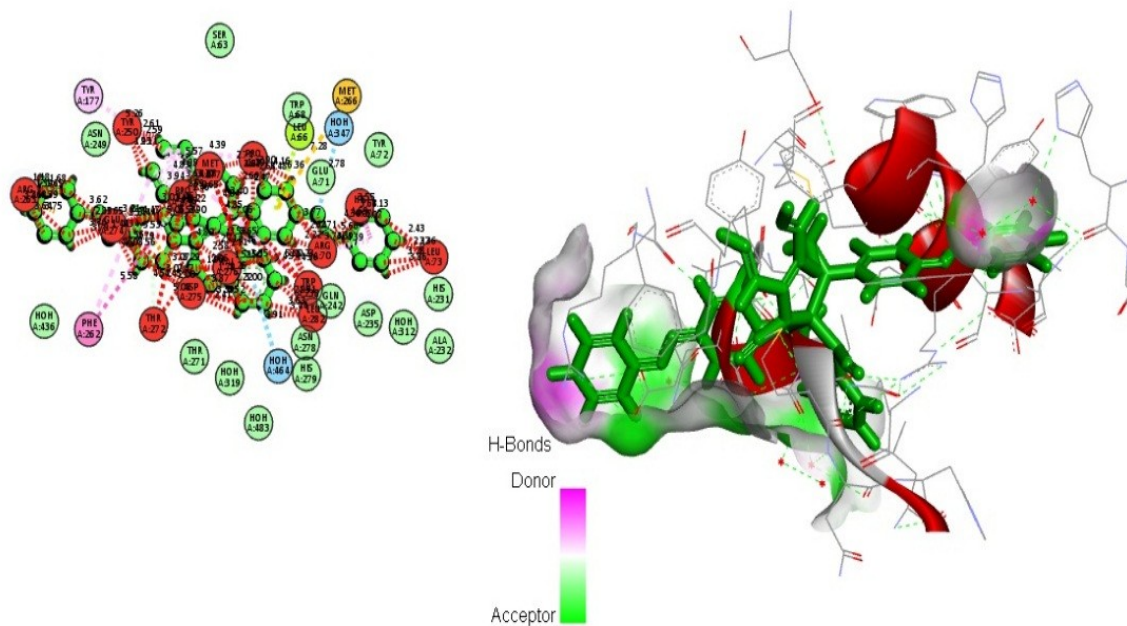
Figure 7: 3D and 2D binding interactions of Co(II) complex with PDB: 1STE.
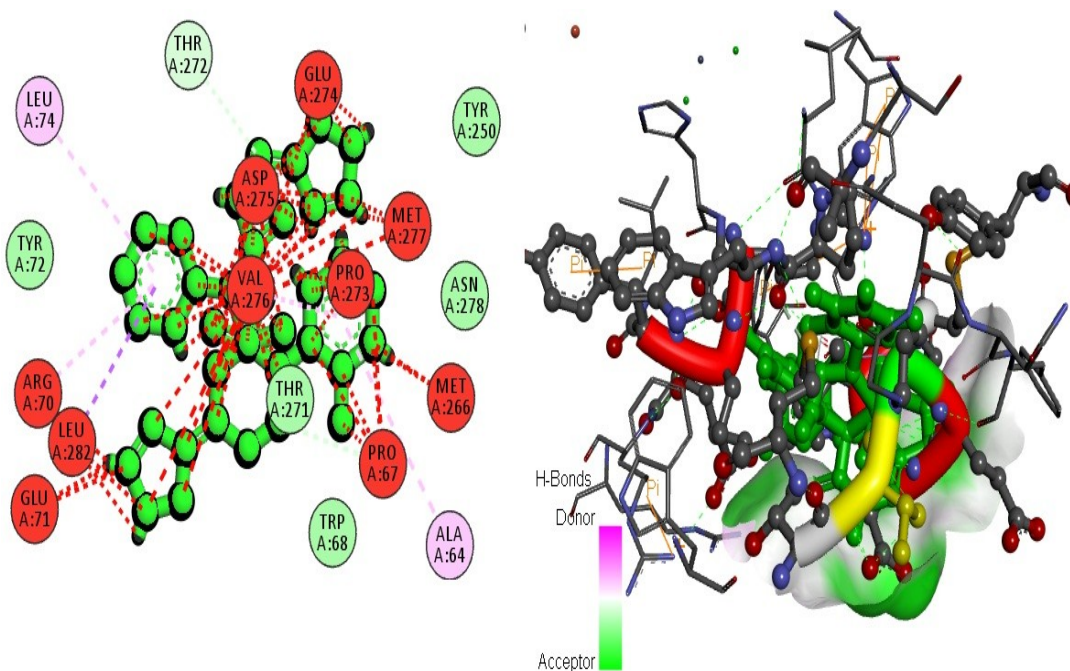
Figure 8: 3D and 2D binding interactions of Ni(II) complex with PDB: 1STE.

CONCLUSION

The synthesized compounds were characterized by spectral and analytical data. The bidentate nature of ligand indicates the bonding through (-OH) and (C=N) groups. The Co(II), Ni(II) complexes suggest the octahedral geometry and the Cu(II) complex exhibits The theoretical study using DFT/B3LYP supports experimental pieces of evidence of the binding sites of the ligand, geometry and the stability of the complexes. B. subtilis and K. pneumonia showed the highest

activity towards Ni(II) and Cu(II) complexes, The docking study revealed that Co(II) and Cu(II) complexes showed binding interaction with several amino acid residue of the protein receptor SEC2 (PDB: 1STE) in Staphylococcus aureus.

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Spectral, DFT studies,molecular docking and antibacterial activity of Schiff base derived from furan-2-carbaldehyde and their metal (II) complexes

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Sarvajith M S[4]

Supplementary Figures
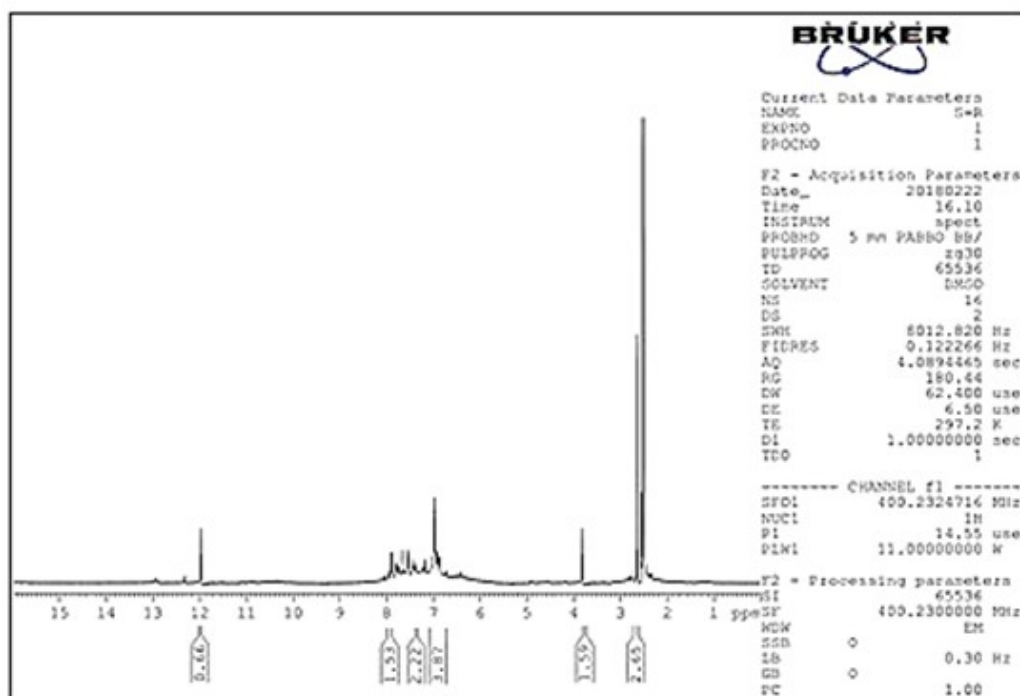
Figure S1: 1H NMR spectrum of AF.
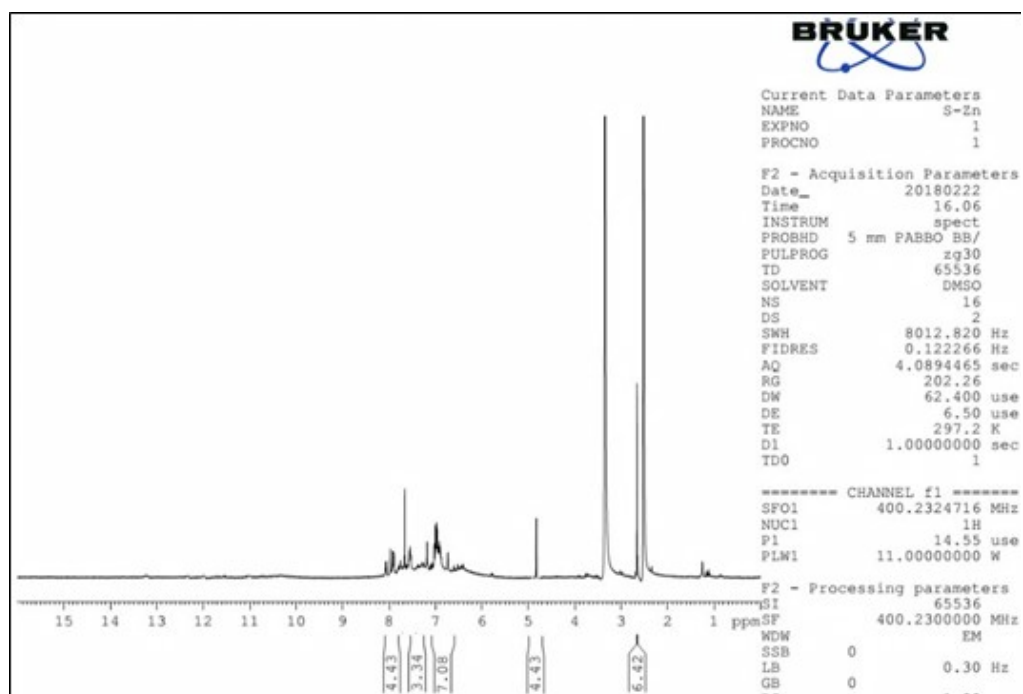
Figure S2: 1H NMR of Zn(II) complex.
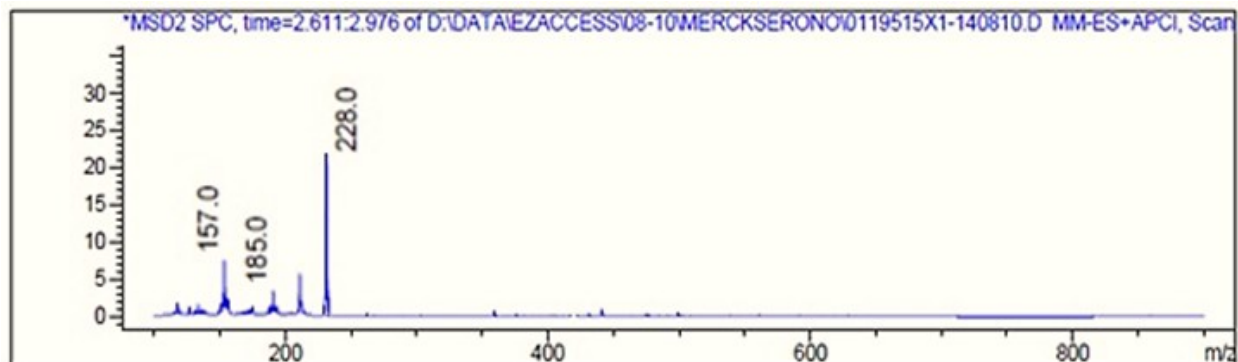
Figure S3: LCMS spectrum of Ligand AF.
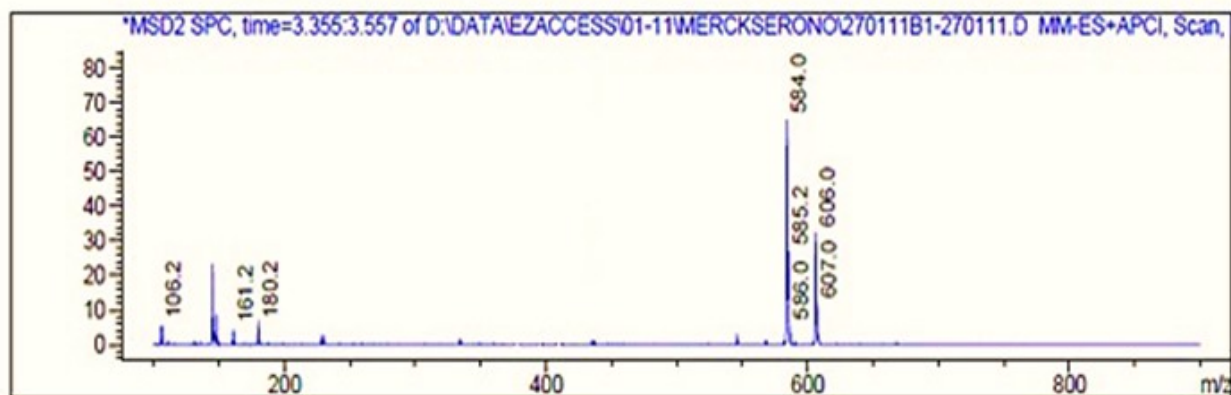
Figure S4: LCMS spectrum of Ni(II) complex .
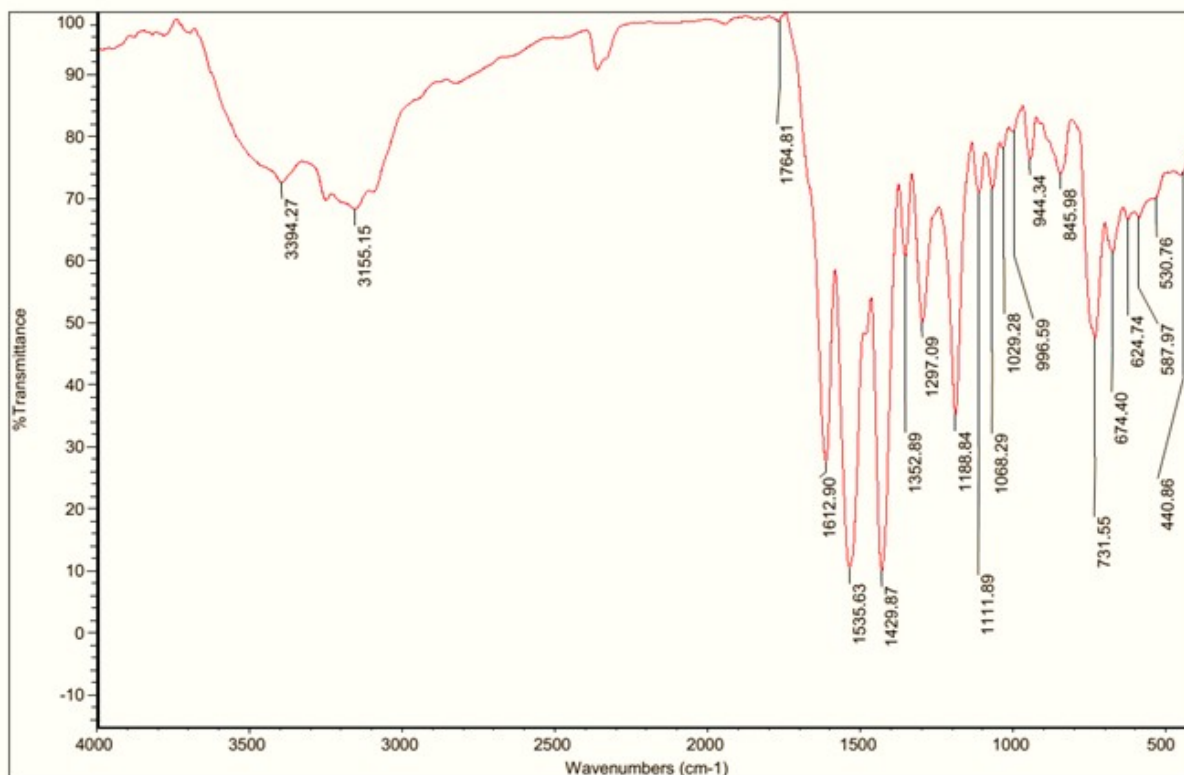
Figure S5: FT-IR Spectrum of Ligand AF .
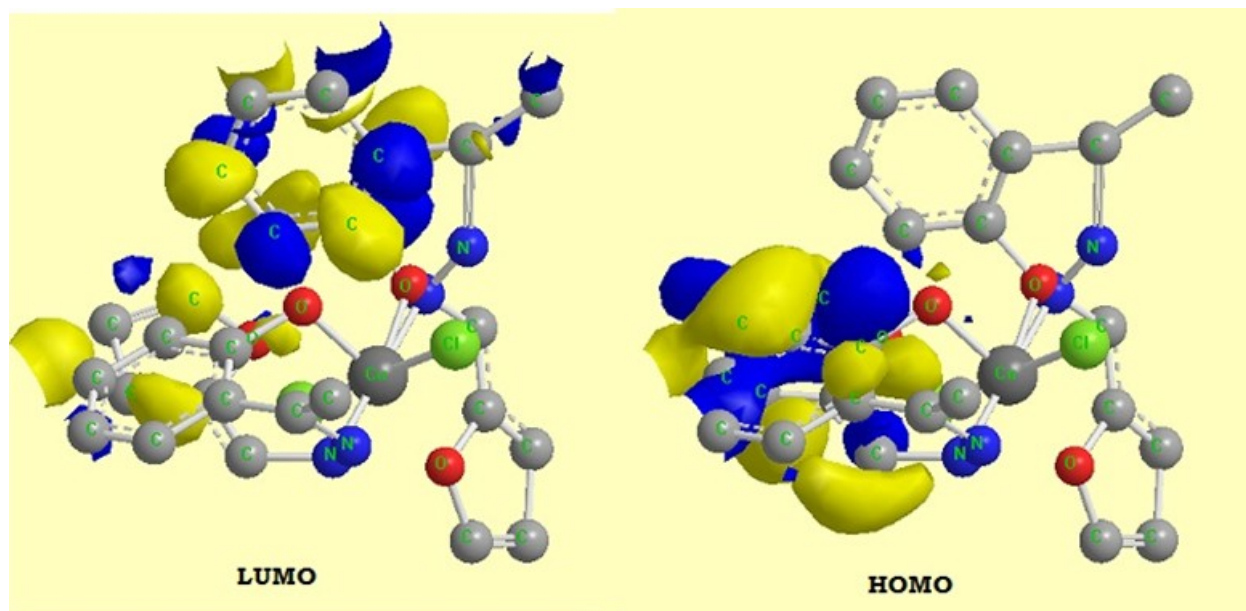
LUMO

HOMO

Figure S6: E_{LUMO}-E_{HOMO} [-5.114 eV and -2.231eV] molecular orbitals of Cu(II) complexes.
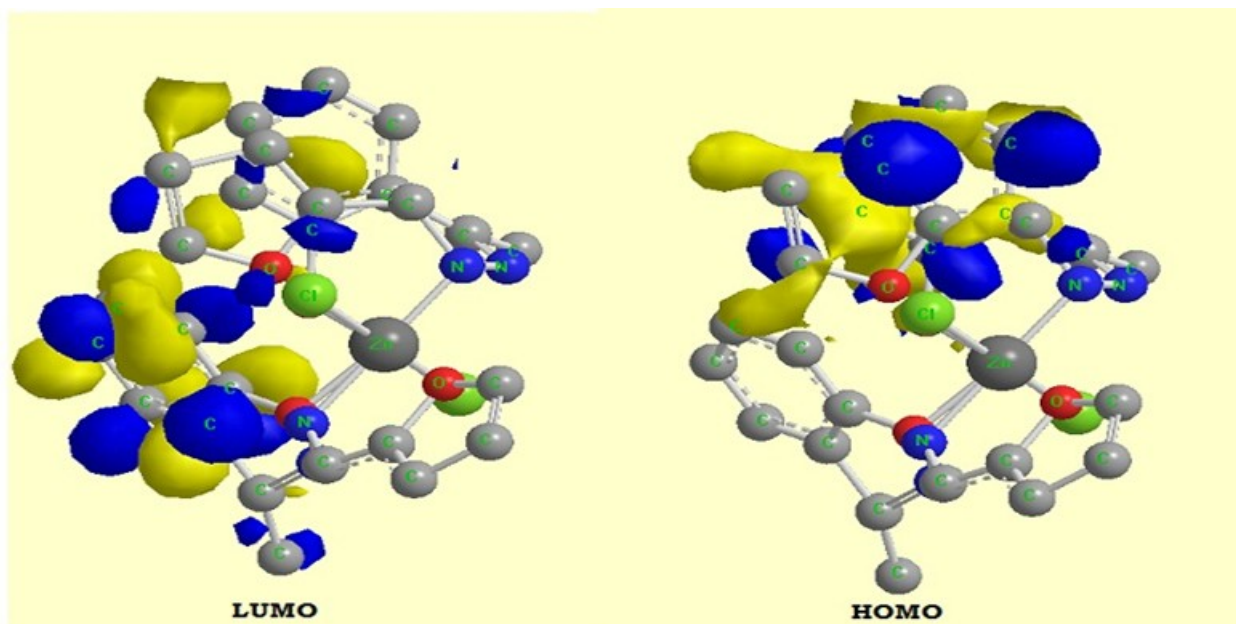
Figure S7: E_{LUMO}-E_{HOMO} [-6.221 eV and -4.241eV] molecular orbitals of Zn(II) complexes.

Dyeing Polyester, Cotton and Wool with Some Schiff Bases Derived from 4-chloroaminophenol and Various Benzaldehydes

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Abstract: Dyeability of ten Schiff bases obtained from 4-chloroaminophenol and various benzaldehydes including methyl, chloro, bromo, nitro, methoxy, and hydroxy groups were investigated on polyester, cotton and wool fibers. The dry/wet rubbing and washing fastness of the compounds were measured. It is observed that the rubbing and washing fastness values are varying at the 4 – 5 range (1: weak, 5: excellent). The lightness (**L**), red-greenness (**a**), and yellow-blueness (**b**) properties of the compounds were also investigated. It was observed that the dyeing properties of the compounds change depending on the substituent positions, and the substituent effect is observed on wool mostly. Especially, 3- and 5-methyl derivatives (**VIII** and **IX**) exhibit similar behavior toward polyester and cotton while they behave differently on wool in point of the **L**, **a,** and **b** values. On the other hand, it can be claimed that chlorine substituent increases the **L** value on wool.

INTRODUCTION

Schiff bases have considerable interest due to a large number of biological activities, including anticancer (1-3), antibacterial (4-6), antiviral (7-9), antifungal (10-12), antimalarial (13), urease inhibition (14-15), antioxidant agents (16-17) and DNA binding ability (18-19). The presence of C=N functional group in Schiff bases is responsible for the biological activities (20). Intramolecular hydrogen bonding between the phenolic group and the azomethine nitrogen atoms of Schiff bases derived from aromatic o-hydroxy aldehydes impacts the properties of various molecular systems and plays an essential role in many biochemical reactions (21).

Besides, the synthesis of Schiff bases has been increasing attention in synthetic organic chemistry, because of their potential applications in biological

modeling, catalysis, design of molecular magnets and materials chemistry (22-24) and also widely used as chelating agents (25-27), as corrosion inhibitors (28-29), as an herbicide (30), as textile dyes (31), as stabilizers in polymer chemistry (32).

Polyester is a synthetic fiber which is the most widely used in the textile industry due to their advantageous properties, including high tenacity, good resistance to chemicals, and deformation. Polyester fiber is preferred because of its hydrophobic nature and has a highly compact structure that permits efficient dyeing at high temperatures with disperse dyes.

The present study reports the investigation of the dyeability of some Schiff bases synthesized from 4-chloroaminophenol and various benzaldehydes. Polyester, cotton, and wool fabrics dyeing with conventional aqueous dyeing (batch dyeing) methods are studied in this context.

EXPERIMENTAL SECTION

General

All reagents were commercially available and used as received without further purification. Elemental analysis data were recorded on a Thermo Finnigan Flash EA 1112 analyzer. The FTIR spectra were obtained with a Bruker Optics Vertex 70 spectrometer (ATR). UV-Visible spectra were measured using a Perkin Elmer Lambda 25 UV/Visible Spectrophotometer. The Electron Spray Ionization-Mass Spectroscopy (ESI-MS) analyses were recorded on a Thermo Finnigan LCQ Advantage MAX LC/MS/MS. Dyeing was performed at a TERMAL Dyeing Lab. Machine. The used polyester (110 g/m^2), wool (120 g/m^2), and cotton (110 g/m^2) fabrics for dyeing procedure are 100% woven. Decacid Sab was used as a dispersing agent.

General Procedure for the Preparation of Schiff Bases

The Schiff bases were synthesized by condensation of 4-chloro-2-aminophenol (1.44 g; 10 mmol) with an appropriate aldehyde (*e.g.,* 2,4-dihydroxybenzaldehyde for **V**, 1.38 g; 10 mmol) in 20 mL of EtOH. This mixture was stirring under reflux for 3h. After the heading, the mixture was allowed to cool to room temperature. The solid product was separated by filtration and then crystallized from CH$_3$OH (33-34).
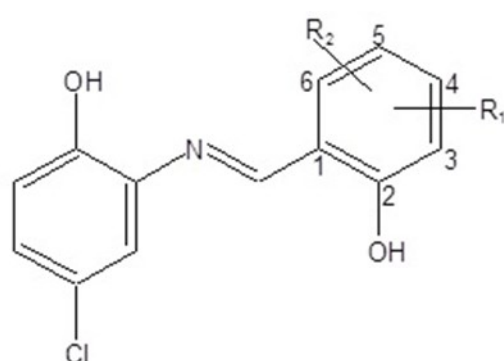
Compound	R₁	R₂
I	H	5-Br
II	H	5-Cl
III	3-Br	5-Cl
IV	3-Cl	5-Cl
V	4-OH	H
VI	3-OCH₃	H
VII	4-OCH₃	H
VIII	3-CH₃	H
IX	H	5-CH₃
X	H	5-NO₂

Scheme 1. The chemical structures of Schiff bases **I** – **X** used in this study.

I: Yellow-orange solid. Yield: 90%, m.p.: 201 °C. Elemental analysis: Calculated for C$_{13}$H$_9$BrClNO$_2$: C, 47.82; H, 3.28; N, 4.56; Found : C, 47.81; H, 2.78; N, 4.29. FT-IR (ATR, v/cm^{-1}): 3071 m,br, 2925 m,br, 2854 m,br, 2587 m, 1628 m, 1607 w, 1504 s, 1288 m, 1231 m, 1177 m, 1123 m, 816 m, 656 m, 525 w. UV (10^{-4} M, in methanol): 456 sh, 364 m,br, 262 m, 214 s. MS (ESI), m/z (%) (MA: 326.57 g/mol): 324.6 (38.0, [M-2]$^+$), 325.4, 326.7, 327.5, 328.6, 329.7.

II: Orange solid. Yield: 96%, m.p.: 203 °C. Elemental analysis: Calculated for C$_{13}$H$_9$Cl$_2$NO$_2$: C, 54.17; H, 3.41; N, 5.08; Found : C, 55.34; H, 3.22; N, 4.96. FT-IR (ATR, v/cm^{-1}): 3072 m,br, 2926 m,br, 2542 m,br, 1628 s, 1452 m, 1208 m, 1154 m, 1093 m, 1018 m, 910 m, 831 s, 698 w. UV (10^{-4} M, in methanol): 452 w,br, 364 m,br, 269 m, 234 sh, 215 s. MS (ESI), m/z (%) (MA: 282.12 g/mol): 280.6 (100, [M-2]$^+$), 281.7 (11.7, [M-1]$^+$), 282.5 (65.6, [M]$^+$), 283.6 (8.1, [M+1]$^+$), 284.5 (9.6, [M+2]$^+$).

III: Orange solid. Yield: 93%, m.p.: 271 °C. Elemental analysis: Calculated for C$_{13}$H$_8$BrCl$_2$NO$_2$: C, 43.40; H, 2.19; N, 3.70; Found : C, 43.25; H, 2.32; N, 3.88. FT-IR (ATR, v/cm^{-1}): 3214 m,br, 3059 m,br, 2925 m,br, 1628 s, 1499 s, 1438 m, 1305 m, 1226 s, 1145 m, 1021 m, 915 m, 845 m, 739 m, 664 m. UV (10^{-4} M, in methanol): 462 w,br, 440 sh, 367 m,br, 269 m, 211 s. MS (ESI), m/z (%) (MA: 361.02 g/mol): 358.8 (41.4, [M-2]$^+$), 359.9 (6.0, [M-1]$^+$), 360.8 (100, [M]$^+$), 361.7 (15.9, [M+1]$^+$), 362.5 (49.7, [M+1]$^+$), 363.2 (43.0, [M+2]$^+$), 364.1 (9.8; [M+3]$^+$), 364.8 (11.7, [M+4]$^+$), 365.7 (5.0, [M+5]$^+$).

IV: Orange solid. Yield: 95%, m.p.: 279 °C. Elemental analysis: Calculated for C$_{13}$H$_8$Cl$_3$NO$_2$: C, 49.18; H, 2.74; N, 4.56; Found : C, 49.32; H, 2.55; N, 4.42. FT-IR (ATR, v/cm^{-1}): 3164 m,br, 3063 m,br, 2921 m, 1628 s, 1508 s, 1438 m, 1217 s, 1149 m, 1034 m, 854 m, 761 m, 660 w. UV (10^{-4} M, in methanol): 463 m,br, 437 sh, 365 m,br, 269 m, 213 s. MS (ESI), m/z (%) (MA: 316.57 g/mol): 314.7 (87.2, M-2), 316.6 (100, [M]$^+$), 317.7 (14.6, [M+1]$^+$), 318.6 (31.5, [M+2]$^+$), 319.5 (5.4, M+3), 320.5 (4.4, M+4).

V: Dark yellow solid. Yield: 73%, m.p.: 221 °C. Elemental analysis: Calculated for C$_{13}$H$_{10}$ClNO$_2$: C, 58.89; H, 3.84; N, 5.50; Found : C, 59.22; H, 3.82; N, 5.31. FT-IR (ATR, v/cm^{-1}): 3178 m,br ●(OH), 3072 m,br, 2926 m, 2553 m,br, 1637 s, 1592 m, 1269 s, 1228 s, 1131 m, 989 m, 915 m, 849 m, 790 m, 662 w. UV (10^{-4} M, in methanol): 422 m,br, 352

m,br, 269 m, 228 sh, 212 s. MS (ESI), m/z (%) (MA: 263.67 g/mol): 264.5 (38.0, $[M+1]^+$), 265.8 (17.2, $[M+2]^+$), 509.3 (100, $[2M-OH]$), 511.3 (71.9), 525.5 (56.7), 526.5 (20.8), 527.5 (22.0).

VI: Bright yellow solid. Yield: 85%, m.p.: 219 °C. Elemental analysis: Calculated for $C_{14}H_{12}ClNO_3$: C, 60.82; H, 4.46; N, 5.01; Found : C, 60.55; H, 4.36; N, 5.04. FT-IR (ATR, v/cm^{-1}): 3071 m,br, 2970 m,br, 2909 m,br, 1649 s, 1621 s, 1592 m, 1525 m, 1504 s, 1479 s, 1429 s, 1278 m, 1226 s, 903 m, 831 m, 800 m, 772 m, 656 m. UV (10^{-4} M, in methanol): 446 sh, 426 m,br, 351 s,br, 304 m,br, 285 sh, 242 s,br, 215 sh. MS (ESI), m/z (%) (MA: 277.50 g/mol): 276.67 (77.6, $[M-1]^+$), 278.69 (100, $[M+1]^+$), 280.16 (30.4, $[M+3]^+$).

VII: Red solid. Yield: 50%, m.p.: 145 °C. Elemental analysis: Calculated for $C_{14}H_{12}ClNO_3$: C, 60.75; H, 4.48; N, 5.00; Found : C, 60.55; H, 4.36; N, 5.04. FT-IR (ATR, v/cm^{-1}): 3054 m,br, 3002 m,br, 2930 m,br, 1623 s, 1594 m, 1504 s, 1443 m, 1251 m, 1140 m, 906 m, 827 m, 799 m, 646 m. UV (10^{-4} M, in methanol): 482 w,br, 373 m,br, 354 sh, 273 s, 252 sh, 236 s,br, 219 sh. MS (ESI), m/z (%) (MA: 277.50 g/mol): 276.72 (100, $[M-1]^+$), 278.71 (71.3, $[M+1]^+$), 280.37 (24.4, $[M+3]^+$).

VIII: Light orange solid. Yield: 97%, m.p.: 220 °C. Elemental analysis: Calculated for $C_{14}H_{12}ClNO_2$: C, 64.20; H, 4.83; N, 5.42; Found : C, 64.25; H, 4.62; N, 5.35. FT-IR (ATR, v/cm^{-1}): 3071 m,br, 2920 m, 2673 m,br, 2562 m,br, 1624 s, 1595 sh, 1441 m, 1232 s, 1162 m, 1036 m, 817 m, 762 m, 662 w. UV (nm, 10^{-4} M, in methanol): 452 w,br, 352 m,br, 269 m,br, 215 s. MS (ESI), m/z (%) (MA: 261.70 g/mol): 262.6 (100, $[M+1]^+$), 263.5 (30.7, $[M+2]^+$).

IX: Orange solid. Yield: 85%, m.p.: 173 °C. Elemental analysis: Calculated for $C_{14}H_{12}ClNO_2$: C, 64.34; H, 4.51; N, 5.11; Found : C, 64.25; H, 4.62; N, 5.35. FT-IR (ATR, v/cm^{-1}): 3428 m, 2925 m, 1631 s, 1585 m, 1508 s, 1438 m, 1294 m, 1240 m, 1162 m, 922 m, 822 m, 656 w. UV (nm, 10^{-4} M, in methanol): 451 w,br, 359 m,br, 269 m, 237 sh, 214 s. MS (ESI), m/z (%) (MA: 261.70 g/mol): 262.5 (31.6, $[M+1]^+$), 261.6 (10.5, $[M]^+$), 260.6 (100, $[M-1]^+$).

X: Yellowish orange solid. Yield: 74%, m.p.: 268 °C. Elemental analysis: Calculated for $C_{13}H_9ClN_2O_4$: C, 53.44; H, 3.26; N, 9.74; Found : C, 53.35; H, 3.10; N, 9.57. FT-IR (ATR, v/cm^{-1}): 3066 m, 2925 m,br, 2774 m,br, 2557 m,br, 1649 s, 1623 s, 1446 m, 1391 m, 1296 s, 1238 m, 1192 m, 1151 m, 1100 m, 1038 m, 901 m, 837 m, 755 m, 639 m. UV (nm, 10^{-4} M, in methanol): 461 sh, 426 m,br, 350 m,br, 269 m, 262 m, 211 s. MS (ESI), m/z (%) (MA: 292.67 g/mol): 290.3 (100, $[M-2]^+$), 292.4 (65.7, $[M]^+$), 293.5 (29.3, $[M+1]^+$) 294.5 (28.6, $[M+2]^+$).

Dyeing Polyester Fabrics
The polyester samples were dyed with an HT sample dyeing machine. Dyebath was prepared with dye, which was synthesized, nonionic dispersant (0.5 g/L), and CH_3COOH (pH~5) with a liquor ratio of 1:20. The temperature was raised to 130 °C at a rate of 3 °C/min. Within 30 min. and the dyeing continued for 60 min. After completion of dyeing, reduction cleaning of the samples were rinsed with warm water and were subjected to neutralization with a solution containing $Na_2S_2O_4$ and NaOH (2 g/L each) at 70 °C for 10 min. To remove the superficial dye, the polyester was rinsed with cold water at 50 °C for 10 min. and washed with water and air-dried.

Dyeing Cotton Fabrics
The cotton samples were dyed with an HT sample dyeing machine. Dyebath was prepared with dye, which was synthesized, nonionic dispersant (0.5 g/L), and Na_2CO_3 (pH~10) with a liquor ratio of 1:20. The temperature was raised to 90 °C at a rate of 6 °C/min within 30 min, and the dyeing continued for 90 min. After completion of dyeing, the samples were rinsed two times for 10 min. with water at 60 °C and air-dried.

Dyeing Wool Fabrics
The wool samples were dyed with an HT sample dyeing machine. Dyebath was prepared with dye, which was synthesized, nonionic dispersant (0.5 g/L), and CH_3COOH (pH~5) with a liquor ratio of 1:20. The temperature was raised to 80 °C at a rate of 8 °C/min and then held at this temperature for 90 min. and the dyeing continued for 60 min. After completion of dyeing, the samples were removed, thoroughly rinsed with water for 10 min. at 40 °C and air-dried.

Color Fastness Tests
The reduction cleaning of the dyed samples was measured using the ISO 105 C06 standard method, and ECE was used as washing detergent for this test. The Greyscale was used for evaluating the change in shade and staining of adjacent multifiber test fabrics. Color fastness to rubbing test was evaluated according to the ISO105–X122002 standard method. The color of the dyed fabrics was measured using lSO7724/1 standard method

RESULTS AND DISCUSSIONS

The Schiff bases used in this study were reported previously (33- 34). The physicochemical and spectroscopic data of the Schiff bases are given at Experimental Section.

Tables 1, 3, and 5 show the washing and rubbing fastness test results of dyes on polyester, cotton, and wool fabrics. Washing and rubbing fastness values of the compounds for polyester, cotton, and wool are detected as 4 or 5, which considered that these values are specified at the 1 – 5 range (1:

weak, 4: good, 5: excellent). It can be said that the compounds give good fastness properties for washing and rubbing levels as well.

The lightness (**L**), red-greenness (**a**), and yellow-blueness (**b**) properties of the compounds, containing different substituents on polyester, wool, and cotton fibers are shown in Tables 2, 4, and 6.

The following points are found by examining the lightness (**L**), red-greenness (**a**) and yellow-blueness (**b**) values of the compounds for polyester fabric (Table 2): The **L** value of the compound **VI** (3-methoxy derivative) is highest, that of **III** (3-Br,5-Cl derivative) is lowest although all **L** values are close to each other (between 68.11 and 53.61). Similarly, all the **a** and **b** values are close to each other, also. The highest **a** and **b** values are belonging to 3-Me (**VIII**) and 5-Br (**I**) derivatives, 14.52 and 43.34; the lowest ones are 10.27 (**VII**: 4-methoxy) and 30.01 (**III**: 3-Br,5-Cl), respectively.

It is seen that the **L** and **b** values of 5-Br derivative (**I**) are higher than those of 5-Cl derivative (**II**),

whereas the **a** value is lower (Table 2). The bromo substituent at the ortho position (**III**: 3-Br,5-Cl) decreases the **L**, **a,** and **b** values comparing to the chloro derivative (**IV**: 3-Cl,5-Cl) on polyester fabric. It is observed that the **L** value of the 5-Br derivative (**I**) is highest among the compounds having chloro and bromo substituents, namely **I**, **II**, **III,** and **IV**. The **L** and **a** values of the 4-methoxy derivative (meta position, **VII**) are lower (66.73 and 10.27, respectively) than those of 3-methoxy derivative (**VI**; L: 68.11, a: 13.41) whereas the **b** value is higher (42.43) than that of **VI** (36.39) towards to polyester fabric (Table 2). The **L** value of the 3-Me derivative (**VIII**: methyl group at ortho position) is lower than that of 5-Me derivative (**IX**: methyl group at the para position) whereas the values of **a** and **b** are higher than that of 3-Me derivative.

It is seen that the highest **L** value is belonging to 5-Br derivative (**I**) for cotton fabrics (Table 4), whereas the nitro derivative (**X**) shows the lowest **L** value. The highest **a** and **b** values are shown by the compound **VIII** (3-Me derivative); the lowest **a** and **b** values are exhibited by **I** and **II** (5-Br and 5-Cl derivatives), respectively.

Table 1. Color fastness of the compounds on polyester fabric.[1]

Compound	Washing fastness							Rubbing fastness	
	Color Change	Staining[2]						Dry	Wet
		AC	CO	PA	PES	PAN	WO		
I	4-5	4-5	4	4-5	4-5	4-5	4-5	4-5	4
II	4	4-5	4	4	5	5	4	4-5	5
III	4	5	4	4	5	5	4	4-5	5
IV	4	4-5	4	4	5	5	4	4-5	5
V	4	4-5	4	4-5	5	4-5	4	5	4
VI	5	5	4	5	5	5	5	5	4-5
VII	5	5	4	4-5	5	5	4-5	5	5
VIII	4	4	4	4	4	4	4	4	4
IX	4	4	4	4	4	4	4	4	4
X	4-5	5	4	4	5	5	4	4-5	5

[1] The changes are graded with marks 1 - 5 (1: weak, 5: excellent)
[2] AC: Acetate cellulose, CO: Cotton, PA: Polyamide, PES: Polyester, PAN: Polyacrylonitrile, WO:wool

Table 2. Lightness (L), red-greenness (a) and yellow-blueness (b) values for polyester (D65).[1]

Compound	L [2]	a [2]	b [2]	Compound	L [2]	a [2]	b [2]
I	64.01	10.59	43.34	VI	68.11	13.41	36.39
II	55.52	14.00	37.55	VII	66.73	10.27	42.43
III	53.61	11.74	30.01	VIII	64.64	14.52	39.08
IV	60.84	12.18	36.51	IX	63.78	12.63	33.42
V	56.32	14.37	43.04	X	59.75	12.68	37.54

[1]: D65: daylight lamp.
[2] **L**: Lightness (low or negative value: darkness, high or positive value: lightness); **a**: Red-greenness (low or negative value: green, high or positive value: red); **b**: Yellow-blueness (low or negative value: blue, high or positive value: yellow).

Table 3. Color fastness of the compounds on cotton fabric.[1]

Compound	Washing fastness							Rubbing fastness	
	Color Change	Staining[2]						Dry	Wet
		AC	CO	PA	PES	PAN	WO		
I	5	4-5	4	4	4-5	5	4	4-5	4
II	5	4-5	4	4-5	4-5	4-5	4	4-5	4
III	4-5	4	4	4	5	5	4	4-5	4
IV	4-5	4	4	4	4	3-4	4	4-5	4
V	4	4-5	4	4	4-5	5	4	5	4
VI	4	4-5	4	4	4-5	5	4	5	4
VII	4	4-5	4	4	4-5	5	4	5	4
VIII	4	4	4	4	4	5	4	5	4
IX	4	4	4	4	4	4	4	4	4
X	4	4	4	4	5	5	4	4	4

Table 4. Lightness, red-greenness and yellow-blueness values for cotton (D65).[1]

Compound	L [2]	a [2]	b [2]	Compound	L [2]	a [2]	b [2]
I	85.20	2.68	11.45	VI	82.66	4.39	16.96
II	82.24	5.03	10.04	VII	82.04	4.08	18.66
III	77.16	6.47	25.64	VIII	74.28	7.86	26.53
IV	81.53	4.96	15.73	IX	84.30	2.78	16.26
V	77.96	6.16	14.65	X	71.22	4.95	13.47

Table 5. Color fastness of the compounds on wool fabric.[1]

| Compound | Color Change | Staining[2] | | | | | | Rubbing fastness | |
		AC	CO	PA	PES	PAN	WO	Dry	Wet
I	4	4	4	4	4	4	4	5	4
II	4	4	4	4	4	4	4	5	4
III	4	4	4	4	5	5	4	5	4
IV	4	4	4	4	4	4	4	5	4
V	4-5	4-5	4	4-5	4-5	5	4-5	4-5	4
VI	4-5	4-5	4	4	4-5	5	4	4-5	4
VII	4	4-5	4	4	4-5	5	4	4-5	4
VIII	4	4	4	4	4	5	4	4	4
IX	4	4	4	4	4	4	4	4	4
X	4	4	4	4	5	5	4	4	4

Table 6. Lightness, red-greenness and yellow-blueness values for wool (D65).[1]

Compound	L [2]	a [2]	b [2]	Compound	L [2]	a [2]	b [2]
I	54.83	12.20	41.08	**VI**	52.70	14.71	39.60
II	90.46	2.02	-6.25	**VII**	49.83	15.05	40.31
III	64.86	8.37	10.52	**VIII**	46.34	14.41	35.83
IV	90.94	1.73	-5.10	**IX**	90.58	0.38	-2.07
V	37.48	18.01	38.86	**X**	38.16	14.93	33.15

It is observed that the **L** and **b** values of 5-Br derivative (**I**) are higher than those of 5-Cl derivative (**II**), whereas the **a** value is lower for cotton fabric (Table 4). On the other hand, the chloro substituent at ortho position (**IV**: 3-Cl,5-Cl) increases **L** value whereas decreases the **a** and **b** values compared to those of the bromo derivative (**III**: 3-Br,5-Cl). The **L** value of the 5-Br derivative (**I**) is highest among the compounds having chloro and bromo substituents, namely **I**, **II**, **III,** and **IV** (similarity to the polyester fabric). In the compounds **VI** (3-methoxy, ortho position) and **VII** (4-methoxy, meta position), the **L** and **a** values are close to each other, whereas the methoxy group at the meta position (**VII**) increases the **b** value according to the ortho position. It is seen that the methyl group at the para position (**IX**) increases the **L** value according to the ortho position methyl group (**VIII**) (84.30 and 74.28 respectively) whereas decreases **a** and **b** values (Table 4).

The **L**, **a,** and **b** values for wool fabrics (Table 6) are in a wide range according to the polyester and cotton. These ranges are 90.94 – 37.48, 18.01 – 0.38, and 41.08 – (-6.25) for **L**, **a,** and **b**, respectively. The highest **L**, **a,** and **b** values belong to **IV**, **V,** and **I**, respectively, whereas the lowest **L**,

a, and **b** values are exhibited by the compounds **V**, **IX,** and **II**, respectively. Interestingly, compound **V** (4-OH derivative) has the highest **a** and lowest **L** values.

By comparing **I** and **II** for wool fabric (Table 6), it is seen that the **L** value of **II** (5-Cl derivative) is relatively high than that of **I** (5-Br derivative) (90.46 and 54.83, respectively). The **a** and **b** values of **II** (2.02, -6.25) are relatively low, according to those of **I** (12.20 and 41.08). There is a similar situation for **III** (3-Br,5-Cl) and **IV** (3-Cl,5-Cl): The **L** value of **IV** is considerably high (64.86 and 90.94 for **III** and **IV**, respectively) and the **a** and **b** values (1.73 and -5.10 for **IV**, respectively) are considerably low according to those of **III** (8.37 and 10.52). From these results, it can be claimed that chloro substituent increases **L** value whereas decreases the **a** and **b** values in case of wool. The **L**, **a,** and **b** values are close to each other for 3-methoxy and 4-methoxy derivatives (**VI** and **VII**) on wool. The **L** values are 90.58 and 46.34 for **IX** and **VIII**, respectively: The methyl group at para position (**IX**) increases the **L** value whereas decreases the **a** and **b** (0.38 and -2.07) values considerably according to the methyl group at ortho position (**VIII**: 14.41 and 35.83).

Tables 2, 4, and 6 reveal that the **a** and **b** values are higher for polyester and wool fabrics according to that of cotton fabric. In addition, it is interesting that the **b** values of the compounds **III**, **IV,** and **IX** are negative. It is considered that the **b** value of **IX** (5-Me derivative) is negative (-2.07) for wool, although that of the **VIII** (3-Me derivative) is a reasonably positive value such as 35.83. These two compounds (**VIII** and **IX**) show similar behavior toward polyester and cotton while behaving differently on wool in point of the **L**, **a,** and **b** values. Contrarily for wool, there is no significant difference in the polyester and cotton fabrics comparing their **L**, **a,** and **b** values.

As a conclusion, it is observed that the compounds have **L**, **a,** and **b** values at a wide range of wool fabric. It means that the substituent effect is seen more clearly in wool fabrics. Besides, it can be concluded that the position of the substituents is significant on dyeing properties even if slightly, comparing the **VI** with **VII** and **VIII** with **IX**, the derivatives including the methyl and methoxy groups.

It is possible to say that comparing Tables 2, 4, and 6, the **L** values are inversely proportional to **a** and **b** values, generally: If the **L** values are high, **a** and **b** values are low.

CONCLUSIONS

In this study, dry/wet rubbing and washing fastnesses of some Schiff bases derived from 4-chloroaminophenol and various benzaldehydes including methyl, chloro, bromo, nitro, methoxy, and hydroxy groups were investigated on polyester, wool and cotton fibers. The compounds exhibit good or excellent rubbing and washing fastness characteristics. Also, the lightness (**L**), red-greenness (**a**), and yellow-blueness (**b**) properties of the compounds were investigated. Dyeing properties of the compounds depend on the substituent positions, and the change in dyeing properties resulted from substituent effect is observed on wool mostly. Interestingly, the **L**, **a,** and **b** values of 3-Me and 5-Me derivatives (**VIII** and **IX**) are close to each other on polyester and cotton; however, they show significant differences in wool fabric (substituent position effect).

On the other hand, it can be claimed that chlorine substituent increases **L** value on wool. Schiff base substituents cause various effects on the fabrics. These result from interactions between the functional groups on the molecule and the fabrics depending on their structure.

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RESEARCH ARTICLE

Design of Novel Benzimidazole Derivatives as Potential α-amylase Inhibitors by 3D-QSAR Modeling and Molecular Docking Studies

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Abstract: The enzyme α-amylase belongs to the highly conserved glycoside hydrolase family which is regarded as a good target for the discovery of antidiabetic agents. Following a 3D-QSAR study on forty-five 2-aryl benzimidazole derivatives which have been reported as potential insulin-independent antidiabetic agents. The results revealed that the CoMFA values were found to be 0.696 and 0.860 for Q^2 and R^2 respectively; while for the CoMSIA, the Q^2 and R^2 values were found to be 0.514 and 0.852 respectively. Both models were derived from a training set of thirty-seven compounds based on an appropriate method of alignment, while the predictive ability was approved by a test set containing eight compounds with r_{ext}^2 values of 0.990 and 0.987, respectively. Moreover, the contour maps generated from CoMFA and CoMSIA models provided much helpful information to figure out the structural requirements that influence the activity. To further reinforce the 3D-QSAR results, the molecular docking method was implemented which led to the design of new potential insulin-independent antidiabetic compounds with high predicted activity values.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic syndrome of multiple etiology characterized by hyperglycemia as its initial symptoms and glucose intolerance. The disease can be simply classified into insulin secretion (type 1 diabetes) or insulin action (type 2 diabetes) (1,2). The digestive enzyme, α-Amylase, is the main form of amylase belonging to the family of glycoside hydrolases and found principally in pancreatic fluid and saliva. It is responsible for the biosynthesis of glycoproteins with an important role in the digestion of carbohydrate in the small intestine. Acute renal necrosis, cancer of urinary bladder, and active pyelonephritis are some urinary disorders and causes due to increasing the level of this enzyme(3). The glycoside hydrolases family can be isolated from many organisms including humans (4), animals (5,6), plants (7), and bacteria (8).

Benzimidazoles are a class of heterocyclic compounds that attracts much attention as promising bioactive molecules. Furthermore, benzimidazole is one of the privileged pharmacophores in drug discovery acting as a good ligand towards the transition metal ions using the nitrogen atom (9). This is due to their various reported anti-tumor (10), anti-fungal (11), anti-inflammatory (12), antiviral (13), anti-ulcerative (14), and antimicrobial activities (15).

The structural parameters along with the application of the 3D-QSAR modeling and molecular docking

methods have proved to be a promising avenue for structure-based drug design that can provide quantitative forecasts of activity and predict the activity of new organic compounds with a lowered economic investment costs (16). The current study aimed to explore the 3D-QSAR and molecular docking approaches (17) on a series of benzimidazole derivatives against α-amylase in order to develop a correlation between their inhibitory activities and structural characteristics. This would eventually facilitate the design and optimization of new potential α-amylase inhibitors with potent activities.

MATERIALS AND METHODS

Dataset

A set of forty-five compounds with their reported α-amylase inhibition activities, IC_{50} was obtained from the literature (18). And the corresponding IC_{50} values (uM) were converted into the pIC_{50} values (Table 1). The dataset was split into the training set (37 molecules) and test set (8 molecules). Both sets were selected at random to create the quantitative model.
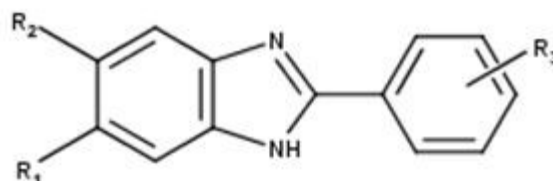
Figure 1. The structural template of the benzimidazole derivatives, **1-45**.

Table 1. IC_{50} values of the reported 2-Aryl benzimidazole derivatives against α-amylase.

Training/ Test	Comp.	R_1	R_2	R_3	pIC_{50}
Test	**1***	H	H	2-Cl-3-OCH_3	5.562
Test	**2***	H	H	2-OH-3-OCH_3-6-Br	5.605
Training	**3**	H	H	3,4-diOCH_3-6-Br	5.821
Training	**4**	H	H	2-OH-3-Br-5-Cl	5.555
Training	**5**	CH_3	CH_3	3,6-diOH	5.524
Training	**6**	CH_3	CH_3	4-OCH_3-6-F	5.555
Training	**7**	CH_3	CH_3	5-OCH_3-6-Cl	5.546
Training	**8**	CH_3	CH_3	4,5,6-triOCH_3	5.638
Test	**9***	CH_3	CH_3	2-OH-3-OCH_3-6-Br	5.585
Training	**10**	CH_3	CH_3	3,4-diOCH_3-6-Br	5.829
Training	**11**	CH_3	CH_3	3,5-diOCH_3-4-Br	5.790
Training	**12**	CH_3	CH_3	3-I-4-OH-5-OCH_3	5.751
Training	**13**	CH_3	CH_3	3-Cl-5-Br-6-OH	5.530
Training	**14**	CH_3	CH_3	3,5-diCl-6-OH	5.520
Training	**15**	H	F	4,6-diOH	5.520
Training	**16**	H	F	4-OCH_3-6-F	5.790
Test	**17***	H	F	2-OCH_3-5-Br	5.585
Test	**18***	H	F	5-OCH_3-6-Cl	5.580
Training	**19**	H	F	4-OAc-5-OCH_3	5.770
Training	**20**	H	F	4,5,6-triOH	5.595
Training	**21**	H	F	3,5-diOCH_3-4-OH	5.603
Training	**22**	H	F	2-OH-3-OCH_3-6-Br	5.663
Training	**23**	H	F	3,4-diOCH_3-6-Br	5.804
Training	**24**	H	F	4,5-diOCH_3-6-Cl	5.591
Training	**25**	H	F	3,5-diCl-6-OH	5.542
Training	**26**	H	F	3-Cl-5-Br-6-OH	5.543
Training	**27**	H	Cl	4-OAc-5-OCH_3	5.774
Training	**28**	H	Cl	5-OCH_3-6-Cl	5.555
Training	**29**	H	Cl	2-OCH_3-5-Br	5.581
Test	**30***	H	Cl	4-OCH_3-6-F	5.562
Training	**31**	H	Cl	3,4-diOCH_3-6-Br	5.761
Training	**32**	H	Cl	3-I-4-OH-5-OCH_3	5.793
Test	**33***	H	Cl	3-Cl-5-Br-6-OH	5.543
Training	**34**	H	Cl	4,5-diOCH_3-6-Cl	5.821
Training	**35**	H	Cl	3,5-diOCH_3-4-Br	5.790
Training	**36**	H	Cl	2-Br-5-OCH_3-6-OH	5.655
Training	**37**	H	Cl	3,5-diOCH_3-4-OH	5.598
Training	**38**	H	NO_2	4-OAc-5-OCH_3	5.761

Training/ Test	Comp.	R_1	R_2	R_3	pIC_{50}
Training	**39**	H	NO_2	4-OCH_3-6-F	5.560
Training	**40**	H	NO_2	5-OCH_3-6-Cl	5.556
Training	**41**	H	NO_2	2-Br-5-OCH_3-6-OH	5.640
Training	**42**	H	NO_2	3,4-diOCH_3-6-Br	5.749
Training	**43**	H	NO_2	3-I-4-OH-5-OCH_3	5.764
Training	**44**	H	NO_2	3-Cl-5-Br-6-OH	5.542
Test	**45***	H	NO_2	2-F-5-Br	5.634

Molecular modeling

The studied molecules were constructed and minimized using the SKETCH option in SYBYL-X 2.0 program package (19) running on a windows 10, 64 bit workstation/ASUS, under the Tripos standard force field (20) with Gasteiger-Hückel atomic partial charges (21) by the Powell method with a convergence criterion of 0.01 kcal/mol Å. Sybyl program provides a molecular design environment to identify, predict and analyze molecular behavior.

Molecular alignment

Molecular alignment aims to build an efficiency of the 3D-QSAR models. The studied molecules were stratified on the common core; 2-aryl benzimidazole using the simple alignment protocol in Sybyl (22). Compound **10** was selected as a representative template for superimposing structures as shown in Figure 2.
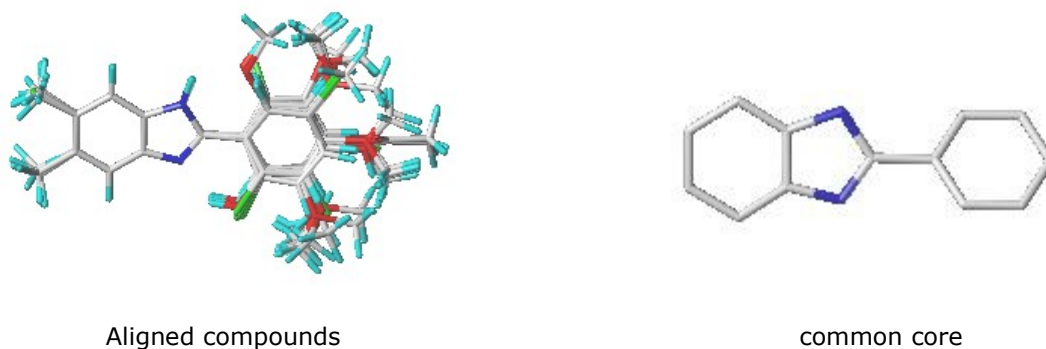

Aligned compounds

common core

Figure 2. 3D-QSAR aligned molecules using compound 10 as a template.

3D QSAR analyses

On the basis of molecular alignment protocol which is considered as one of the most crucial factors that affects the accuracy of 3D-QSAR models, comparative molecular field analysis (CoMFA) (23) and comparative molecular similarity indices analysis (CoMSIA) (24) models were developed to derive a linear relationship between the obtained QSAR models and α-amylase enzyme inhibitory activities. 3D-QSAR study was performed with the default parameters; the minimum sigma (column filtering) is set to 2.0 kcal/mol and the energy cutoff values of 30 kcal/mol (25).

The partial least squares (PLS) regression method was adopted to derive 3D-QSAR models (26). Hence the PLS with leave-one-out cross-validation was implemented to produce the coefficient of cross-validation correlation (Q^2) with an optimum number of components (N). And the non-cross validation was carried out to get the correlation coefficient (R^2), F-test value (F) and the standard error of estimate (SEE). Consequently, the best QSAR model is selected based on the high Q^2 value ($Q^2 > 0.50$) and R^2 value ($R^2 > 0.60$), an optimal number of component values, and low standard error estimation (SEE). Furthermore, the external

validation was performed to evaluate the sturdiness of the generated 3D-QSAR models using eight molecules as a test set. Where the best value of $r_{ext}^2 > 0.6$ indicated that the predictive power of derived QSAR model was good (27).

Molecular Docking

The docking was carried out to further explore the binding interactions of the molecules in the active site of the α-amylase enzyme and confirm the contour maps results of CoMFA as well as the CoMSIA using the Surflex-Docking method (19). Further, the results were analyzed using PyMol (28) and Discovery studio 2016 softwares (29). The crystal structure of the α-amylase enzyme (PDB code: 1HNY) with the co-crystallized ligand removed was utilized for this study. Using the Discovery Studio 2016, the protein was prepared by the addition of polar hydrogens and Kohlman charges while removing water molecules from the 1HNY receptor. The 3D structure of the representative compound **10** was geometrically optimized and energetically minimized using default parameters and consequently docked into the active site of the α-amylase enzyme.

Y-Randomization test

The Y-Randomization test was performed to value the vigor of both models (30), and the inhibition activities (-logIC$_{50}$) were shuffled many times. A new 3D-QSAR model was is generated for each iteration. Besides, the low Q^2 and R^2 values pointed out that the results were not due to a chance correlation.

RESULTS AND DISCUSSION

3D-QSAR

Based on the 3D-QSAR modeling, it appeared that the models might play substantial roles in predicting new potent insulin-independent antidiabetic agents as contained in the summary of PLS analyses (Table 2). Obviously, for the CoMFA model, the coefficient of cross-validated, Q^2 value was 0.696 with 2 as the optimized component, N. While the R^2, SEE and F-

test values were 0.860, 0.041 and 104.72, respectively. It thus implied that the high Q^2, R^2 and F values along with low SEE value, suggest a good statistical correlation and reasonable predictive capability of the obtained models. Furthermore, the high r$_{ext}^2$ (0.990) value of the external validation exhibits a high level of reliability in the current study. Similarly, the ratio of steric to electrostatic contributions were set at 51:49, which means that both fields are much explanatory.

The most accurate CoMSIA model which involved the steric, electrostatic, hydrophobic, H-bond acceptor and donor fields showed Q^2, N, R^2, SEE, and F values of 0.514, 3, 0.852, 0.042 119.904 respectively. The r$_{ext}^2$ value obtained was 0.987 which approved the prediction ability of the obtained model. The experimental and predicted pIC50 are shown in Table 3.

Table 2. PLS statistical parameters.

Model	Q^2	R^2	SEE	F	N	r$_{ext}^2$	Fractions				
							Ster	Elect	Hyd	Acc	Don
CoMFA	0.696	0.860	0.041	104.72	2	0.990	0.514	0.486	-		-
CoMSIA	0.514	0.852	0.042	63.55	3	0.987	0.084	0.471	0.229	0.138	0.077

Table 3. Experimental and predicted pIC$_{50}$ of forty-five benzimidazole derivatives (*:test set)

Comp.	Experimental pIC$_{50}$	predicted pIC$_{50}$		Comp.	Experimental pIC$_{50}$	predicted pIC$_{50}$	
		CoMFA	CoMSIA			CoMFA	CoMSIA
1*	5.562	5.559	5.558	22	5.663	5.663	5.653
2*	5.605	5.647	5.645	23	5.804	5.846	5.847
3	5.821	5.861	5.858	24	5.591	5.655	5.658
4	5.555	5.563	5.562	25	5.542	5.574	5.574
5	5.524	5.493	5.493	26	5.543	5.578	5.578
6	5.555	5.634	5.630	27	5.774	5.751	5.752
7	5.546	5.541	5.542	28	5.555	5.588	5.587
8	5.638	5.664	5.659	29	5.581	5.553	5.549
9*	5.585	5.568	5.601	30*	5.562	5.557	5.557
10	5.829	5.817	5.816	31	5.761	5.755	5.758
11	5.790	5.755	5.756	32	5.793	5.760	5.760
12	5.751	5.714	5.713	33*	5.543	5.586	5.586
13	5.530	5.523	5.525	34	5.821	5.721	5.716
14	5.520	5.521	5.521	35	5.790	5.738	5.740
15	5.520	5.518	5.521	36	5.655	5.663	5.658
16	5.790	5.685	5.684	37	5.598	5.693	5.695
17*	5.585	5.544	5.541	38	5.761	5.736	5.739
18*	5.580	5.578	5.570	39	5.560	5.655	5.6657
19	5.770	5.749	5.750	40	5.556	5.556	5.557
20	5.595	5.513	5.517	41	5.640	5.56	5.655
21	5.603	5.688	5.686	42	5.749	5.715	5.720
				43	5.764	5.726	5.727
				44	5.542	5.493	5.557
				45*	5.634	5.616	5.617

Interpretation of graphical contours
The graphical contour maps were created to visualize areas where the activity can be increased or decreased. Figures 3 and 4 show the CoMFA and CoMSIA contour maps respectively for compound **10** as a representative molecule.

CoMFA contour maps
The electrostatic fields are presented with red (20% contribution) and blue (80% contribution) contours, while the steric fields are presented with green (80% contribution) and yellow (20% contribution) contours. The bulky substituents are preferred around green areas, whereas yellow areas' bulky substituents are not preferred (Figure 3).

As depicted in Figure 3, a relatively big yellow contour was observed around the phenyl group which could be favored to activity while the small-sized green contour around the C-3 position indicated that less bulky group at this position would

not be favorable for increasing the activity, which could be demonstrated by compounds **4**, **13**, **14**, **25**, **26**, **33** and **44**. For example, the compounds **13** (pIC$_{50}$ = 5.530) and **14** (pIC$_{50}$ = 5.520), with halogens at the 3 and 5 positions of the phenyl moiety, showed low activities than that of compound **10** (pIC$_{50}$ = 5.829), with OCH$_3$ and H groups at the same positions, respectively.

Moreover, figure 3 revealed that that phenyl group with the electropositive substituents may increase the activity around the blue contours. As such three major space-favorable blue regions were distributed around the ortho, meta and para positions of the phenyl ring. This indicated that the existence of these electron donor groups at these positions could increase the activity. Therefore, electropositive and less bulky groups are required at these positions which could easily reach the binding pocket in the enzyme active sites and present high α-amylase inhibition affinity.
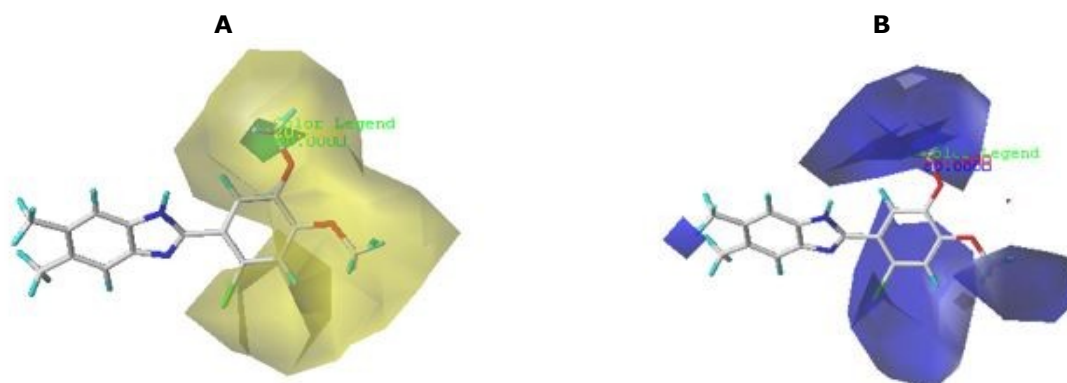
A   **B**

Figure 3. Analysis of CoMFA contour maps. **(A)** Steric field; **(B)** Electrostatic field.

CoMSIA contour map
The CoMSIA contour maps for the steric and electrostatic fields were found to be similar to the corresponding CoMFA fields. Therefore, the focus was directed towards the remaining parameters. Hence according to the CoMSIA fractions presented in Table 2, the electrostatic (0.471); H-bond acceptor (0.138); and hydrophobic fields (0.229) were found to be the major fields that could describe the activity.

The contour map for the hydrophobic field (Figure 4(a)) is depicted in yellow domains (80% contribution) and white domains (20% contribution) in which hydrophobic substituents are predicted to enhance the biological activity in yellow contours, whereas in white domain, hydrophobic groups were not favored. The yellow contour near the ortho position of the phenyl group brought to light that the hydrophobicity could play a crucial role in the activity. For example, compound **23** (pIC$_{50}$ = 5.804) with a –Br group at the C-2, which is falls within the

yellow contours, exhibited higher activity than those with no hydrophilic groups such as; compounds **15** (pIC$_{50}$ = 5.520) and **26** (pIC$_{50}$ = 5.542), which lead into increase of activity. Moreover, two white contours near the C-3 and C-5 positions of the phenyl group pointed that the presence of a hydrophobic group especially halogens in this region may not be beneficial to the biological activity. For instance, compounds **4** (pIC$_{50}$ = 5.555) and **33** (pIC$_{50}$ = 5.543) with halogens groups at C-3 and C-5 position of the phenyl moiety exhibit lowest activities than that of the corresponding compound **10** (pIC$_{50}$ = 5.829).

The CoMSIA hydrogen bond acceptor field (Figure 4(b)), is displayed in magenta (80% contribution) and red (20% contribution) contours, representing favorable and unfavorable positions for H-bond acceptors respectively. A relatively big red contour was observed around C-2, C-3 and C-4 positions of the phenyl group which has been proven to be a disadvantage to activity due to the existence of H-

bond acceptor substituent at one or two of these positions. Whereas, the magenta contour around the C-5 position of the phenyl moiety is crucial for significant inhibitory activity. These characteristics may explain significant α-amylase inhibitory activity for compounds with no hydrogen bond acceptor groups such as; compound **11** and compound **10**, respectively. For instance, compound **11** (pIC$_{50}$ = 5.790) without any substituent and compound **10** (pIC$_{50}$ = 5.829) with a -Br showed high activities.

Y-randomization test
Table 4 shows the results of seven random shuffles for the Y-randomization test. For every iteration, a set of Q^2 and R^2 of new QSAR models is generated.

In the contour map of H-bond donor field, as shown in Figure 4(c), the cyan (80% contribution) and the purple (20% contribution) contours represent areas with favorable and unfavorable H-bond donor groups respectively. A big-sized cyan contour is found around the phenyl ring, indicating that H-bond donor substituents in this area are predicted to enhance biological activity.

As a result, the Q^2 and R^2 values were low. Thus allowing to conclude that the probability of random correlations was excluded.

Table 4. Q^2 and R^2 values of the Y-randomization test.

Iteration	CoMFA		CoMSIA	
	Q^2	**R^2**	**Q^2**	**R^2**
1	0.082	0.128	0.048	0.134
2	0.125	0.317	0.121	0.242
3	0.199	0.421	0.178	0.391
4	0.167	0.360	0.142	0.308
5	-0.214	0.248	-0.199	0.195
6	0.099	0.126	0.092	0.102
7	0.137	0.301	0.129	0.284

Molecular docking
To reinforce the 3D-QSAR results, the molecular docking technique was applied to investigate the different interactions of benzimidazole derivatives in the active site of α-amylase (PDB code: 1HNY) using the Surflex program. The potent compounds were found to be those that contain ortho Cl or Br atom, para and meta methoxy groups on the phenyl ring which could be responsible for these compounds' inhibitory activity. As depicted in Figure 5, the representative compound **10** which is originally reported to be the most effective compound was docked in the α-amylase enzyme active site. This enabled the understanding of the hydrophobic and

H-bond interactions between the active molecule and 1HNY receptor.

The blue and pink colors around C-2, C-3 and C-4 positions of the phenyl ring indicated that substituents with hydrophilic character and H-bond donor substituents are favored in these positions. The R1 and R2 substituents were found in the brown area which implied that the groups with the hydrophobic characters at these positons would be favored. The observations obtained from the hydrophobicity and H-bond surfaces are in agreement with the contour maps of CoMSIA model.

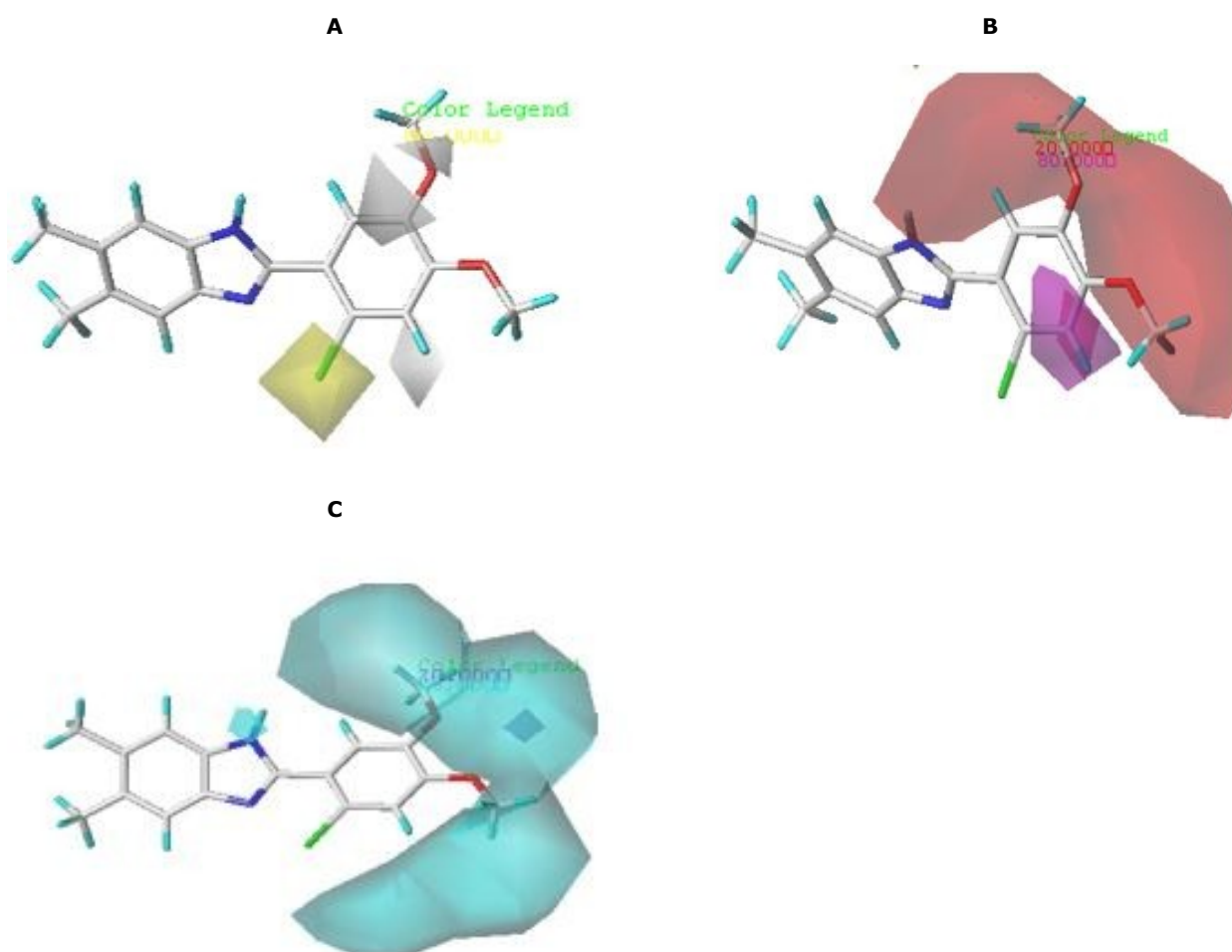**A**

B

C

Figure 4. Analysis of CoMSIA contour maps. **(A)** Hydrophobic field; (**B**) H-bond acceptor field; (**C**) H-bond donor field
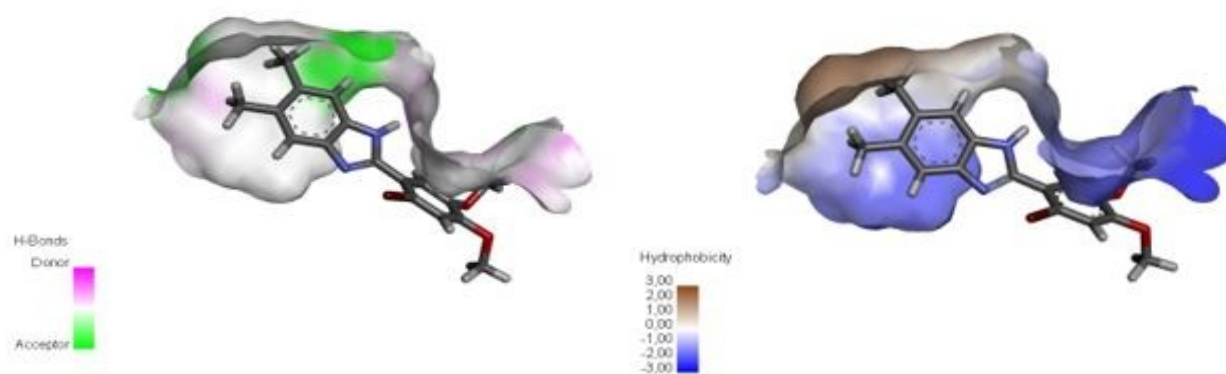

Figure 5. The docking pose of the active molecule and 1HNY receptor.

SAR summarized results

Figure 6 summarized the information derived from the 3D contour maps that satisfactorily matched the results of molecular docking, which could be much helpful to propose new molecules with high predictive activities.
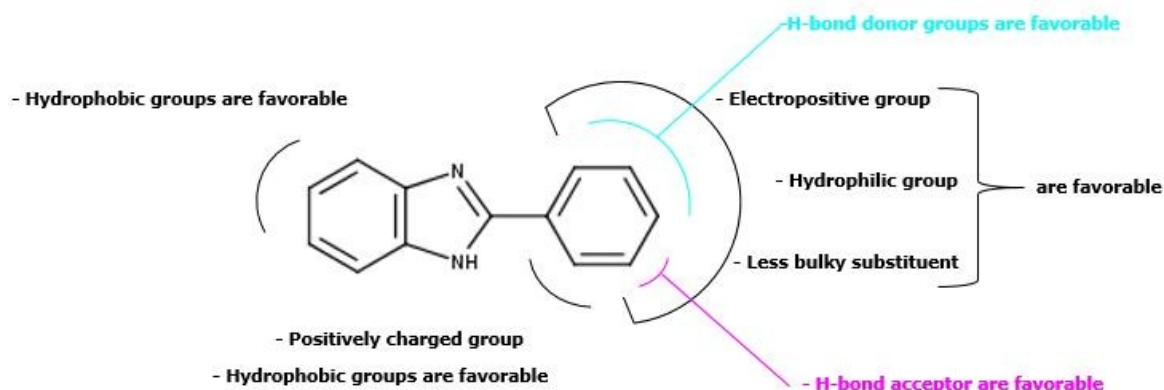

Figure 6. SAR-summarized results from the present work.

Newly designed compounds

Based on the information obtained from the 3D contour maps and molecular docking studies, five new 2-aryl-benzimidazole derivatives which exhibited high pIC_{50} values were designed. The newly predicted compounds A1, A2, A3, A4 and A5 showed exceptionally improved inhibitory activity and higher total score (binding affinities) than that of compound 10 for both models, indicating their potential as new α-amylase inhibitors as shown in Table 5.
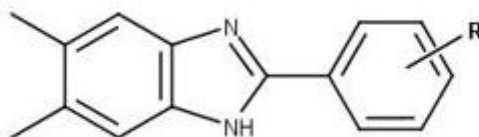
Figure 7. The structural template of the newly designed molecules.

Table 5. The pIC_{50} values of newly designed 2-arylbenzimidazole derivatives as α-amylase inhibitors.

N°	R	Predicted pIC_{50}		Total scoring
		CoMFA	CoMSIA	
Comp 10	3,4-diOCH₃-6-Br	5.817	5.816	1.7149
A1	2-OH-3-NHCH₃	6.353	6.340	3.2841
A2	2-NH₂-3-OH	6.326	6.325	3.2719
A3	2,3,6-triOH	6.299	6.297	3.2722
A4	2-OH-3-NH₂	6.092	6.101	2.9940
A5	2,4,6-triNH₂	6.052	6.076	2.9866

CONCLUSION

The current study has provided some valuable information on the design of new potential insulin-independent antidiabetic agents. This has been achieved through investigating forty-five benzimidazole derivatives retrieved from the literature by molecular docking and 3D-QSAR studies. Graphical analyses of the optimal results obtained from the contour maps brought to light the main structural requirements that may influence the activity. Moreover, the molecular docking analyses matched well with the results obtained from the contour maps which led to the design of five potent insulin-independent antidiabetic molecules and successfully predicted their α-amylase inhibitory activities.

El Khatabi et al. JOTCSA. 2020; 7(2): 471-480.

RESEARCH ARTICLE

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SYNTHESIS, ANTIBACTERIAL ACTIVITY AND DOCKING STUDIES OF BENZYL ALCOHOL DERIVATIVES

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Abstract: Benzyl alcohol derivatives were synthesized, and characterized using NMR and FTIR spectroscopic techniques. For the first time, the antibacterial activities of the synthesized compounds were examined using disc diffusion method by measuring the diameter of the zones of inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results demonstrated that the activity was concentration dependant, and that the compounds were generally potent against *P. aeruginosa*. Only two of the compounds were active against *S. aureus.* In terms of broad spectrum activity, compound **2d** (35 mm) was found to exhibit a promising efficacy which surpassed that of the standard drug (amoxicillin).The binding of compounds **2a-e** to the glucosamine-6-phosphate synthase (GlcN-6-P) active-site revealed that all the synthesized compounds fitted into the GlcN-6-P active-site receptor cavity, exhibited potential hydrogen-bonding interactions with the proximal amino acid residues and aligned similar to amoxicillin. Interestingly, it has been found that the most active compound, **2d** also appeared to have a relatively low binding energy (-52.8901 kcal/mol).

INTRODUCTION

The continuous increase in the antibiotics resistance and the attendant scarcity of new antimicrobial agents is arguably one of the major challenges in public health today, especially in the developing countries where up to one-half of deaths are attributed to infectious diseases (1-4). This could particularly be seen in the level of antibiotics resistance associated with a number of staphylococcus species which causes respiratory and skin infections, as well as pseudomonas species which are also responsible for the gastrointestinal and urogenital diseases (5). For instance, the clinical isolates of staphylococcus aureus — the leading cause of nosocomial infections — are becoming resistant to a range of popular antimicrobial agents such as vancomycin, trimethoprim-sulfamethoxazole, chloramphenicol, erythromycin, clindamycin, ciprofloxacin, penicillin, amikacin, tobramycin, and gentamicin (6).

In an attempt to address the aforementioned concerns, a number of workers have reported various bioactive molecules built around some promising scaffolds and assessed their antibacterial or antimicrobial potentials. These includes nitroimidazole derived oxazolidinones (7),

ciprofloxacin derivatives (8-11), icariin derivatives (12), 1-monolaurin (13), (*E*)-stilbene derivatives (14), benzimidazole-incorporated sulfonamide analogues (15), chalcone derivatives (16), 3-phenyl-1-methylquinolin-2-one derivatives (17), oxysterols (18), 2-thiazolylimino-5-arylidene-4-thiazolidinones (19), sulfonamide and carbamate derivatives of 5-Nitro-1H-indazole (20), polyfluorinated 4-thiazolidinone and α-aminophosphonic acid derivatives (21). Interestingly, we have noted a known bioactive scaffold that aparently escaped the attention of workers — benzyl alcohol.

Benzyl alcohol (Figure 1) is an aromatic alcohol commonly found in various essential oils such as jasmine, ylang-ylang, and hyacinth (22). Due to its antibacterial and antifungal activities, it is widely used as an ingredient in the manufacture of soaps, topical creams, skin lotions, shampoos, and facial cleansers. Unfortunately however, benzyl alcohol has been reported to be a contact allergen (23). It thus prompted us in this work to prepare benzyl alcohol derivatives in order to evaluate their structure activity relationships with the aim of developing lead compounds that could offer safe and efficacious oral or topical antibacterial agents.
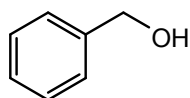

Figure 1. Structure of benzyl alcohol.

EXPERIMENTAL SECTION

Materials

All reagents and solvents were purchased from Aldrich and used as received. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. IR spectra were recorded on a Perkin–Elmer FT-IR Spectrum BX spectrophotometer. Melting points were determined on an Electrothermal melting point apparatus and are uncorrected.

Methods

Chemistry

General Procedure for the preparation of benzyl alcohol derivatives (2a-e)

The aldehydes (13.2 mmol) were placed in a 250 mL round bottom flask containing ethanol (4 mL) and stirred at room temperature to obtain homogenous solutions. The resulting solutions were cooled on an ice bath prior to the addition of the reducing agent. Subsequently NaBH$_4$ (13.2 mmol) dissolved in 1M NaOH (3.8 mL) was slowly added over a period of 10 minutes. The resulting

mixture was stirred at room temperature for 10 minutes after which it was cooled and then treated with 6M HCl dropwise until the evolution of H$_2$ gas stopped. The pH was checked to make sure the solution was acidic, and further stirred for another 10 minutes to allow the appropriate product to precipitate. The products were collected by filtration, washed twice with ice-cold water and then transferred to a dry piece of filter paper and air dried. But in the case of compound **2d**, the crude mixture was extracted with chloroform which after evaporation afforded the product as a film-like solid.

4-hydroxy-3-methoxybenzyl alcohol, 2a
White powder, 93% yield, mp 112-114°C. ^{1}HNMR (400MHz, DMSO$_4$-d$_6$) δ ppm 3.71 (3H, s, OCH$_3$), 4.37 (2H, s, CH$_2$), 5.14 (1H, s, OH), 6.70 (2H, d, *J* = 4, Ar-H), 6.88 (1H, s, Ar-H), 8.88 (1H, s, Ar-OH); ^{13}CNMR (100MHz, DMSO$_4$-d$_6$) δ ppm 39.52, 55.80, 63.36, 111.32, 1115.35, 119.51, 133.72, 145.54, 147.67; IR 3503, 1509, 1259cm^{-1}.

4-hydroxybenzyl alcohol, 2b
White powder, 87% yield, mp 260-265°C; ^{1}HNMR (400MHz, DMSO$_4$-d$_6$) δ ppm 4.37 (2H, s, CH$_2$), 5.07 (1H, s, OH), 6.71 (2H, d, *J* = 8, Ar), 7.11 (2H, d, *J* = 8, Ar), 9.36 (1H, s, Ar-OH); ^{13}CNMR (100MHz, DMSO$_4$-d$_6$) δ ppm 39.52, 63.09, 63.12, 115.10, 115.12, 128.43, 128.46, 132.94, 156.41, 156.42; IR 3377, 1513, 1233 cm^{-1}

4-nitrobenzyl alcohol, 2c
Yellow powder, 93% yield, mp 131-134°C. ^{1}HNMR (400MHz, DMSO$_4$-d$_6$) δ ppm 3.56 (2H, s, CH$_2$), 5.61 (1H, s, OH), 7.57 (2H, d, *J* = 8, Ar), 8.17 (2H, d, *J* = 4, Ar); ^{13}CNMR (100MHz, DMSO$_4$-d$_6$) δ ppm 39.52, 62.21, 123.49, 127.25, 148.50, 150.89 IR 3503, 1505, 1230 cm^{-1}

4-methoxybenzyl alcohol, 2d
Film-like white solid; 94% yield; mp 212-215°C; ^{1}HNMR (400MHz, DMSO$_4$-d$_6$) δ ppm 3.71 (3H, s, OCH$_3$), 4.40 (2H, s, CH$_2$), 5.15 (1H, s, OH), 6.87 (2H, d, *J* = 8, Ar), 7.22 (2H, d, *J* = 8, Ar); ^{13}CNMR (100MHz, DMSO$_4$-d$_6$) δ ppm 39.52, 55.22, 62.79, 113.67, 128.21, 128.82, 131.14, 134.60, 158.36, IR 3326, 1513, 1244 cm^{-1}.

4-bromobenzyl alcohol, 2e
Yellow powder; 95% yield; mp 212-215°C; ^{1}HNMR (400MHz, DMSO$_4$-d$_6$) δ ppm 4.46 (2H, s, CH$_2$), 5.38 (1H, s, OH), 7.27 (2H, d, *J* = 8, Ar), 7.48 (2H, d, *J* = 8, Ar); ^{13}C-NMR (100MHz, DMSO$_4$-d$_6$) δ ppm 39.52, 62.40, 62.43, 119.81, 119.83, 128.79, 131.12, 142.06; IR 3272, 1483, 1203 cm^{-1}.

Antibacterial Assay

Sourcing of Bacteria for Assay

The bacteria used for the assay were obtained from the Department of Microbiology, Umaru Musa Yar'adua University, Katsina, Nigeria. The Gram-positive bacterium, *Staphylococcus aureus* was cultured in the medium prepared from mannitol salt agar. While the Gram-negative *Pseudomonas aeruginosa* was cultured in the medium prepared from nutrient agar.

Preparation of culture media

50 g of nutrient agar was dissolved in 1800 mL distilled water, autoclaved at 121°C for 15 minutes, spread into eighty (80) petri dishes and allowed to solidify. The Gram positive and negative bacteria were each inoculated into forty (40) prepared petri dishes (24).

Preparation of the test concentrations

Twenty milligrams (20 mg) of the compounds were dissolved in 1 mL of DMSO to afford the stock solution. Using serial double dilution, six different concentrations (10^{-1} mg/mL, 10^{-2} mg/mL, 10^{-3} mg/mL, 10^{-4} mg/mL, 10^{-5} mg/mL, 10^{-6} mg/mL) of each compound were prepared (24).

In vitro assay of the activity of the synthesized compounds on Staphylococcus aureus and Pseudomonas aeruginosa culture

Discs were made from filter paper and dipped into the different concentrations prepared from the five synthesized compounds. The discs were then transferred into the six (6) prepared petri dishes for the Gram-positive and negative bacteria which gave a total of thirty (30) petri dishes for each

bacterium. The activity for each compound was determined by measuring the zones of inhibition (mm) of each bacterium after 24 hours of incubation (24).

Molecular Docking studies

Discovery studio 2018 software was used to investigate the possible orientation and interactions of **2a-e** with glucosamine-6-phosphate GlcN-6-P synthase (25). The model structure of GlcN-6-P synthase, ID: 1XFF, was downloaded from the protein data bank. The target protein for molecular coupling was prepared based on the CHARMm force field with help of DS 2018. Structural and energy optimization of **2a-e** as ligands were prepared in DFT/B3LYP/6-31G base set using Gaussian 09 software. The docking studies estimate the binding site in the target model as Glcn-6-p synthase and the affinity of **2a-e** as ligands. CDOCKER was performed to place **2a-e** at the selected target binding sites. Docking results were evaluated with three-dimensional amino acid interactions and Binding energy values. Five possible structures of the ligands were generated and the best exposure was defined according to the lowest energy values for each complex.

RESULTS AND DISCUSSION

Chemistry

Benzyl alcohol derivatives, **2a-e** were synthesized (Scheme 1) by reacting the appropriate aldehyde with the basified solution of sodium borohydride, $NaBH_4$/NaOH(aq).

	R
a	4-OH, 3-OCH$_3$
b	4-OH
c	4-NO$_2$
d	4-OCH$_3$
e	4-Br
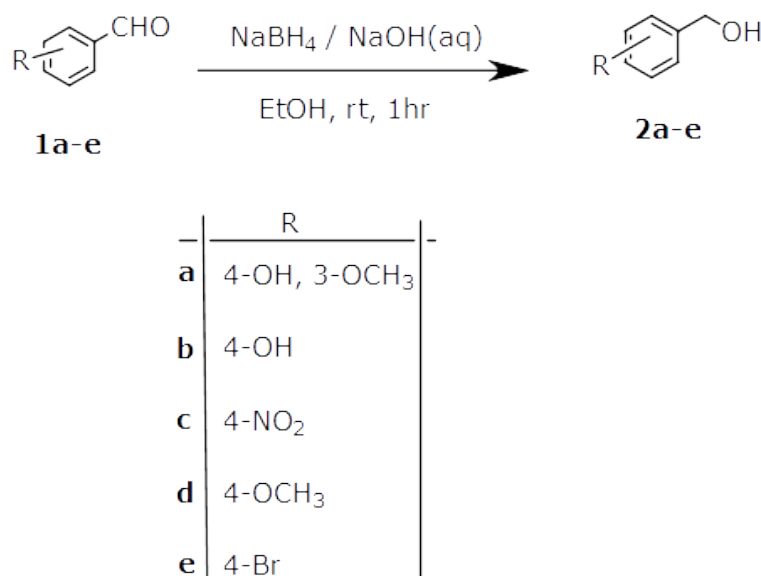
Scheme 1: Synthesis of benzyl alcohol derivatives.

Typically, the $NaBH_4$ solution in aqueous sodium hydroxide was slowly added to the appropriate

solution of aldehyde in ethanol. The resulting mixture was stirred at room temperature for a few minutes. This was followed with the addition of aqueous HCl which resulted in the evolution of hydrogen gas signifying the quenching of the excess $NaBH_4$. The corresponding alcohol product precipitated and collected by filtration. After additional washings, the product was air-dried and analyzed. The Nuclear magnetic resonance (NMR) spectroscopic data of compounds **2a-e** revealed the positions as well as the type of the proton and carbon in each compound. While the IR data gave the functional group present, particularly the hydroxyl (OH) functionality. Typically, the ^1H NMR spectrum of **2a** revealed a signal at 4.37 ppm assigned to the two protons of the methylene group, ($-CH_2$). Further upfield, the signal at 6.70 ppm appeared in the aromatic region of the spectrum and was integrated and assigned to the two aromatic protons closer to the methylene group. The ^1H NMR spectrum revealed that the signal at 3.71 ppm was for the three protons of the

methoxy group, ($-OCH_3$). Also the -OH proton attached to carbon-1 resonated at 5.14 ppm. The ^{13}C NMR spectrum showed a distinct signal at 55.80 ppm which corresponded to the carbon atom of the methoxy group. The signal at 66.36 ppm was assigned to the methylene carbon, while the signals at 111.32 ppm, 115.35 ppm, 119.51 ppm, 133.72 ppm, 145.54 ppm and 147.67 ppm were assigned to the carbons of the benzene ring. From the IR spectrum of compound **2a**, it was deduced that the band at 3503 cm^{-1} was due to the -OH stretching, while the C-O stretch (alcohol) was found at 1259 cm^{-1}.

Antibacterial Activity

The synthesized compounds were screened for *in vitro* antibacterial activity against Gram-positive and negative bacteria species, *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively using disc diffusion method. The result for the activity was expressed as the length of diameter of the zones of inhibition as given in Table 1.

Table 1: Antibacterial activity of benzyl alcohol derivatives[a]

Compound	Organism	Zone of Inhibition (mm) Conc (mg/mL)					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
2a	*S. aureus*	08	00	00	00	00	00
	P. aeruginosa	27	11	11	09	08	08
2b	*S. aureus*	10	00	00	00	00	00
	P. aeruginosa	11	09	08	08	08	07
2c	*S. aureus*	08	08	07	00	00	00
	P. aeruginosa	07	00	00	00	00	00
2d	*S. aureus*	12	11	11	09	00	00
	P. aeruginosa	35	26	23	22	00	00
2e	*S. aureus*	00	00	00	00	00	00
	P. aeruginosa	12	11	07	00	00	00

[a]Amoxicillin disc (30μg/mL): *S. aureus* = 24 mm; *P. aeruginosa* = 13 mm

Generally, the antibacterial activities of all the compounds appeared to be concentration dependant. In the case of Gram-positive bacterium (*S. aureus*), it was found that at the highest concentration (10^{-1}), compound **2d** showed a moderate activity (12 mm). While all other compounds relatively exhibited weak activities. Specifically, compounds **2b**, **2a** and **2c** have 10 mm, 08 mm and 08 mm zones of inhibition respectively. Compound **2e** did not show any activity at all concentrations. Regarding the Gram-negative bacterium (*P. aeruginosa*), all of the compounds except **2c** exhibited some degree of activities at more than one concentration. It was interestingly found that compound **2d**

demonstrated an excellent zone of inhibition (35 mm), and nearly matched the control antibiotic, amoxicillin even at a low concentration of 10^{-3} (23 mm). Compound **2a** has also showed a better activity at 10^{-1} concentration (27 mm) than the control drug. Overall, it could be commented that compound **2d** appeared quite promising as it demonstrated the ability to inhibit the growth of both Gram-positive and negative bacteria.

Molecular Docking Studies

The enzyme glucosamine-6-phosphate (GlcN-6-P) synthase is responsible for the catalysis of first and rate-limiting step in hexosamine biosynthesis which give rise to uridine 5'-diphospho N-

acelglucosamine (UDP-GlcNAc). Considering that UDP-GlcNAc is an essential building block in the fungal and bacterial cell walls, it thus becomes an interesting target for antifungal and antibacterial drug discovery (25).

Amoxicillin as the standard drug binds with GlcN-6-P synthase with binding affinity of -169.504 kcal/mol. The binding sites of amoxicillin on GlcN-6-P synthase target included twelve residues involved in the hydrogen bond, five residues involved in hydrophobic interaction and three residues involved in other interactions (Figure 2, left panel). There were two strongest hydrogen bonds that occurred with GLY99 with functional group O16 (bond length = 2.09381) and H27

(bond length = 2.72711). then with TRP24 through functional group H34 (bond length = 2.7042) and H41 (bond length = 2.9026). Similarly, another type of hydrogen bond designated as Carbon Hydrogen Bond has also occurred with GLY99 through functional group H43 (bond length = 2.10665).

The binding of compounds **2a-e** to the GlcN-6-P synthase active-site revealed that all the synthesized compounds fitted into the GlcN-6-N active-site receptor cavity, exhibited potential hydrogen-bonding as well as hydrophobic interactions with the proximal amino acid residues (Figure 2, right panel).
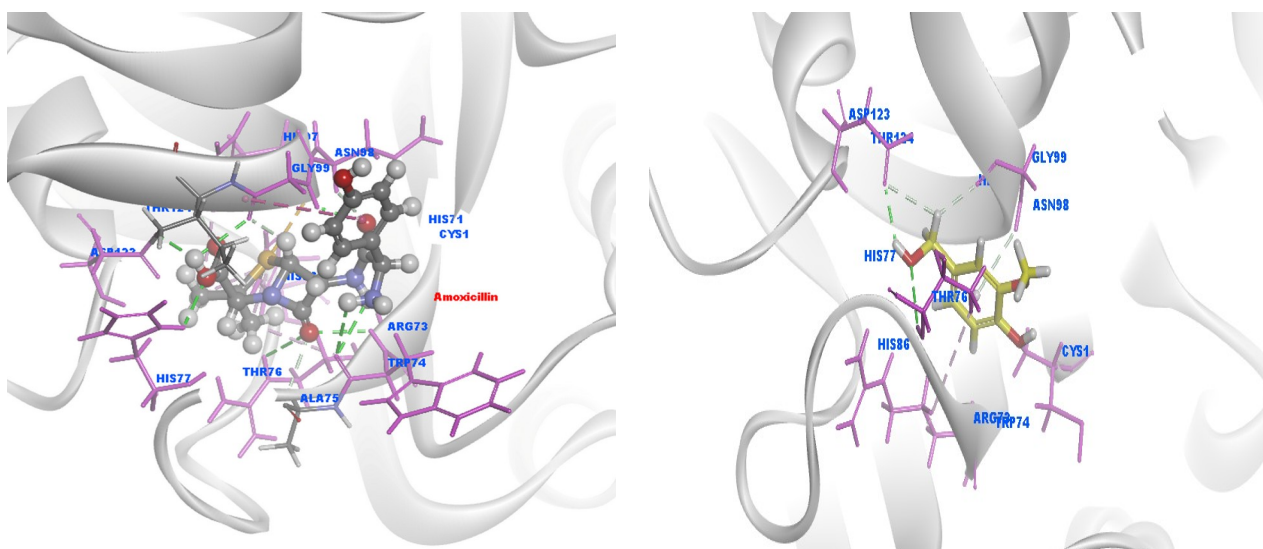

Figure 2. 3D interactions of amoxicillin as reference compound in the GlcN-6-P synthase binding region (left panel); Compound **2a** superimposed in the binding site of GlcN-6-P synthase (right panel).

Compound 2a was found to dock at seven sites (binding energy = -28.0758 kcal/mol). There were two hydrogen bond interactions with ASP123 through functional group H17 and H15 (bond length = 2.126 and 2.874 respectively). Another two hydrogen bonds were revealed with GLY99 through functional groups H15 and HN (bond length = 2.300 and 3.067 respectively). Other types of hydrogen bond as well as the hydrophobic interactions accounted for the remaining binding sites.

Compound 2b exhibited a total of nine docked interactions (binding energy = -34.4725 kcal/mol). One hydrogen bond with the residue ARG73 through functional group O9 (bond length = 2.456) and hydrophobic interaction with the same residue (bond length = 5.05659) were found. But for the residue TRP74, it was found that the conventional hydrogen bonds have occurred through functional groups O8 and H16 (bond length = 2.141 and

2.55708 respectively). The rest of the interactions have occurred with other residues through various functional groups.

Compound 2c docked with the enzyme (binding energy = -66.1382 kcal/mol) with lower energy than 2a and 2b. This might be due to the fact that there were some residues that each interacted multiple times with the compound. For instance, CYS1 interacted three times using different bonding types through functional groups O17, O18 and O19 (bond length = 4.4659, 1.86553 and 3.06222 respectively). Also the residue ARG73 doubly interacted using hydrogen bond through functional group O8 (bond length = 1.87337) and a hydrophobic interaction. Similarly, the residue ASP123 interacted using hydrogen bond through functional groups H15 and H13 (bond length = 3.08947 and 2.27452 respectively). In total, there were ten various interactions observed with compound 2c.

Sulaiman M et al. JOTCSA. 2020; 7(2): 481-488.

RESEARCH ARTICLE

Compound 2d (binding energy = -52.8901 kcal/mol) docked at a total of eight sites. It exhibited double interactions with ARG73, a hydrogen bonding through functional group O8 (bond length = 1.87521) and a hydrophobic interaction (bond length = 5.00408). The rest are single interactions with the remaining seven residues.

Compound 2e docked with the enzyme (binding energy = -74.6045 kcal/mol) at six sites which are fewer than all the compounds. It demonstrated double hydrophobic interactions with the residue HIS86 (bond length = 4.83674 and 4.91763). The remaining residues interacted singly through various bonding types.

Apparently, in terms of the binding energy, compound **2e** demonstrated the least value (-74.6045 kcal/mol) second to the standard drug, amoxicillin (-169.504 kcal/mol). This is in disagreement with the in vitro assay where compound **2d** exhibited the best zone of inhibition of the bacterial strains even better than the standard drug. Though it also possessed some significant low binding energy (-52.8901 kcal/mol). Mismatching of the results of biological assay with the docking studies had been observed in some works. For instance, a standard drug with the most potent in vitro bioactivity was found to exhibit a lesser docking score compared to the tested compounds and vice versa (26). Furthermore, some tested compounds exhibited similar *in vitro* bioactivities but appeared to differ in their docking results (27).

We opined that these discrepancies might be due to the fact that the tested compounds could be hitting targets other than the enzyme being studied. Other contributing factors might be due to the solubility issues, change in binding conformation of proteins, temperature and ionic strength of the buffer which influence entropy but often neglected in the docking software.

CONCLUSIONS

Benzyl alcohol derivatives were synthesized using simple $NaBH_4$ reduction. Although the compounds are known, but for the first time, their antibacterial activities have been investigated. And the results demonstrated that the activity was concentration dependant, and that the compounds were generally potent against *P. aeruginosa*. Only two of the compounds were active against *S. aureus.* In terms of broad spectrum activity, compound **2d** was found to exhibit a promising efficacy which surpassed that of the standard drug. In order to understand the mode of interaction of the compounds, an *in silico* studies were carried out by docking the compounds against glucosamine-6-phosphate synthase (GlcN-6-P) as potential target. Interestingly, all the compounds fitted into the cavity and demonstrated a close alignment of the ligands with the enzyme active site. Of note was the binding energy of the most biologically active compound, **2d** which appeared to have a relatively favorable binding energy.

This work recommends further studies on the pharmacokinetic properties of compound **2d.** Based on the information obtained, it may also be subjected to *in vivo* studies to fully explore its potential as a possible lead that could be optimized to clinical trials in order to have a new antibacterial agent.

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RESEARCH ARTICLE

Source Analysis of Heavy Metals and Polycyclic Aromatic Hydrocarbons from a Popular Dumpsite, Lagos State, Nigeria

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Abstract: In this study, landfill leachates and plants from Olususun landfill in Ojota area of Lagos State, Nigeria were investigated. Physicochemical parameters, concentrations of heavy metals (HMs – Pb, Cd, Cr, Ni, Mn and Zn) and polycyclic aromatic hydrocarbons (PAHs) were determined in leachates while HMs and PAHs were determined in plants. Nitric and Perchloric acid mixtures were used for digesting both leachates and plant samples before quantification by atomic absorption spectrometry. Cold extraction was used for extracting PAHs and was quantified using gas chromatography-mass spectrometry. The range of results on HMs analysis were: leachates (mg/L) - Pb: 0.60 - 1.10, Cd: 0.04 - 0.05, Cr: 0.30 - 0.40, Ni: 0.13 - 0.19, Mn:0.53 - 0.74 and Zn: 2.24 - 3.72; plants (mg/kg) – Pb: 1.0 – 6.0, Cd: 0.3 – 0.5, Cr: 0.0 – 4.0, Ni: 0.2 – 1.6, Mn: 10.1 – 41.2 and Zn: 0.0 – 13.2 respectively. For PAHs, the concentrations (mg/L) ranged from 0.10 - 0.24 and 0.09 - 0.31 mg/kg for leachates and plants respectively. Pearson correlation and principal component analysis identified the main sources of pollution in Olususun landfill as anthropogenic. Proper management of the landfill is advised to prevent contamination of groundwater and surface waters.

Keywords: Landfill leachate, heavy metals, Olususun, polycyclic aromatic hydrocarbons, plants.

INTRODUCTION

Rapid urbanization and increasing population growth increase waste generation (1). Management of solid waste is an environmental challenge in many urbanized areas of the world (2). Landfilling is a standard method of waste disposal in several developing countries (3). However, the quality of many landfill sites is far from recommended standards mainly because the landfill systems are not correctly engineered and suited with the requisite leachate collection facilities and installation. These landfills thus serve as repositories for many toxic substances (4,5). The extent of the threats posed by these landfills depends on the depth of the water table, the direction of the groundwater flow, permeability of the geologic strata, the concentration and level of pollutants in the leachates, and municipal solid waste type (6).

Leachates are mixtures of dissolved inorganic and organic compounds as well as microbes generated via the percolation of water through disposed of

solid wastes in a landfill (5). Several factors are responsible for leachates composition, including the kind of waste deposited on the landfill, degree of compaction, particle size, the composition of the wastes, the age of the landfill, the climate, hydrology of the site and other various peculiar conditions such as landfill design and type of liner used (7). Leachates can thus contain substantial amounts of organic (polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs)) and inorganic (heavy metals) pollutants (8,9). They can also serve as indicators of environmental pollution through their ability to leak, percolate, and contaminate groundwater (10). There has been a growing interest regarding the possible harmful effects of PAHs released into the environment from anthropogenic sources as several PAHs have been reported to exhibit mutagenic and carcinogenic properties (3,11). Furthermore, uncontrolled inputs of heavy metals into the environment are unpleasant and undesirable due to non-biodegradability and bioaccumulation in soil, underground water, or plants; and the toxicity of several heavy metals is well documented in the literature. Leachate production via decayed solid waste materials mixed with precipitates of surface waters can bring about contamination of plants, surface and sub-surface water collection systems, and transportation of the contamination to the farther point of the ecosystem (12). While the volume of leachate generated in a landfill depends on the amount of solid waste deposited, water availability, condition of the landfill, and deposited refuse, as well as the soil condition (3). Leachate outflow and infiltration are reported to be the primary source of groundwater contamination from existing landfills (13). In Lagos metropolis, three dumpsites – Olusosun, Solus, and Abule-Egba – are in use, with Olusosun being the most active in terms of quantity of waste generated daily (14). There are several reports on the state of different landfill sites in Lagos with particular interest on contamination of leachates and surrounding soils by heavy metals (10,15,16) and PAHs (17). However, the impact of heavy metals and PAHs from leachates on plants growing on the dumpsite is lacking. Therefore, in this study, the physicochemical properties, level of heavy metals and polycyclic aromatic hydrocarbons in leachates and plants from Olusosun dumpsite, were investigated

MATERIAL AND METHODS

Description of study area
Olusosun landfill is located in the Ojota area of Lagos State, Nigeria (Figure 1). It is known to be the most giant landfill in Lagos State and ranked among the biggest in the world. It has been in existence since 1978 (18), occupies 42 hectares of land, and receives approximately 40% of the total waste deposit Lagosians (19). It lies between latitude 6°. 20' N and longitude 3°. 20' E. Various human activities take place around this vicinity, including automobile repairs, petrol fueling stations (18), and motor parks. The landfill does not have any protective bottom layer constructed to protect leachate plumes from migrating into groundwater.

Sample collection
Five Leachate samples were collected from each of the three leachate streams flowing out of the base of the landfill to make a composite (L1). Another five leachate samples (L2) were collected from the main pond located within the vicinity of the landfill. Each of the composite samples was placed in 1L pre-cleaned plastic bottles with the cap securely tightened, preserved in ice-chest, taken to the laboratory, and stored in a refrigerator at 4 oC pending analysis. Sample bottles were rinsed with the leachates before sample collection.
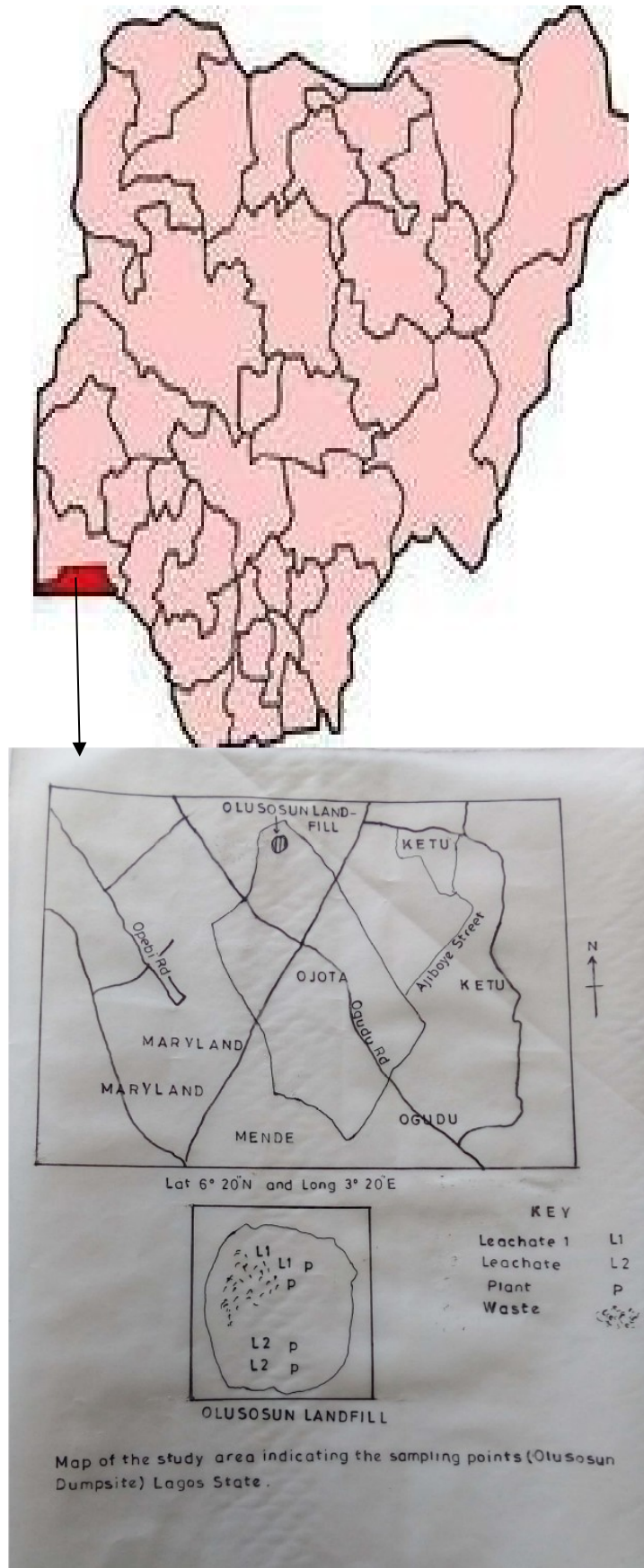
Figure 1: Map of the Study Area. (the red portion in the south-west quadrant).

The leaves of three different plants - pawpaw (*Carica papaya*, P_1), pepper (*Capsicum annum*, P_2) and banana (*Musa acuminata*, P_3) - were collected within the vicinity of L1 and L2. The collected plant leaves were placed in pre-cleaned polyethylene bags and transported to the laboratory. The plants were identified at the Department of Plant Biology, University of Ilorin, Nigeria. In the laboratory, the leaves were rinsed with tap water and distilled water and air-dried at room temperature. The dried samples were ground using an agate mortar and pestle and sieved using a 2 mm mesh size sieve.

Physico-chemical parameters
The pH of composite leachate samples was measured in-situ using a portable digital pH meter (pHep® Hanna, USA), which had been previously calibrated with buffer solutions of pH 4 and 7, respectively. The temperature was also determined in-situ using a thermometer. Electrical conductivity (EC) was measured with a conductivity meter calibrated with a potassium chloride solution. Colour was determined using colorimetry. Total dissolved solids (TDS) was determined gravimetrically by evaporating a known volume of leachate to dryness in a pre-weighed crucible on a steam bath. Total suspended solids (TSS) were also determined gravimetrically by evaporating a known volume of filtered leachate to dryness in a pre-weighed crucible on a steam bath. Biochemical oxygen demand (BOD), chemical oxygen demand (COD), chloride (Cl-), and hardness were determined titrimetrically. The concentration of sulfate (SO_4^{2-}) was determined turbidimetrically, phosphate (PO_4^{3-}) was determined via the colorimetric method, and nitrate (NO_3^-) was determined using the brucine method (20).

Analysis of heavy metals in leachates and plant samples
50 mL of each leachate samples were treated with 10 mL of concentrated HNO_3 and 5 mL of $HClO_4$. The entire mixture was heated in a digestion block at 125 °C until white fumes are observed to confirm the complete utilization of the acid, and the volume was reduced to about 25 mL. The mixture was removed from the block, cooled, filtered using a Whatman No. 40 filter paper, transferred to a 50 mL volumetric flask and made up to mark with distilled water (21). Atomic absorption spectrophotometer (Buck Scientific Model 210 VGP, USA) was then used for quantification of the following metals: Pb, Cd, Cr, Ni, Mn, and Zn. All samples were prepared in duplicate.

For plant samples, approximately 5.00 g of each of the powdered leaf samples was placed in a 25 mL conical flask. 10 mL of concentrated HNO_3 was added, followed by 5 mL of $HClO_4$. The contents of the flask were heated at 150 °C for 1 hour in a fuming hood and then cooled to room temperature. After that, 20 mL of distilled water was added, and

the mixture was filtered using filter paper in a 50 mL volumetric flask (21). Atomic absorption spectrophotometer was also used for quantification of the following metals: Pb, Cd, Cr, Ni, Mn and Zn. All samples were prepared in duplicate.

Analysis of PAHs in leachate and plant leaves
For extraction and clean-up of PAHs in leachates, 100 mL of thoroughly mixed leachate sample was filtered using clean cotton wool to attain a clear solution without particles into a separating funnel. A 100 mL mixture of n-hexane and dichloromethane 1:1 (v/v) was added, and the separating funnel was left undisturbed on a retort stand for about 30 minutes. The separating funnel was then opened to drain the water phase while the organic phase was collected in a glass funnel containing 20 g of anhydrous sodium sulfate plugged with a ball of cotton wool. A second extraction was carried out on the organic phase and was re-extracted using a 50 mL mixture of dichloromethane and n-hexane 1:1 (v/v). The extract was concentrated to an absolute 20 mL prior to the determination of PAHs (22,23).

For plant leaves, a 2.00 g of each ground plant sample was weighed into a clean extraction container. 10 mL of dichloromethane was added and left for 20 minutes. Another 20 mL of dichloromethane was added again to the mixture and was then filtered into an extraction bottle. The extracted sample was concentrated to 3 mL for separation. The column was packed with 1 g of moderately packed cotton wool. 3 g of dried activated silica gel was then poured into the column, and 0.6 mL of sodium sulfate was added. The column was rinsed with 20 mL of dichloromethane to allow it to run through the silica gel. Pre-elution with dichloromethane (20 mL) was allowed to flow through the column for about 2 minutes, and 3 mL of the plant sample was poured immediately into the column. The stop-cork of the chromatographic column was opened, and the eluent was collected at intervals with a 10 mL graduated cylinder. Due to exposure of sodium sulfate to air, dichloromethane was added at intervals to the column in increments of 5 mL. The concentrated fractions were then transferred in a glass bottle with rubber crimps caps for GC-MS analysis (24). All samples were prepared in duplicate.

Extracts from leachates and plant leaves were analyzed for 16 PAHs by gas chromatography-mass spectrometry (GC-MS, Agilent 7890B GCMS, USA).

Data Analysis
All the experimental data were subjected to further statistical analyses such as Analysis of variance (ANOVA), Pearson correlation matrix, and principal component analysis to determine the source of pollution from the studied landfill (Olusosun) using Origin pro 2018.

RESULTS AND DISCUSSION

Physico-chemical properties of leachate
Physico-chemical properties of leachates obtained from Olususun dumpsite are presented in Table 1. The pH values, ranging from 7.3 - 7.5, indicates slight alkalinity of the leachates. Leachate pH values are reported to increase with age and are usually greater than 7 (10,17,25). Values of pH greater than 7 may be attributed to high salinity, an increase in the utilization of unionized free volatile fatty acids by bacteria, and a decrease in short-chain fatty acids (26). The pH also serves as an indicator of the chemical state in which an environmental species will be present, and pH-dependent species such as heavy metals may become environmentally available at low pH (27). Hence, the metals' bioavailability might be low. Similarly, alkaline values have been reported for landfill leachates (10,16,17,26). The TDS values ranged from 5880 - 6500 mg/L. High TDS values imply the presence of a large concentration of soluble ions, leaching of the ions, and dissolved organic matter from the landfill caused by the hydrogeological, physical, and chemical conditions from the landfill, including chlorides, carbonates, phosphates, nitrates among others (27). The range of concentration values for TSS, BOD, COD, NO_3^-, PO_4^{3-}, Cl^-, TH and SO_4^{2-} are: 1260-1805 mg/L, 640-658.2 mg/L, 2788-2866 mg/L, 18.4-21.2 mg/L, 9.0-9.8 mg/L, 3439-3471 mg/L, 7305-7670 mg/L and 107-150 mg/L, respectively. All but SO_4^{2-} exceeded their respective limits set by WHO (29) and NESREA (30). A high BOD level implies that the leachate cannot undergo self-purification, and therefore require some form of pre-treatment. Similarly, a BOD/COD ratio greater than 0.2 indicates that the leachate is old and relatively stable (17). For Cl^-, no harmful level is reported in humans, but high concentrations exceeding the 250 mg/L permissible limit may be harmful to and cause damage to plants. The concentration of sulfate at high amounts in landfill leachate is reported to depend on depends on the following factors: landfill age, composition of waste, the biological, physical, and chemical composition (31). Nitrate level concentration values are important factors in determining the extent of pollution in leachates. Phosphate at high concentrations can lead to oxygen depletion of water bodies via eutrophication with a response to an increasing level of nutrients due to the presence of phosphates and nitrates (32). The color of the leachates was found to be very black, which is a result of the changes in the oxidation state of Fe^{2+} (ferrous ions) to Fe^{3+} (ferric ions). The ferric ion forms ferric hydroxide colloids and fulvic complexes contributing to the blackish color of the leachates (26).

Table 1: Physico-chemical properties of leachates

| Parameters | Leachates | | | |
	L_1	L_2	*	**
Color	Black	Black	Colorless	-
pH	7.5±0.10	7.3±0.10	6.0 - 9.0	6.5 - 9.5
EC (µS/cm)	724.0±0.40	740.0±0.90	_	1000.0
TDS (mg/L)	6500.0±0.90	6880.0±1.30	500.0	500.0
Temperature (°C)	17.0±0.90	18.7±0.10	40.0	_
BOD (mg/L)	640.0±0.51	658.2±0.90	50.0	_
COD (mg/L)	2788.0±0.20	2868.0±1.10	90.0	_
Cl^- (mg/L)	3439.0±0.80	3471.0±1.11	250.0	250.0
TH (mg/L)	7305.0±0.90	7670.0±0.71	_	180.0
TSS (mg/L)	1260.0±1.10	1505.0±0.92	25.0	_
SO_4^{2-} (mg/L)	107.0±0.30	150.0±0.90	250.0	250.0
NO_3^- (mg/L)	18.4±0.01	21.2±0.20	10.0	50.0
PO_4^{3-} (mg/L)	8.0±0.50	9.8±0.03	2.0.0	_

*NESREA, 2011

**WHO, 2007

Table 2: Pearson correlation matrix of the physicochemical parameters of leachate from Olususun Landfill

	pH	EC	TDS	Temp.	BOD	COD	Cl⁻	TH	TSS	SO₄²⁻	NO₃⁻	PO₄³⁻
pH	1											
EC	-0.4096	1										
TDS	-0.8504	0.8283	1									
Temp.	-0.5903	0.9782	0.9267	1								
BOD	-0.8361	0.8429	0.9996	0.9363	1							
COD	-0.7219	0.9270	0.9780	0.9847	0.9832	1						
Cl-	-0.8072	0.8692	0.9970	0.9530	0.9987	0.9912	1					
TH	-0.7665	0.8999	0.9897	0.9709	0.9932	0.9978	0.9978	1				
TSS	-0.8085	0.8681	0.9972	0.9523	0.9988	0.9909	0.9965	0.9977	1			
SO₄²⁻	-0.7672	0.8994	0.9899	0.9706	0.9933	0.9977	0.9979	0.9968	0.9978	1		
NO₃⁻	-0.9209	0.7329	0.9883	0.8583	0.9838	0.9346	0.9735	0.9563	0.9740	0.9565	1	
PO₄³⁻	-0.9226	0.7298	0.9876	0.8560	0.9830	0.9330	0.9724	0.9549	0.9729	0.9552	0.9999	1

Two-tailed significance is used.

The physicochemical parameters for all the leachates were analyzed with Origin pro 2018 using one-way ANOVA (Analysis of variance), which shows that all the physicochemical parameters analyzed are significantly different ($p > 0.05$ level). Also, Pearson correlation was used to analyze the physicochemical parameters, as shown in Table 2 above. The pH shows a negative correlation with EC ($p = 0.7313$), TDS ($p = 0.3527$), Temp. ($p = 0.5980$), BOD ($p = 0.3697$), COD ($p = 0.4865$), TH ($p = 0.4440$), TSS ($p = 0.4006$), Cl^- ($p = 0.4020$), SO_4^{2-} ($p = 0.4434$), NO_3^- ($p = 0.2550$), PO_4^{3-} (0.2521). There was a positive correlation among other physicochemical parameters with which includes EC vs. TDS, SO_4^{2-} vs. NO_3^-, TH vs. PO_4^{3-} BOD vs. Cl^-, Temp. vs. SO_4^{2-}, COD vs. TH, Cl^- vs. TH, TDS vs. Temp., TDS vs. BOD, Temp. vs. COD, EC vs. Temp., Cl^- vs. SO_4^{2-}, BOD vs. TSS, TDS vs. PO_4^{3-}, COD vs. SO_4^{2-}, TDS vs. TSS, BOD vs. COD, Temp. vs. TH, TSS vs. NO_3^-, BOD vs. TH, EC vs. NO_3^-, Cl^- vs. Temp., BOD vs. TSS, EC vs. TSS, TDS vs. NO_3^- TH vs. SO_4^{2-}, Temp. vs. PO_4^{3-}, TH vs. NO_3^-, TSS vs. SO_4^{2-}, EC vs. BOD, and NO_3^- vs. PO_4^{3-}. With the results from Pearson correlation,

it can be deduced that the landfill is heavily polluted with diverse kinds of wastes dumped from the residents in this State.

Heavy metal concentrations in leachates and plant leaves

The concentrations of heavy metals (mg/L) in leachate samples were: 0.60 - 1.10 for Pb, 0.04 - 0.05 for Cd, 0.30 - 0.40 for Cr, 0.13 - 0.19 for Ni, 2.24 - 3.72 for Zn and 0.53 - 0.74 for Mn, respectively as shown from Figure 2 below. The increasing order of metals concentration metals in the leachate samples followed the order: Zn>Pb>Mn>Cr>Ni>Cd. These values are similar to the results obtained elsewhere (17) but slightly higher than those reported elsewhere (28). All analyzed metals in leachates samples exceeded their respective permissible limits set by WHO (29) and NESREA (30) except Cd, which was lower than the NESREA (30) permissible limit. The presence of heavy metals in both leachates can be attributed to the type of waste being deposited on the landfill site, which in turn is a function of the industrialized nature of the study areas.
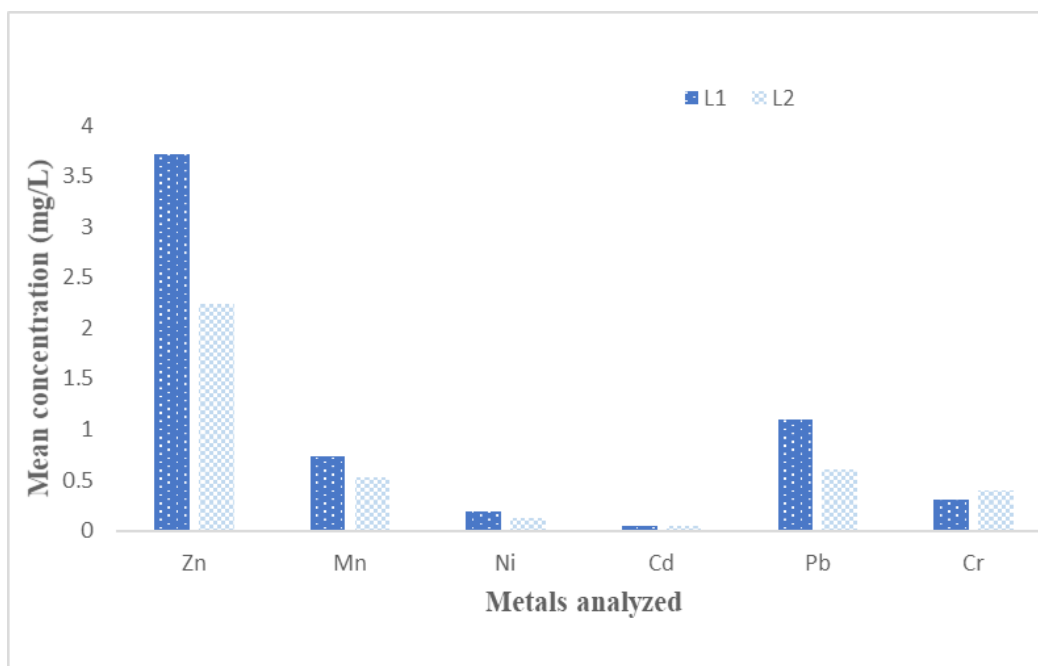

Figure 2: Metal concentrations in leachate samples.

The mean concentrations of heavy metals in plant samples analyzed are shown in Figure 3 below. The increasing order of metals concentration in plant samples followed the order: Mn>Zn>Pb>Cr>Ni>Cd.

The concentration of heavy metals in all plant samples were all below their respective permissible limits set by WHO (33) (50 mg/kg for Zn, 10 mg/kg for Ni, 0.02 mg/kg for Cd, 2 mg/kg for Pb and 1.3 mg/kg for Cr).
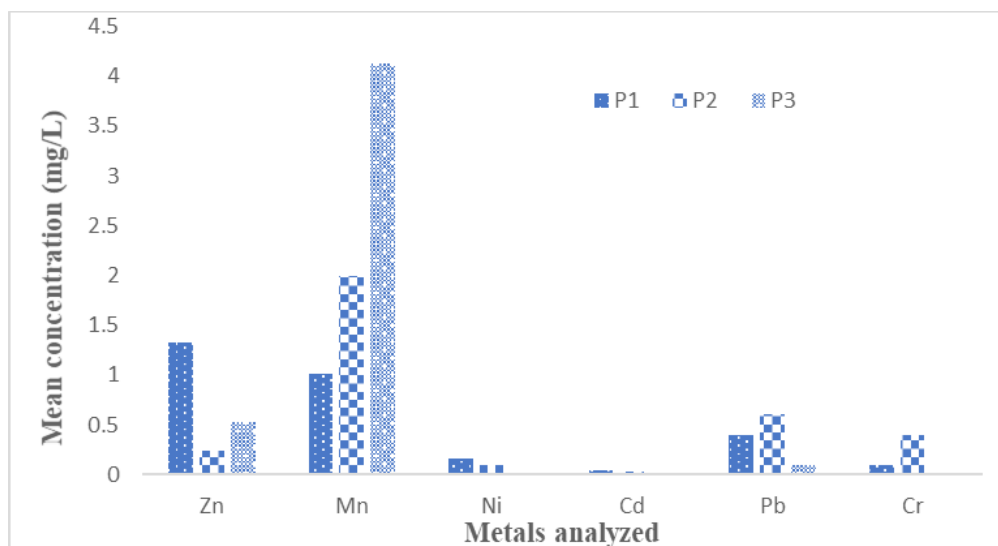
Figure 3: Metal concentrations in plant leaves.

Table 3: Pearson correlation matrix of heavy metals from leachates Samples in the Olususun landfill.

	Zn	Mn	Ni	Cd	Pb	Cr
Zn	1					
Mn	0.9411	1				
Ni	0.7827	0.9471	1			
Cd	0.7278	0.4530	0.1429	1		
Pb	0.8967	0.9936	0.9774	0.3491	1	
Cr	0.1477	-0.1956	-0.5000	0.7857	-0.3054	1

Two tailed ($p > 0.05$ level)

Table 4: Pearson correlation matrix of heavy metals from the plant samples in the Olususun landfill.

	Zn	**Mn**	**Ni**	**Cd**	**Pb**	**Cr**
Zn	1					
Mn	-0.5434	1				
Ni	0.7067	-0.9780	1			
Cd	0.7067	-0.9780	0.6215	1		
Pb	-0.1469	-0.7506	0.5960	0.5960	1	
Cr	-0.5171	-0.4376	0.2402	0.2402	0.9226	1

Two tailed ($p > 0.05$ level)

From Table 3 above, there is a strong positive correlation between Zn with other metals such as Mn (p =0.2197), Ni (p = 0.4277), Cd (p = 0.4811), Pb (p= 0.2919) and Cr (p = 0.9057). Also, there is a positive correlation in Mn versus Ni (p = 0.2080), Mn versus Pb (p = 0.0723)), Ni versus Pb (p = 0.1357), and Cd versus Cr (p = 0.4246) and slight correlation between Ni versus Cd (p = 0.9087), Mn versus Cd (p = 0.7007) and Cd versus Pb (p = 0.7730). A negative correlation was identified between Mn versus Cr (p = 0.8747), Ni versus Cr (p = 0.6667) and Pb versus Cr (p = 0.8024). The Pearson correlation of the plant samples is shown in Table 4 above. A negative correlation was identified between Zn vs. Mn (p = 0.6343), Zn vs. Cr (p = 0.6540), Mn vs. Ni (p =0.1339), Mn vs. Pb (p = 0.4596), Mn vs. Cr (p= 0. 0.7117) and slight correlation exist between Ni vs. Pb (p = 0.8456) and very strong correlation exist between Zn vs. Ni and Zn vs. Cd having the same values (p = 0.5004), Ni vs. Cd, Cd vs. Pb are also having the same values (p = 0.5935). The correlation between the metals analyzed was from a similar source (34), and this confirmed that the landfill was contaminated with different composition of solid wastes dumped on this landfill, which percolated into soil and then contaminated the plants grown from this site. The relatively high concentrations recorded for Mn in plant leaves compared with leachate concentrations could be attributed to different degrees in bioaccumulation of Mn by the different plant laves studied.

The eigenvalues obtained from any data are used to determine components that are retained. A set of data has eigenvalues greater than unity indicates a component that is retrained, but when it is less than unity, the component is not retained (35, 36). The scree plot of heavy metals in leachates and plants were shown from Figure 4 below. There were five

components from each scree plot of leachates and plants. Components 1 and 2 are retained with eigenvalues of 4.00 and 1.97 respectively for both leachates and plants, while other components (3-6) are not retrained. However, components 1 and 2 are retrained for leachates and plants because the eigenvalues are greater than unity, as shown in Figure 5. It is similar to the previous research from the assessment of heavy metals from roadside dust in Ilorin (36). The two principal components analysis (PC 1 and PC 2) from heavy metals in leachate samples has a variance of 66.99 % and 33.01%, respectively, as shown from Figure 6 below.

In the plant samples, the PC 1 and 2 have 66.05% and 33.95% variance, respectively, as shown from

Figure 6 below. PC 1 is strongly correlated with these metals (Zn, Mn, Ni, Cd, Pb) in leachates and negatively correlated with Cr while PC 2 is strongly correlated with Zn, Cd, and Cr, but negatively corrected with Mn, Ni, and Pb as shown from Table 5 below. Table 6 below indicates the PC 1 and PC2 of heavy metals in plant samples. PC 1 is strongly correlated with Zn, Ni, Cd, Pb, and Cr but negatively correlated with Mn. While PC 2 is strongly correlated with Mn, Pb, and Cr but negatively correlated with Zn, Ni, and Cd. With the statistical data from the principal component analysis, it can be deduced that the heavy metals from this landfill are heavily polluted by anthropogenic sources (17, 34, 37).
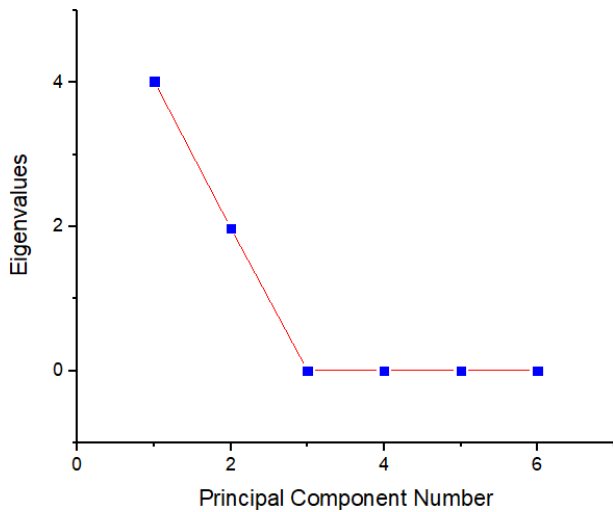

Figure 4: Screen plot of heavy metals in both plants and leachate samples.
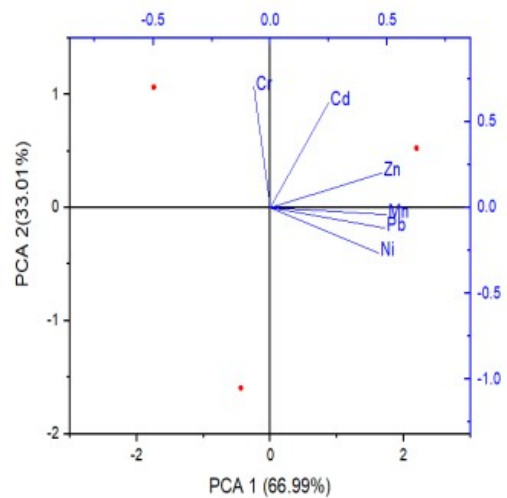
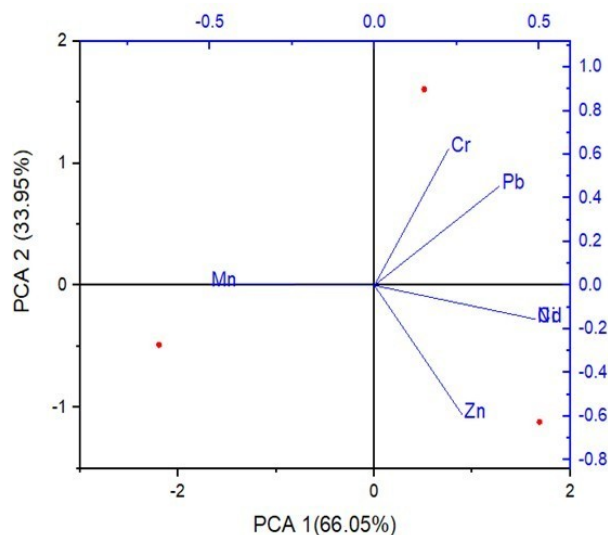**Figure 5:** Bi-plot of heavy metals in plants leachates.

Figure 6: Bi-plot of heavy metals in leachates from Olusosun landfill.

Table 5: Extracted eigenvalues of the heavy metals in leachate samples.

	Coefficients of PC1	Coefficients of PC2
Zn	0.4781	0.2026
Mn	0.4980	-0.0397
Ni	0.4627	-0.2653
Cd	0.2504	0.6145
Pb	0.4916	-0.1198
Cr	-0.0701	0.7035

Table 6: Extracted eigenvalues of the heavy metals in leachate samples.

	Coefficients of PC1	Coefficients of PC2
Zn	0.2675	-0.5931
Mn	-0,5023	0.0091
Ni	0.4899	-0.1551
Cd	0.4899	-0.1551
Pb	0.3813	0.4562
Cr	0.2256	0.6260

Concentrations of PAHs in leachates and plant leaves

Table 7 shows the concentration of PAHs in leachate samples. Sixteen PAHs are recognized by the United States Environmental Protection Agency (EPA) and are classified as low molecular weight (LMW, petrogenic), medium molecular weight (MMW), and high molecular weight (HMW, pyrogenic). The following PAHs were detected in leachate and plant samples: HMW - chrysene, benzo(b)fluoranthene, and benzo(a)anthracene; MMW - pyrene and fluoranthene and LMW - phenanthrene. The concentration range (mg/L) for the detected PAHs in landfill leachates were: LMW: 0.17 - 0.22; MMW: 0.15 - 0.24 and HMW: 0.10 - 0.19 mg/L, respectively. The presence of LMW PAHs in the environment usually results from the emission of oil spills, noncombustible matter, and agricultural sources, while MMW and HMW PAHs are emitted mainly from sources like incomplete combustion/pyrolysis of organic matter and solid wastes, and incorporated into landfill leachate by rainwater (3). Municipal and waste discharge from industrial sites are part of the factors causing a high concentration of PAHs in leachates (38). The result of PAHs concentration in plant leaf samples is shown in Table 8. The concentration of PAHs in plant samples ranged between 0.09 - 0.31 mg/kg. Since P1 is far away and P2 and P3 are close, no PAHs were detected in P1, and some levels of PAH were found in P2 and P3.

Table 7: Concentration of polycyclic aromatic hydrocarbons found in leachates.

PAHs	Concentration (mg/L/) L1	L2	PAHs	Concentration (mg/L/) L1	L2
Anthracene	BDL	BDL	Pyrene	0.15	0.19
Acenaphthene	BDL	BDL	Perylene	BDL	BDL
Phenanthrene	0.22	0.17	Chrysene	0.10	0.13
Acenaphthylene	BDL	BDL	Benzo(b)fluoranthene	0.19	0.15
Naphthalene	BDL	BDL	Benzo(k)fluoranthene	BDL	BDL
Fluorene	BDL	BDL	Benzo(a)an thracene	0.14	0.16
Fluoranthene	0.21	0.24	Indeno(1,2,3-cd)pyrene	BDL	BDL
	Σ=1.01	Σ= 1.04			

Table 8: Concentration of PAHs in plant leaves.

	Concentration (mg/kg)		
PAHs	P1	P2	P3
Naphthalene	BDL	BDL	BDL
Acenaphthylene	BDL	BDL	BDL
Acenaphthene	BDL	BDL	BDL
Fluorene	BDL	BDL	BDL
Phenanthrene	BDL	0.31	0.19
Anthracene	BDL	BDL	BDL
Fluoranthene	BDL	0.16	0.14
Pyrene	BDL	0.24	0.11
Benz(a)anthracene	BDL	0.17	0.14
Chrysene	BDL	0.09	0.13
Benzo(b)fluoranthene	BDL	0.11	0.17
Benzo(k)fluoranthene	BDL	BDL	BDL
Benzo(a)pyrene	BDL	BDL	BDL
Indeno(1,2,3-cd)pyrene	BDL	BDL	BDL
Dibenzo(a,h)anthracene	BDL	BDL	BDL
Benzo (g,h,i) perylene	BDL	BDL	BDL
	Σ=0.0000	Σ= 1.08	Σ= 0.88

Table 9: Pearson correlation matrices of PAHs from leachate samples in the Olususun landfill.

	Phe	Flu	Pyr	Chy	Benzo(b)F	Benzo(a)A
	1					
Phe	0.0576	1				
Flu	0.8572	0.5636	1			
Pyr	0.8934	0.5000	0.9972	1		
Chy	0.4018	-0.8910	-0.1273	-0.0524	1	
Benzo(b)F	-0.7825	0.5766	-0.3500	-0.4193	-0.8846	1
Benzo(a)A	0.7924	0.6547	0.9934	0.9820	-0.2402	-0.2402

Phe- Phenanthrane; Flu- Fluoranthene; Pyr- Pyrene; Chy- Chrysene; Benzo (b)- Benzo(b)fluoranthene; Benzo(a)A- Benzo(a)anthracene

Table 10: Pearson correlation matric of PAHs from plant samples in the Olususun landfill.

	Phe	**Flu**	**Pyr**	**Chy**	**Benzo(b)F**	**Benzo(a)A**
Phe	1					
Flu	0.9477	1				
Pyr	0.7341	0.4790	1			
Chy	0.9995	0.9368	0.7559	1		
Benzo(b)F	-0.3192	3.61E-17	-0.8778	-0.3499	1	
Benzo(a)A	-0.4039	-0.0908	-0.9177	-0.4336	0.9959	1

The Pearson correlation of PAHs in leachates in shown from Table 9 above. A positive correlation exists between some PAHs found in leachates with significant difference >0.05 which includes: Pyr/Phe, Flu/Phe, Pyr/Flu, Chy/Phe, Benzo(a)A/flu, Benzo(b)F/ Flu, Benzo(a)A /Phe and a negative correlation exists between Benzo(b)F /Phe, Chy/Flu, Benzo(b)F/Phe, Benzo(b)/ Pyr, Benzo(a)A/ Benzo(b)F and Chy/Pyr. Also, a positive correlation exists between Phe/Flu, Chy/Phe, Flu/Pyr, Benzo(b)F/ Benzo(a)A Pyr/Phe, Chy/Flu, Phe/Chy, Pyr/Chy, and negative correlation occurred between Phe/ Benzo(b)F, Phe/ Benzo(a)A, Chy/ Benzo(b)F, Benzo(a)A, Chy, Benzo(a)A/Pyr with a significant difference >0.05 in plants as shown in Table 10 above. It indicates that the source of contamination in this study is a result of different anthropogenic activities that take place in the landfill.

Table 11: Extracted eigenvalues of the PAH in leachate samples.

	Coefficients of PC1	Coefficients of PC2
Phenanthrene	0.4812	-0.1872
Fluoranthene	0.4716	0.2317
Pyrene	0.4825	0.1806
Chrysene	0.1013	-0.6957
Benzo(b)fluoranthene	-0.3174	0.5481
Benzo(a)anthracene	0.4498	0.3072

Table 12: Extracted eigenvalues of the PAH in plant samples removed from the plants.

	Coefficients of PC1	Coefficients of PC2
Phenanthrene	0.4415	0.3221
Fluoranthene	0.3495	0.5156
Pyrene	0.4706	-0.2109
Benz(a)anthracene	0.4483	0.3004
Chrysene	-0.3454	0.5217
Benzo(b)fluoranthene	-0.3757	0.4727

The variance of PAHs in leachate and plant samples analyzed are 66.98 %, 33.02 %; 69.02%, 30.98% for PC1 and PC2, respectively, as shown from Figures 7 and 8 below. The scree plot of PAHs in plants and leachates proved the components that are retrained, as shown in Figure 8 below. Components 1 (4.34) and 2 (1.77) are retained because it is greater than unity (35, 36) while other components (3, 4 and 5) are not retained. There is a strong positive correlation between the PAHs in PC1 for leachate samples includes; Phenanthrene, Fluoranthene, Pyrene, Chrysene, and Benzo(a)anthracene but negative correlation occurred at Benzo(b)fluoranthene. While the positive correlation was seen between the PAHs in PC2 includes; Fluoranthene, pyrene, Benzo(b)fluoranthene and Benzo(a)anthracene but negative correlation exists in Phenanthrene and Chrysene as shown from Table 11 above. For plant samples, a positive correlation exists between Phenanthrene, Fluoranthene, pyrene, Benzo(a)anthracene, and a negative correlation exists between Chrysene and Benzo(b)fluoranthene for PC1. A positive correlation exists between these PAHs (Phenanthrene, Chrysene, Fluoranthene, Benzo(b)fluoranthene and Benzo(a)anthracene) and pyrene only had a negative correlation in PC2 as shown from Table 12 above. However, the statistical data from this study confirmed that the source of PAHs was through resistance to environmental degradation, surface run-off (17), and type of waste deposited on the landfill.
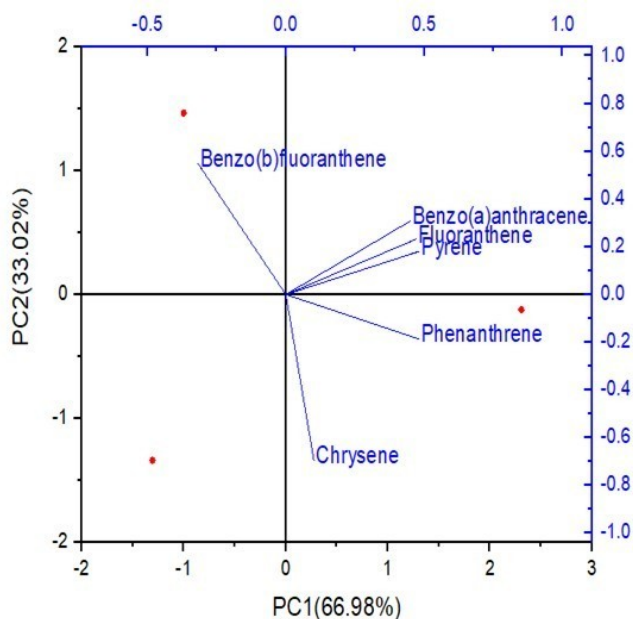

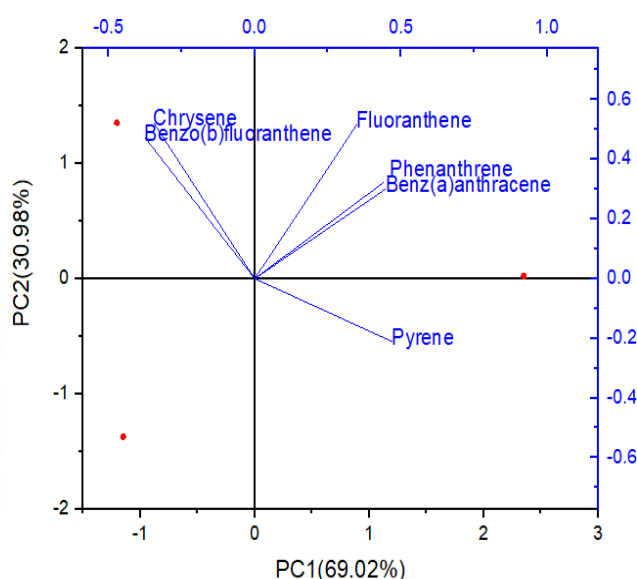**Figure 7:** Biplot of the PAH in leachate samples.

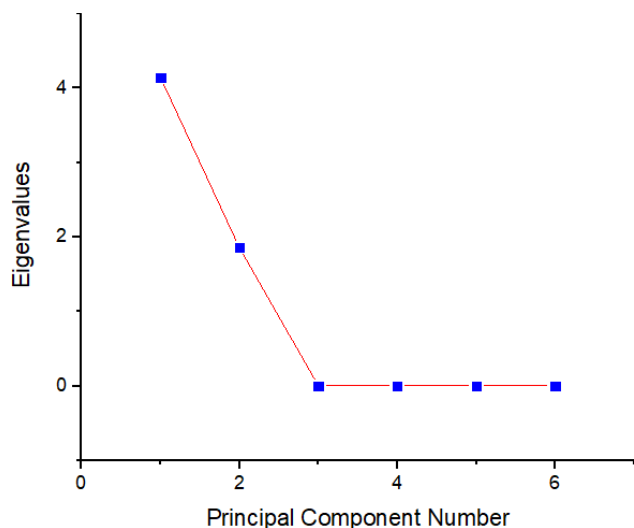**Figure 8:** Bi-plot of PAH in plant samples.

Figure 9: Screen plot of PAHs in plants.

CONCLUSION

Six (6) PAHs each were detected in both the plant and leachate samples analyzed, which includes the following: Chrysene, Benzo (b)fluoranthene, Benzo (a) anthracene, Phenanthrene, Pyrene, and Fluoranthene. Heavy metals such as Zinc (Zn), Lead Pb, Chromium (Cr), Manganese (Mn), Cadmium (Cd), and Nickel (Ni) were also detected. The majority of the physicochemical parameters analyzed were found to exceed their permissible limits set by WHO and NESREA. The source of pollution in this study is the same, and it is confirmed by the statistical analysis used. With the results obtained from this study, it could be deduced that the landfill is heavily polluted, and adequate management needs to be put in place by various agencies.

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CONFLICT OF INTEREST
All the authors declare no conflict of interest regarding this manuscript

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RESEARCH ARTICLE

A novel selective "turn-on" fluorescent sensor for Hg^{2+} and its utility for spectrofluorimetric analysis of real samples

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Abstract: A novel anthracene-based dipodal Schiff base "turn-on" fluorescent sensor (**FS**) was designed and synthesized by accessible and straightforward Schiff base reaction of salicylaldehyde and 9,10-bis(aminomethyl)anthracene with high yield. The chemical characterization of fluorescent sensor **FS** was performed by standard spectroscopic techniques (MALDI-MS, FT-IR, ^{1}H, and ^{13}C NMR), and photophysical properties were exanimated by UV-vis and fluorescent spectroscopies. The fluorescent sensor **FS** can coordinate with Hg^{2+} via Schiff base moiety when analytical signal as a "turn on" fluorescent response was obtained via anthracene moiety after coordination. Also, spectrofluorimetric analysis of Hg^{2+} was carried out using fluorescent sensor **FS** in environmental water samples after optimization required experimental conditions such as pH, the time before measurements, and photostability. According to obtained results, the presented fluorescent sensor can be used for selective and sensitive spectrofluorimetric determination of Hg^{2+}.

INTRODUCTION

Although industrial and technological activities increase the quality of our life, it causes severe problems for environmental pollution and human health. Contamination of the environment with heavy metal ions led to potential damage to mankind due to the highly toxic effect of these ions even at low concentrations (1). Between these metal ions, Hg^{2+} is one of the most hazardous heavy metal ions because it can inhibit the activity of some biological species such as enzymes and proteins (2). Besides, it can be accumulating to the human body via the food chain and causes severe diseases of the central nervous system (3-5). Therefore, the United States Environmental Protection Agency (EPA) and World Health Organization (WHO) strongly limited the permissible concentration of Hg^{2+} ions in drinking water in the range of several ppb (μg/kg) (6, 7). Although a significant number of analytical methods are used for detection and determination of Hg^{2+} ions up to now such as capillary electrophoresis (CE), liquid chromatography (LC), atomic absorption spectrometry (AAS), gas chromatography (GC), and atomic fluorescent spectrometry (AFS), these methods generally require preconcentration processes with extra chemicals (adsorbents or eluents), applied by expert users. In addition, they are not applied to in-field analysis (8-11). Fluorescent sensors, handling these disadvantages, attract attention for detection and determination of Hg^{2+} ions due to their several advantages such as simple operation, high sensitivity and selectivity, low cost, and real-time monitoring in-field analysis (12-14). Notably, the development of "turn-on" fluorescent sensors, which are selective for Hg^{2+}, has become an

important research topic due to their increased sensitivity compared to "turn-off" fluorescent sensors. Because they can detect analytes via fluorescent signal increment, which led to more easy monitoring than fluorescent sensors that can detect analytes with fluorescent quenching (15-17), another important phenomenon is that heavy metal ions such as Hg^{2+} are known as quencher due to spin-orbit coupling (18). Therefore, developing a selective, sensitive, new, and simple "turn-on" fluorescent sensor for Hg^{2+} ions, which can be practicable to the spectrofluorimetric analysis of the environmental and biological sample is still an essential research area (19, 20).

Schiff bases, which are the essential class of organic compounds, can be obtained with the moderate reaction of primary amines with aldehydes/ketones. These kinds of compounds generally offer one-step synthesis processes in moderate conditions with high yield. Also, an imine bond, known as carbon-nitrogen double bond (-C=N-), is obtained as a result of Schiff base reaction can readily be used for coordination with metals (7, 21, 22). After coordination with metal ions, inhibition of the C=N isomerization cause substantial fluorescent enhancement for such molecules, which is used as obtaining analytical signals (23). Also, according to previously published reports, it is well-known that via sulfur, oxygen, or nitrogen atoms, mercury ions can coordinate with fluorescent sensors (7, 13, 24). Therefore, Schiff base preparation methods can be used for obtaining Hg^{2+} ion-selective fluorescent sensors that containing anthracene with proper cavity and donor atoms due to its well-known optical and metal-binding properties (7, 25, 26). There are a few papers that existed in the literature regarding the develop fluorescent sensors, which are used for selective and sensitive detection of Hg^{2+} based on anthracene containing Schiff base systems (23, 27). However, some deficiencies regarding selectivity and applicability were observed in these reports (27). Thus, in this study, simple, accessible, and non-sulfur anthracene-based dipodal Schiff base fluorescent sensor for selective, sensitive, and simple quantification of Hg^{2+} ion in environmental water samples was prepared by the reaction of salicylaldehyde and 9,10-bis(aminomethyl)anthracene with high yield. The nitrogen and oxygen donor atoms provide the coordination capacity for Hg^{2+} ions (7, 13, 24) when anthracene core acts as a fluorophore for obtaining analytical signals. After full characterization of novel fluorescent sensor by MALDI-MS, FT-IR, NMR spectroscopies, required conditions were optimized and spectrofluorimetric determination of Hg^{2+} ions were carried out in environmental water samples.

EXPERIMENTAL

Materials and Instrumentation
The reagents and solvents which are used for preparation and fluorescent sensor application of **FS** purchased from commercially as follows: Anthracene, dichloromethane, 2-bromoethanol, paraformaldehyde, cetyltrimethylammonium bromide (CTAB), glacial acetic acid from Merck, 2,5-dihydroxybenzoic acid (MALDI-MS matrix) from Fluka, deuterated DMSO from Sigma-Aldrich and they were used as received. Inert argon atmosphere was used for the synthesis of **FS**.

Varian Eclipse spectrofluorometer and Shimadzu 2101 UV spectrophotometer were used for recording fluorescent and the electronic absorption spectra of **FS** in the UV-vis region. Also, spectrofluorimetric analysis in environmental water samples where 5 nm of slit width and 1 cm of path-length were used for all fluorescent measurements at 25°C were performed by Varian Eclipse spectrofluorometer. NMR (^1H and ^{13}C NMR), FT-IR, and mass analysis of **FS** were recorded with Varian INOVA 500 MHz spectrometer, Perkin Elmer Spectrum 100 spectrophotometer, and Bruker Daltonics Microflex MALDI-TOF mass spectrometer, respectively. Deuterated DMSO was used for all NMR measurements. The non-linear regression calculations were performed using Sigma-Plot 14.0.

All water samples were collected from Kocaeli/Sakarya in Turkey and filtered with filter paper (blue band). After that, they were acidified with nitric acid and kept at 4°C in a refrigerator before the spectrofluorimetric analysis. The presented spectrofluorimetric analysis performed without any enhancement processes via sensitive and selective complexation of **FS** with Hg^{2+} ion. The fluorescent signal of **FS** that raised via anthracene proportionally and gradually increased after treatment with 0.05-120.00 μM of Hg^{2+}. Spectrofluorimetric determination of Hg^{2+} ion in real samples was performed via analytical signals that were obtained by complexation of Hg^{2+}-**FS,** and the calibration curve was used for analysis with relative fluorescent response change. For determination of Hg^{2+}, 6.25×10^{-5} M of **FS** was prepared in DMSO. After taking 0.80 mL of **FS** from this solution, 1.70 mL of DMSO, 0.25 mL of Britton–Robinson buffer solution (pH 8.0), and 0.25 mL of sample was added onto volumetric flask (5 mL). After that, the volume of the flask was completed with deionized water to 5 mL and carefully shaken for 60 seconds. All spectrofluorimetric measurements were recorded at 426 nm.
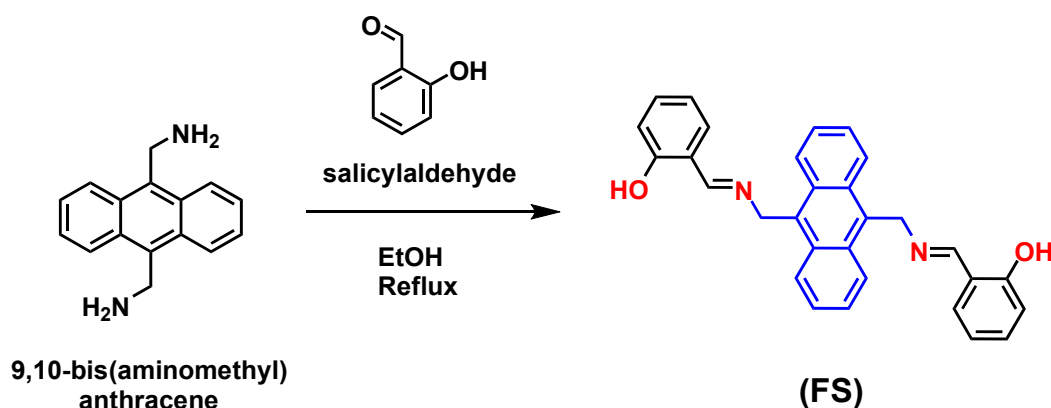
Synthesis

9,10-bis(aminomethyl)anthracene was synthesized and characterized according to literature (28) and **FS** was obtained as follows: 2.460 g of salicylaldehyde (0.0201 mol) dissolved in absolute ethanol in the stream of argon gas and refluxed for 30 minutes. Then, 4.750 g of 9,10-bis(aminomethyl)anthracene (0.0201 mol) and several drops of glacial acetic acid were added onto the solution of salicylaldehyde. The reaction mixture was refluxed during a day. Then the yellow precipitate was filtered with blue filter paper and washed three times with ethanol and n-hexane. **FS** was obtained as a yellow solid with 95% yield (8.488 g). FT-IR; 3271 cm^{-1} (-OH), 3061-3050 cm^{-1} (-ArH), 2917-2867 cm^{-1} (-C-H), 1634 cm^{-1} (-CH=N), 1600-1400 cm^{-1} (-C=C-), 1444.1-1335.1 cm^{-1} (-C-H-). [M]$^{+}$: 444.654 m/z (calc. [M]$^{+}$: 444.530). ^{1}H NMR in DMSO-d_6 at 25°C, δ_{ppm}; 13.38 (s, 2H), 8.90 (s, 2H), 8.58 (dd, J = 6.7, 3.2 Hz, 4H), 7.67 (dd, J = 6.7, 3.2 Hz, 4H), 7.42 (d, J = 7.8 Hz, 2H), 7.27 (t, J = 7.9 Hz, 2H), 6.85 (t, J = 7.5 Hz, 2H), 6.77 (d, J = 8.2 Hz, 2H), 5.88 (s, 4H). ^{13}C NMR in in DMSO-d_6 at 25°C, δ_{ppm}; 166.10,

160.27, 132.41, 131.72, 130.37, 129.82, 126.31, 125.05, 118.65, 116.41, 53.65.

RESULT AND DISCUSSION

Structural Characterization

The fluorescent sensor (**FS**) having a di-functional primer amine derivative of anthracene core was prepared by following the literature procedure (28). Briefly, 9,10-bis(bromomethyl)anthracene was obtained with high yield (96%) by the reaction of anthracene and HBr in the presence of paraformaldehyde and CTAB. After that, 9,10-bis(aminomethyl)anthracene, which is the di-functional primer amine derivative of anthracene core, was prepared in high yield (95%) by reaction of 9,10-bis(bromomethyl)anthracene and hexamethylenetetramine. Finally, the presented fluorescent sensor (**FS**) was prepared with high yield (95%) as a result of the Schiff base reaction of 9,10-bis(aminomethyl)anthracene and salicylaldehyde in ethanol (Scheme 1). During the preparation of **FS** with high yield, any purification process was not used except for washing and precipitation.

Scheme 1: The synthetic route of **FS**.

The chemical structure of the novel fluorescent sensor was investigated by FT-IR, MALDI-MS, and NMR (^{1}H and ^{13}C) spectroscopies. All results point out that the proposed structure of **FS** was consistent with characterization data. For instance, The FT-IR spectra of 9,10-bis(aminomethyl)anthracene, salicylaldehyde, and **FS** were depicted in Figure 1a. 9,10-bis(aminomethyl)anthracene demonstrated peaks at 1550 cm^{-1}, 2956-2867 cm^{-1}, 3078-3045 cm^{-1} and 3256-3167 cm^{-1} which assigned to stretching vibrations of -C=C-, -C-H, -ArH and -NH$_2$, respectively. Besides, -C=C-, -OH and -C=O stretching of the aldehyde functional groups of salicylaldehyde were observed at 1555 cm^{-1}, 3142 cm^{-1}, and 1662 cm^{-1}, respectively. As can be seen from FT-IR spectra of **FS**, stretching vibrations of -C=C-, -CH=N, -C-H, -ArH and -OH were observed

at 1575 cm^{-1}, 1634 cm^{-1}, 2917-2867 cm^{-1}, 3061-3050 cm^{-1} and 3271 cm^{-1}, respectively (13, 29). Importantly, -C=O stretching of salicylaldehyde (1662 cm^{-1}) and -NH$_2$ stretching of 9,10-bis(aminomethyl)anthracene (3256-3167 cm^{-1}) were disappeared after the formation of **FS** which were important evidence for the proposed structure of **FS**. The ^{1}H NMR spectrum of **FS,** which recorded in DMSO-d_6, was demonstrated in Figure 1b. The peak observed at 5.88 ppm was attributed to aliphatic methylene protons, which exist in the anthracene core, and aromatic protons of **FS** were observed between 8.59-6.76 ppm. Also, the imino proton was observed at 8.90 ppm while -OH proton existed at 13.38 ppm as a singlet peak. The integration value ratio of aromatic protons to methylene protons was 4: 1, which is an evidence

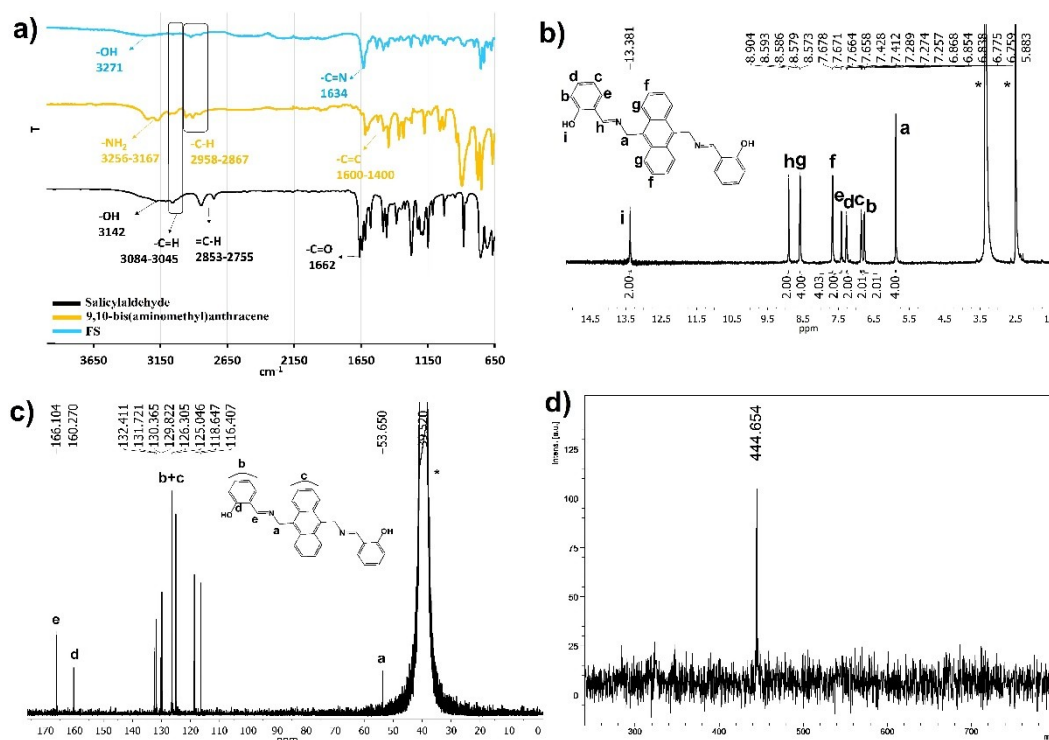of compatibility with the proposed chemical structure.

Figure 1: a) FT-IR spectra of 9,10-bis(aminomethyl)anthracene, salicylaldehyde, and **FS**, **b)** ^{1}H NMR (in DMSO-d$_6$), **c)** ^{13}C NMR (in DMSO-d$_6$) and **d)** MALDI-MS spectrum of **FS**.

Photophysical Investigation

The photophysical properties of **FS** were investigated using electronic absorption in the UV-Vis region and fluorescent experiments. The effect of various solvents on UV-Vis absorption of 10 µM **FS** was investigated with various solvents such as n-hexane, THF, dichloromethane (DCM), acetonitrile (ACN), ethanol, DMSO, DMF, and water (Figure 2a). The UV-vis electronic absorption of **FS** reached maxima at 258 and 378 nm, which can be assigned to π–π* transition of anthracene moieties (30). Also, UV-Vis electronic absorption properties of **FS** were not changed by solvents except for absorbance values, which are presumably dissolution differences in various solvents. The fluorescent sensor presented has high solubility in THF, dichloromethane, acetonitrile, DMSO, DMF, while it has low solubility in n-hexane, ethanol, and water only. UV–Vis electronic absorption properties of **FS** were examined at different concentrations in DMSO (Figure 2b). According to Figure 2b, absorbance values were proportionally decreased with decreasing concentrations of **FS** in UV–Vis electronic absorption spectra. The same situation was observed with other tested solvents, and molar absorptivities (ε) of compounds in various solvents were calculated. The fluorescent quantum yield (ΦF) and other photophysical properties were summarized at Table 1.
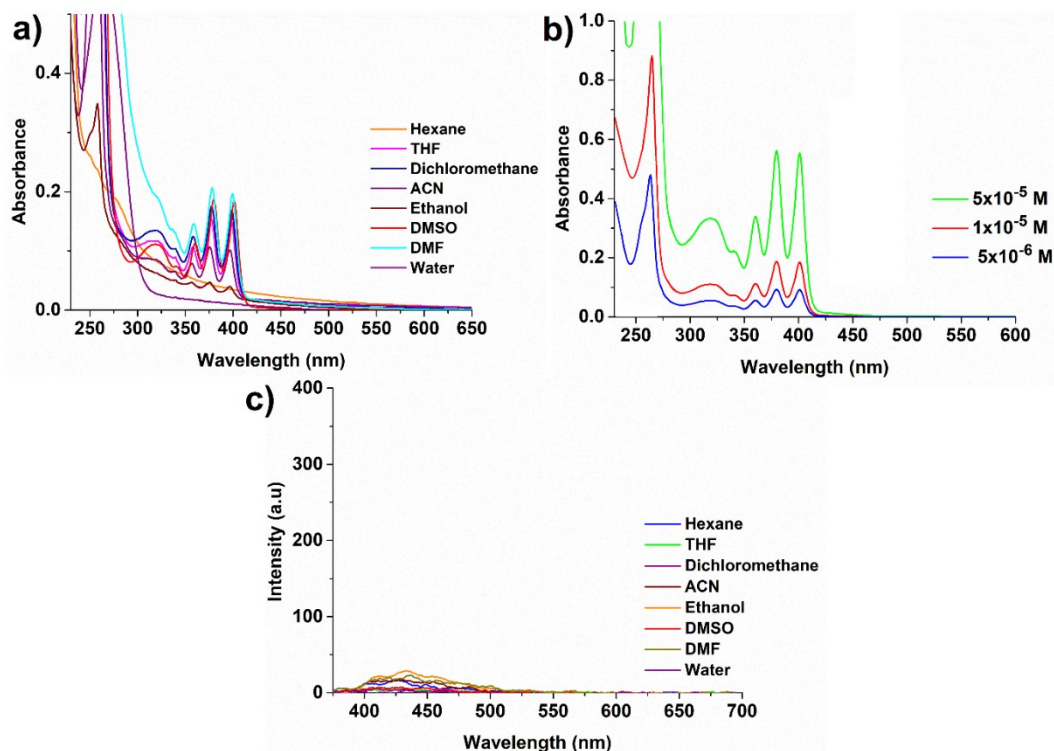
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Figure 2: a) UV-Vis electronic absorption of 10 µM **FS** in different solvents, **b)** UV-vis electronic absorption of different concentrations of **FS** in DMSO, and **c)** fluorescent emission spectra of 10 µM **FS** in different solvents.

After the evaluation of the electronic absorption properties of **FS**, fluorescent emission properties of **FS** were examined with steady-state fluorescent measurements under the same conditions with UV-Vis absorption studies (Figure 2b). All fluorescent emission spectra of **FS** were recorded at 10 µM when excited at 360 nm. The effect of various solvents on fluorescent emission of **FS** was investigated with the same solvent systems. In Figure 2b, the fluorescent sensor **FS** showed a very weak fluorescent signal at ~420 nm when excited at 360 nm. It is a well-known phenomenon for Schiff base due to photoinduced electron transfer (PET) process from Schiff base to fluorophore group of sensor (7, 23, 27, 29).

Table 1: Photophysical properties of **FS**.

	λ_{abs} (nm)	λ_{ems} (nm)	THF	DCM	ACN	Ethanol	DMSO	DMF	Φ_f (%)
			ε (L.mol⁻¹.cm⁻¹) x10³						
FS	258, 378	426 (very weak)	15.1	17.4	9.4	4.3	17.7	19.7	1.44

The fluorescent quantum yield (Φ_f) of **FS** was determined with the comparative method (Eq. (1)) (31). The quinine sulfate was used as a standard for the calculation of Φ_f, because Φ_f of quinine sulfate was calculated as 0.54 in the literature (in 0.1 M H_2SO_4) (32).

$$\emptyset\, F = \emptyset\, F_{Std} \frac{F . A_{Std} . n^2}{F_{Std} . A . n_{Std}^2}$$ **Eq. (1)**

In Eq. (1), F_{Std}, A_{Std}, F and A represent the areas of fluorescent emission and electronic absorption curves of standard and **FS**, respectively at the excitation wavelengths. Besides, n_{std} and n demonstrate the refractive indices of solvents used for the standard and **FS**, respectively.

Fluorescent Sensor Property of FS and Optimization Studies

Fluorescent sensor property and selectivity of 10 µM **FS** was investigated in DMSO:water (pH 8.0, 1:1 v/v) against 120 µM of various metal ions (as nitrate salts) such as Mn^{2+}, Al^{3+}, Cs^+, Ba^{2+}, Fe^{3+}, Ca^{2+}, K^+, Cu^{2+}, Pb^{2+}, Hg^{2+}, Zn^{2+}, Mg^{2+}, Na^+, Li^+,

Ni^{2+}, Ag^+, Fe^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+} by UV-Vis and fluorescent spectroscopies. The UV-Vis electronic absorption spectrum of **FS** significantly changes upon the addition of 120 µM Hg^{2+} ion when other metal ions did not cause any change under identical analytical conditions (Figure 3a). The absorption of **FS,** which at long wavelength centered at 378 nm, decreased (~50%) and other absorption, which centered at 258 nm red-shifted as 16 nm and observed at 274 nm. As a result of the electronic reorganization, an important color change was observed from yellow to colorless at daylight (Figure 3b). These changes in the UV-Vis absorption spectrum occurring by the addition of Hg^{2+} to **FS** can be assigned to the electronic reorganization after coordination of **FS** with Hg^{2+} (25, 33).

After the evaluation of UV-Vis response of **FS** towards various metal ions, fluorescent sensor properties of **FS** were investigated using fluorescent spectroscopy under identical analytical conditions with UV-Vis experiments (see Figure 3c); **FS** has a minimal fluorescent signal due to PET process from Schiff base to anthracene moiety when excited at 360 nm (34). After selective complexation of **FS** with the Hg^{2+} cation, the

fluorescent emission of **FS,** which originated from blue monomer emission of anthracene moiety, importantly increased at 426 nm. The change for fluorescent emission can be attributed to the chelation-enhanced fluorescence (CHEF) process, which occurred with selective complexation of **FS** with Hg^{2+} cation, and it inhibited the PET process (34). Importantly, except for Hg^{2+}, other tested metal ions did not demonstrate this effect and cause any "turn on" response on the fluorescent signal of **FS** (Figure 3c). As significant property of fluorescent sensors, selectivity of 10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v) over other competitive metal cations and anions was investigated with addition of Mn^{2+}, Al^{3+}, Cs^+, Ba^{2+}, Fe^{3+}, Ca^{2+}, K^+, Cu^{2+}, Pb^{2+}, Hg^{2+}, Zn^{2+}, Mg^{2+}, Na^+, Li^+, Ni^{2+}, Ag^+, Fe^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cl^-, F^-, NO_3^-, I^-, CO_3^{2-}, SO_4^{2-}. The selectivity studies were performed where concentration of other competitive species was 2400 µM (Figure 4). As seen in this figure, the effect of different competitive ions on relative fluorescent signal change of 10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v) were insignificant when compared with response of Hg^{2+} ion that clearly showed selectivity of **FS** against to Hg^{2+}.
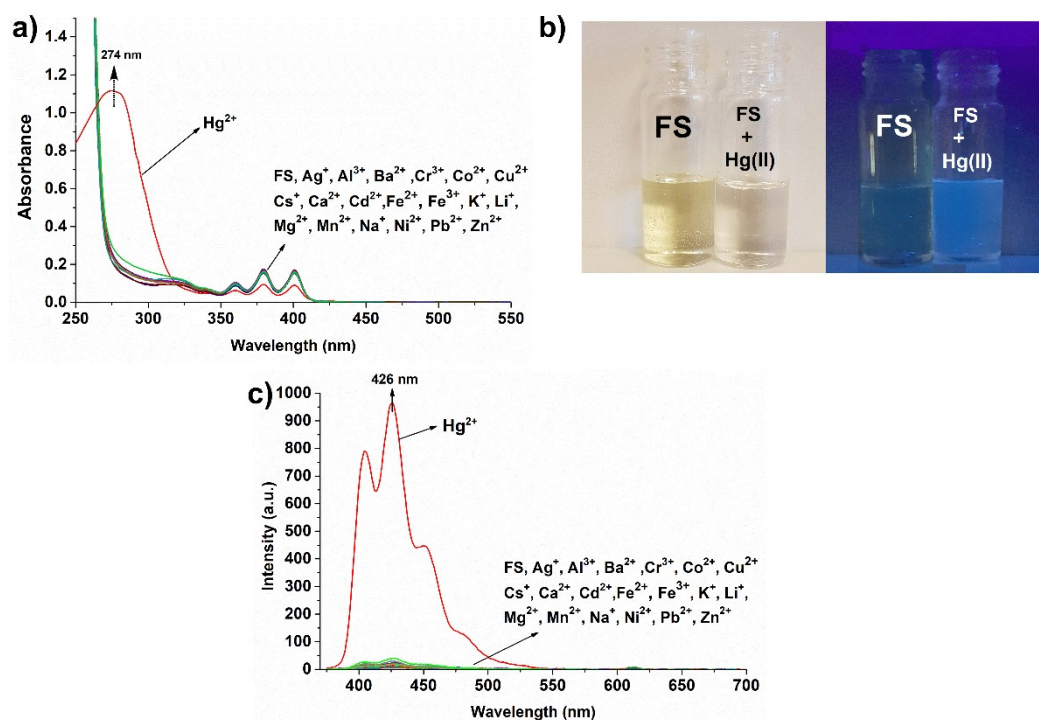

Figure 3: a) UV-vis electronic absorption, **b)** color change (daylight and 365 nm light source) and **c)** fluorescent emission spectra of 10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v) upon addition of 120 µM Hg^{2+}.
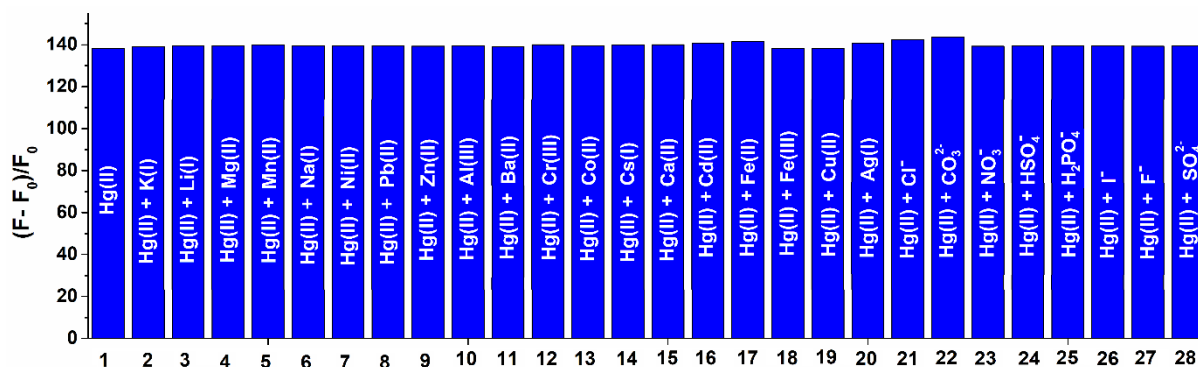
Figure 4: Relative fluorescent signal changes of 10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v, λ_{ex}=360 nm) upon addition of 120 µM Hg^{2+} + 2400 µM various metal ions.

The pH of analysis medium for fluorescent sensors which were designed for detection or determination of metal ions was important parameter, therefore, the effect of pH on relative fluorescent signal change of 10 µM **FS** in DMSO: water (1:1 v/v) with the addition of Hg^{2+} was investigated between pH 4 and 11 (29, 33) when other conditions kept steady. For that purpose, pH of 10 µM **FS** in DMSO: water (1:1 v/v) was adjusted by Britton-Robinson buffer (Figure 5a). As seen, the **FS**-Hg^{2+} complex is nearly pH-independent between pH 6.0–9.0 when considering the relative fluorescent signals. Therefore, the optimum pH was determined as 8.0 for the rest of the study, which demonstrated that **FS** could be used as a fluorescent sensor for spectrofluorimetric analysis of Hg^{2+} under neutral physical conditions (35). Then, the optimization of pH, effect of response time before the measurement was investigated for **FS** in DMSO: water (1:1 v/v) with the addition of Hg^{2+} ion when other parameters kept steady (Figure 5b). For that investigation, pH

of 10 µM **FS** DMSO:water (1:1 v/v) was adjusted 8.0 and 120 µM Hg^{2+} ion was added in this solution. Thereafter, obtained solution was carefully stirred up to 120 seconds and fluorescent spectra were recorded periodically. The results demonstrated that, after 60 seconds relative fluorescent signals remained nearly steady. Therefore, optimum time for measurement was determined as 60 second for determination of Hg^{2+}. To obtain accurate results in a spectrofluorimetric determination of a real sample using fluorescent sensors, the photostability of the sensor is highly essential. Accordingly, the photostability of **FS** and **FS**+Hg^{2+} was investigated under optimized conditions. For that purpose, 120 µM of Hg^{2+} was added to 10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v) then stirred vigorously for 60 seconds and fluorescent responses observed between 1-60 minutes under daylight. As can be seen from Figure 5c, both **FS** and Hg^{2+} complex of **FS** was immensely photostable.
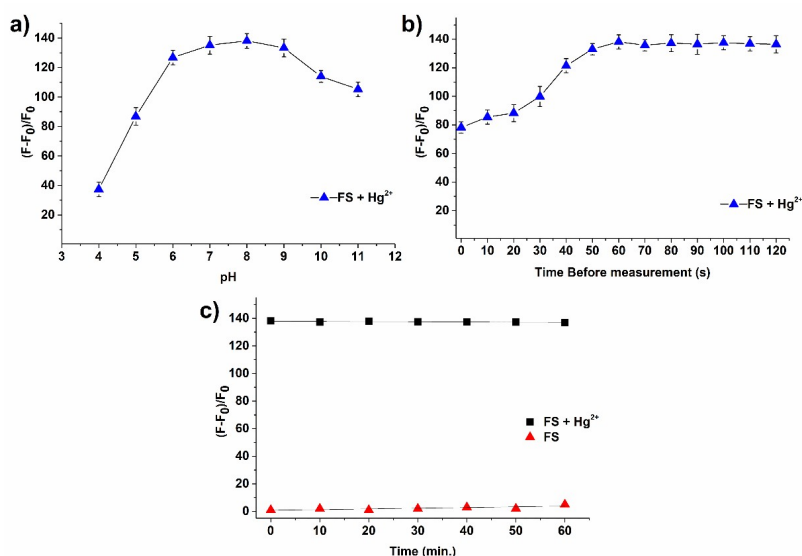

Figure 5: The effect of **a)** pH and **b)** measurement time on on relative fluorescent signal of **FS**+Hg^{2+} and **c)** photostability of **FS** and **FS**+Hg^{2+} (10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v, λ_{ex}=360 nm); 120 µM Hg^{2+}).

Complex formation of **FS** with Hg²⁺ ion was investigated by Job's plot (continuous variation) and non-linear curve fitting analysis in a buffered solution at pH 8.0 (36, 37). As can be seen from Figure 6a, maximum signal change was obtained at 0.5 in Job's plot, which revealed that stoichiometry of the **FS**-Hg²⁺ complex was 1:1. Besides, non-linear curve fitting analysis showed an inflection point at 1.0, which supports 1:1 binding mode (Figure 6b). With FT-IR analysis, we

obtained more information about the complexation of Hg²⁺ with **FS**, (Figure 6c). In the FT-IR spectrum of **FS**, -OH and C=N vibration peaks were observed at 3271 cm⁻¹ and 1634 cm⁻¹, respectively. After treatment with Hg²⁺, -OH vibration peak of **FS** disappeared, and the C=N vibration peak shifted to 1610 cm⁻¹ proving that Hg²⁺ and **FS** had coordination via O-Hg²⁺-N bond. According to the obtained results, the proposed binding mechanism for **FS**-Hg²⁺ was given in Figure 6d.

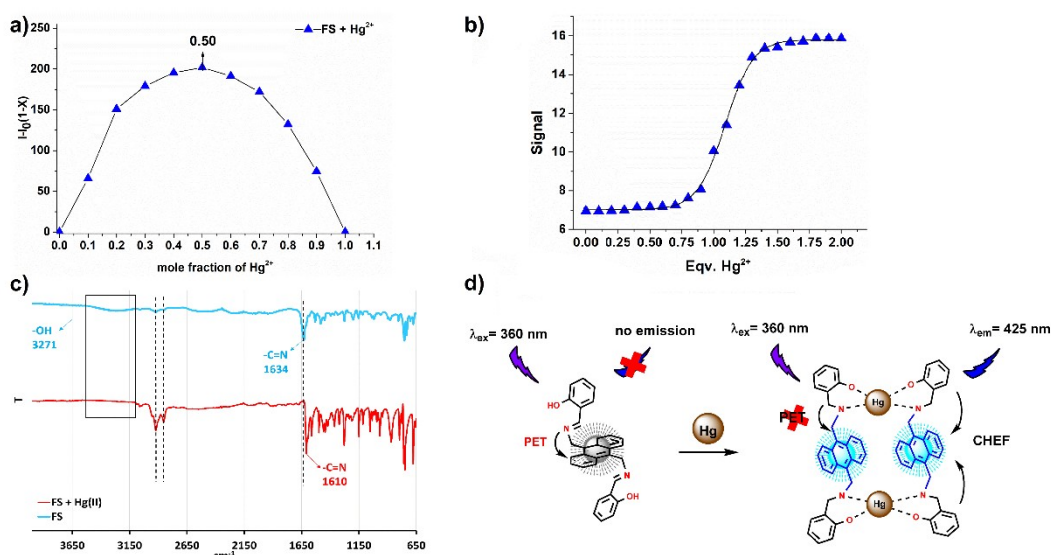**Figure 6: a)** Job's Plot, **b)** non-linear curve fitting, **c)** FT-IR spectral analysis of **FS**+Hg²⁺ and **d)** proposed binding mode for **FS**+Hg²⁺ (10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v, λₑₓ=360 nm).

Analytical Performance

The fluorescent response of **FS** towards to Hg²⁺ was also evaluated by fluorescent titration experiments under the optimized conditions (Figure 7a). As shown in Figure 7a, after gradual addition increased amount of Hg²⁺ to 10 µM **FS** in DMSO: water (pH 8.0, 1:1 v/v), fluorescent emission signal of **FS** at 426 nm was gradually and proportionally increased between 0.05-120.00 µM

Hg²⁺ which indicates the formation of **FS**+Hg²⁺ complex. The calibration curve of **FS** for Hg²⁺ was given in Figure 7b, which demonstrated a good linear relationship (R^2=0.9938) between the relative fluorescent signal change and Hg²⁺ concentration between 0.05-120.00 µM. Also, linear regression equations for Hg²⁺ was determined as (F-F₀)/F₀=1.1839[Hg²⁺]-8.3709.
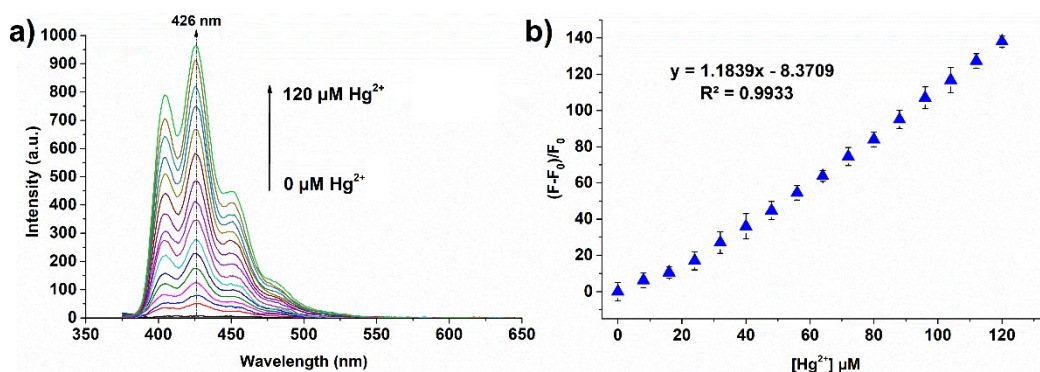

Figure 7: a) Fluorescent emission response of 10 µM **FS** in DMSO:water (pH 8.0, 1:1 v/v, λₑₓ=360 nm) upon gradually addition of Hg²⁺ ion and **b)** calibration curve for Hg²⁺ ion.

The LOD, limit of detection, which calculated as dividing three times of the standard deviation of

the blank sample by the slope of the calibration graph (3σ/K) determined as 0.082 µM for Hg²⁺,

whereas the limit of quantification, LOQ, calculated as 0.246 µM for Hg^{2+}. Under optimized experimental conditions, the precision of the developed spectrofluorimetric determination method (N=10) was calculated as 2.58%, which assigned high precision and stability. All analytical parameters of **FS** for Hg^{2+} ion determination were given in Table 2. To evaluate the applicability of presented fluorescent sensor for spectrofluorimetric analysis for Hg^{2+} in environmental water samples, **FS** was applied to seawater, lake, and tap water samples without any enhancement processes and obtained results given at Table 3. As seen in Table 3, obtained results for the spectrofluorimetric analysis of Hg^{2+} demonstrated high percentage recovery in environmental water samples and in a good agreement with to the spiked concentrations.

Table 2: Analytical parameters of **FS** for Hg^{2+} ion determination.

Excitation wavelength (nm)	360
Emission wavelength (nm)	426
Limit of detection (LOD) (µM)	0.082
Limit of quantification (LOQ) (µM)	0.245
Linear range (µM)	0.05-120
Optimum pH	8.0
Ligand concentration (µM)	2
Final volume (mL)	5
Solvent	DMSO:water (1:1 v/v)
Time before measurement (second)	60
Correlation coefficient (R2)	0.9938
Precision (RSD%)	2.58

Table 3: Spectrofluorimetric determination of Hg^{2+} in environmental water samples using **FS**.

Water sample	Spiked concentration of Hg^{2+} ions (µM)	Found (µM)	Recovery (%)
Seawater	20.00	20.59±0.52	102.95
	40.00	40.32±0.96	100.80
Lake water	20.00	20.29±0.35	101.45
	40.00	39.52±0.84	98.80
Tap water	20.00	19.20±0.28	96.00
	40.00	38.92±1.01	97.30

CONCLUSION

In summary, simple, efficient, and accessible anthracene-based dipodal Schiff base "turn-on" fluorescent sensor for Hg^{2+} ion was designed and synthesized via high yield Schiff base reaction of salicylaldehyde and 9,10-bis(aminomethyl)anthracene. The characterization of **FS** was performed by MALDI-MS, FT-IR, ^{1}H and ^{13}C NMR spectral methods. The photophysical and fluorescent sensor properties of **FS** were investigated by UV-Vis and fluorescent spectroscopies in different solvents. The presented fluorescent sensor demonstrated excellent selectivity and sensitivity towards Hg^{2+} over different competitive ions with "turn-on" response. The PET-triggered weak fluorescent response of **FS** was inhibited by coordination of Hg^{2+} *via* CHEF processes, and importantly "turn-on" fluorescent response was obtained at 426 nm. The binding mechanism of **FS** with Hg^{2+} was examined by electronic absorption and fluorescent spectroscopies. The LOD and LOQ were found to be 0.082 µM and 0.246 µM for Hg^{2+}, respectively. Finally, after the optimization of experimental conditions, spectrofluorimetric analysis of Hg^{2+} ions was carried out in environmental water samples. According to obtained results, it can be said that **FS** is a selective and sensitive "turn-on" fluorescent sensor candidate for Hg^{2+} detection in aqueous media.

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RESEARCH ARTICLE

Radionuclide Potential of Holocene Sediments in the West of Marmara Sea (Turkey)

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Abstract: Radionuclides that cause radioactive pollution descend to the bottom in marine and water environments such as heavy metals and accumulate in bottom sediments. It is useful to determine the radionuclides in these environments to control the radionuclide release and its damage. Radioactive pollution can harm people's life directly or through the food chain. In this study, natural and artificial radionuclide values were measured in Recent sediment samples taken from the seabed in the western part of the Marmara Sea. Gamma spectrometry method was used in radionuclide examinations. In gamma spectrometry studies of sediments, values of radionuclides (^{40}K, ^{137}Cs, ^{226}Ra, ^{54}Mn, ^{95}Z, and ^{232}Th) were determined. Sea depths where 18 analyzed seafloor sediments are taken vary between 15-50 m. The determined radionuclide concentration activity values of the study area are ^{137}Cs (0.9 - 9.4 (Bq / kg)), ^{232}Th (18.9 - 86 (Bq / kg)), ^{226}Ra (10 - 50 (Bq / kg)), ^{40}K (24.4 - 670 (Bq / kg)), ^{54}Mn (0.71-0.9 (Bq / kg)) and ^{95}Zr (0.18 - 0.19 (Bq / kg)). These values were correlated with the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR). The ^{226}Ra series, ^{232}Th series, and 40K radionuclides accumulate naturally, and their concentrations increase gradually due to anthropogenic impurities. ^{226}Ra values obtained across the study areas are within normal limits according to UNSCEAR values. ^{40}K and ^{232}Th values were higher than UNSCEAR values in all locations. ^{137}Cs (0.9 - 9.4 (Bq / kg)) from almost all locations reveals a risky situation in terms of ambient conditions because this element cannot be found in the natural environment and can be found artificially as an end of radioactivity.

INTRODUCTION

Humanity has lived with radiation continuously from the beginning of our existence. With the formation of the Earth, very long-lived (order of billion years) radioactive elements, inherently a part of nature, have created a natural and inevitably accepted natural radiation level in the environment (1).

Natural and anthropogenic radioactivity is related to the primary sources of radioactive composition encountered in marine, terrestrial, and atmospheric environments.

Natural airborne radiation is caused either by radioactive gases from various cracks in the earth (especially from radon gas) or by cosmic rays. The radioactivity in the soil is due to uranium, thorium, and other radioactive substances included in the

decomposition series of those radioisotopes. Radiation in water is due to the interaction of the radioactive sources in both the air and the soil with water (2).

Natural radionuclide concentrations are continually increasing in terrestrial and mainly marine environments. The reason for that is the rapid increase in the use of artificial fertilizers, fossil fuels, detergents, and pesticides and also the proliferation of phosphate processing plants (3). The most critical radioactive substances found in natural waters are uranium, thorium, radium, radon, strontium, potassium, carbon, and hydrogen (4). Radionuclides that cause artificial radioactivity are spread to the environment as a result of radiological activities, radioactive waste heaps, or nuclear accidents. Although those activities create a variety of different radionuclides, the ones that play an essential role in the radiation exposure of humans are the products of diffusion and activation (^{137}Cs and others). Those radionuclides may lead to internal and external radiation damage in the human body. Therefore, the radioactivity levels in environmental samples must be measured with an acceptable error so that the exposure dose can be accurately assessed (5).

Nuclear weapons trials that began in 1945 and continue to date, along with the Chernobyl (1986) and Fukushima (2011) nuclear reactor accidents, have resulted in high levels of artificial radionuclides spreading to the atmosphere.

Among radionuclides spreading to the environment since 1945 are the long-half-life radionuclides, including ^{14}C, ^{137}Cs, and ^{90}Sr, which are important for environmental and human health. Those radionuclides accumulate in the marine environment and the living body, damaging both living spaces and living things (6).

In Turkey, Aközcan (2012) (7) studied the distribution of natural radionuclide concentrations in sediment samples in Didim and Izmir Bay. In his study, the activity concentrations of ^{238}U, ^{226}Ra, ^{232}Th, and ^{40}K in sediments collected from two different locations in the Aegean Sea have been defined. The highest activity concentrations of ^{238}U and ^{232}Th were observed in sediments from the Gulf of Izmir. The highest activity concentrations of ^{40}K in sediments were measured from Didim (7). Aytaş et al. (2012)(8) evaluated thirty sediment samples from different distances along the Maritza and Tundja River. These samples were analyzed by gamma spectroscopy for ^{238}U, ^{232}Th, and ^{40}K.

The measured average activity concentrations of ^{238}U and ^{232}Th are above the world average, but the average activity concentration of ^{40}K is within the world average value (8).

Natural and artificial radioactivity assessment of dam lakes sediments in the Çoruh River (Turkey) was studied by Kobya et al. (2015) (9). In the study, ^{238}U, ^{232}Th, ^{40}K, and ^{137}Cs activity concentrations were determined. The mean concentrations of ^{238}U, ^{232}Th, ^{40}K, and ^{137}Cs were found to be 15.8, 13.9, 551.5 and 18.1 Bq/kg respectively in Deriner Dam Lake (zone 1); 3.7, 12.5, 473.8 and 6.8 Bq/kg in Borçka Dam Lake (zone 2); 14.4, 30.0, 491.7 and 18.2 Bq/kg in Muratlı Dam Lake (zone 3) was determined. The fact that the activity concentration values of radionuclide are maximum in the Deriner Dam Lake, where the sediment deposition is at its maximum level (9).

This study aims to determine natural and artificial radionuclide distribution in both intense industrialization and non-industrialized areas of the West of Marmara Sea by using gamma spectrometry and bring out the risky areas for human life.

MATERIALS AND METHOD

The study area covers the area between Silivri (Istanbul) and Tekirdağ-Gelibolu (Çanakkale) in the north and between Lapseki (Çanakkale) and Bandırma (Balıkesir) in the south in the Western Marmara Sea (Figure 1).

The bathymetry of the sea from which the samples were taken ranges from 20 m to 35 m. In the Marmara Sea, the Anatolian (Southern Marmara) and Thrace (Northern Marmara) shores show different characteristics. Thrace shores, which meet the sea with large plateaus, feature gulfs (Tekirdağ and Silivri Gulfs) with wide arcs and beaches with small coves that have turned into lagoons. On the other hand, the Anatolian coast features the gulfs (Izmit, Gemlik, Bandirma, and Erdek) extending in the east-west and south-north directions and the islands (Kızıladalar, İmralı, and Marmara Islands) located in front of the rugged coasts (10). Core samples taken from 29 locations (sediment from the seafloor) were used in the study (Figure 2). The samples were taken between 15.10.2016 and 20.10.2016, and the coordinates and depth information of the samples are given in Table 1.
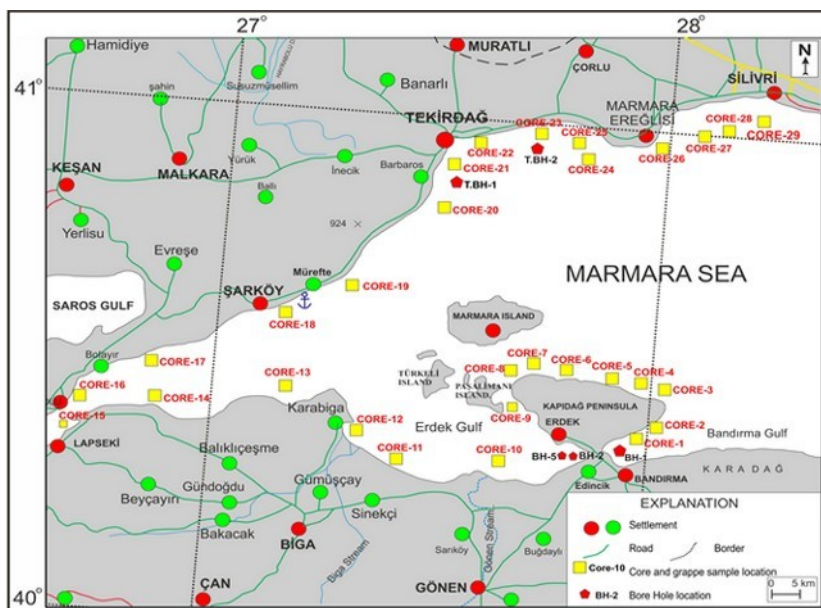
Figure 1. Location map of sample and study area.

Radionuclide analyses of the samples were made at the Çekmece Nuclear Research and Training Center. Samples were first dried at room temperature and prepared for analysis by sieving with 100-micron mechanical sieve. The samples were kept in the dry state for forty days were provided balance in radium, thorium, and other decomposition products. Concentration values of the radionuclides were analyzed using a Canberra GX5020 device.

The gamma activity peaks were: ^{238}U series, 609.3 keV; ^{232}Th series, 583 keV; ^{228}Ac, 911.2 keV; ^{226}Ra, 185.7 keV; ^{137}Cs, 661.7 keV; and ^{40}K, 1460.8 keV . In the gamma spectrometry analysis method, gamma ray-emitting radionuclides that irradiate at the energy range of 40 keV to 2000 keV are usually measured directly, without being separated from the sample matrix (such as air, water, soil, sediment, plant material, vegetable, and animal food.). Therefore, it is one of the most widely-used methods in the environmental analysis (5).

RESULTS

The range of radionuclide activity concentrations in the sediment samples, in Bq/kg, was 0.9–9.4 for ^{137}Cs; 18.9–86 for ^{232}Th; 10–50 for ^{226}Ra, and 24.4–670 for ^{40}K. Tables 2 and 3 present core sample radionuclide activities. The values were interpreted in comparison with the international average values as given in Table 4 (11).

In all locations, the analyses for ^{40}K were obtained in the core samples, the ^{40}K levels were above the world limits, whereas the ^{232}Th levels were below the limit in eleven locations and above world standard in eighteen locations. The values of ^{226}Ra were above the limit value at only one location (K-10, 50 Bq/kg). It is noteworthy that the ^{232}Th values (86–186 Bq/kg) reach the highest level in the Core-10 sample. One of the radioactive isotopes of cesium, ^{137}Cs, which is an alkali metal with similar chemical properties to potassium, reaches the highest value (9.4 Bq/kg) at the Core-20 location. That isotope has a considerably long physical half-life of 30.17 years; therefore, it is considered to be biologically hazardous for the ecosystem (12). The distributions of artificial and natural radionuclides at sea may differ from region to region, depending on various factors. The number of radionuclides deposited in marine environments vary with the physical and chemical properties of the radionuclide, dry or wet accumulation pattern, and topographic and meteorological environmental conditions (13).

Therefore, numerical results obtained from sediment samples show large variations. Figures 2, 3, 4, and 5 show the distribution of radionuclides for all sample locations in the west of the Marmara Sea. Figure 2 shows the map of ^{40}K, and radionuclides have been produced with the kriging method. Similarly, Figure 3 shows ^{226}Ra and Figure 4 shows ^{232}Th and Figure 5 shows ^{137}Cs radionuclides**.**

Table 1. Geographic coordinates of core samples.

CORE SAMPLE NUMBER	GPS-NO	DEPTH (M)	GEOGRAPHIC POSITION (WGS-84)		CORE SAMPLE NUMBER	GPS-NO	DEPTH (M)	GEOGRAPHIC POSITION (WGS-84)	
			East	North				East	North
CORE 1	116	30	058 14 59	447 46 77	Core 14a/b	142	29	049 02 82	447 33 47
CORE 2	117	29	058 49 85	447 66 92	Core 15	143	36	047 39 14	446 99 71
CORE 3	119	30	058 81 05	448 16 39	Core 16	144	43	047 29 08	447 24 87
CORE 3	120	35	058 62 29	448 27 80	Core 17	145	26	048 80 99	448 10 25
CORE 4A	121	30	058 23 93	448 35 51	Core 18	146	41	051 10 08	449 48 09
CORE 4B	123	32	058 22 60	448 39 37	Core 19	147	30	540197	4522435
CORE 4C	124	35	057 98 35	448 54 62	Core 20	148	33	542591	4530386
CORE 5	125	38	057 74 01	448 53 35	Core 21	149	32	546815	4533986
CORE 6A	126	40	056 68 40	448 63 34	Core 22	150	30	560139	4536953
CORE 7	127	48	056 21 01	448 68 10	Core 23	151	32	569767	4535792
CORE 8	128	39.5	055 81 97	448 48 62	Core 24	152	33	580368	4533824
CORE 9A	129	28	055 82 82	448 01 29	Core 25	153	33	588549	4541739
CORE 9B	137	32	055 83 61	447 97 91	Core 26	154	32	596782	4544493
CORE 10	138	19	055 62 81	446 36 87	Core 27	155	30	603222	4545684
CORE 11A/B	139	26	054 24 36	446 46 34	Core 28	156	31	540197	4522435
CORE 12	140	18	052 87 96	447 06 48	Core 29	157	35	542591	4530386
CORE 13	141	46	051 44 52	447 87 84					

Table 2. Results of gamma spectrometric analyses of core samples.

Sample	Radionuclide	Activity ± Bq/kg	Sample	Radionuclide	Activity ± Bq/kg
CORE-1	^{40}K	490	CORE-10	^{137}Cs	3.5
	^{137}Cs	2.5		^{226}Ra	50
	^{226}Ra	30		^{232}Th	86
	^{232}Th	42		^{155}Eu	3.2
CORE-2	^{137}Cs	1.9	CORE-11	^{40}K	670
	^{226}Ra	19.8		^{232}Th	57
	^{232}Th	27.1		^{226}Ra	34
CORE-3	^{226}Ra	25		^{137}Cs	3.8
	^{232}Th	28		^{54}Mn	1.1
CORE-4	^{40}K	24.4	CORE-12	^{232}Th	49
	^{137}Cs	1.4		^{226}Ra	28.2
	^{232}Th	21.6		^{137}Cs	4.9
	^{54}Mn	0.71	CORE-13	^{232}Th	45
CORE-5	^{40}K	470		^{226}Ra	25.7
	^{137}Cs	1.8		^{137}Cs	2.2
	^{226}Ra	21.5		$^{95+}$Zr	0.27
	^{232}Th	30	CORE-14	^{40}K	470
CORE-6	^{40}K	450		^{232}Th	36
	^{137}Cs	1.3		^{226}Ra	25.2
	^{226}Ra	27		^{137}Cs	0.90
	^{232}Th	26	CORE-15	^{137}Cs	3.2
CORE-7	^{137}Cs	1.1		^{226}Ra	19.5
	^{226}Ra	24		^{232}Th	36
	^{232}Th	39	CORE-16	^{40}K	600
	$^{95+}$Zr	0.2		^{137}Cs	4.7
CORE-8	^{137}Cs	1.1		^{226}Ra	24.9
	^{226}Ra	18.6		^{232}Th	37
	^{232}Th	30	CORE-17	^{137}Cs	1.6
CORE-9	^{40}K	470		^{226}Ra	18.9
	^{137}Cs	0.9		^{232}Th	26
	^{226}Ra	19			
	^{232}Th	33			

Table 3. Results of gamma spectrometric analyses of core samples (continuation of Table 2)

Sample	Radionuclide	Activity ± Bq/kg	Sample	Radionuclide	Activity ± Bq/kg
CORE-18	^{137}Cs	4.8	CORE-24	^{226}Ra	10
	^{226}Ra	29		^{232}Th	21
CORE-19	^{232}Th	36	CORE-25	^{40}K	550
	^{40}K	650		^{137}Cs	1
	^{137}Cs	2.3		^{226}Ra	22
	^{226}Ra	27.9		^{232}Th	38
CORE-20	^{232}Th	39		$^{95+}$Zr	0.19
	^{40}K	630	CORE-26	^{40}K	480
	^{137}Cs	9.4		^{232}Th	24
	^{226}Ra	20.5		^{226}Ra	19.2
	^{232}Th	35		^{137}Cs	0.8
CORE-21	^{137}Cs	3.7	CORE-27	^{137}Cs	1.3
	^{226}Ra	20.6		^{226}Ra	16.1
	^{232}Th	30		^{232}Th	21.7
CORE-22	^{137}Cs	5.1	CORE-28	^{40}K	580
	^{226}Ra	27		^{137}Cs	1
	^{232}Th	20.7		^{226}Ra	18.1
CORE-23	^{137}Cs	1.7		^{232}Th	24.8
	226Ra	13.7	CORE-29	^{226}Ra	20.9
	^{232}Th	18.9		^{232}Th	31

Table 4. International average values of radionuclides (11)

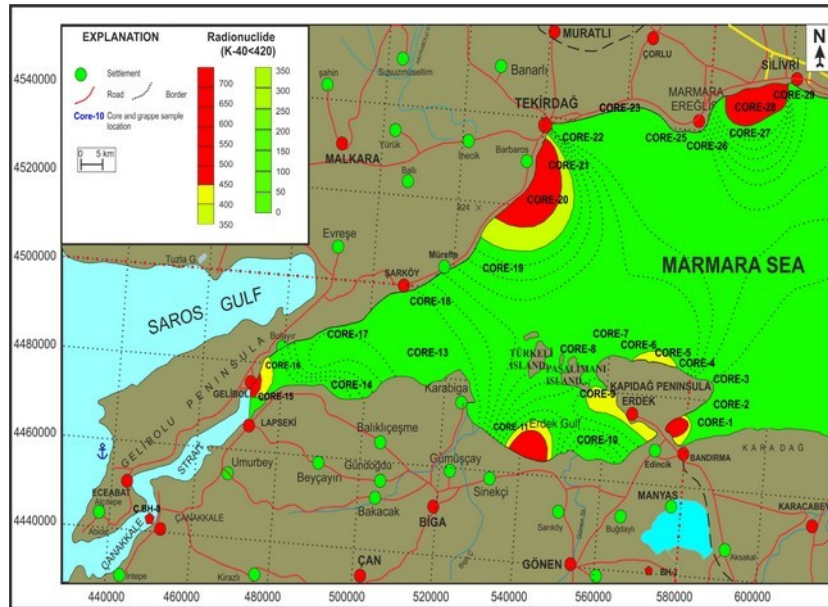| Radionuclides	International Average Limit Values
^{232}Th	30 Bq/kg
^{226}Ra	35 Bq/kg
^{40}K	400 Bq/kg

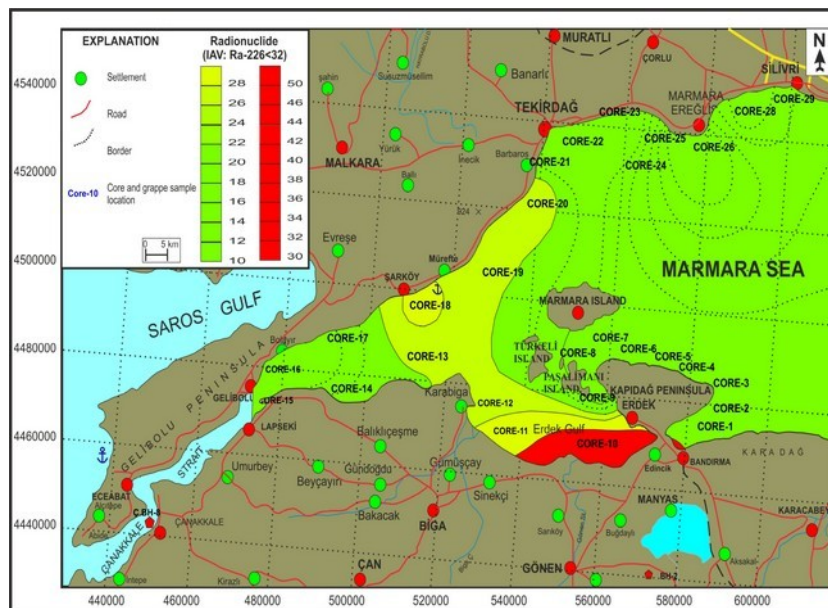**Figure 2.** Distribution of the activity concentrations of ^{40}K (Bq/kg)

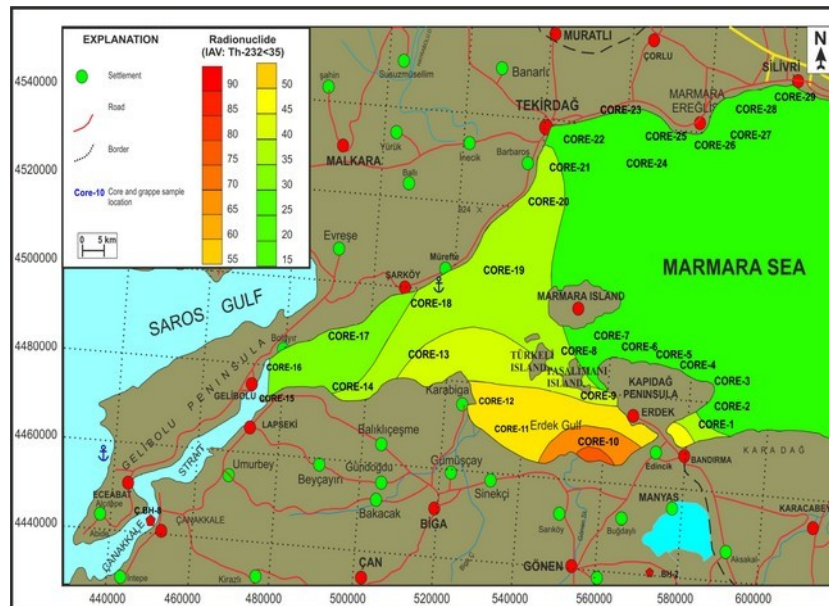**Figure 3.** Distribution of the activity concentrations of ^{226}Ra (Bq/kg).

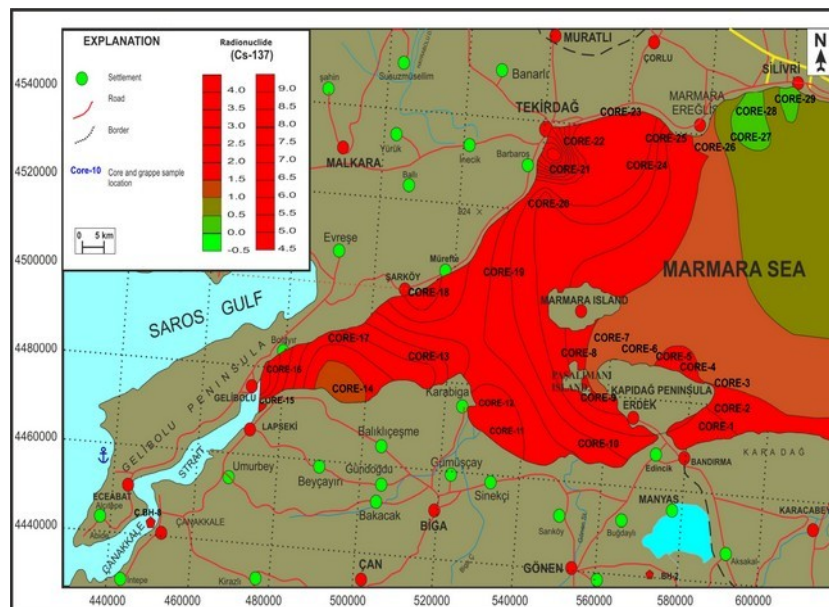**Figure 4.** Distribution of the activity concentrations of ^{232}Th (Bq/kg).

Figure 5. Distribution of the activity concentrations of ^{137}Cs (Bq/kg).

DISCUSSION AND CONCLUSION

In this study, core samples were collected from 29 different locations in the Marmara Sea for radioactivity analyses for ^{40}K, ^{232}Th, ^{137}Cs, ^{226}Ra, ^{54}Mn, and $^{95+}$Zr on the western coast of the Sea of Marmara. The Gamma Spectrometric Analysis method was used to determine the radioactivity properties of the sediments. The radionuclide concentration activity values in the sediment samples obtained from the locations, in Bq/kg, were ^{137}Cs, 0.9–9.4; ^{232}Th, 18.9–86; ^{226}Ra, 10–50; ^{40}K, 24.4–670; ^{54}Mn, 0.71–0.9; and ^{95}Zr, 0.18–0.19. These values were compared with data from the Turkish Atomic Energy Agency (TAEK) and the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), and environmental analysis was also carried out. As a result of the analyses ^{40}K values were found to be zero in Core-2, Core-3, Core-4, Core-7, Core-8, Core-10, Core-12, Core-13, Core-15, Core-17, Core-18, Core-21, Core-23, Core-24, Core-27, and Core-29. The ^{137}Cs value was zero in Core-3 and Core-24, whereas it was determined to be 9.4 (Bq/kg) in Core-20. In the Core-11 sample taken from the Sea of Marmara (between Gönen Creek and Karabiga), the ^{40}K value was higher than those of other locations and higher than the average world value. On the contrary, ^{226}Ra values measured in all the core samples were below the limit values.

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Simple and sensitive detection of quercetin antioxidant by TEOS coated magnetic Fe₂O₃ core-shell

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Abstract: In this study, α-Fe₂O₃ and tetraethyl orthosilicate (TEOS) coated Fe₂O₃ (TEOS@Fe₂O₃) was synthesized by the sol-gel method. The morphological properties and electrochemical detection of quercetin antioxidant with Fe₂O₃ and TEOS@Fe₂O₃ nanomaterials were evaluated. TEOS@Fe₂O₃ material was modified on a glassy carbon electrode (GCE) for the detection of quercetin with a linear range of 1.0-9.0 µM by square wave voltammetry (SWV). TEOS@Fe₂O₃/GCE sensor *ca* 1.6 and 2.5 times more sensitive than Fe₂O₃/GCE and GCE. The quantification (LOQ) and detection (LOD) limits were found to be 306 and 92 nM for quercetin antioxidant on the TEOS@Fe₂O₃ modified electrode. Quercetin was also determined in an apple tea sample with a standard addition method, and the recovery of quercetin was obtained to be 104.16% and 106.04%. The results obtained from this study show that the TEOS@Fe₂O₃ modified electrode examined as a voltammetric sensor was found to be sensitive and straightforward to quercetin.

INTRODUCTION

Magnetite nanoparticles have attracted attention due to potential application in numerous biomedical applications and special physicochemical features, such as contrast agents in magnetic resonance imaging, carriers for targeted drug delivery, the magnetic separation in microbiology, biochemical sensing (1-3). Iron oxides nanoparticles have found extensive interest recently, and these materials often show unique magnetic, physical, catalytic, and optical features (4-6). These non-toxic and cheap nanomaterials are potential applications in high technology and used for enhanced the properties of storage media (7), in drug delivery, biosensors (8), and catalysts (9). The size and magnetic anisotropy of the particles are related to magnetization values of iron oxide nanostructures (10). Fe₂O₃ nanoparticles are exceptionally eco-friendly, rich in magnetite nanoparticles, economical, and highly resistant to corrosion, and they were also found to

be stable at room temperature (11). Therefore, Fe₂O₃ nanoparticles are applied to catalysts and a variety of soft and hard magnetic materials, and they have good biocompatibility (12, 13).

Recent studies have been shown that there is increasing interest in preventing diseases such as obesity, cardiovascular, inflammatory processes, cancer, and neurodegenerative disorders (14). These disorders can accelerate as people grow older, and their effects may be decreased by the intake of vegetables and fruits (15, 16). A diet containing Vitamin A and polyphenolic compounds, consideration of active nutrients in the prevention of disease because of oxidative stress, may decrease the effect (17). Moreover, the determination of antioxidants has increased to significant importance to protect from these disorders (18, 19). Flavonoids are naturally found in fresh vegetables and fruits, and they generally used as a component of health products and food additives (20). They show anti-

inflammatory and antioxidant properties concerning radical scavenging features of the compounds. Quercetin is a member of the flavonoid family, found in apples, onions, sweet pepper, grapes, celery, and honeysuckle. The antioxidant properties of quercetin have been extensively explored in the past studies (21, 22) due to other biological advantages such as anti-tumor, anti-viral, anti-allergic activities, reducing blood lipids and blood pressure (16, 23, 24). For this reason, developing sensitive, fast and straightforward determination methods of quercetin is vitally important.

Quercetin determination was carried out by traditional methods such as ultra-performance liquid chromatography (25), spectrofluorimetry (26, 27), and mass spectrometry (28), which are highly selective and sensitive. However, they are time-consuming, expensive, and complicated sample preparation. Voltammetric techniques are potentially applied for the determination of quercetin because of the electrochemical activity to overcome these difficulties (29). Therefore, Fe_2O_3 was coated TEOS to obtain $TEOS@Fe_2O_3$, which is a spherical material for the determination of quercetin via the electrochemical method for the first time. Due to higher surface area, well-designed material shape, and electroactive metal content, $TEOS@Fe_2O_3$ was tested in quercetin determination via signal amplification.

Herein, a magnetic nanoparticle of $TEOS@Fe_2O_3$ has been synthesized and successfully immobilized onto a GCE using an electrochemical sensor. The electrode showed impressive electrocatalytic action for the oxidation of quercetin, which is characterized by the enhancement of the highest current in both cyclic and square wave voltammetry techniques. After optimization, the electrode was used for quantification of quercetin in apple tea sample extracts. Experiments indicated that the $TEOS@Fe_2O_3$ based sensor was shown excellent electrocatalytic activity to quercetin.

EXPERIMENTAL

**Materials and Characterization Techniques**

$Fe(NO_3)_3.9H_2O$ (98%), citric acid (98%) and quercetin (99%) were supplied from Sigma Aldrich, and stock solution of quercetin (concentration of 1 mM) was prepared daily. 0.1 M Britton-Robinson (BR) buffer solution, which was used as an electrolyte and prepared by boric, orthophosphoric, and acetic acids in pure water and kept at 4.0 °C. pH value of buffer solutions was adjusted by 5 M NaOH with a pH meter (Mettler Toledo, Switzerland). Voltammetric studies were carried by an electrochemical analyzer system of CH Instruments (CHI440B model, USA) at 25 °C. A three-electrode cell consisting of a counter, reference, and working electrodes are platinum wire, Ag/AgCl, and $TEOS@Fe_2O_3$/GCE, respectively.

Surface morphological properties of Fe_2O_3 and $TEOS@Fe_2O_3$ materials were performed by scanning (SEM) and transmission electron microscopic methods (TEM). The morphology of the synthesized hybrids was studied using an FEI (PHILIPS) XL30 SFEG scanning electron microscope. TEM images were obtained using an FEI TALOS F200S TEM instrument (accelerating voltage of 200 kV).

Preparation of Fe_2O_3 and $TEOS@Fe_2O_3$

Hematite (α-Fe_2O_3) nanoparticles were prepared according to a simple method given in the literature with a slight modification (30, 31). Briefly, 10 mmol $Fe(NO_3)_3.9H_2O$ and 12.5 mmol citric acid was dissolved in 60 mL of pure water and mixed ultrasonically for about 20 minutes. Then, 10 mL of ethanol was added, and the pH was adjusted to 5 with ammonia. The solution was taken into a beaker and boiled until obtaining a gel. It was transferred into an oven at 95 °C for drying. The dried gel was purged with pure N_2 gas for 30 minutes and placed to a tube furnace annealed to 400 °C for 3 hours. The final product was 1.5 g of α-Fe_2O_3. The prepared α-Fe_2O_3 particles were coated with silica using a sol-gel method (32). In a typical procedure, 100 mg of Fe_2O_3 was dispersed in absolute ethanol solution by ultrasonic bath, and 750 μL of NH_4OH and 400 μL of TEOS have added the suspension (Scheme 1, inset). The morphological analysis of samples was characterized by SEM and TEM.
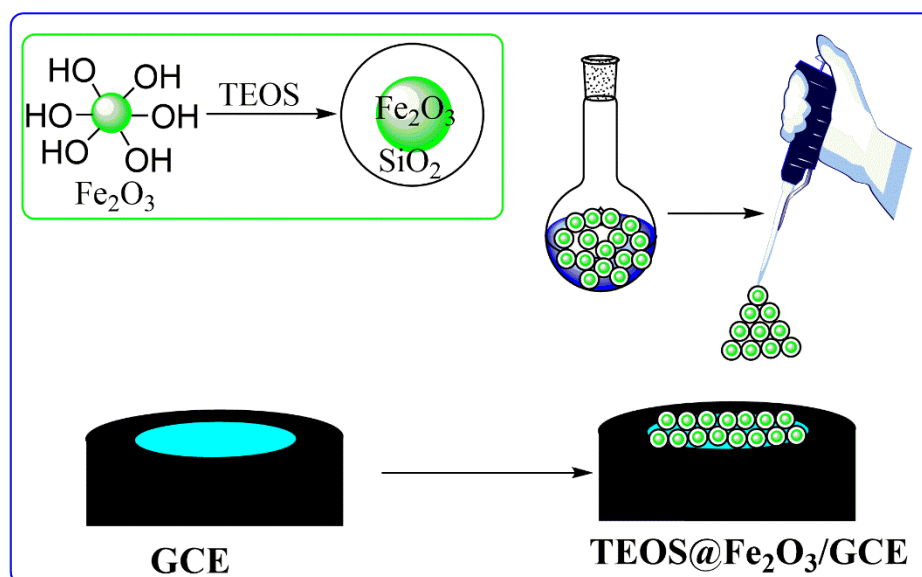
Scheme 1. Schematic representation of the synthesis of TEOS@Fe$_2$O$_3$ material and fabrication of the sensor.

Preparation of the TEOS@Fe$_2$O$_3$ modified GCE

Scheme 1 depicts the fabrication procedure of the sensor based on the investigated TEOS@Fe$_2$O$_3$. The mirror-like clean surface of GCE was obtained with different size alumina powders ranging between 1.0 to 0.05 µm. Then GCE was cleaned after each polishing with ultra-pure water and ultrasonicated in acetone, HNO$_3$ solution (1:1, v/v), and double-distilled water, respectively. The cleaned GCE was then dried at room temperature until use. The TEOS@Fe$_2$O$_3$ modified GCE was obtained by drop-casting method. 2 mg of TEOS@Fe$_2$O$_3$ was placed into 1.0 mL ultra-pure water and stirred in an ultrasonic bath for 15 min to obtain well-dispersed 2 mg/mL suspension. An amount of 10 µL brownish-red suspension was transferred onto the cleaned surface of the electrode and dried at 40 ℃. The obtained sensor electrode was taken as TEOS@Fe$_2$O$_3$/GCE.

Real samples assay procedure

Apple tea sample was purchased from a local market and weighed as 0.5 g and dissolved in ethanol (10.0 mL). The sample solution was well dispersed by an ultrasonic bath for 20 min. The supernatant of the dispersion was separated by centrifugation for 4 min at 3000 rpm. The sample solution was used to determination of quercetin without any further treatment. Electrochemical determination of quercetin was performed in the solution by the SWV technique from 0.0 to 1.0 V. All real sample detection measurements were applied three times at room temperature, and average values were taken to calculate the amount of quercetin antioxidants.

RESULTS AND DISCUSSION

Morphological Characterization of Fe$_2$O$_3$ and TEOS@Fe$_2$O$_3$

Morphological characterization of Fe$_2$O$_3$ and TEOS@Fe$_2$O$_3$ were investigated by scanning SEM TEM (Figure 2). Figure 2a demonstrates a typical SEM image of the α-Fe$_2$O$_3$ samples. As seen in this figure, pores structure can be monitored with an average size of 50-60 nm. SEM images also clearly showed the nanostructural homogeneities and remarkably unique neck-structured morphology, which could be the advantage of the penetration of antioxidant molecules. Figure 1b depicts the SEM image of TEOS@Fe$_2$O$_3$ particles. A large number of iron particles are monitored on the Si substrate with diameters in the range of 250-400 nm. Figure 2c and 2d show TEM images of the α-Fe$_2$O$_3$ and TEOS@Fe$_2$O$_3$ particles. Although Fe$_2$O$_3$ nanoparticles can be obtained as the shaping of many rough surfaces and different sizes, TEOS@Fe$_2$O$_3$ particles have almost uniform shapes. The spherical TEOS@Fe$_2$O$_3$ particle sizes were also confirmed by TEM. Besides, elemental analysis of the spherical structures, EDX analysis (installed in SEM) was carried out. The EDX analysis of the α-Fe$_2$O$_3$ particles represents the atomic percentage (%) of the O, Fe is 2:1, and the percentage of Fe, O, and Si in TEOS@Fe$_2$O$_3$ are 2:5:3.

Electrochemical Sensor Examination Tests of TEOS@Fe$_2$O$_3$ Material to Quercetin

The cleaned GCE was coated by 10 µL drop of the TEOS@Fe$_2$O$_3$ suspension (2 mg/mL) in ultra-pure water to provide the TEOS@Fe$_2$O$_3$/GCE sensor. The electrochemical sensor properties of TEOS@Fe$_2$O$_3$/GCE towards 6 µM quercetin were investigated by SWV methods with bare GCE, Fe$_2$O$_3$/GCE in a BR buffer solution (pH = 2) comparatively.

TEOS@Fe_2O_3/GCE sensor *ca* 2.5 and 1.6 times more sensitive than pristine GCE and Fe_2O_3/GCE, respectively (Figures 1a and 1b).
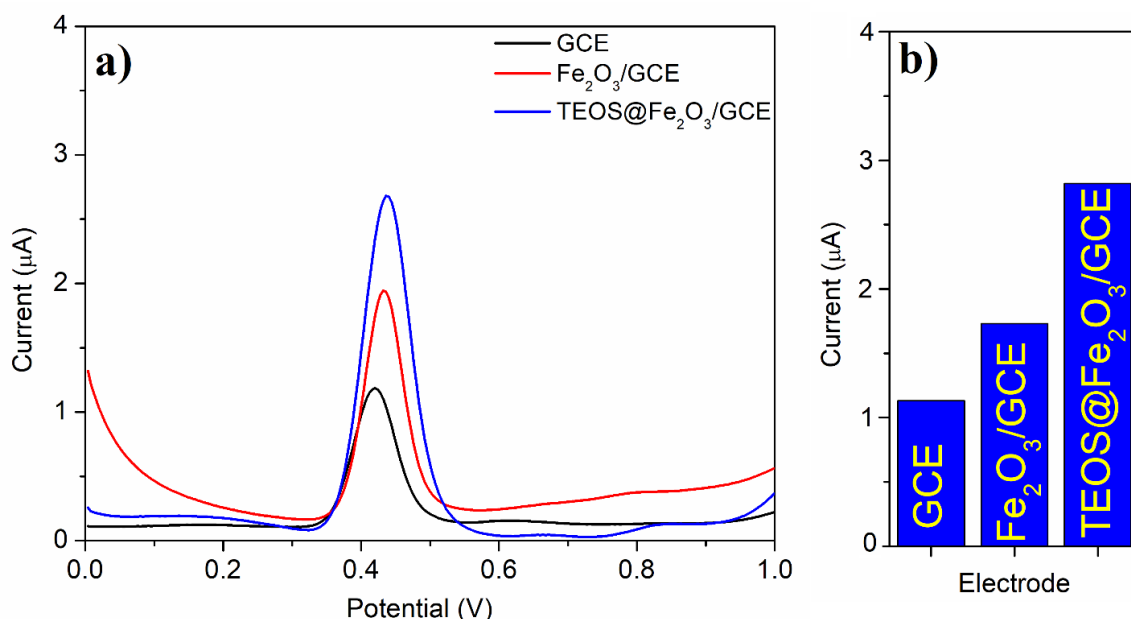

Figure 1. a) SWV response to 6 µM quercetin (pH=2) TEOS@Fe_2O_3/GCE in comparison with bare GCE and Fe_2O_3/GCE, b) their column graph.
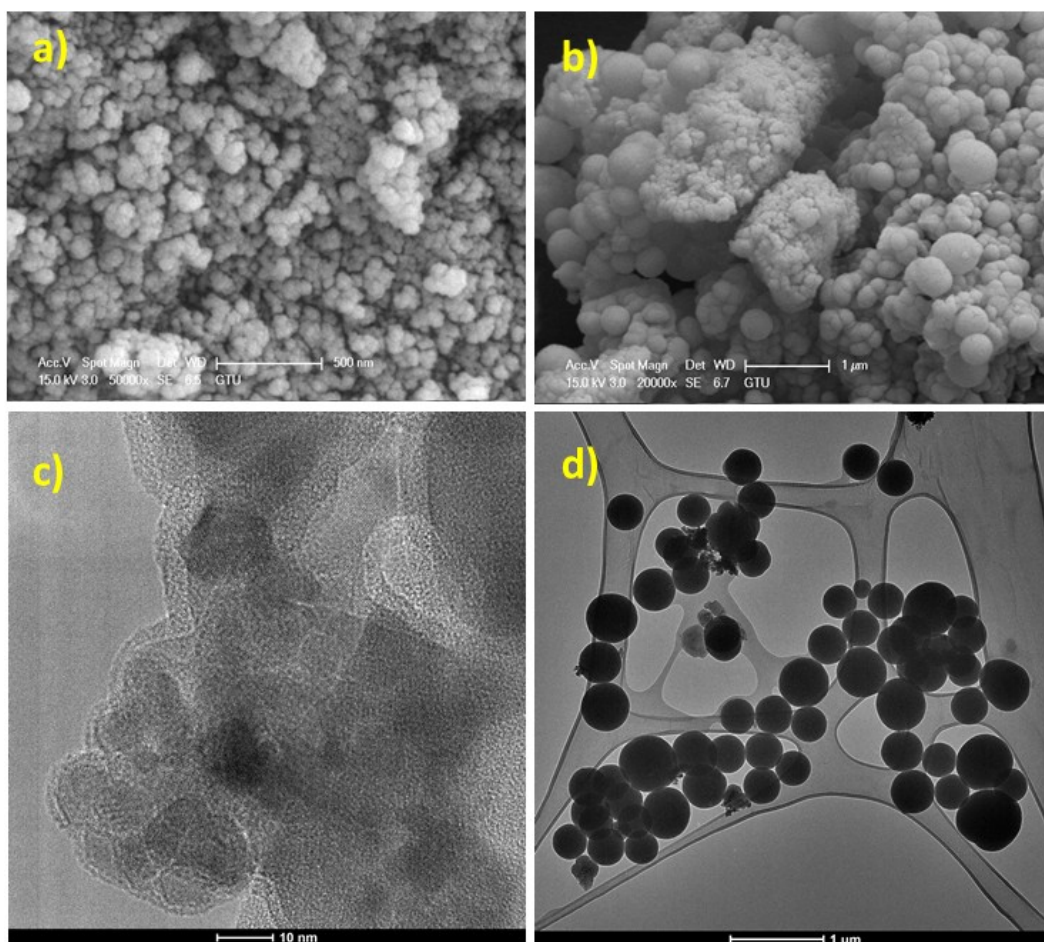

Figure 2. a) and b) SEM images, c) and d) TEM of images of Fe_2O_3 and TEOS@Fe_2O_3

The investigation of pH impact on voltammetric sensor features was also evaluated in BR buffer solution, and adjustments of pH were performed by NaOH solution (5.0 M). Figure 3a depicts the SWV responses of quercetin in the BR solutions with different pH values (2-7). The optimal pH value is found to be 2 because of better and higher peak current. When the acidic pH value comes out, the interaction between –OH functional groups in quercetin and electron-deficient boron atoms of in the BR are increased (33). Thus, it can be said that the reaction between the quercetin and the TEOS@Fe_2O_3 is a pH-dependent reaction.

Moreover, higher hydrophobic interaction and hydrogen bond formation occurs between spherical TEOS@Fe_2O_3 nanomaterial. Also, mass transfer of quercetin via diffusion happened very hard because

the at basic and neutral pH can limit the surface of the electrode and causes a decrease of the peak current. Due to the inhibition of quercetin antioxidant transfer by the absorption with OH functional groups, peak current values decreased suddenly. Quercetin's oxidation peak currents are almost invisible at high pH, along with a decrease of interaction between the buffer solution and OH of the quercetin (34). Therefore, the relationship between peak current and pH values (range of 2 to 7) was shown in **Figure 3b,** which demonstrates a linear decrease with a slope of 60.80 mV/pH. This value is very close to 59 mV/pH (Nernstian behavior) (29, 35). This voltammetric response has the same numbers of proton and electron (y=-0.0608x+0.5896, R^2 = 0.9961) and in harmony with the electro-oxidation mechanism of quercetin (29).
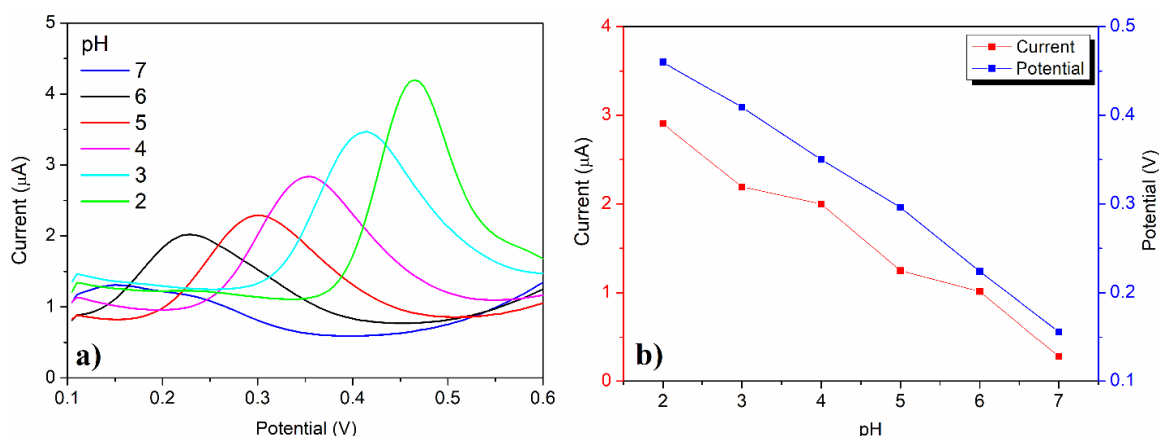

Figure 3. a) SWV responses at different pH values in the range of 2-7 and b) changes in pH with peak currents and peak potentials.

Not only electrokinetic behavior, both also analytical features performed by SWV and CV techniques. Figure 4a demonstrates the SWV responses obtaining by different concentrations of quercetin on the TEOS@Fe_2O_3/GCE at the supporting electrolyte as a pH 2.0 BR buffer solution. As seen, this figure

with a concentration of quercetin ranging from 1.0 to 9.0 µM was linearly increasing of peak current (Figure 4b). The LOQ and LOD of quercetin antioxidant were calculated as 306 and 92 nM (y =0.4632x-0.0391, R^2 = 0.9956) respectively.
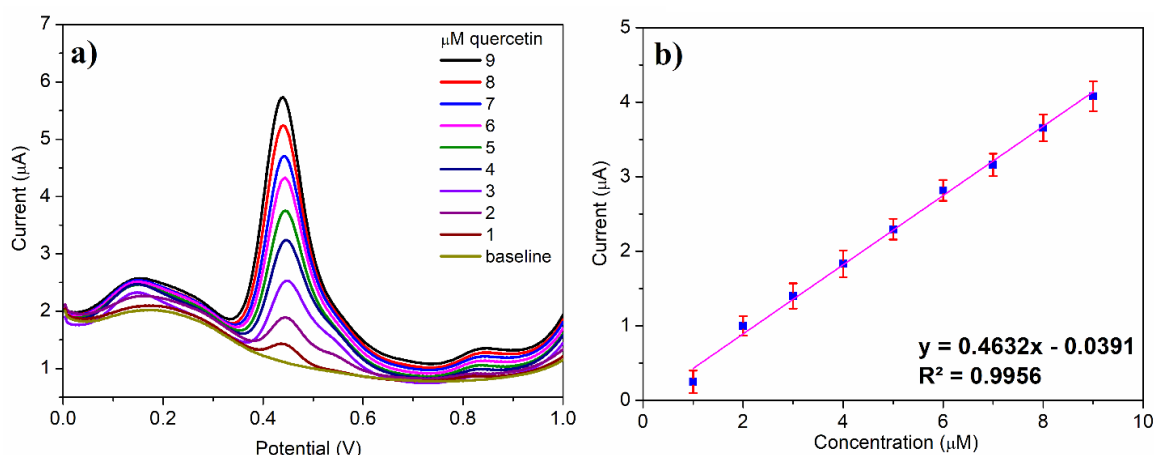

Figure 4. a) SWV responses at increasing concentrations of quercetin on the TEOS@Fe_2O_3 modified GCE and b) calibration graphs corresponding to (a).

CV measurements were carried out to determine the impact on the redox reaction mechanism of quercetin by the scan for gaining further information about the relationship between the scan rate and peak current. Figure 5a demonstrates the electrochemical measurements containing 6 µM quercetin, which was performed at the several scan rates (in the ranging from 25 to 500 mV/s) in the buffer (pH=2.0). As seen in Figure 5b, the peak current was increased linearly with an increase of the scan rate (R^2 values were found to be 0.9979 and 0.9989 for oxidation and reduction). This linear correlation was pointed out that the electro-oxidation process of quercetin is controlled by adsorption in agreement with the previous works (36). Also, the quercetin has reversible electrode processes because it exhibited two peaks, which are an anodic peak at 490 mV and a cathodic peak at 430 mV.
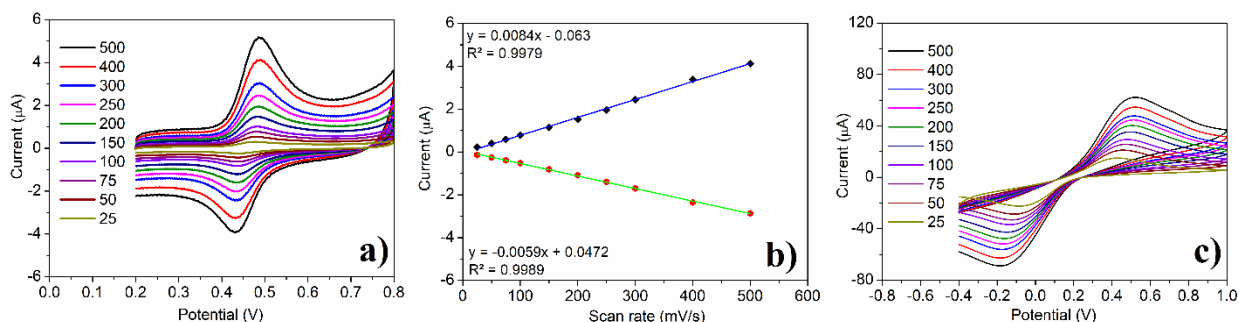
Figure 5. a) CV responses of 6 µM quercetin at various scan rates, b) Plot of Ipa and Ipc and c) CV of TEOS@Fe_2O_3 /GCE in a solution containing $K_3[Fe(CN)_6]$ and KCl at different scan rates in the ranging from 25 to 500 mV/s.

CV at different scan rates was performed to estimate the specific surface area of TEOS@Fe_2O_3 modified GCE in a solution containing $K_3[Fe(CN)_6]$ and KCl by Randles-Sevcik equation (Figure 5c). The concentrations of KCl and $K_3[Fe(CN)_6]$ were 0.1 M and 5.0 mM. where A, D_0, and C_0 are the specific surface area (cm^2) of the TEOS@Fe_2O_3 modified GCE, the concentration of $K_3[Fe(CN)_6]$ (5.0 mM) and diffusion coefficient ($7.6x10^{-6}$ cm^2/s). The activated surface area of TEOS@Fe_2O_3-GCE was estimated as 0.103 cm^2 from the Randles-Sevcik equation by calculating the slope of Ip vs $u^{1/2}$ plot. This surface area was more remarkable than the unmodified GCE (0.071 cm^2).

Repeatability, Stability, Selectivity, and Comparison Of TEOS@Fe_2O_3/GCE Sensor
Anions and cations such as SO_4^{2-}, NO_3^-, K^+, Na^+, Mg^{2+}, Ca^{2+}, Fe^{3+}, Ni^{2+} were examined to show the selectivity of the TEOS@Fe_2O_3 modified GCE sensor at 25 ℃. Selectivity experiments were carried out by SWV method using 10 mM of anions and metal ions stock solutions in ultra-pure water. Besides, another interfering study was done in the presence of adenine purine base and ascorbic acid, and glucose solutions at concentration of 1 mM in water. Anions, metal ions and other interfering molecules had no significant effect (< 4%) on the electrochemical response on 6 µM quercetin (Figure 6 a and b).
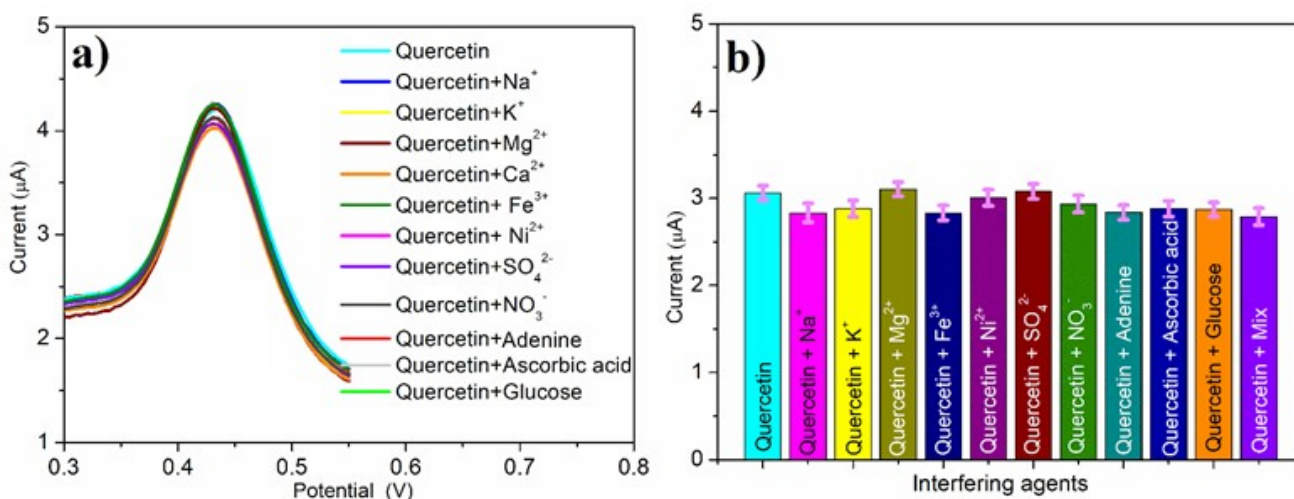

Figure 6. a) DPV curves in the presence of anions, cations, and bioanalytes and **b)** their column graph.

SWV technique was performed in the stability experiment of the sensor every weekday for the determination of quercetin. The electrochemical responses measured in a pH of 2 on TEOS@Fe$_2$O$_3$ modified GCE at 25 °C, which did not demonstrate any significant changes in comparison with the beginning response. RSD (Relative Standard Deviation) of the peak current values was found to be 4.1%. Moreover, the repeatability test was done by 7 successive SWV of 6.0 μM quercetin on TEOS@Fe$_2$O$_3$/GCE, and the RSD was calculated to be 3.5%. It can be concluded that the results from these measurements TEOS@Fe$_2$O$_3$/GCE sensor showed high repeatability and stability for the determination of quercetin.

Table 1. Comparison of present electrochemical sensors with other reported methods for the determination of quercetin in various samples.

Methods	Working Electrode	LOD (nM)	Sample	References
SWV	TEOS@Fe$_2$O$_3$/GCE	92	Apple Tea	**This work**
SWV	Co nanoparticle/GCE	100	Tablet and urine	(37)
SWV	NiO-CNT/CPE	30	Onion, apple and capsule	(38)
DPV	MIP-GO/GCE	48	Apple juice	(39)
DPV	Carbon@Fe nanoparticle/GCE.	150	Human urine	(40)

MIP-GO: Molecularly imprinted polymer incorporated graphene oxide, CNT: Carbon nanotube, CPE: Carbon paste electrode.

TEOS@Fe$_2$O$_3$/GCE sensor system was compared with the other techniques data on the determination of quercetin given in Table 1. The obtained outcomes in this study demonstrated that TEOS coated Fe$_2$O$_3$ sensor system had excellent properties for the selective and sensitive determination of quercetin. The suggested TEOS@Fe$_2$O$_3$/GCE had some benefits in terms of LOD and linear range. In most quercetin determination studies, electrode modifications were done by carbon nanotube, graphene oxide, and metal nanoparticles. As known, carbon nanotubes have high toxicity, and graphene is expensive and challenging to synthesize. This sensor system has more sensitivity with a high surface area, but their stabilities are very low.

Moreover, some other metal nanoparticles containing studies were available in the literature among them. The TEOS@Fe$_2$O$_3$/GCE was suggested in this study. TEOS@Fe$_2$O$_3$ modified GCE demonstrated very high stability to evaluate quercetin in the apple tea sample. Moreover, the TEOS@Fe$_2$O$_3$ modified GCE was easy to prepare, cheaper, and fast electron transfer between quercetin and its surface compared to the electrodes.

Sample Analysis
The applicability of the TEOS@Fe$_2$O$_3$ modified GCE sensor in a commercially available apple tea sample was performed *via* standard addition method, as reported in (41). Tea sample was weighted as 0.5 g and thrown in a 10 mL ethanol. For better dissolution, the suspension was kept in an ultrasonic bath for an hour and centrifuged with 3000 rpm speed. Then the supernatant of the sample suspension was separated from the suspension, and the solution was used to determine quercetin without any treatment by SWV technique from 0.0 to 1.0 V. 100 μL of the apple tea sample solution was added in a voltammetric cell to determine the amount of quercetin, and 1.0 μM of quercetin was added to the cell. The addition process of quercetin was repeated two times to 100 μL sample containing BR buffer solution. Average data and RSD (<5%) were estimated through successive measurements (**Table 2**). Moreover, the recovery of the measurements on the detection of quercetin was calculated as 104.16% and 106.04%. According to the real sample measurements, the suggested the TEOS@Fe$_2$O$_3$ modified GCE is fast, accurate, and efficient for the determination of quercetin.

CONCLUSION

In this study, TEOS-coated Fe2O3 to obtain spherical nanomaterials. A voltammetric determination of low concentrations of quercetin was performed using TEOS@Fe2O3 material modified GCE. The selectivity of TEOS@Fe2O3 modified GCE sensor to quercetin was examined in the presence of different anions, cations, and bio-analytes at the optimum operating conditions. The suggested protocol shows the successful application of TEOS@Fe2O3 modified GCE for the determination of quercetin in real samples with excellent selectivity and sensitivity due to its high synergistic effect of metal nanoparticles and TEOS. Also, reproducibility and stability of the sensor are excellent.

Table 2. Examples of quercetin detection by SWV on TEOS@Fe_2O_3 modified GCE in apple tea sample.

Addition (µM)	Measured (µM)	Recovery (%)	RSD (%, n=3)
0.00	1.18±0.04	-	-
1.00	2.22±0.04	104.16	4.19
2.00	3.30±0.05	106.04	4.07

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RESEARCH ARTICLE

Effect of Calcium Stearate on the Thermal Conductivity of Geopolymer Foam

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Abstract: Geopolymers are considered an alternative to conventional cement recently. The use of fly ash and blast furnace slag in geopolymer, which are waste products considered as an environmentally friendly product due to the solution to the storage of wastes also. Geopolymer concrete production is also reported to be 44-64% less than the cement that causes the most CO_2 emissions. CO_2 emissions are reduced due to the minimum processed natural minerals and industrial waste products used in the geopolymer system. For this reason, this study comes to the fore in terms of the evaluation of wastes. Production of porous geopolymers is potential in use in many industrial applications such as filtering, thermal insulation, light structural material, and catalysis. By controlling the pore type, pore size distribution, pore connectivity, and shape of porosities, potential usages are differentiated. In this study, closed porosity geopolymer foams were produced by the geopolymerization technique with the help of hydrogen peroxide and calcium stearate (CaS) as a surfactant. The thermal conductivity, density, and strength values was correlated with the changing pore size distribution depending on the amount of surfactant and foaming agent. In this study, porous geopolymers with density values 450-500 kg/m^3, 0.069 W/mK thermal conductivity, and 2.1 MPa strength value was reached. The reduction in pore sizes due to CaS increase was analyzed. However, we did not observe a decrease in thermal conductivity values due to the reduction of the pore size. Exciting results for CaS content on thermal conductivity were reported.

INTRODUCTION

An incredible acceleration is seen in the construction sector in recent years, and the necessity of the technically superior parameters and values of the construction materials to be used pave the way for the use and application of many new construction materials. Significantly, the sustainability concept that arises due to the rapid consumption of fossil energy resources, air pollution caused by greenhouse gas, the effort to

minimize the amount of energy use, and the lack of proper use of the produced materials accelerates the research and development of these materials (1–3).

Today, in parallel to the increasing environmental awareness, national and international environmental policies are becoming more stringent. Kyoto Protocol has been signed by many countries, and studies have been started to reduce CO_2 emissions. The World Business Council for

Sustainable Development says it is responsible for the cement sector for about 5% of CO_2 emission. It is an indication that the cement sector has a significant impact on the increase in CO_2 emissions. (4). The concrete and cement sector has been criticized for not only high emissions of carbon dioxide but also consuming large amounts of natural resources and energy mainly from cement production. These justified criticisms are tried to be overcome by alternative solutions. With the addition of fly ash, industrial waste in cement-based systems (such as plaster, mortar, concrete) can be minimized, and carbon dioxide emission can be minimized, and the need for natural resources is reduced (5–12).

In our country, resources such as petroleum, coal, and natural gas are used for energy production. As presented in TÜIK 2015 and 2016 reports, coal use for energy production is expected to increase gradually by 2040 (13,14). It means that by-products such as fly ash and bottom ash formed as a result of the combustion of coal will also increase gradually. While Japan recycled 96% of fly ash in the context of cyclic economy and England, India, America, Australia recycles about 50% of the fly ash; this ratio remains 15% in Turkey (14). One of the other objectives of this study is the fact that the fly ash, which is generated as a waste of millions of tons per year, can be converted into added-value products.

Secondly, energy efficiency in buildings is always an important issue. Demand for energy, which is indispensable for human life, is increasing rapidly with population growth, industrialization, and urbanization. Meeting the increasing energy consumption in the world where there is limited energy supply has been a problem for many countries, and the dependence on foreign energy has increased (15). In Turkey, a large part of the energy consumption consists of housing under the name of city consumption. Heat insulation is one of the most critical measures that can be taken to ensure energy efficiency in houses.

Thermal insulation materials used in buildings are the most effective solution in reducing heat losses. In this way, it contributes to the almost zero energy target by reducing the heat energy requirement in buildings. It is necessary to produce a foam geopolymer with homogeneously distributed and closed porosity to provide thermal isolation (16,17).

Many articles on porous geopolymer production have been published. In the direct foaming method, which is the most commonly used method, foam materials are produced by adding gas-forming additives to a suspension or liquid medium. Foaming is usually done by adding H_2O_2,

Al powder, metallic Si, and sodium perborate as a foaming agent to the geopolymeric mixture and mixing them mechanically. After the decomposition of additives in an alkaline slurry, a bubble forms and a porous structure is formed (7,18–21). Besides, the amount of porosity, which depends on the amount of foaming agent used, also depends on whether the foams can remain stable in the mixture. Additives are needed to ensure that thermodynamically unstable gas bubbles are kept in the mixture and are homogeneously distributed. These additives are referred to as surfactants. Many studies have been conducted on butyric acid, valeric acid, butyl gallate, propyl gallate, hexylamine, oleic acid, and albumin as surfactants in foam geopolymer production (22–31). However, there are only limited numbers of publications on CaS, and its effect on the thermal conductivity properties. Cui et al. reached a minimum thermal conductivity value of 0.096W / mK from their work, depending on the amount of CaS. They observed that although samples containing a fixed amount of stearate reached a minimum value of 0.048W/mK by changing the water/binder amount, this negatively affected the mechanical properties (24,32).

This study was carried out on the development of porous geopolymer wall elements for insulation with the help of CaS as a surfactant. This study gains importance in CO_2 emission, cyclic economy, and energy fields thanks to the reduction of cement use, recycling of fly ash, and the development of thermally insulated wall elements. In this study, the effect of CaS on thermal conductivity is examined from a different perspective. CaS, which reduces the pore size, negatively affected the thermal insulation feature.

MATERIALS AND METHODS

The mixed composition of foam geopolymer includes fly ash, metakaolin, sodium hydroxide, sodium silicate, expanded perlite, water, and chopped polypropylene fiber (Table 1). Hydrogen peroxide was used as a foaming agent (%35), and CaS was used to obtain foam stabilization. Fly ash (FA) was taken from the Seyitömer Thermal Power Station, Turkey, and metakaolin was supplied from the Czech Republic. Chemical compositions of metakaolin and fly ash are presented in Table 2.

The principle of homogeneous mixing of solid components and liquid components in separate places was followed. First, the NaOH flakes were completely dissolved in water to obtain a 10M environment and then mixed with sodium silicate solution. Geopolymeric slurry was added by adding an alkaline solution to the mixture of metakaolin, fly ash, exploded perlite, and polypropylene fibers mixed separately. After the geopolymer slurry

became homogeneous, foaming agent and surfactant were added and mixed. Finally, a foam mixture was poured into 100x100x100 mm molds and cured at 60°C.

The compressive strength tests were conducted with a compressive testing machine on the samples whose dimensions were 100 × 100 × 100 mm. Compressive strength test was applied.

Table 1. Composition of foam geopolymer mixtures.

Code	Fly Ash(%)	Metakaolin (%)	CaS* (%)	Perlite (%)	Fiber (%)
1				0	
2	90	10	0.15	2	0.21
2				4	
4				8	
5				0	
6	90	10	0.45	2	0.21
7				4	
8				8	
9				0	
10	90	10	0.75	2	0.21
11				4	
12				8	

Table 2. Chemical content of Fly ash and Metakaolin.

	Fly Ash	Metakaolin
SiO_2	50.30	54.10
Al_2O_3	19.10	41.10
CaO	4.55	0.13
Fe_2O_3	12.40	1.10
MgO	4.67	0.18
K_2O	2.16	0.80

After drying, the unmolded samples were stored in the laboratory conditions for 28 days. The thermal conductivity coefficient λ was calculated with a modified transient plane source. The bulk density of the product was calculated as the mass to volume ratio of the sample.

RESULTS AND DISCUSSION

Density and Porosity
According to scanning electron microscopy analysis, samples have closed porosity, pore morphology is pretty homogeneous, and pore dimensions are getting smaller following the surfactant amount (Figure 1). Measurements were taken from perlite-free samples. Samples contain 0.15%, 0.45% and 0.75% CaS, respectively. The pore sizes varying depending on the amount of

CaS are 928.8 μm, 659.5 μm, and 573.5 μm, respectively. Pore sizes were observed to change inversely proportional to the amount of CaS.

Density was effected directly proportional to the amount of surfactant in the foam geopolymer mixture. Utilizing H_2O_2, the gas bubbles formed in the geopolymer slurry were further kept in mixture with the effect of the increased amount of surfactant, and therefore the density decreased. Density values also gradually decreased inversely proportional to the amount of perlite added to the mixture. The lowest density value at different Ca stearate concentrations were obtained in samples containing 8% perlite. The highest density values were also measured in samples without perlite (Figure 2).
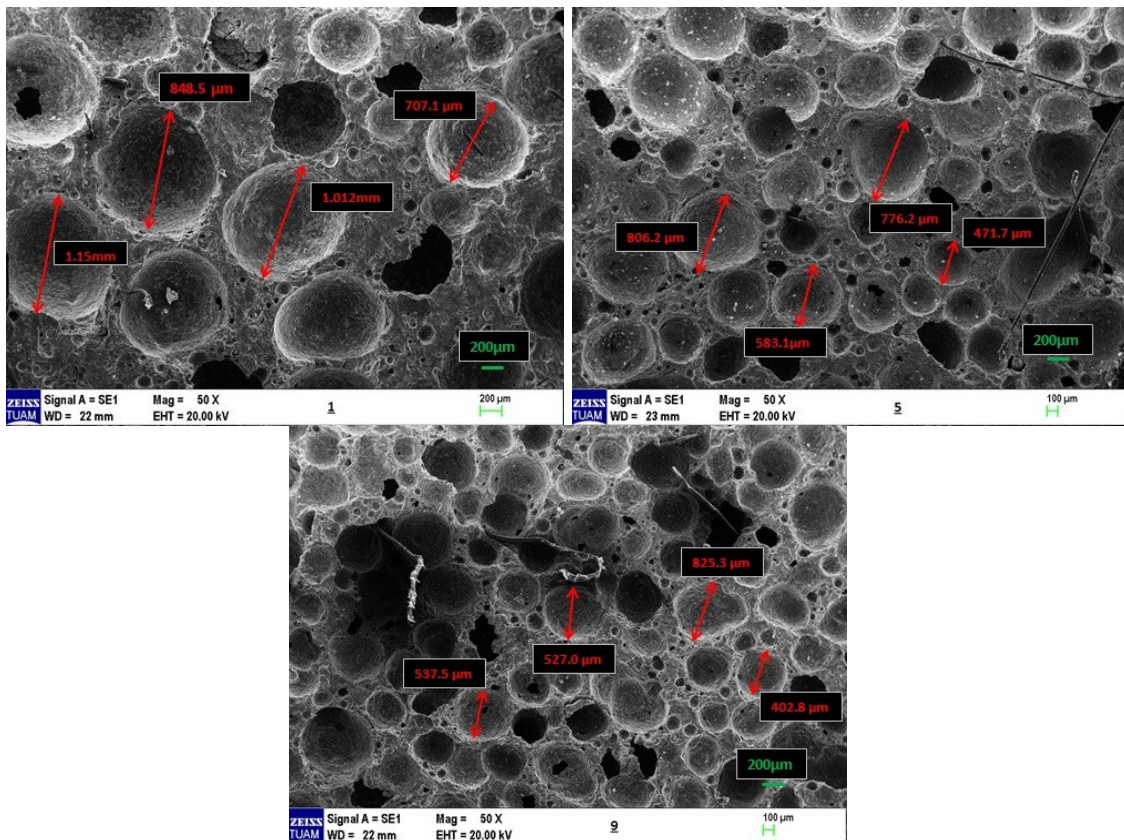
Figure 1. Scanning electron microscopy images of geopolymer foams at 50X magnification. Foams contains upper row, left) 0.15%, upper row, right) 0.45%, and lower row, center) 0.75% CaS.

Compressive Strength

The compressive strength values recorded from an average of three cubic specimens with dimensions of $100 \times 100 \times 100$ mm^3 cured during 28 days were tested. The results of the compressive strength of geopolymer foams are shown in Figure 3. The perlite-free foams had higher strength values compared to the samples containing 8% perlite. A gradual decrease in compression strength values was observed with the increase of perlite input into the composition. This decrease in compressive strength is due to the lower strength (0.1-0.4MPa) and porosity of perlite (33). It is also due to the highly crushable behavior of expanded perlite during compression (34). Besides, our work suggests that this is because of non-bonding between perlite and geopolymer foam.
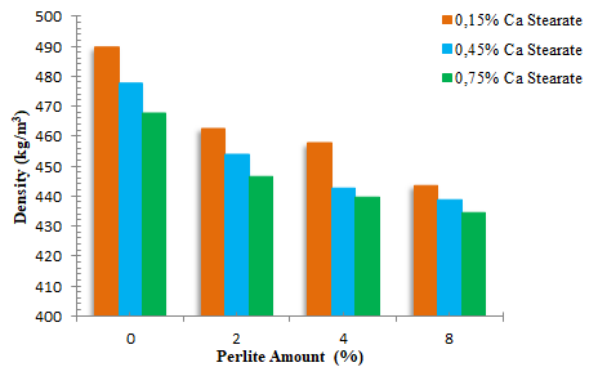
Figure 2. Density values of foam geopolymer samples according to perlite amount and CaS concentration.

In the samples without perlite, the strength values changed depending on the amount of CaS. The strength value of the sample without perlite containing 0.75% CaS was 2.14 MPa, while the strength of the sample containing 0.15% CaS was 1.82 MPa.
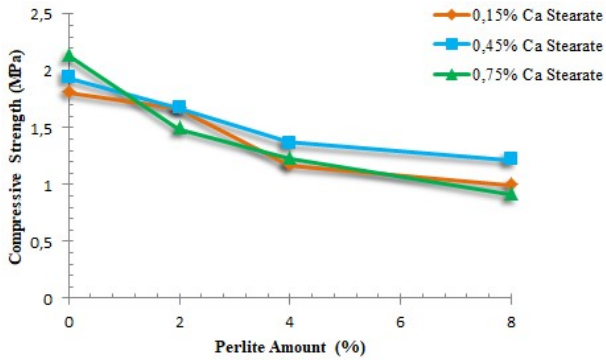
Figure 3. Compressive strength values of foam geopolymer samples according to perlite amount and CaS concentration.

Thermal Conductivity
Figure 4 presents the thermal conductivity performances of geopolymer mixtures. Thermal conductivity tests were carried out on all twelve geopolymer mixtures. It is evident that the perlite input into the mixture gradually reduces the thermal conductivity values for all samples. The minimum thermal conductivity value was obtained at 8% perlite input for all samples.

Considering the amount of CaS, surprising results were obtained when the thermal conductivity values were examined. Considering the Knudsen effect (35,36), thermal conductivity is expected to decrease as the pore size decreases. According to theory, when the pore diameter of the material becomes less than the average free length of the path of gas molecules, the molecules only collide with pore surfaces without transferring energy. Baetans et al. (44) also summarized this statement and said that reducing cell sizes is a very effective way to reduce the thermal conductivity of insulating material. However, an increase was observed in contrast to the theory in thermal conductivity with cell reduction due to the increasing CaS amount. This situation most probably because water repellant property of CaS (45). As a result of this situation, CaS makes it challenging to remove the water in the sample from the sample during drying. **Table 3.** Foam geopolymer studies reported in the literature and the findings of this study.

Table 3. Foam geopolymer studies reported in the literature and the findings of this study.

Aluminosilicate Precursor	Activator	Admixture	Aggregate	Foaming Agent	Pore Size	Strength (MPa)	Density (kg/m³)	Thermal Cond (W/mK)	Refs
Fly Ash	Water Sodium Silicate	-	-	H_2O_2	-	0.4-1.43	270-375	0.071-0.092	(37)
Fly Ash Blast Furnace Slag	NaOH Sodium Silicate Distilled Water	-	-	Foaming Agent	-	3.0-50.0	600-1500	0.15-0.5	(38)
Fly Ash	NaOH Sodium Silicate Tap Water	-	-	H_2O_2	-	1.9-3.4	400-650	0.083-0.127	(39)
Fly Ash Metakaolin	NaOH Sodium Silicate Distilled water	-	-	H_2O_2	-	0.26-21	440-1100	0.082-0.227	(40)
Metakaolin Fly Ash	NaOH Sodium Silicate	-	-	H_2O_2	-	1.23	560	0.107	(12)
Fly Ash Metakaolin	NaOH Sodium Silicate Distilled Water	-	Construction and demolition waste	H_2O_2	0.198-31.88 µm	3.6-11.9	1000-1700	0.19-0.44	(41)
Fly Ash Blast Furnace Slag	NaOH Sodium Silicate Water	SDS	-	H_2O_2	0-2mm	3.2-44.8	140-1020	0.183-0.646	(20)
Fly Ash Metakaolin	NaOH Sodium Silicate Water	Surfactant	-	Al	-	0.6-403	430-850	0.079-0.170	(42)
Fly Ash Metakaolin	NaOH Sodium Silicate Distilled Water	-	-	H_2O_2	0.57-1.13mm	0.68-2.23	150-300	0.0622-0.0852	(43)
Fly Ash	-	Calcium Stearate	-	H_2O_2	140-4563 µm	1.45-1.65	310-360	0.095-0.139	(24)
FlyAsh-Metakaolin	NaOH Sodium Silicate Water	Calcium Stearate	Expanded Perlite	H_2O_2	573-928 µm	0.925-2.14	435-490	0.069-0.1052	This work
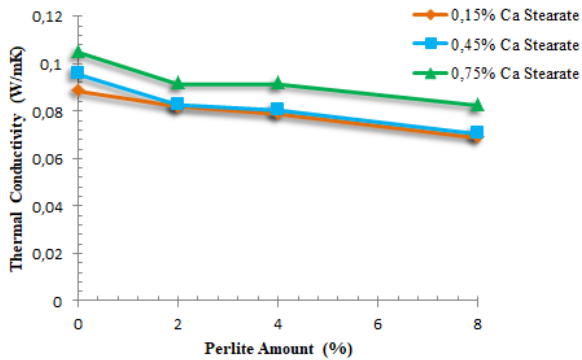
Figure 4. Thermal insulation performances of foam geopolymer samples according to CaS and perlite amount.

Table 3 gives the foam geopolymer studies reported in the literature. As mentioned earlier, there are not too many foam geopolymer studies using CaS as a surfactant. Cui et al. obtained the lowest conductivity when using CaS as a surfactant in fly ash based geopolymer foams is 0.096W/ mK (24). Minimum detectable thermal conductivity value among fly ash based foam studies for samples with a density of 150-300kg/ m^3, 0.0622W / mK has been reached by Wu et al. (43). The best thermal conductivity value was recorded as 0.069W/mK in a sample containing 0.15% CaS and 8% perlite in this study.

CONCLUSION

The results show that hydrogen peroxide with CaS is useful in obtaining homogeneous pore morphology with low thermal conductivity. Density was effected directly proportional to the amount of surfactant in the foam geopolymer mixture. Also, the density decline continued with perlite added to the mixture. Although in the construction area, perlite is widely used as an aggregate because of its outstanding insulation characteristics and low weight, it decreases the compressive strength of the foam. The strength value of perlite-free samples increased in direct proportion with the amount of CaS.

An interesting result was encountered in thermal conductivity measurements. Contrary to what the thermal conductivity theorem states, there is no result that thermal conductivity decreases with decreasing pore size. On the contrary, the increased amount of CaS impairs the insulating properties of the sample. This situation was caused by the water repellent feature of CaS. The presence of perlite as an aggregate gave the material better-insulating properties in all samples where thermal conductivity decreased with increasing porous perlite input. The best thermal conductivity value was recorded as 0.069W/mK in a sample containing 0.15% CaS and 8% perlite in this study.

Foamed fly ash geopolymers have good potential in an application for lightweight building materials. These geopolymer mixtures where the amount of perlite is maximum 2% appear to meet the values specified in TSE 13655 (> 1.5MPa, d <450-550kg/ m^3 and ʎ <0.12W / mK).

This study shows an environmentalist approach in terms of converting fly ash into value-added products and reducing the use of cement that causes CO_2 emission. It supports the protection of the limited energy resources by improving the thermal insulation feature of the walls that have the most energy loss in buildings.

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An improved isolation of trimyristin from *Myristica fragrans* as a renewable feedstock with the assistance of novel cationic gemini surfactant

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Abstract: In the present work, a surfactant-assisted convenient extraction method was developed for the isolation of trimyristin from nutmeg. Commercially available monomeric surfactants and novel readily synthesized cationic dimeric surfactants were used as auxiliary chemicals. The improved isolation method herein revealed that the combination of dimeric surfactant with n-hexane allows selective extraction (without colored polar components of nutmeg) and maximum yield of triglyceride. Besides, the developed method is more practical than existing protocols and provides higher yields of trimyristin in a short period.

INTRODUCTION

The necessary chemical raw materials required by the chemical, pharmaceutical, textile, and food industries have been provided for a long time from crude oil. However, the depletion of oil reserves, the cost of production and derivatization processes of different petrochemicals and excessive energy requirements have led both the academic and the industrial community to the search for new sources of raw materials. It is becoming increasingly popular to find more environmentally friendly, biodegradable, and renewable raw material resources. Among these feedstock sources, animal and vegetable-originated fats and oils are particularly remarkable (1, 2). Vegetable oils are biomass that has the potential to replace many chemicals in oil refineries, and the main component of this biomass is triglycerides, also

known as triacylglycerol (3). Triglycerides are receiving increased interest owing to their potential as a starting material for the production of biofuel and as a source for valuable renewable raw chemicals (4-6). As a result of pyrolysis or thermal and catalytic cracking processes performed with triglycerides, bio-based versatile chemicals can be prepared suitable for different industrial fields (7-9).

Lipids are often found in tissues with carbohydrates and proteins, which makes their isolation difficult. Triglycerides and many other natural compounds of the lipid class are generally isolated from plant sources like canola and *Jatropha curcas* by extraction techniques, and supercritical carbon dioxide is the widely used solvent along with other supercritical fluids (10-12). The triglyceride ratios and compositions of

lipid extracts obtained from several vegetable sources show variability. For instance, *Myristica fragrans* seeds (nutmeg) are rich in myristicin as one of the necessary components of its essential oil and trimyristin as a major triglyceride component (13-15). As known, trimyristin is a neutral lipid component, and non-polar solvents such as ether, n-hexane, and chloroform are suitable for its extraction. Trimyristin is a yellowish-white solid at room temperature, formed by esterification of glycerol with myristic acid as a saturated fatty acid. It is a raw material for the soap and oleochemical industries and widely used in the cosmetic industry (16). In the cosmetic industry, trimyristin can be classified as the following: emollient, refatting, skin conditioning, solvent, and viscosity controlling agent. It is used in makeup products, deodorants, creams and lotions, suntan and sunscreen products, hair conditioners, skincare, and skin cleansing products. Therefore, it is crucial to develop efficient extraction procedures to obtain this particular triglyceride with high yields. Unfortunately, it can be grown on an industrial scale only in regions with a tropical climate.

Beal reported a convenient ether-based isolation method with Soxhlet extractor, which requires a long time (24 to 72 hrs.), and the yield of the purified trimyristin was approximately 53% based on the mass of crude nutmeg extract (17). According to a procedure recommended by Ikan, extraction was carried out with chloroform as a solvent, and after the purification stage, trimyristin was obtained with a yield of 20% based on the mass of crushed nutmeg materials (18). Lugemwa used water-organic solvent mixtures for the isolation of trimyristin from nutmeg and obtained the desired triglyceride with a yield of only 8% at the end of 1 hour of extraction (19). On the other hand, some isolation methods designed as general chemistry experiments have also been developed but with a lower yield of trimyristin based on the mass of crude nutmeg extract (20, 21).

As is known, surfactants are both hydrophilic and lipophilic compounds which make them unique tools in isolation of various natural compounds via extraction processes (22-30). At the extraction stage, the combination of solvent with surfactant molecules facilitates the penetration of solvent molecules throughout seed cell walls. To the best of our knowledge, there has been no work investigating the surfactant-assisted isolation of trimyristin from nutmeg. In this work, we report the synthesis of a novel cationic gemini surfactant and its evaluation in an efficient extraction process of trimyristin from nutmeg.

EXPERIMENTAL SECTION

Reagents and chemicals
All reagents and solvents were purchased from either Merck or Sigma-Aldrich (St. Louis, MO) and used without further purification. Thin-layer chromatography (TLC) was performed using silica gel plates (60 F_{254}, Merck, Darmstadt, Germany).

Analytical techniques
Melting points were recorded by BÜCHI melting point B-540 apparatus (BÜCHI Labortechnik AG in Flawil, Switzerland). The Fourier Transformed Infrared (FTIR) spectra were measured using a PerkinElmer Spectrum 100 spectrometer. The Nuclear Magnetic Resonance (NMR) spectra were measured using Bruker Avance NEO 600 MHz NMR spectrometer (Santa Clara, CA, USA). Chemical shifts (δ) are reported in ppm, and J values in Hertz. A Hitachi 3100H dual-beam spectrophotometer (Tokyo, Japan) was used for the spectrophotometric analyses. Conductivity measurements were performed using a Thermo Scientific ORION 3 STAR digital conductometer (Madison, WI, USA). The elemental analyses were performed using a LECO CHNS-932 elemental analyzer (Saint Joseph, MI, USA).

Preparation of the surfactant

N^1,N^1,N^6,N^6-tetramethyl-N^1,N^6-bis(4-((10-(5-(octylthio)-1,3,4-oxadiazol-2-yl)decyl)oxy)benzyl)hexane-1,6-diaminium chloride
N,*N*,*N'*,*N'*-Tetramethyl-1,6-hexanediamine (0.15 g, 0.87 mmol) and two equivalents of 2-(10-(4-(chloromethyl)phenoxy)decyl)-5-(octylthio)-1,3,4-oxadiazole (31) (0.85, 1.72 mmol) are heated in MeCN at 80 ºC for 24 h. After completion of the reaction, the mixture was cooled, and the excess of solvent was removed under reduced pressure. The residue is washed successfully with n-hexane. A white waxy product is obtained quantitatively in a sufficient purity.
Waxy solid (mp: 35–36 °C); FTIR (ATR): v_{max} 3351, 2919, 2852, 1667, 1612, 1586, 1514, 1484, 1474, 1433, 1367, 1306, 1252, 1183, 1146, 1043, 1018, 983, 958, 928, 865, 825, 795, 742, 718, 682, 617, 555, 522 cm^{-1}; ^{1}H NMR (600 MHz, CDCl$_3$) δ 7.50-7.47 (*m*, 4H, Ar), 6.87-6.85 (*m*, 4H, Ar), 4.74 (*s*, 4H, 2 x ArC$\underline{H}_2$N$^+$), 3.90 (*t*, *J* = 6.4 Hz, 4H, 2 x ArOC$\underline{H}_2$-), 3.60 (*t*, *J* = 7.4 Hz, 4H, 2 x (CH$_3$)$_3$N$^+$C$\underline{H}_2$CH$_2$-), 3.18 (*t*, *J* = 7.4 Hz, 4H, 2 x HetSC$\underline{H}_2$CH$_2$-), 3.15 (*s*, 12H, 2 x –N$^+$(C$\underline{H}_3$)$_2$), 2.77 (*t*, *J* = 7.4 Hz, 4H, 2 x HetC$\underline{H}_2$CH$_2$-), 2.04-2.01 (*m*, 4H, 2 x HetSCH$_2$C$\underline{H}_2$CH$_2$ -), 1.77-1.70 (*m*, 12H, 6 x C$\underline{H}_2$), 1.43-1.32 (*m*, 16H, 8 x C$\underline{H}_2$), 1.28-1.23 (*m*, 32H, 16 x 1.36 C$\underline{H}_2$), 0.84 (*t*, *J* = 7.1 Hz, 6H, 2 x -CH$_2$C$\underline{H}_3$); ^{13}C NMR (150 MHz, CDCl$_3$) δ 167.91,

164.24, 160.77, 134.57, 119.12, 114.90, 68.13, 67.27, 64.17, 49.24, 42.96, 32.47, 31.69, 29.42, 29.31, 29.29, 29.20, 29.11, 29.04, 29.03, 28.92, 28.91, 28.53, 26.36, 25.96, 25.35, 24.96, 22.56, 21.70, 14.03; Anal calc for $C_{64}H_{110}Cl_2N_6O_4S_2$ (1162.64): C 66.12, H 9.54, N 7.23, S 5.52. Found: C 66.15, H 9.49, N 7.27, S 5.50.

Conductivity measurements
Conductivity measurements were performed using a Thermo Scientific ORION 3 STAR digital conductometer apparatus (Madison, WI, USA). To measure the specific conductivity at each surfactant, we followed the step-by-step dilution–extraction method. The critical micellar concentration (cmc) value was estimated from the breakpoint on the curve of conductivity versus surfactant concentration.

Surface tension measurement at cmc
The capillary rise method is used to measure the surface tension of aqueous solution of the synthesized cationic surfactant at cmc (32, 33). The corresponding value was measured at 20 °C.

Emulsion stability
Emulsion stability was determined by mixing the aqueous solution of the surfactants with the mineral oil in a graduated cylinder with a plastic stopper. An aqueous solution of the surfactant at the cmc (10 mL) was poured into a 100 mL cylinder containing 10 mL mineral oil. The glass cylinder was closed with a stopper. The mixture was shaken by turning the glass cylinder vigorously up and down for 30 s at a constant speed, and then the time for the separation of 9 mL of clean surfactant solution was noted (34).

Ultraviolet (UV) spectrophotometric analysis
A Hitachi 3100H dual-beam spectrophotometer was used for spectrophotometric analysis. The spectra of the compounds were viewed at a wavelength range of 1100-190 nm. Since no peak was observed in the near IR and Visible (VIS) regions, comparative spectra were taken at a range of 500-190 nm. For the cationic surfactant, spectra of solutions with various concentrations (1.0×10^{-6} – 2.5×10^{-5}) M and separately 1.0×10^{-4} M on the curve were taken.

General extraction and purification procedure for trimyristin

The *Myristica fragrans* seeds were ground using a porcelain mortar and pestle at ambient temperature just before extraction to isolate triglycerides more efficiently. From this undried crushed sample, exactly 1 gram was weighed, placed in a 50 mL single-necked flask, and was extracted at 50 ºC for 20 min with 20 mL of the solvent indicated in the present work. In the case of surfactant-assisted extractions, 5 mg of the related surfactant was also added into the flask. After that, the obtained mixture was filtered, and the filtrate was evaporated in a vacuum. The residue was weighed and recrystallized from acetone to afford pure trimyristin as a white solid with mp: 55–56 ºC. However, the triglyceride is sufficiently pure and can be used for many purposes without further purification.

RESULTS AND DISCUSSION

Synthesis and physicochemical characterization of the surfactant
1,3,4-oxadiazole based long-chain benzyl chloride derivative (31) was treated with *N*, *N*, *N´*, *N´*-tetramethyl-1,6-hexanediamine, as shown in Scheme 1, to prepare the cationic gemini type surfactant. The structure of the novel surfactant was confirmed by FTIR, ^1H NMR, and ^{13}C NMR spectroscopic methods (Appendix A. Supplementary material, S2). The evaluation of ^1H NMR spectrum showed that (ArC$\underline{H}_2$N$^+$) benzyl protons appear as a singlet at 4.74; methylene protons of (ArOC$\underline{H}_2$-) group also appear as a triplet peak at 3.90 ppm. The methylene protons next to the heterocyclic ring sulfur atom (HetSC$\underline{H}_2$CH$_2$-) have a chemical shift of 3.18 ppm as a triplet peak. The methyl protons attached to the quaternary N atom [N$^+$(CH$_3$)$_2$] have a chemical shift of 3.15 ppm as a singlet peak, which are the essential characteristic peaks of this compound (Figure 1). The surfactant is not soluble in water at room temperature but is readily soluble by mild heating. Physicochemical parameters such as foam height and foam stability could not be determined because this compound did not form a permanent foam layer in water. However, various parameters like density (ρ), cmc, surface tension at cmc (γ_{cmc}), surface effectiveness (π_{cmc}), Gibbs free energy of micellization (ΔG_{mic}), and hydrophilic-lipophilic balance (*HLB*) value were measured and/or calculated for the surfactant. The results are summarized in Table 1.
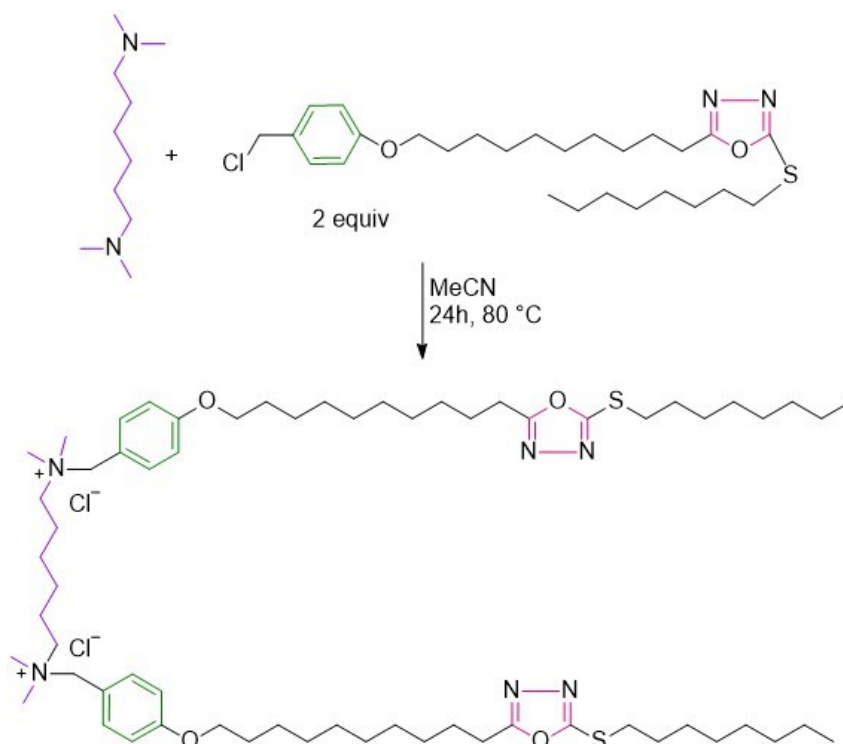
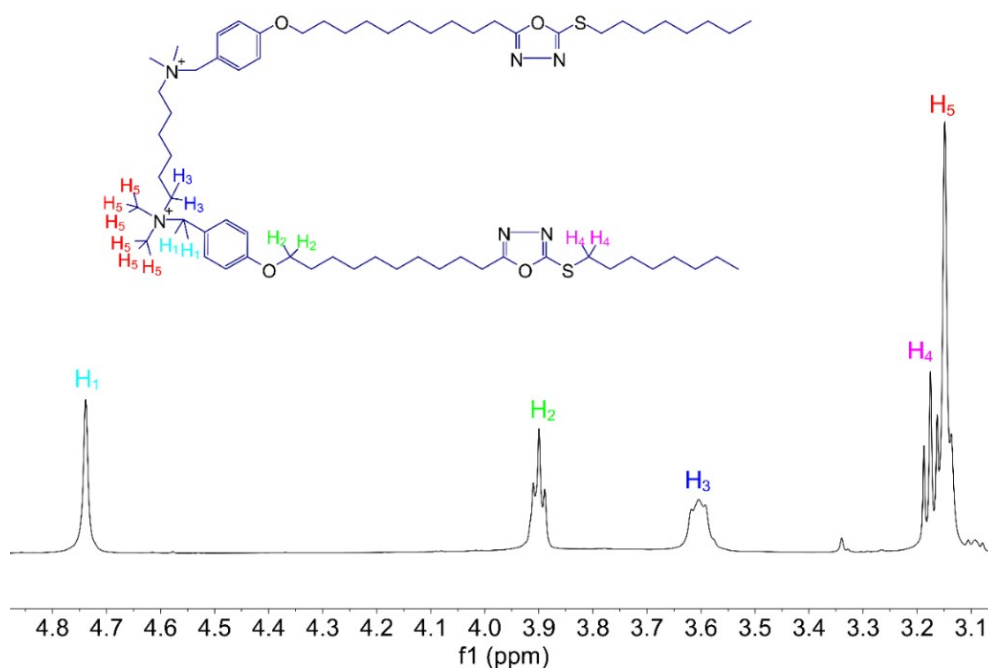**Scheme 1:** Synthesis of symmetrical heterocyclic gemini-type surfactant.

Figure 1: Some characteristic ^{1}H NMR peaks of the surfactant molecule.

Table 1: Experimental and calculated physicochemical properties of the surfactant catalyst.

ρ (g mL^{-1})[a,b]	cmc (x 10^{-5} mM)[a]	γ_{cmc} (dyn cm^{-1})[a]	π_{cmc} (dyn cm^{-1})	ΔG_{mic} (kj mol^{-1})	ES_1 (s)[a,b,d]	ES_2 (s)[a,c,d]
1.1227	26.36	33.01	39.79	- 36.9	164	38

HLB (Hydrophilic-lipophilic balance) value of the surfactant molecule
Hydrophilic and hydrophobic group contributions[e]
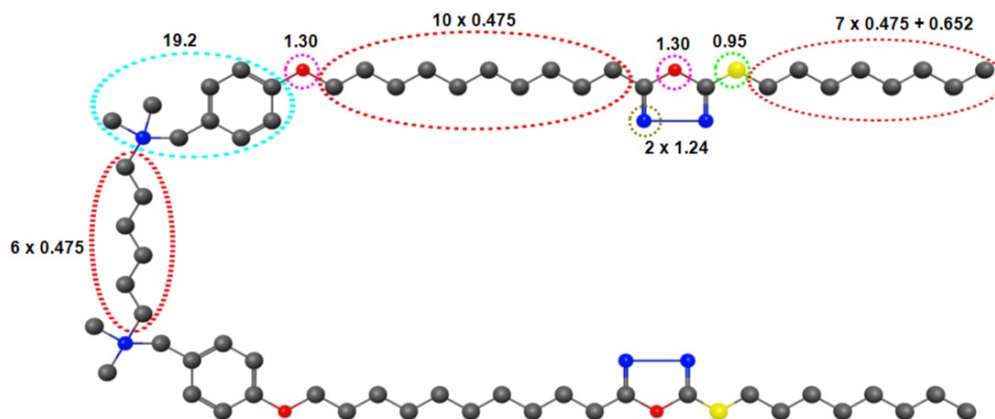
HLB = Σ (Hydrophilic group contributions) − Σ (Hydrophobic group contributions) + 7
= (19.2 x 2 + 4 x 1.30 + 4 x 1.24 + 2 x 0.95) − (40 x 0.475 + 2 x 0.652)
= 30.2

[a] Value measured at 20 °C.
[b] Value measured at 1,0 x 10^{-4} M of surfactant concentration.
[c] Value measured at critical micelle concentration of the surfactant.
[d] Emulsion stability in seconds.
[e] Contributions were received from references (35, 36).

The cmc of the surfactant was determined by conductivity measurements. As seen in Figure 2, with the increase in conductivity, the concentrations also showed a linear increase. Besides, the concentration was determined as 26.36x10^{-5} mM at which the slope was changed. This concentration is the critical micelle concentration of the synthesized gemini surfactant and is relatively low in comparison with cmc values of previously synthesized cationic gemini surfactants (37). The surfactant's cmc plays an essential role in the Gibbs free energy of micellization. The value of Gibbs free energy of

micellization, which is calculated by the following equation, proves that as the cmc of the surfactant decreases, the tendency of the substance to form micelle increases. The negative value of Gibbs' free energy of micellization shows that micelle formation occurs spontaneously.

$$\Delta G_{mic} = RT \ x \ ln\left(cmc\right)$$

Where ΔG_{mic} is the Gibbs free energy of micellization, R is the ideal gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature (293 K).
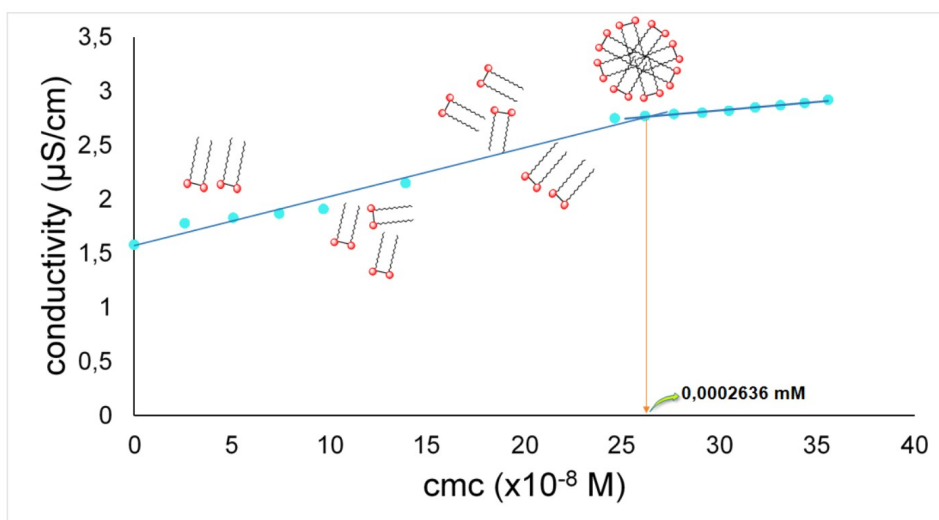
Figure 2. Conductivity as a function of surfactant concentration.

The surface tension was measured to evaluate the surface activity of the aqueous solution of the surfactant at cmc with a capillary rise method in accordance (32, 33). The effectiveness in surface tension reduction (π_{cmc}) is the amount of reduction in surface pressure attained at the cmc (38). It is defined by using the following equation:

$$\pi_{cmc} = \gamma_0 - \gamma_{cmc}$$

Where, γ_0 is the surface tension of pure solvent (water, 72.8 dyn cm^{-1} at 20 °C) and γ_{cmc} is the surface tension of the solution at the cmc. This parameter (π_{cmc}) indicates the maximum reduction of surface tension caused by the dissolution of surfactant molecules and hence becomes a measure for the effectiveness of the surfactant to lower the surface tension of the solvent (39). The effectiveness value was calculated as 39.79 dyn cm^{-1}. This result indicates that the synthesized surfactant has the potential to reduce the surface tension in the aqueous system (40). The *HLB* value is also calculated for the synthesized surfactant according to the Davies method (41).

One of the essential properties of surfactant molecules is emulsion stability, and it reflects the capability of a surfactant to maintain an emulsion for a longer duration. It was determined as a time required to break down the emulsion formed between surfactant solution and mineral oil. The emulsification is realized by the effect of adsorption, and it forms a layer of adsorptive film in the interface of dispersed droplets. The emulsion stability of the synthesized surfactant was measured as the time required for separation of 9 mL of pure water from the emulsion formed between surfactant solution at cmc and mineral oil (10/10 mL). As depicted in Table 1, the emulsion stability was measured at two different concentrations of the synthesized surfactant. ES$_1$ is the emulsion stability measured at 1.0 x 10^{-4} M of surfactant concentration, and ES$_2$ is the emulsion stability measured at cmc. Although the emulsion stability ES$_1$ was found higher than ES$_2$, the results in Table 1 showed that the synthesized compound has a low emulsification tendency, and therefore, it can be used safely in various applications.

The UV spectra of the surfactant measured for different concentrations of aqueous solutions are given in Figure 3a. The UV spectrum of the slightly more concentrated surfactant solution (1,0x10^{-4} M) was also taken (Figure 3b). In these spectra, a maximum absorbance value was measured in the wavelength of 232 nm, and two narrow bands were also detected in the wavelength range of 272—280 nm.

As known, dimeric (gemini) surfactants are compounds consisting of more than one hydrophilic head group and a hydrophobic tail group linked by a rigid or a flexible spacer at or near the head groups. The spacer group may also possess hydrophobic or hydrophilic groups, short or long alkyl groups, rigid or flexible groups (42). These surfactants appear to be superior to the corresponding conventional monomeric surfactants. They have much lower cmc, are more efficient in lowering the surface tension of water, and have better wetting and lime-soap dispersing properties (43). Gemini surfactants have widespread use, some of which are using as a phase transfer catalyst and finding applications in adsorption (44), analytical separations (45), solubilization processes (46), nanotechnology (47) and oil recovery (48). They also find use in the dissolution of dyes and pigments in the spinning and weaving industry (49).
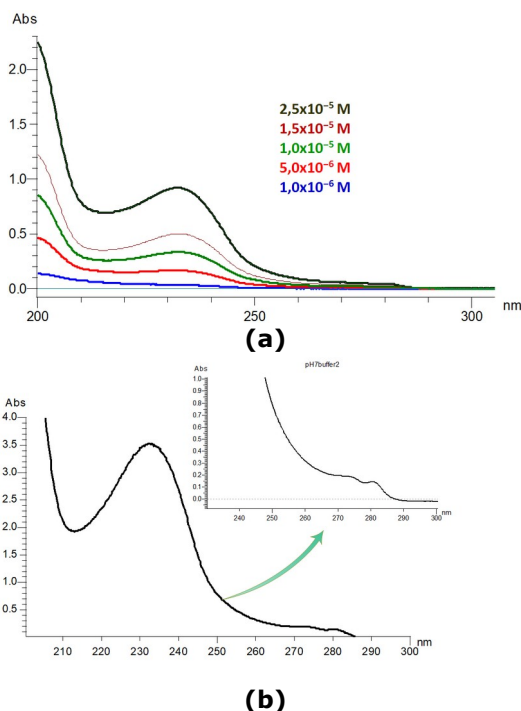
Figure 3. (a) UV spectra of aqueous solutions of the surfactant at different concentrations. Figure (b) UV spectrum of an aqueous solution of the surfactant at 1.0×10^{-4} M.

Extraction of trimyristin

In this study, a series of extraction studies were carried out for the isolation of trimyristin from nutmeg (Scheme 2). The extractions were performed by slightly polar solvents ($CHCl_3$ and THF) and n-hexane as a non-polar solvent, all of which have a relatively low boiling point. Cationic and anionic monomeric surfactants such as cetyltrimethylammonium bromide (CTAB), cetylpyridinium bromide (CPB), and sodium dodecyl sulfate (SDS) respectively were selected, and their effectiveness was compared with the gemini surfactant (GS), to determine surfactant assistance in the isolation process (Table 2). It should be noted that the percent recovery yield of trimyristin is the yield after crystallization from acetone and is based on the recovered crude extract. As can be seen from the table, n-hexane, including gemini surfactant, is the more efficient system comparing to the other extracting neat solvents and surfactant-based solvent systems. The crude extract obtained at the end of the extraction with n-hexane is quite clear and colorless, so the purification of trimyristin becomes a simple task. On the other hand, orange-colored extracts obtained by extraction processes with the other slightly polar solvents, indicating that some moderately polar components of nutmeg essential oil are also transferred to the solvent phase along with trimyristin.
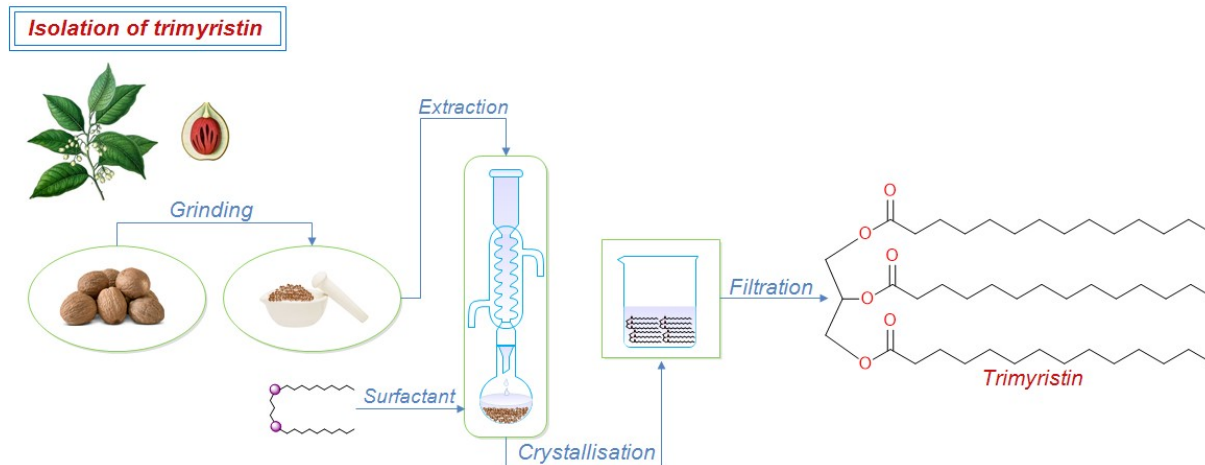
Scheme 2. Schematic representation of the trimyristin isolation process.

The possible role of the cationic surfactant during the extraction process can be expressed as in Figure 4. According to this figure, the surfactant molecules form a channel-like cavity for easier penetration of the non-polar solvent molecules from the semipermeable phospholipids based cell membrane. However, intriguing results were encountered in extractions with SDS as an anionic surfactant. As seen from Table 2, the amount of recovered trimyristin slightly decreased with the use of CHCl3 or n-hexane in combination with SDS compared with the same neat solvent (entries 10, 12, and entries 1, 3 respectively). The main possible reason for this result may be that the surface of the cell membrane composed of phospholipids and is negatively charged (50), which has a repellent effect against anions of SDS surfactant.

Table 2. Experimental conditions for the extraction of trimyristin.

Entry	Surfactant	Solvent	Surfactant (mg)	Time (min)	Temperature (ºC)	Recovery yields based on crude extract (%)[f]
1	-	CHCl$_3$	5	20	50	60
2	-	THF	5	20	50	62
3	-	n-hexane	5	20	50	63
4	CTAB	CHCl$_3$	5	20	50	64
5	CTAB	THF	5	20	50	72
6	CTAB	n-hexane	5	20	50	69
7	CPB	CHCl$_3$	5	20	50	71
8	CPB	THF	5	20	50	74
9	CPB	n-hexane	5	20	50	67
10	SDS	CHCl$_3$	5	20	50	42
11	SDS	THF	5	20	50	69
12	SDS	n-hexane	5	20	50	57
13	GS	CHCl$_3$	5	20	50	67
14	GS	THF	5	20	50	66
15	GS	n-hexane	5	20	50	76

[f]After crystallization from acetone.
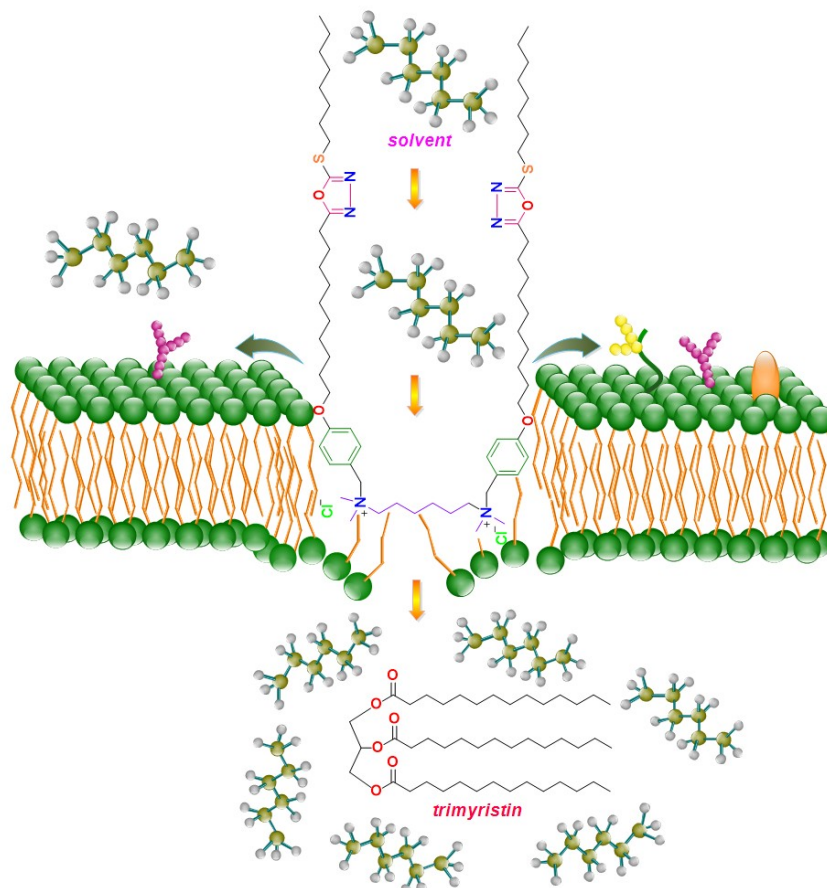
Figure 4. Plausible role of the surfactant molecule in the extraction process.

On the other hand, Table 2 shows that the ideal solvent and surfactant in the extraction process are n-hexane and GS, respectively. Accordingly, optimization studies were performed and the results obtained are shown in Figure 5. When these results are evaluated, it can be concluded that the optimum conditions for the extraction of trimyristin with n-hexane should be as given in Figure 5, entry 5. As a result, the most apparent advantages of the method developed in this study compared with the existing ones are that it allows obtaining triglyceride with more practical, selective, and high yields.

YILDIRIM A et al. JOTCSA. 2020; 7(2): 545-560.

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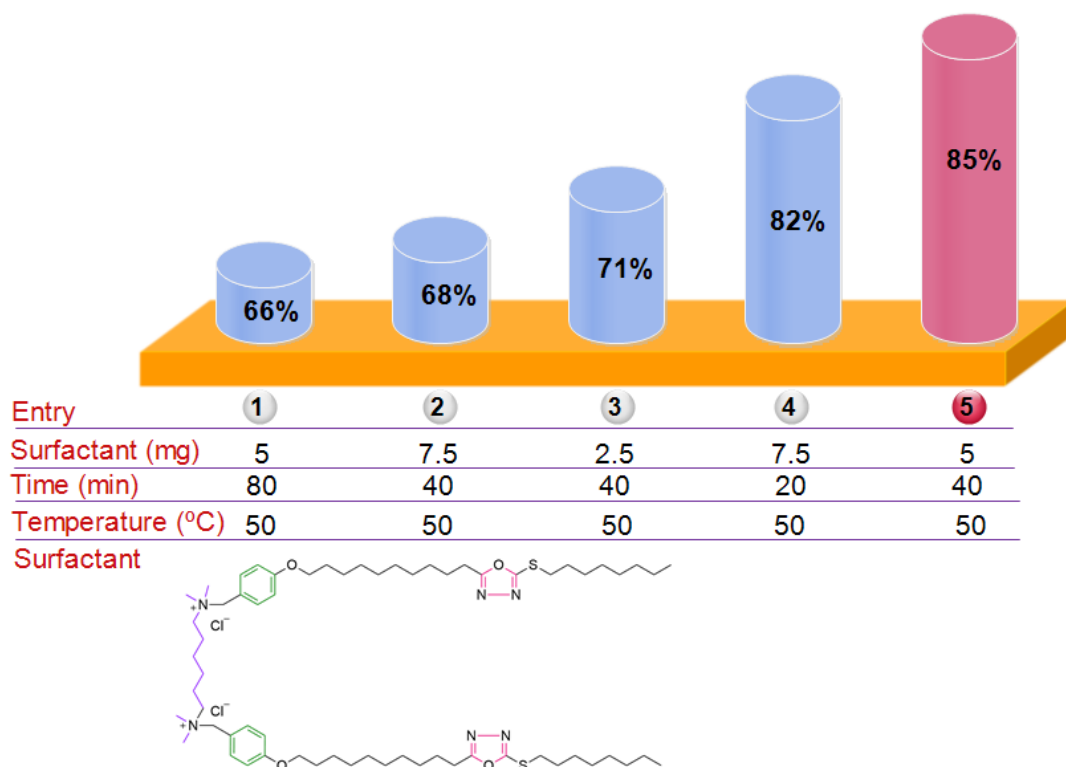
Figure 5. Optimization parameters for the recovery of trimyristin (percent yields are based on the crude extract after crystallization from acetone).

CONCLUSIONS

Herein, an extractive isolation method was developed for trimyristin from nutmeg as a valuable renewable raw material, and this method is more practical and effective than the existing isolation methods. Several different surfactants in combination with organic solvents, were used to make extraction more effective. It has been observed that monomeric and dimeric cationic surfactants, especially the newly synthesized gemini type surfactant, are more effective than the anionic surfactant (SDS) in the extraction process. The performed optimization studies revealed that to obtain the highest yield of the triglyceride, n-hexane and gemini surfactant are ideal as an organic solvent and as an auxiliary agent, respectively.

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RESEARCH ARTICLE

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An improved isolation of trimyristin from *Myristica fragrans* as a renewable feedstock with the assistance of novel cationic gemini surfactant

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Supplementary material

Table of Contents:
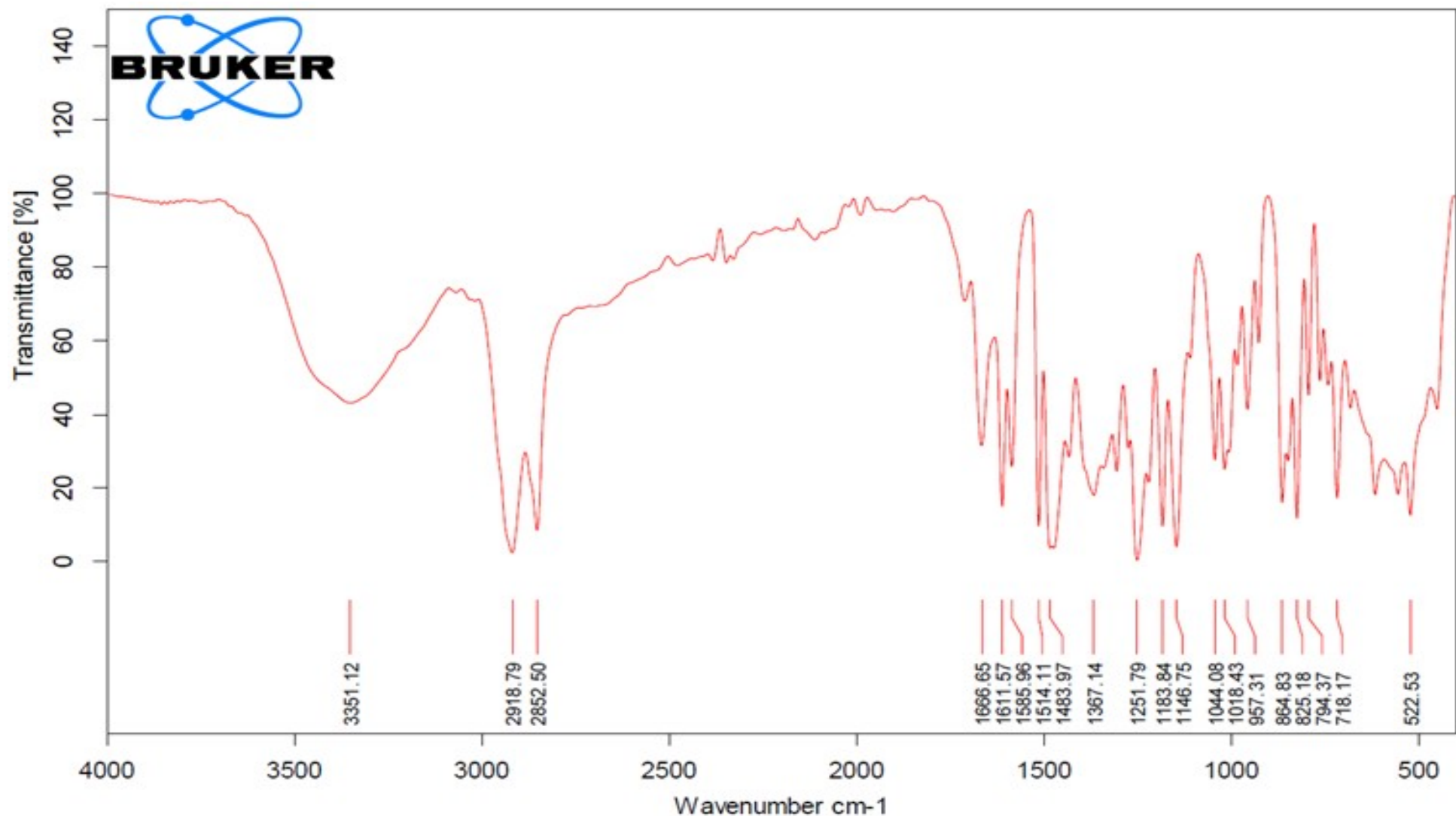
Figure S1. FTIR Spectrum.

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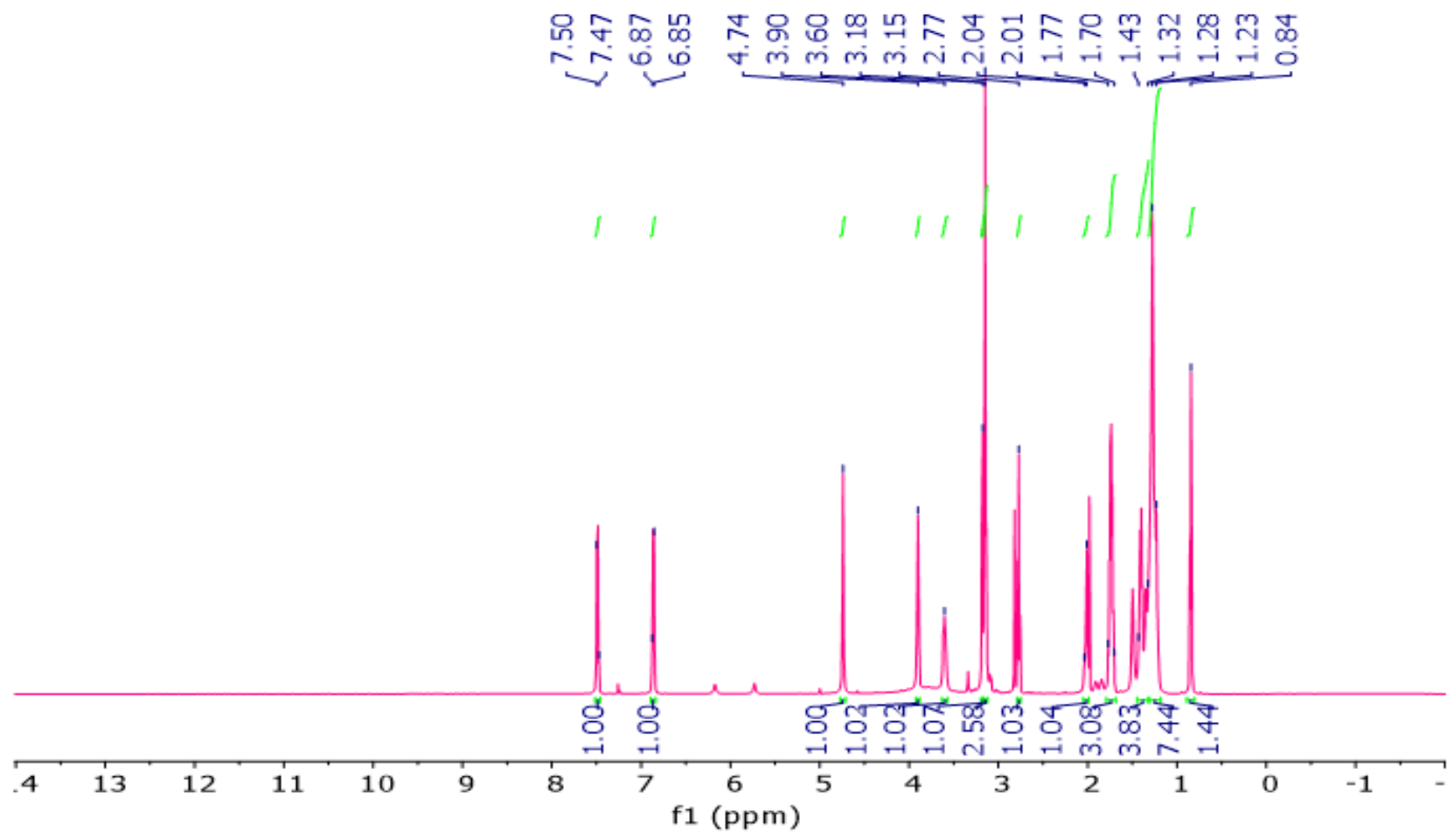
Figure S2. ¹H NMR Spectrum.
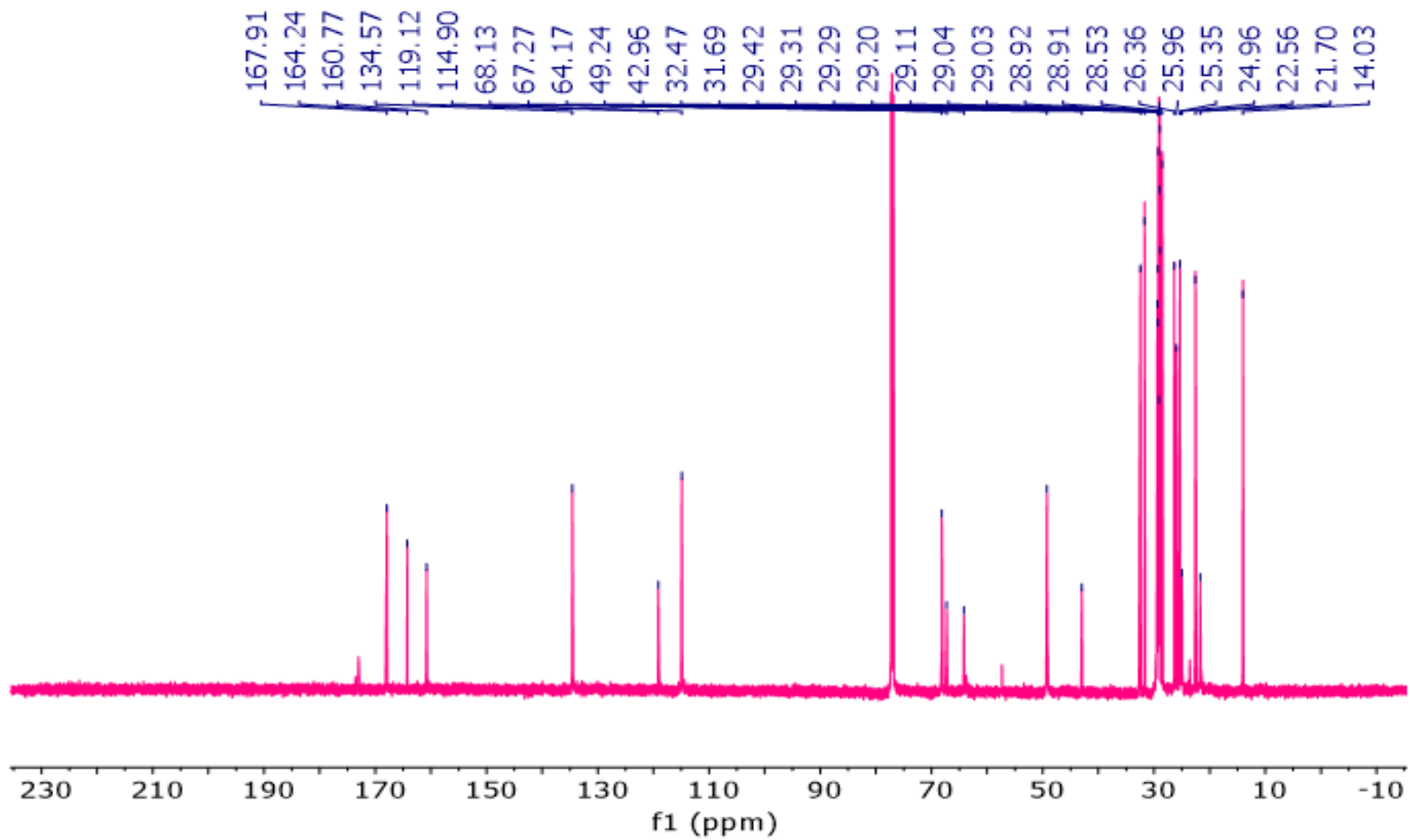
Figure S3. ^{13}C NMR Spectrum.

RESEARCH ARTICLE

Copper Oxide Nanoparticles: Synthesis, Characterization, Antimicrobial Activities and Catalytic Reduction of Methylene Blue

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Abstract: In recent years, metal nanoparticles have been applied in many areas due to their attractive properties. Copper oxide nanoparticles, in particular, have drawn much attention owing to their electrical, catalytic, optical, antibacterial, and antifungal properties. In this study, copper oxide nanoparticles were synthesized using *Onosma Sericeum Willd (Boraginaceae)* extract with a simple, economical, and eco-friendly method for the first time. The synthesized nanoparticles were characterized using ultraviolet-visible spectrophotometry, field emission scanning electron microscopy, and X-ray diffraction. The particle size distribution and zeta potential measurements of the copper oxide nanoparticles were measured with the dynamic light scattering technique. It was determined that the copper oxide nanoparticles with a particle size of less than 100 nm showed a catalytic effect in the reduction of methylene blue (MB). At room temperature, up to 90% MB dye degradation was achieved. Also, the antimicrobial properties of the copper oxide nanoparticles were investigated in this study. The results of the study showed that synthesized copper oxide nanoparticles could be used as a promising agent in nanotechnology applications.

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INTRODUCTION

Metal nanoparticles are used in various fields due to properties including high surface area/volume ratio, differences in chemical reactivity and catalytic properties, and selective drug delivery to target organs and tissues with easy surface modification. They can be used in electrochemical processes and hydrogen production, electronic devices and communication systems, sensor design, and biomedical and medical imaging technologies. They also have the potential to be used in many different fields, including material chemistry (1,2).

Copper oxide nanoparticles (CuONPs) attract attention with their catalysis (3), sensor (4), and superconducting applications (5) due to their superior physicochemical properties. These structures are also considered to be semiconductor materials with photocatalytic (6)

and photovoltaic (7) properties. Some studies have shown that CuONPs can be antibacterial agents (8) and used in the treatment of cancer (9).

CuONPs can be synthesized by different chemical methods such as chemical reduction (10), microwave radiation (11), and thermal decomposition (12). However, synthesis procedures performed with these methods involve the adsorption of toxic chemicals to the surface. It can cause serious side effects, especially in medical applications. The use of toxic chemicals, which pose a risk to the environment and human health, in chemical methods has led researchers to develop new alternative methods. Green chemistry (13), which is defined as the utilization of a dozen principles that prevent or reduce the emergence and use of substances that pose a danger to the environment and human health in the design, production, and use of chemical products, is an alternative approach that has been extensively researched in recent years. Association of green chemical principles with nanotechnology has been shown by Schmidt (2006) as the key to a sustainable society in the 21st century (14). The high toxicity and long biocompatibility of nanoparticle technologies, which have been known for a long time, have urged scientists to develop new synthetic procedures that are more secure, environmentally friendly, and producing as little waste as possible, using natural and renewable raw materials that can be easily integrated into biomedical applications. With this new green nanotechnology approach, it has become much easier to find solutions to the problems caused by the conventional synthesis procedure.

Green nanotechnology has been reported to use plants and extracts such as *Aloe vera*, *Azadirachta indica (linden)*, *Camellia sinensis (tea), Jatropha curcas (castor nuts), Acalypha indica (castor nettle), Cymbopogon sp (lemongrass)* being preferred in metal nanoparticle synthesis as they are cheap and renewable natural resources. Also, microorganisms such as *Rhodococcus sp., Sargassum wightii, Pseudomonas stutzeri, Candida glabrata, M13 bacteriophage, Fusarium oxysporum,* and *Thermomonospora sp.* are extensively used in the synthesis of metal nanoparticles as natural resources that serve the purpose of green synthesis (15, 16). Particularly in CuONP synthesis, the leaves and stems of the *Gundelia tournefortii (kenger) plant* (17), the leaves of the *Psidium guajava*

plant (18), *lemon juice* (19) and the *Gloriosa superba* L plant (20) are used.

Plants do not require any other agents in the production of metal nanoparticles because of the phenolic species they contain (21). Therefore, in recent years, studies on plants and plant extracts and nanoparticle synthesis have been conducted with increasing acceleration. *Onosma sericeum Willd* is a member of the *Boraginaceae* family and has been known for its wound healing properties for many years among people in Adıyaman, Turkey. The *Boraginaceae* family, found in tropical, subtropical, and temperate regions of the world, is represented by 154 genera and 2500 species (22).

Depending on the chemical composition and ionic features, several classes of dye molecules have been reported. Thiazine dyes such as MB are aromatic, organic, and toxic pollutants (23). Methylene blue, like the other organic dyes, are released by different industries such as cosmetic, textile, pharmaceutical, and plastic industries (24). There are findings in the literature that MB affects the central nervous system (25). Besides, this pollutant can be accumulated in soil and water and threaten human health. Therefore the investigation of catalytic degradation is essential. In recent years nanoparticles have been used in the degradation of the dye. Especially owing to their large surface area, CuONPs showed very high reactivity.

In this study, CuONP synthesis was carried out by using the root extract of the *Onosma Sericeum Willd* plant fort the first time, the wound healing properties of which are well-known, and applying a completely green method without the need for any other organic solvents or stabilizing agents. The characterization of the CuONPs was elucidated using analytical methods. The antimicrobial activities of the obtained CuONPs against a range of Gram-positive and Gram-negative bacteria were evaluated. Its catalytic activity on the reduction of methylene blue, a harmful pollutant for the environment, in the presence of CuONPs was also studied.

MATERIALS AND METHODS

Materials
Copper(II) nitrate ($Cu(NO_3)_2$, 99%), methylene blue ($C_{16}H_{18}ClN_3S$, 95%), sodium borohydride ($NaBH_4$, 99%) were obtained from Sigma. Gram-positive bacterial strains *Staphylococcus*

aureus (ATCC25925) and *Bacillus subtilis (ATCC 6633)*, gram-negative bacterial strains *Escherichia coli (ATCC25923)*, *Acinetobacter baumannii (ATCCS 02026)* and *Aeromonas hydrophila (ATCC95080)* were purchased from the Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey. All the chemicals used in the study were of analytical grade, and 18.2 mΩ of distilled water needed throughout the research was obtained through the Milli-Q A10 water purification system.

Instruments
All spectrophotometric measurements were conducted by using the Shimadzu UV-1800 spectrophotometer. The structural morphology of the CuONPs was obtained using a Zeiss / Supra 55 Field Emission Scanning Electron Microscope (FE-SEM). The XRD of the nanoparticles was studied by a high-resolution Rigaku SmartLab brand X-ray diffractometer (XRD). The size distribution of the CuONPs and zeta potential measurements were performed using a Zetasizer Nano-ZS (Malvern Instruments).

Preparation of Extract
The *Onosma Sericeum Willd* plant was collected from the Adıyaman-Besni region in Turkey between April and July 2018. The identification of the plant species was made by Assoc. Prof. Dr. Rıza Binzet of the Faculty of Arts and Sciences at Mersin University. The collected plant samples were first cleaned from dust and impurities using tap water, followed by distilled water. The plant was left to dry in a clean and dry place. The root portions of the plant were extracted for the CuONP synthesis. For the extraction, 200 mL of distilled water was added to 20 g of roots that were cut into small pieces and subjected to maceration for 6 hours at a temperature that did not exceed 90 °C in a shaking water bath. The extract was filtered *in vacuo* and stored in a refrigerator. Great care was taken to keep the extract as fresh as possible.

Synthesis of the CuONPs
Copper(II) nitrate ($Cu(NO_3)_2$, Sigma) was used as the copper source. 5 mL of the extract (20 g / 200 mL) dropwise was added to the $Cu(NO_3)_2$ solution (25 mL), which was prepared in 100 mM and mixed in the magnetic stirrer. Next, 1000 µL of NaOH (0.1 M) was added to the mixture.

The gray-green color that developed within half an hour indicated the synthesis of CuONP. The resulting CuONPs were separated by centrifugation (10,000 rpm, 20 min), washed three times with deionized water, and dried in an oven at 80 °C for 12 hours. The oven-dried sample was heat-treated in an ash oven at 400 °C for 3 hours (Figure 1). The obtained samples were stored in a cool and dry environment until analysis.
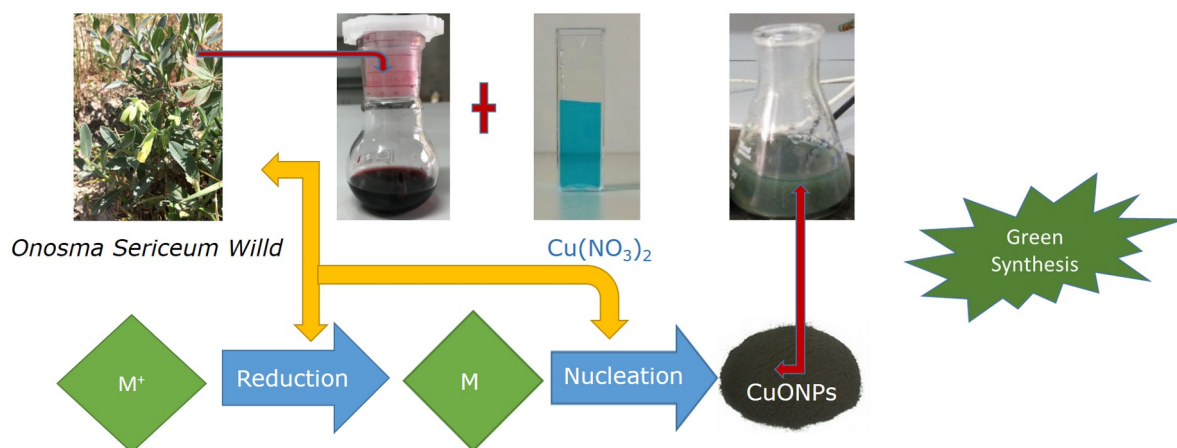
Figure 1. Schematic showing the synthesis procedure and mechanism of CuONPs

Characterization of the Synthesized CuONPs
The optical properties of the synthesized CuONPs were determined by an ultraviolet-visible (UV-Vis) absorption spectrophotometer. All measurements were performed in the range of 200-800 nm, in quartz tubes and with Shimadzu brand UV-1800 spectrophotometer adjusted to 1 nm resolution. The structural morphology of the CuONPs was obtained using a Zeiss / Supra 55 Field Emission Scanning Electron Microscope (FE-SEM). A high vacuum sputter platinum coating device was used for sample preparation. The crystal structure of the

nanoparticles was illuminated by a high-resolution Rigaku SmartLab brand x-ray diffractometer (XRD) using Cu(Kα) radiation (wavelength: 1.54 Å) operated at 40 kV and 40 mA at room temperature. The size distribution of the CuONPs and zeta potential measurements were performed using a Zetasizer Nano-ZS [Malvern Instruments]. Before size distribution and zeta potential measurements, CuONPs were suspended in deionized water at a concentration of 1 mg/10 mL and then sonicated using a sonicator at room temperature for 20 min at 40 W form a homogeneous suspension.

Catalytic Properties

A reduction of MB was observed in the presence of $NaBH_4$ and CuONPs catalysis. 10 mL of water, 1 mL of 10 mM 4MB, and 1 mL of 1mM $NaBH_4$ were mixed in a tube at 25 °C. Then, 1 mL of 0.01% CuONPs solution was added to this mixture. Samples were taken from the obtained mixture at intervals of one minute, and measurements were made between 200-800 nm by UV-Vis region absorption spectrophotometer, and reaction monitoring was performed. Also, MB degradation (%) was calculated using the following equation.

$$\text{Dye degradation (\%)} = \left(\frac{C_0 - C_t}{C_0} \right) \times 100 \quad (1)$$

C_0: Initial concentration of MB
C_t: Concentration of the dye solution after 45 minute
All dye concentrations were measured by UV-Vis absorption spectrophotometer. Results were given as the average of the three measurements.

Antibacterial Activity

In vitro antibacterial activity studies were performed against *Staphylococcus aureus (ATCC 25925), Escherichia coli (ATCC 25923), Acinetobacter baumannii (ATCC 02026), Bacillus subtilis (ATCC 6633)* and *Aeromonas hydrophila (ATCC 95080)* bacteria using the resazurin microplate method (resazurin microtitre assay-REMA) (26). Ampicillin was used as the standard drug in these studies. The color change of resazurin from blue to pink or colorless was considered positive and, therefore, an indication of bacterial growth. The minimum inhibition concentration (MIC) value was determined as the lowest concentration that prevents the resazurin from blue to pink or colorless. All antibacterial activity assays were repeated three times.

RESULTS AND DISCUSSION

UV-Vis Analysis

UV-Vis spectra were taken to evaluate the optical properties of the CuONPs obtained by biosynthesis. It was observed that the solutions of CuONPs obtained in 1 mg / 10 mL water gave characteristic plasmon band at approximately 220 nm at room temperature. Surface plasmon absorbance due to the collective oscillation of the free conduction band electrons was very sensitive to determine CuONPs, which was in good agreement with previous works (27, 28). Figure 2 shows the UV-Vis spectra of the CuONPs and plant extract.
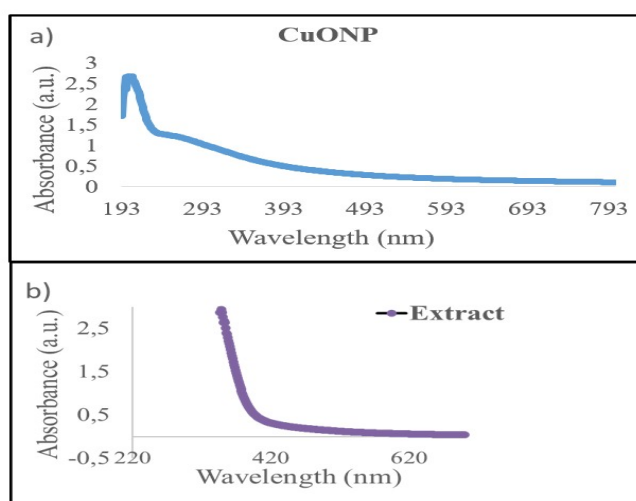
Figure 2. UV-Vis spectra a) CuONPs b) Extract of *Onosma Sericeum Willd*.

FESEM Image and Particle Size Distribution
FE-SEM analyses were performed to determine the surface morphology and particle size of the obtained CuONPs. The FE-SEM images showed that the CuONPs were spherical and approximately 70 nm in size. The dynamic light scattering (DLS) analysis results for size distribution were also in support of the FE-SEM results (Figure 3). The particle size was ranging from 70 ± 6 nm in DLS measurements, which is an excellent agreement to FESEM particle size. DLS results were calculated by taking the average of the three measurements and standard deviation.
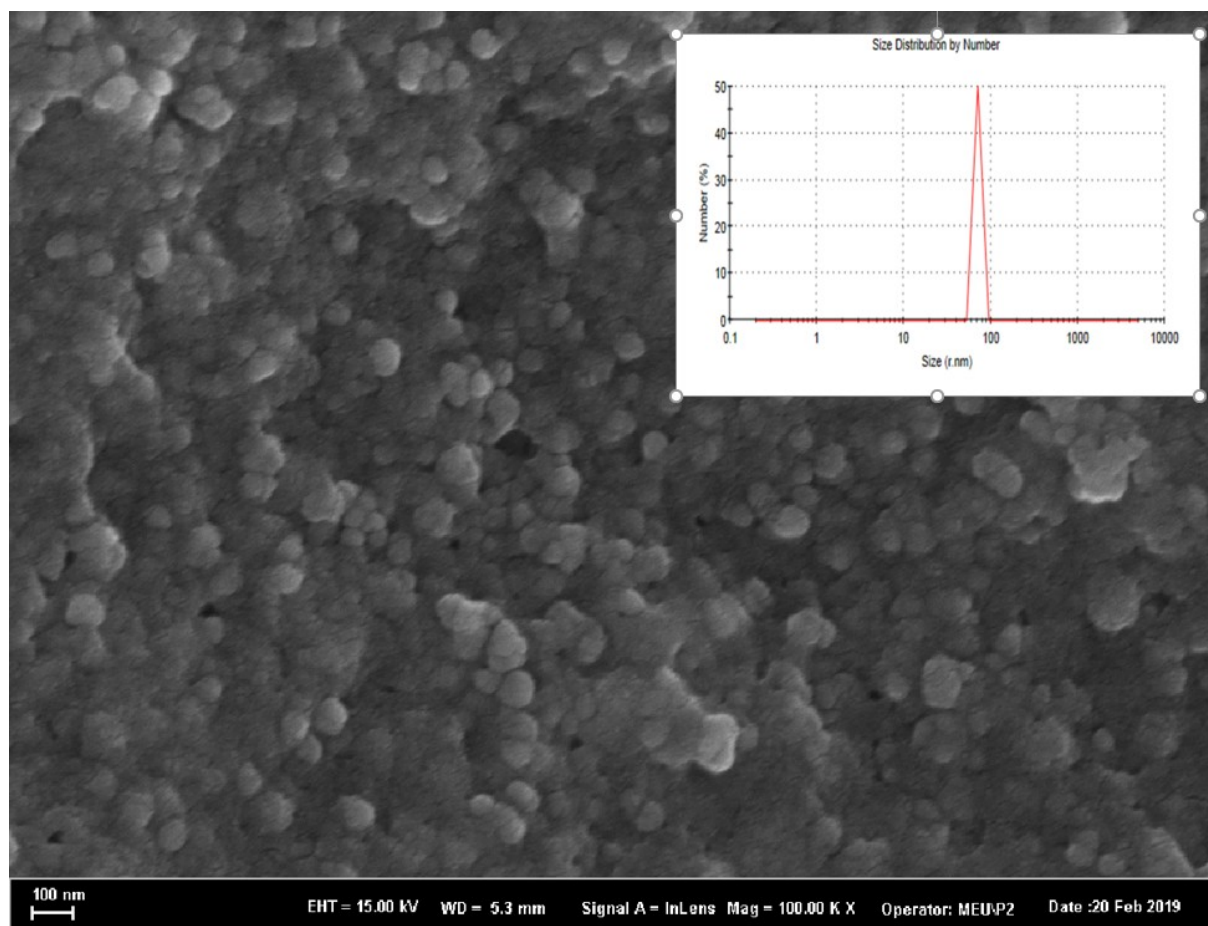
Figure 3. FESEM image and particle size distribution of CuONPs.

Zeta Potential Measurements
Zeta potential is an important parameter related to the stability of nanoparticles. The negative value of the zeta potential reveals that the CuONP negatively charged growths are coated and that electrostatic interaction between these nanoparticles may be responsible for the long-term stability of metal nanoparticles by preventing possible aggregation (28). In this study, the biomolecules present in the *Onosma Sericeum Willd* plant as inducing and stabilizing agents are thought to be responsible for the electrostatic interaction between the species. The zeta potential (ζ-Pot, mV) for the CuONPs was measured as -13.7 ± 1.55 at 25 ° C (Figure 4). Zeta potential results were calculated by taking the average of the three measurements and standard deviation.
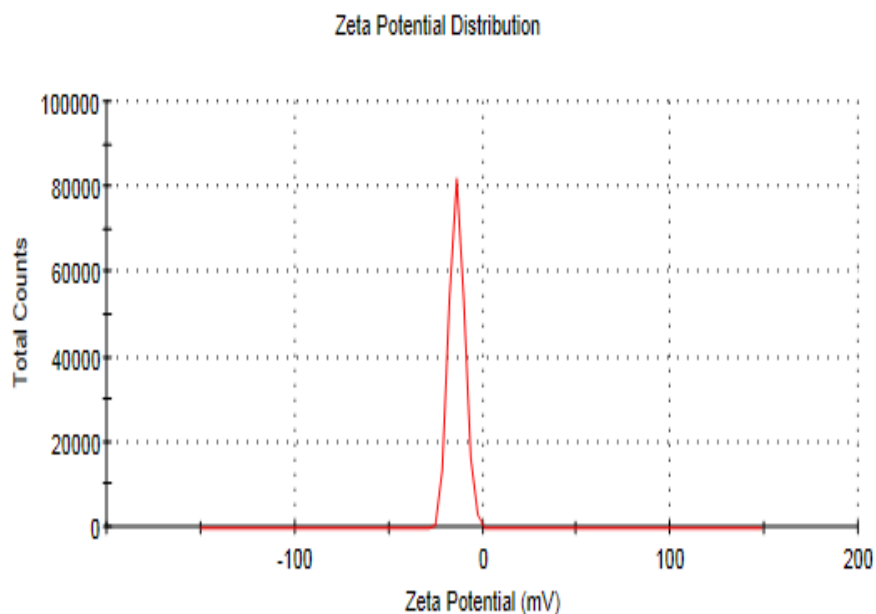
Figure 4. Zeta potential of CuONPs.

XRD Analysis

The powder X-ray diffraction technique was used to illuminate the CuONPs crystal structures obtained by biosynthesis. The diffraction patterns of XRD analyses, which were conducted to clarify the crystal structures, led to peaks observed at (111), (200), (202), (020), (202), (113), (311), (220) and (400).

These peaks confirmed the presence of CuONPs. The obtained XRD patterns were found to match those in the database of the Powder Diffraction Standards Joint Committee (JCPDS) file no: 89-7102. The information obtained was in agreement with the literature (29). The XRD spectrum obtained for the CuONPs is as shown in Figure 5.
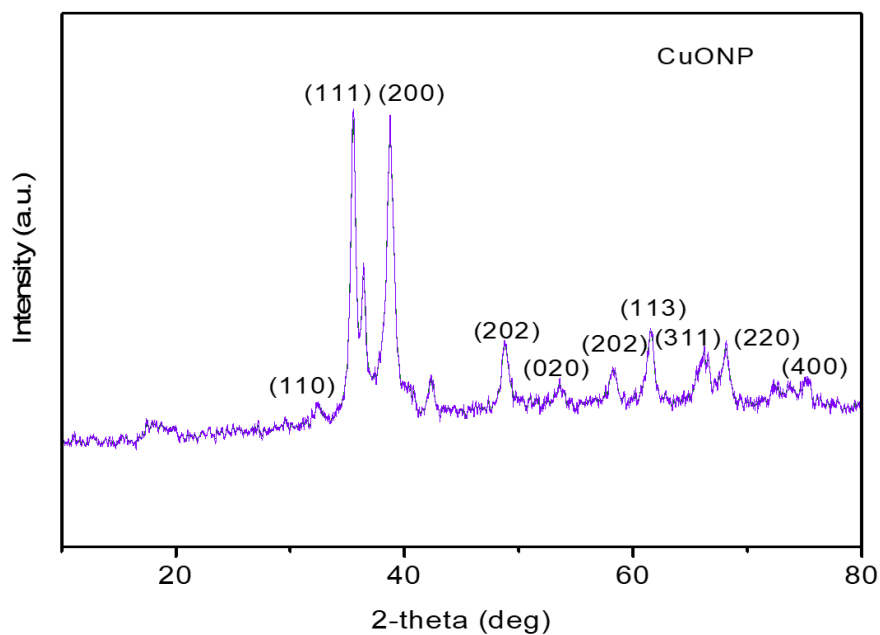
Figure 5. XRD spectra of CuONPs.

Catalytic Activities
As described in catalytic properties, it was found that four characteristic peaks of MB at 246, 292, 613, 664 nm changed only slightly before the addition of CuONPs. However, after adding 0.01% CuONPs solution to the same mixture, the absorbances were reread at specific intervals. In the first 45 minutes, the characteristic blue color of MB was significantly reduced, and the absorbance intensity decreased (Figure 6).
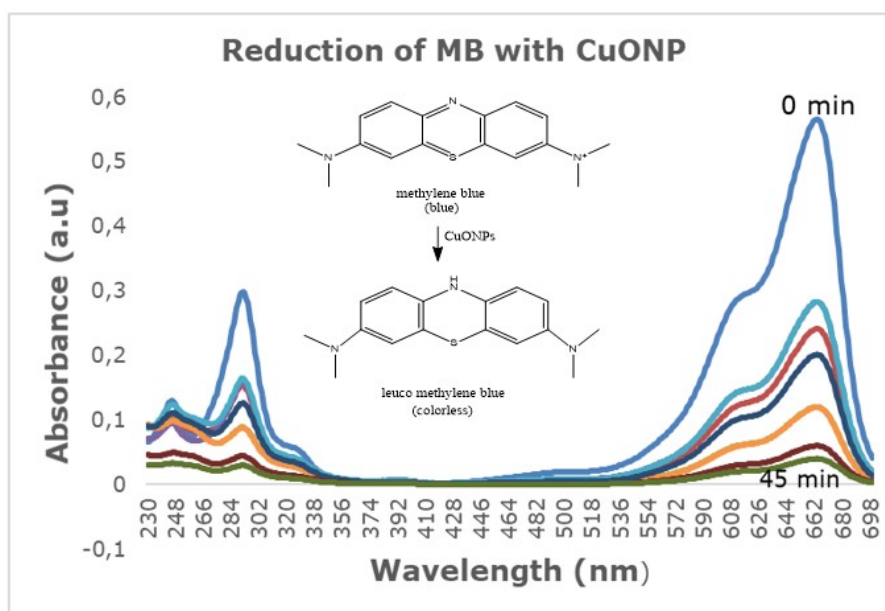
Figure 6. Reduction of MB with CuONP.

As a result of calculations, according to Equation 1, the rate of removal of >90% observed in 45 minutes. Schematic representation of degradation mechanism MB to leuco-methylene blue is given in Figure 7.
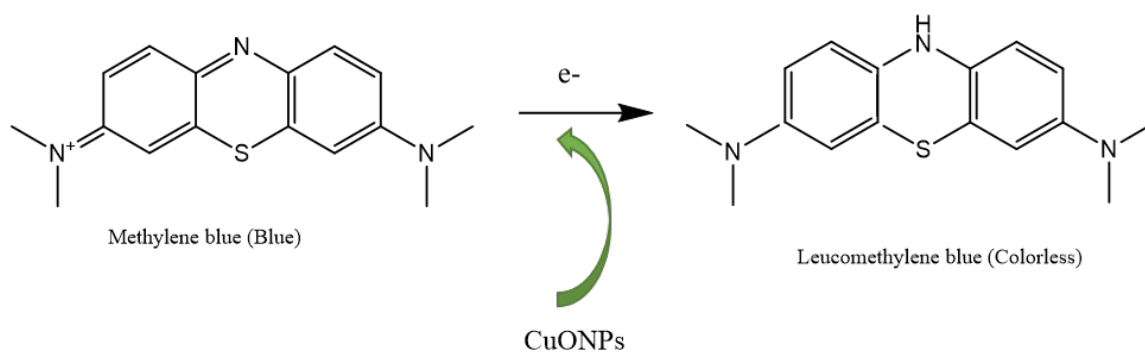
Figure 7. Schematic representation of degradation MB to leuco methylene blue.

MB is a heterocyclic aromatic industrial pollutant-dye, which can be reduced to leuco-methylene blue in the presence of $NaBH_4$, but the reduction rate is prolonged. In this study forty-five minutes after the addition of the CuONPs to the dye, the absorbance is gradually decreased. The results show that CuONPs contribute to electron transfer, which plays an essential role in the reduction of methylene blue to leuco methylene blue. These results are in agreement with the late studies on catalytic degradation of MB using biosynthesized CuONPs (29).

Antibacterial Activity
The results obtained by determining antibacterial activity using the resazurin microplate method are summarized in Table 1. The CuONPs showed antibacterial activity at a concentration of 250 µg / mL for *Bacillus*

subtilis (ATCC 6633), 500 µg / mL for *Staphylococcus aureus (ATCC 25925)*, *Escherichia coli (ATCC 25923)* and *Aeromonas hydrophila (ATCC 95080)* and 500 µg / mL for *Acinetobacter baumannii (ATCC 02026)*. The results determined that the CuONPs showed lower antibacterial activity against the standard drug Ampicillin. However, the increase of

CuONPs concentration also increases the antibacterial effect. It is due to the direct interaction between CuONPs and the external membrane surface of the bacteria (30). However, since high concentrations can cause toxic effects *in vivo*, these effects should be investigated in further studies.

Table 1. The MIC values (µg/mL) of CuONP against the bacteria.

Copper oxide Nanoparticle	*Staphylococcus aureus (ATCC 25925)*	*Escherichia coli (ATCC25923)*	*Acinetobacter baumannii (ATCC 02026)*	*Bacillus subtilis (ATCC 6633)*	*Aeromonas hydrophila (ATCC 95080)*
CuONP	500	500	>500	250	500
Ampicillin	31.25 µg/mL	15.62 µg/mL	125 µg/mL	0.9 µg/mL	31.25 µg/mL

CONCLUSION

In this study, CuONPs were successfully prepared using the extract of *Onosma Sericeum Willd (Boraginaceae)* in a simple, economical, and environmentally friendly way. The synthetic process was advantageous to other conventional methods because this process did not require any organic solvent or toxic reagents. The synthesized nanoparticles were characterized by UV-Vis spectrophotometer, FE-SEM, and XRD. The particle size distribution and zeta potential measurements of the CuONPs were determined by the DLS method. The obtained CuONPs were found to have a spherical structure and an average particle size of 70 nm. The synthesized CuONPs showed antimicrobial activity against *Staphylococcus aureus (ATCC 25925)*, Escherichia coli (ATCC25923), *Acinetobacter baumannii (ATCC 02026)*, *Bacillus subtilis (ATCC 6633)*, *Aeromonas hydrophila (ATCC 95080)* at high concentration. The toxic effect of CuONPs, which increased antibacterial activity following the increase in the dose of CuONP, should be investigated in the future for *in vivo* studies. It also showed that the CuONPs performed excellently in the catalytic reduction of methylene blue. At room temperature, up to 90% MB dye degradation was achieved. In conclusion, it can be said that these nanostructures are candidate molecules for biomedical and biotechnological applications.

FUNDING

ACKNOWLEDGEMENTS

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Development of Gelatin-Alginate-TiO₂-SOD Biosensor for the Detection of Superoxide Radicals

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Abstract: In this work, a biosensor that uses gelatin and alginate hydrogels in addition to titanium dioxide (TiO_2) nanoparticles (NPs) as a sensor matrix was developed to detect superoxide radicals ($O_2^{\bullet-}$), which play a role in carcinogenesis when present in excess levels. Parameters affecting the performance of the biosensor such as the amount of gelatin-alginate ratio, amount of TiO_2 NPs, the concentration of SOD enzymes, and glutaraldehyde (GA) cross-linker were investigated. Chronoamperometry was used as an electrochemical technique for the development of biosensor as well as characterization steps. The developed biosensor exhibited two linear ranges between 0.0009 mM – 0.125 mM and 0.25 mM – 2 mM, which was utilized as calibration curves. The detection limit of the biosensor was found 0.9 μM, which was at the appropriate level for the detection of $O_2^{\bullet-}$ in tumor samples. Finally, the constructed biosensor showed significant analytical performance, such as low detection limit, reusability, and reproducibility.

Keywords: Hydrogel, nanoparticle, biosensor, superoxide dismutase, cancer.

INTRODUCTION

Reactive oxygen species (ROS) are a class of highly reactive reduced oxygen molecules and include various structures such as superoxide anions, hydroxyl radicals, and hydrogen peroxide (H_2O_2). Superoxide radicals, the primary species of ROPs, are present in low concentrations under normal physiological conditions, and they are converted to H_2O_2 and oxygen (O_2) as a result of dismutation with superoxide dismutase (SOD) enzymes (1).

Superoxide anions are often considered to be toxic since they are converted to other radicals in biological systems such as alkoxyl radicals ($RO^{\bullet}$), hydroxyl radicals ($HO^{\bullet}$), and peroxynitrite radicals ($ONOO^-$). Increased superoxide anions result in mutagenesis and consequently, cell death (2-4). Besides, the concentration of superoxide may increase significantly in traumatic brain injuries (5,6). Superoxide radicals can also emerge as a consequence of gradual neurodegenerative

damages such as aging (7), cancer (8), and Parkinson's (9). Taken all together, developing sensitive platforms and tools towards detecting superoxide anions is essential.

So far, different sensing strategies have been followed for constructing biosensor systems aiming for the detection of superoxide anions. Fluorescence (10) and electrochemical (11) techniques are the most widely preferred ones. The biosensors developed by the modification of electrodes with hydrogels, without utilizing NPs, resulted in an insufficient detection limit (12, 13), while other designs containing nano-structures require multi-component recognition material (14, 15) or complex fabrication processes (16), which reduces the feasibility of the developed system. Moreover, a big part of the superoxide biosensors focusses on biosensor design but validating the system with biological samples such as blood or tissue (12, 14). Therefore, we need simple, low-cost, and sensitive biosensing tools for the determination of the level of superoxide anions in

biological samples, which might give information about the development of cancer.

Though enzymes catalyze biochemical reactions with unique celerity, they lack electrical conductivity due to their protein-nature, and therefore coupling enzymes with metal NPs might be an excellent strategy to enhance electron transfer to the electrode. By now, several types of NPs - such as Ag (17), Au (18), ZnO (19), and TiO_2 (20) NPs - have been used to develop biosensors. TiO_2 NPs have attracted a critical interest in their advantages, having a large surface area, high uniformity, and biocompatibility such that TiO_2 NPs have been used as a component of the sunscreen-technology as well as they are well accepted in the food industry. Researchers demonstrated a direct electron transfer between biomolecules and electrodes modified with TiO_2 NPs (21, 22). The improved catalytic activity of the developed biosensors has been attributed to their smaller sizes, enabling a larger surface area for biomolecule immobilization and a faster electron transfer from biomolecule to an electrode surface. All these superior properties make TiO_2 NPs a suitable material for electrode design.

In this work, we focused on creating SOD-based biosensor for fast, specific, easy, and sensitive detection of superoxide anions. To this end, we combined gelatin and alginate biopolymers as a carrier system and utilized from TiO_2 NPs to construct a nanocomposite material that brings various advantages such as high surface area, which enabled us to increase SOD immobilization efficiency, and semi-conductivity. SOD enzymes were bonded onto this nanocomposite material, which was coated onto platinum (Pt) electrode, via crosslinking with glutaraldehyde (GA) reagent. The developed gelatin-alginate-TiO_2-SOD biosensor was then tested for detecting superoxide radicals in cancer tissues.

EXPERIMENTAL SECTION

Preparation of gelatin-alginate-TiO_2-SOD biosensors
Gelatin-alginate-TiO_2-SOD electrodes were prepared following the previously published protocols (12, 23) -with small modifications-. After the gelatin was dissolved at 50 °C, different amounts of alginate and titanium dioxide (obtained from Sigma-Aldrich, with diameter <25 nm, in anatase form) were added and homogenized by vortexing. SOD enzymes dissolved in phosphate buffer were added to different units and re-vortexed for homogenization. Finally, the cross-linker glutaraldehyde was added to obtain stiff immobilization gel. The gels (25 μL) were modified on each surface of the Pt working electrode – square plate having 1 cm^2 surface area- with a total of 50 μL of gel, and were allowed to dry for 6 hours. Un-bonded enzymes were washed away with PBS for 3 times. Each step of electrode modification included coating the electrode with

gelatin-alginate, gelatin-alginate-SOD, gelatin-alginate-TiO_2, and gelatin-alginate-TiO_2-SOD were characterized with electrochemical impedance spectroscopy (EIS) technique. Pt square plate electrode with 1 cm^2 surface area was used as a counter electrode, and Ag/AgCl with saturated KCl was used as a reference electrode, which was introduced into the cell via a Luggin capillary. All electrochemical measurements were performed with Gamry Potentiostat-Galvanostat with Framework Version 5.50.

Optimization of gelatin-alginate-TiO_2-SOD biosensors
Firstly, different gelatin-alginate ratios (0.25, 0.5, 1, 2) were tried to optimize the immobilization conditions. TiO_2, SOD enzyme, and glutaraldehyde concentrations were kept constant in the immobilization gel. To optimize the cross-linking process, different electrodes were prepared by using different glutaraldehyde concentrations (0,001, 0,002, 0,004, 0,01, and 0,016 M). Also, to obtain an efficient electrochemical performance from the developed biosensors, amperometric responses were recorded for electrodes prepared with varying enzyme concentrations that change between 25 and 5000 U, which provided an optimal enzyme concentration. For TiO_2 NPs optimization, different amount of TiO_2 NPs (0.0001 - 0.0005 g) were tested by keeping the gelatin-alginate ratio (1 w/w), SOD concentration (100 U), and glutaraldehyde cross-linker concentration (0.004 M) constant. Finally, the level of xanthine, triggering the dismutation reaction, was optimized (0.0009-2 mM).

Reproducibility and reusability of biosensor
Amperometric measurements were recorded for 6 replica electrodes to examine the reproducibility of the gelatin-alginate-TiO_2-SOD biosensor, and consistency of the amperometric responses was examined. On the other hand, for reusability study of the developed biosensor, amperometric measurements were obtained every day, up to 10 days, against xanthine (2 mM) at +0.65 V. After each use, the electrodes were washed with phosphate buffer (0.05 mM, pH 7.4) and stored in the buffer at +4 °C until further use. Reproducibility and reusability of the biosensor were determined by interpreting the amperometric responses.

Obtaining the calibration graphs
A series of gelatin-alginate-TiO_2-SOD biosensors were prepared, and amperometric measurements were recorded for varying xanthine concentrations at +0.65 V. The obtained current densities were plotted against xanthine concentration to obtain calibration curves and equations. The sensing limit and linear ranges were determined from the obtained graphs. During the calibration study, the electrodes were stored in the phosphate buffer (pH 7.4) at +4 °C until use.

Clinical application of gelatin-alginate-TiO$_2$-SOD biosensor

Cancerous meningioma (grade I) tissue was obtained from Ankara University, Department of Neurosurgery. Biosensor response was tested on healthy and cancerous brain tissues to demonstrate the usability of the developed biosensor in biological applications. After stabilizing the gelatin-alginate-TiO$_2$-SOD biosensors in the electrochemical cell containing phosphate buffer (0.05 M, pH 7.4), homogenized solutions containing healthy or cancerous tissue were added, and the changes in amperometric signal were determined.

RESULTS and DISCUSSION

Characterization of gelatin-alginate-TiO$_2$-SOD electrodes
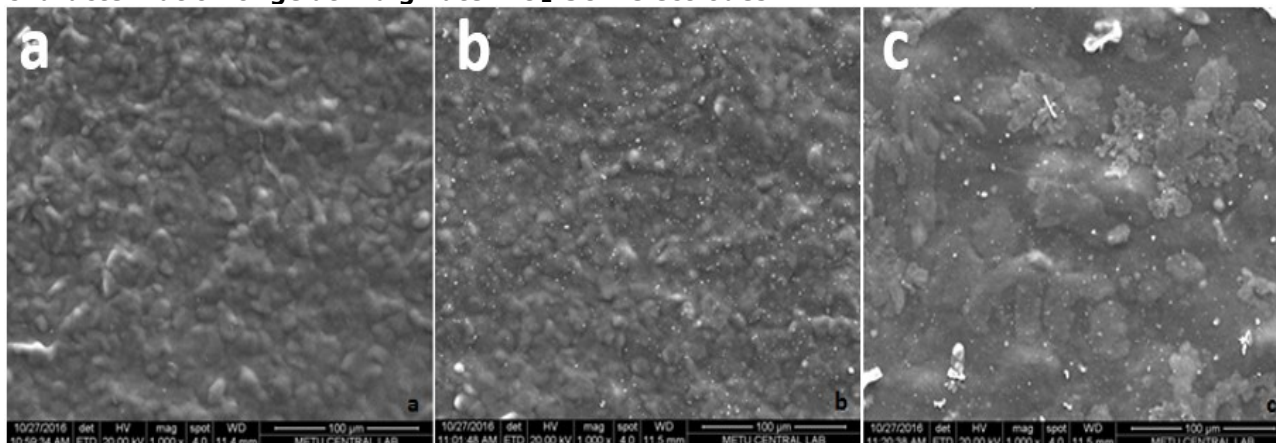
Figure 1. SEM images of gelatin-alginate (a), gelatin-alginate-TiO$_2$ (b), and gelatin-alginate-TiO$_2$-SOD (c) electrodes. Scale bars 100 μm.

The design of surface topography is one of the critical parameters for building a successful biosensor. SEM images were obtained in comparison with gelatin-alginate and gelatin-alginate-TiO$_2$ modified electrodes to examine the surface properties of the developed gelatin-alginate-TiO$_2$ electrode. It was seen in the micrograph that the gelatin-alginate biopolymer mixture covered the electrode surface properly (Figure 1a) and evaluated as a proper matrix in this regard. When TiO$_2$ NPs were included in the biopolymer mixture, microstructures were turned in to lower-scale microstructures (Figure 1b) due to surface enhancing property of nanoparticles, and resulted in a suitable nanocomposite material for biosensor design. Some nanoparticle aggregates were visible on this micrograph. When SOD enzymes included via cross-linking to the system, besides some little salt crystals, SOD enzymes characterized by leaf-shaped appearance seemed efficiently immobilized onto the surface (Figure 1c). Hence, it can be concluded that the gelatin-alginate-TiO$_2$-SOD electrode was successfully prepared, and in terms of surface properties, it seems suitable for biosensing applications.

Electrode modifications were also characterized by an electrochemical technique, namely electroimpedance spectroscopy (EIS). The Nyquist plots of the bare electrode, gelatin-alginate electrode, gelatin-alginate-SOD electrode, gelatin-alginate-TiO$_2$ electrode, and gelatin-alginate-TiO$_2$-SOD electrode were obtained for a Randles circuit model (Figure 2). Each EIS spectrum consists of a semicircle and a linear portion, which correspond to a charge transfer and diffusion process, respectively. The diameter of the semicircle is related to the charge-transfer resistance (R$_{ct}$) at the interface of the electrode surface and solution and can be used to evaluate the processes taking place on the electrode surface. Thus, in our strategy, change in R$_{ct}$ values helps us to understand the success of modification steps. Unless conducting or nano-materials used, R$_{ct}$ increases after electrode modifications due to the decreased electron transfer. While the charge transfer resistance of the bare electrode was nearly 25Ω (Figure 2a), this value increased to 56Ω when the Pt electrode was modified with gelatin-alginate composite, which shows the success of the modification (Figure 2b). SOD immobilization into gelatin-alginate composite hydrogel was further improved the R$_{ct}$ from 56 Ω to 68 Ω (Figure 2c), indicating an efficient immobilization of SOD enzymes into the gelatin-alginate composite structure. Incorporation of TiO$_2$ NPs decreased R$_{ct}$ of gelatin-alginate, 56 Ω, to 48 Ω (Figure 2d) as a result of both enhanced surface area owing to nano-size of the particles and their semi-conductive property. When SOD enzymes were immobilized into gelatin-alginate-TiO$_2$ nanocomposite structure, R$_{ct}$ was increased from 48Ω to 66Ω (Figure 2e), which showed the success of the SOD immobilization. This increase in R$_{ct}$ after SOD immobilization into gelatin-alginate-TiO$_2$ (about 18 Ω) was higher than that of SOD immobilization into gelatin-alginate (about 12 Ω), indicating a more efficient SOD immobilization in gelatin-alginate-TiO$_2$.
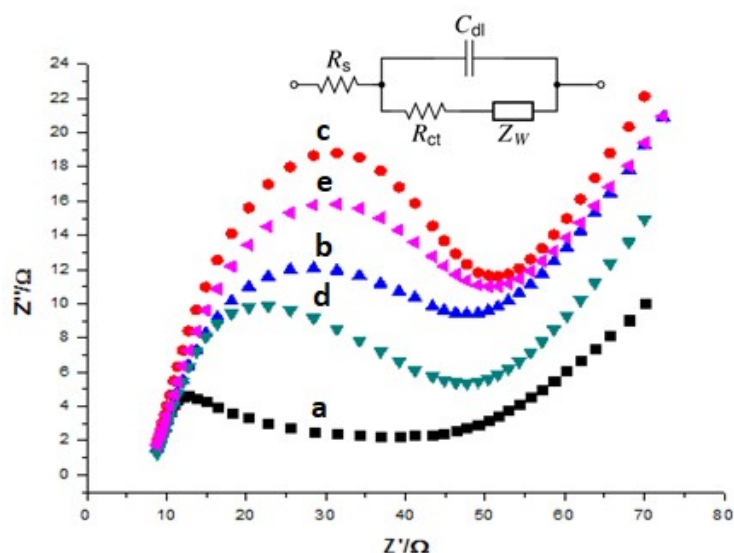
Figure 2. Nyquist Plot showing the change in charge transfer resistance after each modification step: (a) Bare electrode, (b) gelatin-alginate coated electrode, (c) gelatin-alginate-SOD coated electrode, (d) gelatin-alginate-TiO_2 modified electrode, and (e) gelatin-alginate-TiO_2-SOD electrode

Lastly, we obtained the Fourier-Transform Infrared (FTIR) spectra of gelatin-alginate-SOD and gelatin-alginate-TiO_2-SOD for further evaluate the effect of TiO_2 NPs on enzyme immobilization. Both spectra indicated the characteristic peaks of alginate and gelatin biopolymers (Figure 3). The peak near 1028 cm^{-1} was of C-O stretching vibration of pyranose rings constituting the alginate, while the peaks at 1530 and 1637 cm^{-1} related to amide I and II born of gelatin (24). Both spectra exhibited the peaks related to alginate and gelatin, which showed the success of composite preparation. Due to the chemically inert nature of TiO_2 NPs, no specific peak related to TiO_2 NPs was observed. When the spectra of gelatin-alginate-SOD and gelatin-alginate-TiO_2-SOD compared, a slight increase in amide I and II bands in the spectrum of gelatin-alginate- TiO_2-SOD can be seen clearly. This slight increase in amide I and II bands in gelatin-alginate- TiO_2-SOD can be attributed to a higher enzyme immobilization via amide bond formation between gelatin (NH_2-) and enzyme –via glutaraldehyde crosslinker –. This finding consolidated our previous findings and led us to conclude that including the TiO_2 NPs into the system improved enzyme immobilization.
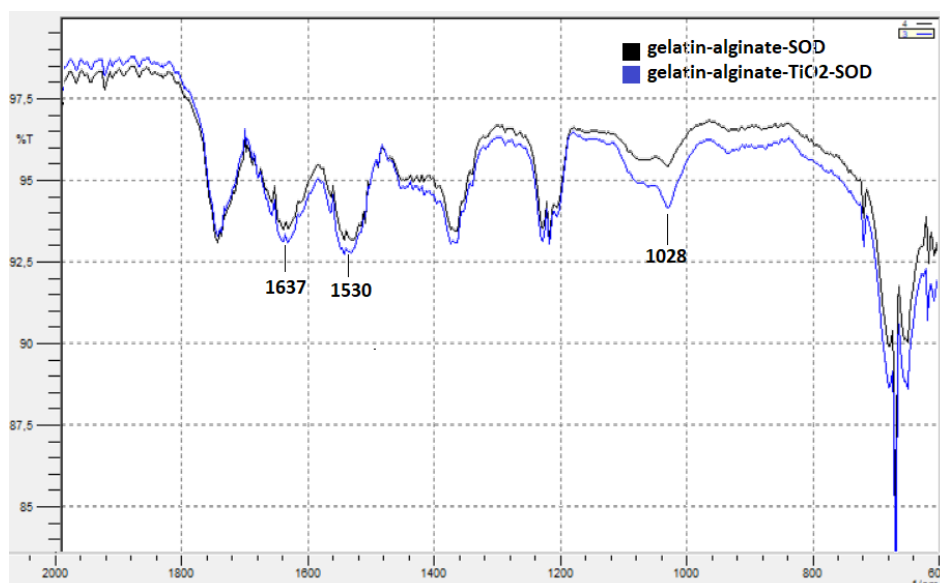
Figure 3. FTIR Spectra of gelatin-alginate-SOD (black) and gelatin-alginate-TiO_2-SOD (blue)

Optimization of gelatin-alginate-TiO_2-SOD biosensor
There are many parameters, namely biopolymer, crosslinker, nanostructure, and bio-recognizing element concentrations which affect biosensor performance, and need to be optimized before further experiments. To this end, we tested different ratios of gelation: alginate biopolymers (0.25-2 w/w) as well as different concentrations of GA cross-linker (0.001-0.016 M), SOD enzyme (25-5000 U), and TiO_2 NPs (0.0001-0.0005 g).

For the optimization of gelatin: alginate ratio, we prepared different electrodes with varying concentrations of gelatin: alginate ratio (0.25-2 w/w) by keeping the GA (0.01 M), SOD (100 U), and TiO_2 (0.0003 g) constant. The optimal gelatin: alginate ratio was found to be 1 (w/w) at which the most efficient amperometric response was recorded (Figure 4a). At higher ratios, as a result of the change in the pore structure/size and thus the reduced electron transfer, a decrease in sensitivity was observed. On the other hand, at the lower ratios (0.25-0.5 w/w), similar reduced amperometric responses were observed unlikely due to mechanical instability. For an efficient enzyme immobilization, different GA cross-linker concentrations (0.001-0.016 M) were tested by keeping the gelatin: alginate ratio (1 w/w), SOD (100 U), and TiO_2 (0.0003 g) constant. 0.004 M GA was found to be the optimal concentration (Figure 4b). The increase in GA concentrations led to more efficient enzyme immobilization, thus a more intense amperometric current as a result of larger electrochemical reactions. However, when we further increased the GA concentration, a decrease in amperometric response was observed mainly due to enzyme inactivation and reduced electron diffusion through electrode surface due to smaller pore size (Figure 4b) (23). The concentration of bio-recognizing elements, SOD enzymes, in this case, is another critical parameter for the electrochemical performance of enzymatic biosensors. Amongst different SOD concentrations (25-5000 U), the optimal value was determined as 100 U (Figure 4c) by keeping the gelatin: alginate ratio (1 w/w), GA (0.004 M), and TiO_2 (0.0003 g) constant. In lower SOD concentrations, lower current density was observed as a consequence of inadequate enzyme concentrations. In contrast, when we increased the SOD concentration over the optimal value, at this time, decreased current density was obtained due to enzyme-enzyme cross-linking in addition to over-saturation of pores with an excessive amount of enzymes that result in not-efficient diffusion of products and electrons through electrode surface (23). Finally, we optimized the amount of TiO_2 NPs, which were used to increase SOD immobilization and electron transfer rate by increasing the surface area. By keeping the gelatin: alginate ratio (1 w/w), GA cross-linker (0.004 M), and SOD (100 U) concentrations constant, we tested different amounts of TiO_2 NPs (0.0001 - 0.0005 g) and found the optimal value to be 0.0004 g (Figure 4d). Low amounts of nanoparticles were not able to increase the surface area as much as necessary, while too high amounts of nanoparticles completely covered the polymer surface, increased resistance, and inhibited electron transfer.
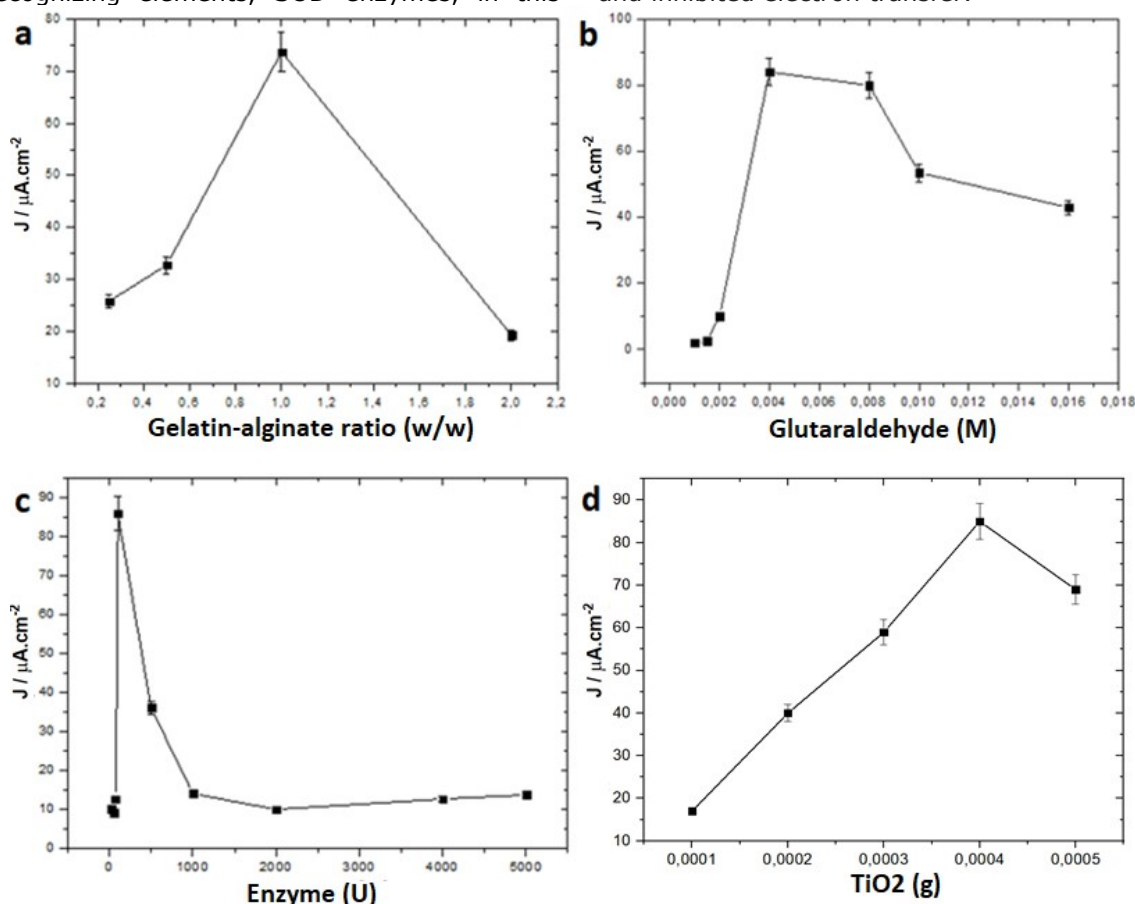

Figure 4. Effect of gelatin: alginate ratio (a), GA concentration (b), SOD concentration (c), and amount of TiO_2 (d) on the amperometric response of biosensor.

Reproducibility and reusability of the developed biosensor

Reproducibility and reusability are two parameters that are important in the practical use and commercialization of biosensors. To investigate the reproducibility and reusability potentials of the developed biosensor, we prepared 6 and 3 replicas, respectively, of a gelatin-alginate-TiO_2-SOD biosensor. For reproducibility study, after the 6 replica electrodes were stored at 4 °C for 1 day, amperometric measurements were performed by adding 2 mM xanthine into the electrochemical cell. Each measurement was performed triplicate. For the reusability study, amperometric measurements were recorded in the presence of 2 mM xanthine up to 10 days. After each use, electrodes were washed with phosphate buffer (pH 7.4) and stored in the refrigerator at 4 °C.

The mean value and standard deviation for amperometric measurements with 6 replica electrodes in the context of reproducibility study were found to be 67.5 $\mu A.cm^{-2}$ and 5.8%, respectively (Figure 5a). This finding showed that gelatin-alginate-TiO_2-SOD electrodes are reproducible with trust. On the other hand, the amperometric response of the gelatin-alginate-TiO_2-SOD electrodes decreased to % 61.6 of its initial performance (Figure 5b). This finding suggests that the developed biosensor has short-term reusability, up to 7 times, after which the response go beyond 70%. One reason why the reusability of the gelatin-alginate-TiO_2-SOD electrode was not found better might be the distortive effect of NPs, which lead to detachment of nanocomposite film from the surface.

Figure 5. Reproducibility (a) and reusability (b) of gelatin-alginate-TiO_2-SOD electrodes.

Calibration study for gelatin-alginate-TiO_2-SOD electrode

Calibration study that defines the lower/higher measurement limits of a biosensor is another key step in biosensor design. To obtain calibration graph for gelatin-alginate-TiO_2-SOD electrode, we tested a broad range of xanthine concentration varying between 0.0009-2 mM. The amperometric current densities obtained within this range showed two linear graphs for 0.0250-2 mM (Figure 6a) and 0.0009-0.0125 mM (Figure 6b). Linear regression equations were expressed for gelatin-alginate-TiO_2-SOD electrode as [J, mM] = (-2.5) + (32.8) x [Xanthine, mM] for high xanthine concentrations (Figure 6a) and [J, mM] = (0.13) + (22.8) x [Xanthine, mM] for low xanthine concentrations (Figure 6b).
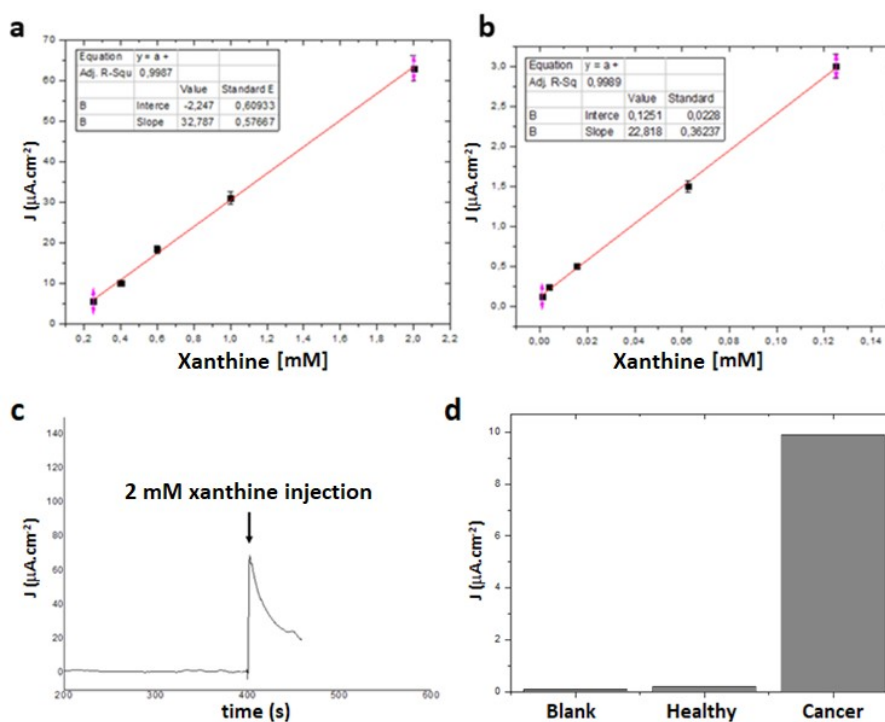
Figure 6. Calibration graphs of gelatin-alginate-TiO$_2$-SOD biosensor for high-range (a) and low-range (b) xanthine concentrations. (c) The amperometric response of gelatin-alginate-TiO$_2$-SOD biosensor after 2 mM xanthine injection. (d) Clinical application of gelatin-alginate-TiO$_2$-SOD biosensor.

The response of the electrode was proportional to the concentration of O$_2$$^{•-}$ and the coefficient of determination (R^2) was found 0.9987 and 0.9989 for high concentration range and low concentration range, respectively. The detection limit for the low linear range was determined as 0.9 µM. The amperometric response of gelatin-alginate-TiO$_2$-SOD biosensor after injection of 2mM xanthin was demonstrated in Figure 6c.

Clinical application of gelatin-alginate-TiO$_2$-SOD biosensor
The ultimate goal of biosensor studies is to apply the developed systems in human clinics to contribute to human health by providing easy-to-use, practical, and low-cost diagnostic tools. To examine the potential of the developed biosensor to be applied in clinics, we tested the electrodes in healthy and cancer tissues to detect superoxide radicals. The gelatin-alginate-TiO$_2$-SOD biosensor exhibited nearly 10-times higher amperometric response to cancer tissue homogenate compared to healthy tissue (Figure 6d). This result confirmed

our anticipation of the higher level of ROS in cancer cells and tissues compared to healthy ones (25, 26). Also, various groups utilized SOD-based biosensors for detecting superoxide radicals in cancer cells (27) and tissues (13, 28), and reported higher levels of superoxide radicals in the cancer samples. For instance, Campanella et al. (2000) has reported an approximately 15 times more intense amperometric response for cancerous renal tissue compared to healthy tissue (13). These reports support and consolidate our findings.

Various published works have lower or higher LOD and linear range compared to our design (Table 1). Our design exhibited a detection limit suitable to be used in superoxide detection; besides, it showed good performance when applied in real samples (cancer tissue). Consequently, the findings we obtained enabled us to conclude that the developed biosensor can be applied in cancer clinics after a comprehensive work, including repetitive biological trials.

Table 1. Comparison of gelatin-alginate-TiO$_2$-SOD sensors sensing with electrochemical (amperometry) with literature.

Electrode	LOD	Linear Range	Application	Reference
Gelatin-SOD	10.0 µM	0.02-2 mM	Cancer tissue	12
GC-PEDOT-CNT-SOD	1.0 µM	20-3000 µM	Wine/Juice	15
PtPd-PDA-RGO-SOD	2.0 µM	0.016-0.24 mM	-	29
GC-MPA-MnSOD	91.1 µM	135.2-1160 µM	-	30
Au-Fc-PEG-SOD	0.001 µM	5-100 µM	Cancer cells	16
Gelatin-Alginate-TiO$_2$-SOD	0.9 µM	0.0009-2 mM	Cancer tissue	This work

GC: Glassy Carbon, PEDOT: Poly(3,4-ethylenedioxythiophene), CNT: Carbon Nanotube, PDA: Polydopamine, RGO: Reduced Graphene Oxide, MPA: 3-mercaptopropionic acid, PEG: Polyethylene glycol, Fc: Ferrocene.

CONCLUSION

Superoxide radicals take place in many biological processes such as cancer, and their detection might be crucial in particularly cancer cases. In this work, it was aimed to develop a sensitive biosensor which uses gelatin-alginate-TiO$_2$ nanocomposite as an electrode material, and SOD enzyme as a biorecognizing element. After optimization and calibration studies, the developed sensor found suitable for detecting O$_2$$^{•-}$ in clinical samples. The developed biosensor might be a promising candidate to be applied in cancer clinics.

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Antioxidant Enzyme Activities in Ascorbic Acid and Selenium Applied Hepatocellular Carcinoma Cells

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Abstract: Ascorbic acid and selenium have potential use in the prevention of cancer. The role of antioxidant enzymes against cancer is to prevent oxidative damage. In this study, the effects of L-selenomethionine (234 µM), SeO_2 (100 µM), ascorbic acid (0,313 mM, and 31,3 mM) on antioxidant enzyme activities in HepG2 cell line were studied. GPx, CAT, and SOD activities of the treated cells were measured spectrophotometrically. There was a significant increase in the activity of GPx in HepG2 cells treated with L-selenomethionine (234 µM), SeO_2 (100 µM), and ascorbic acid (0,313 Mm and 31,3 mM). However, the activity of CAT was found significantly decreased in L-selenomethionine (234 µM), SeO_2 (100 µM), ascorbic acid (31,3 mM) treated HepG2 cells. The SOD levels of HepG2 cells treated with ascorbic acid (0,313 mM and 31,3 mM) were found higher than the control cells. The results of the present study indicated that the antioxidant enzyme activities changed depending on L-selenomethionine, SeO_2 application, and CAT activities changed depending on ascorbic acid doses.

INTRODUCTION

Free radical species are formed as a result of reactions of oxygen and may play a role in tumor formation (1). On the other hand, overproduction of reactive oxygen species (ROS) can also induce the apoptotic pathway in the tumor cells, and this approach has effectively been used as a critical point for anticancer treatment (2). Depending on tumor type and degree, cancer cells exhibit different redox statuses. Because of these differences, the cell may be affected differently by the oxidants (3). Since oxidative stress may involve the etiology of cancer, agents that can prevent oxidative stress such as exogenous antioxidants

have been extensively investigated to prove if they inhibit/reduce oxidative damage and prevent/slow cancer development (4). In addition to their protective effect on cancer, antioxidants like selenium (Se) and ascorbic acid (AA), exhibit pro-oxidant activity in cancer cells (5, 6). Se mediates ROS production in cancer cells, thus induces cytotoxicity (7, 8).

AA, a potent antioxidant, may act as a pro-oxidant when it reacts with metal ions in DNA. High doses of AA may prevent cancer, and it has been shown in limited studies that high dose ascorbate has promising results in the treatment of cancer (9). Because AA contributes to the production of superoxide and

may produce ascorbyl radicals, it is considered as an essential pro-oxidant of free radical reactions (6).

Selenium, which is an essential element for many biological functions, has been shown to have a protective effect against cancer (10, 11). Selenium has various biological activities (12, 13) and can be found as organically bound selenium forms (e.g., selenomethionine, selenocysteine) or inorganic forms (e.g., selenate, selenite) (14). Selenoproteins that contain organically bound selenium are found in living organisms, including humans, and some of their roles are well identified (15, 16, 17).

In the current study, it is aimed to investigate the effect of two different concentrations of AA and two different forms of selenium on antioxidant enzyme activities in the HepG2 cell line.

MATERIALS AND METHODS

Cell Culture

HepG2 cell line was used in this study (ATCC Cat No. HB- 8065). Cells were placed into 75-cm^2 tissue culture flasks and grown in RPMI 1640 Medium containing 10% Fetal Bovine Serum (FBS), 50 mg/l Gentamicin sulfate and 300 mg/l L-glutamine in a cell culture incubator at 37 °C in the presence of 5% CO_2.

For the determination of the effect of compounds in question on antioxidant enzyme activities, 0.313 mM and 31.3 mM AA (Merck, Cat No: 100468), 234 µM L-selenomethionine (Merck, Cat No: 561505), 100 µM SeO_2 (Sigma, Cat no: S-9379) concentrations, which were observed to significantly change the cell viability in previous studies (18, 19), were applied to HepG2 cells. Only RPMI 1640 Medium (containing 10% FBS, 50 mg/l Gentamicin sulfate and 300 mg/l L-glutamine) was applied to the control group.

Determination of Antioxidant Enzyme Activities

After treatments with AA, SeO_2 and L-selenomethionine for 24 h, cells were detached using a sterilized scrapper, lysed in ice-cold lysis buffer (0.1 M Tris/HCl, pH 7.4, containing 0.5% Triton X-100, 5 mM β-Mercaptoethanol, 0.1 mg/ml serine proteases inhibitor Phenylmethylsulfonyl fluoride) and centrifuged at 14.000 × g for 5 minutes at +4 °C. The supernatant was collected and kept at −80°C for CAT, SOD, and GPx analysis.

Superoxide dismutase (SOD) activities were determined using the SOD determination kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. GPx activities were measured by spectrophotometric analysis at 340 nm according to the procedure described by Paglia and Valentine 1967 (20). CAT activity was also measured spectrophotometrically by the method stated by Aebi 1983 (21). This method is based on the measurement of the rate of decrease in the absorbance at 240 nm as the catalase enzyme breaks hydrogen peroxide down into water and oxygen. The rate decrease in absorbance is proportional to catalase enzyme activity. The specific activity of catalase is defined as k/g protein. k value was calculated from the equation k=0.1175/t (t= Time in seconds, which is required for reduction of absorbance from 0.45 to 0.40).

Protein concentration in the cell lysate was determined by Bradford assay (22). All enzyme activity values were divided by protein concentration for specific activity determination. All experiments were repeated in triplicate for each group. Mean values were shown in Table 1.

Statistical Analysis

Statistical analysis between treated and control groups was performed with variance analysis (ANOVA), and differences between the groups were determined with the Duncan post hoc test. P values <0.05 were considered statistically significant.

RESULTS

GPx and SOD Enzyme Activities in HepG2 cells

GPx activities were significantly increased in HepG2 cells treated with 0.313 and 31.3 mM AA, 234 µM L-selenomethionine, and 100 µM SeO_2 compared to the control group (Figure 1). SOD activities were significantly increased in HepG2 cells at 0.313 and 31.3 mM of AA and 234 µM L-selenomethionine concentration compared to the control group (Figure 2). These results may indicate that increasing SOD activity 0.313 and 31.3 mM of AA and 234 µM L-selenomethionine have antioxidant effects in this concentration. Also, GPx levels are higher in L-selenomethionine-treated concentration than SeO_2. Hence, we may conclude that L-selenomethionine is less toxic or has more potent antioxidant properties than SeO_2, which is the inorganic form of selenium. The SOD activities of HepG2 cells treated with different doses of L-selenomethionine, SeO_2 and AA in comparison to the control group are presented in Figure 2.
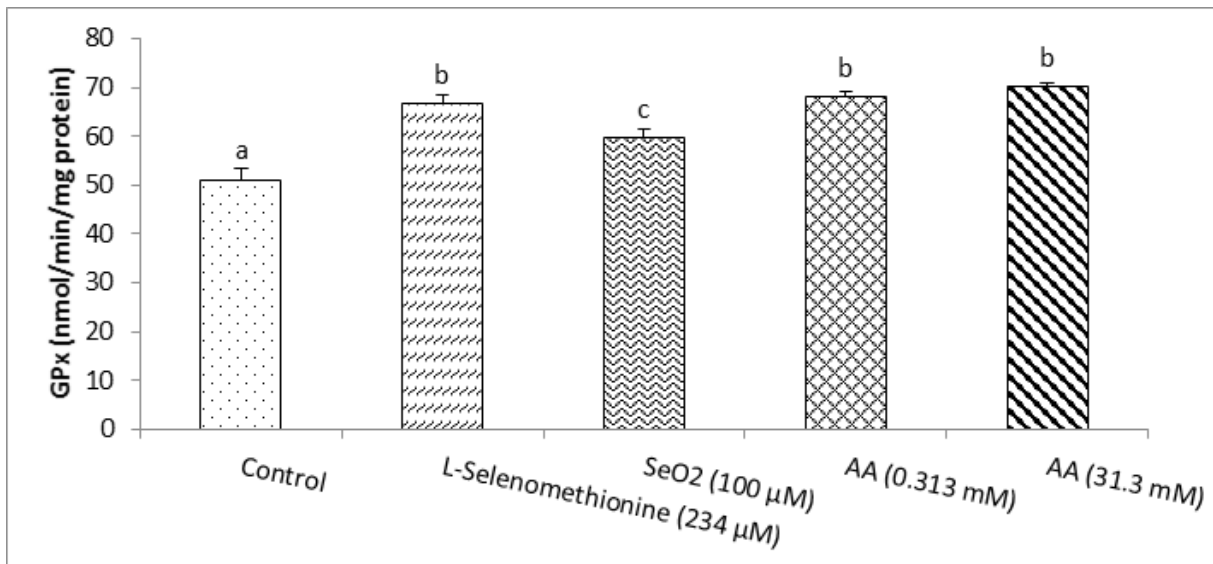
Figure 1. The GPx activities in HepG2 cells treated with 234 µM L-selenomethionine, 100 µM SeO$_2$, 0.313 mM, and 31.3 mM AA (Different letters indicate the differences between the groups).
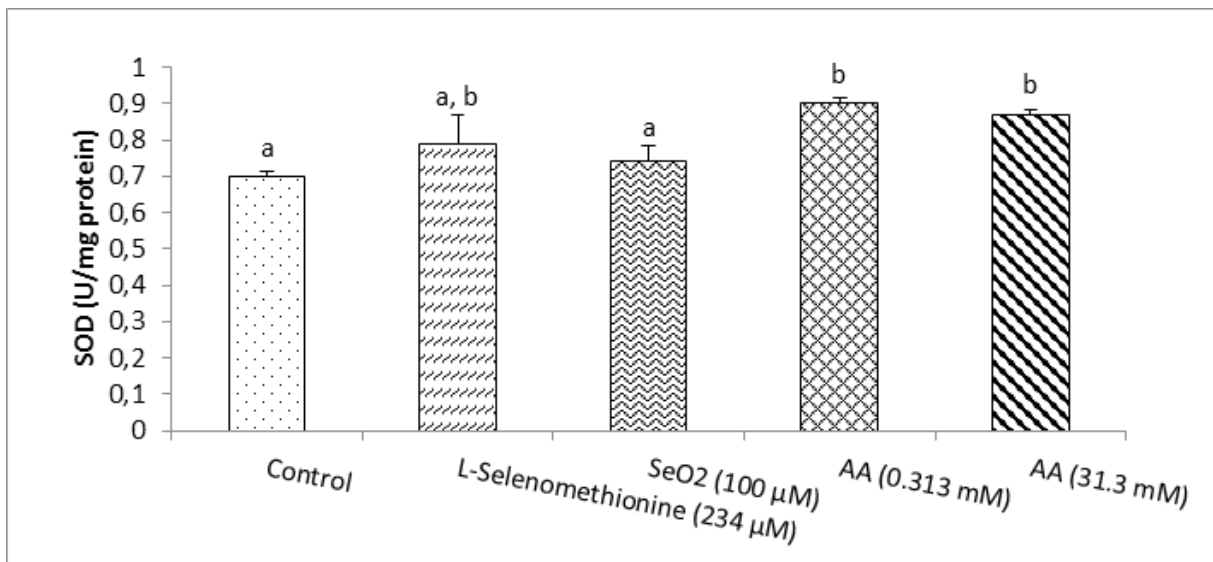

Figure 2. The SOD activities in HepG2 cells treated with 234 µM L-selenomethionine, 100 µM SeO$_2$, 0.313 mM, and 31.3 mM AA (Different letters indicate the differences between the groups).

CAT Enzyme Activities in treated HepG2 cells

CAT activities were significantly increased in HepG2 cells only at 0.313 mM AA concentration. CAT activities were significantly decreased in 31.3 mM AA, L-selenomethionine, and SeO$_2$ applied HepG2 cells compared to the control group (Figure 1). These results may indicate that depending on its dose, AA may have either antioxidant or pro-oxidant effects on HepG2 cells. Decreased activity of catalase represents the conversion of hydrogen peroxide to more toxic hydroxyl radicals. The CAT activities of HepG2 cells which were treated with different doses of L-selenomethionine, SeO$_2$ and AA in comparison to the control group are presented in Figure 3.

0.313 mM and 31.3 mM AA (Different letters indicate the differences between the groups). The results of the study are summarized in Table 1.
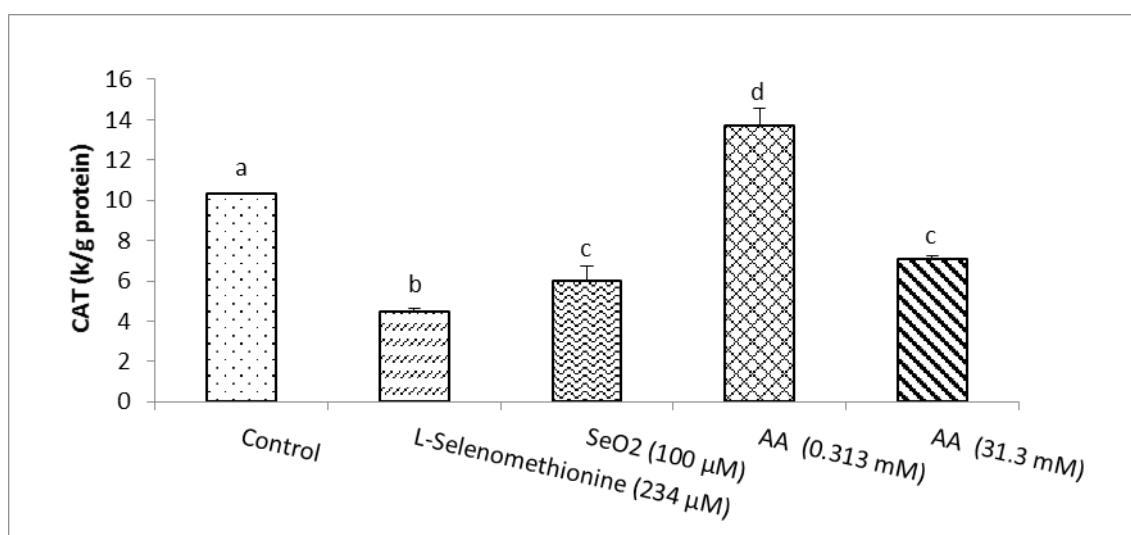
Figure 3. The CAT activities in HepG2 cells treated with 234 µM L-selenomethionine, 100 µM SeO₂, 0.313 mM and 31.3 mM AA (Different letters indicate the differences between the groups)

Table 1. The GPx, SOD and CAT enzyme activities of HepG2 cells treated with 234 µM L-selenomethionine, 100 µM SeO₂, 0.313 mM and 31.3 mM AA.

GROUPS	GPx (X±SD)	SOD (X±SD)	CAT (X±SD)
Control group	51.1 ± 2.32^a	0.70 ± 0.014^a	10.3 ± 0.035^a
L-Selenomethionine (234 µM)	66.7 ± 1.85^b	$0.79 \pm 0.077^{a,b}$	4.47 ± 0.16^b
SeO₂ (100 µM)	59.7 ± 1.79^c	0.74 ± 0.042^a	5.99 ± 0.714^b
AA (0.313 mM)	68.1 ± 0.9^b	0.9 ± 0.014^b	13.7 ± 0.86^d
AA (31.3 mM)	70.1 ± 0.9^b	0.87 ± 0.011^b	7.09 ± 0.15^c

Data are presented as (Mean±SD). SD: Standard deviation. Statistical analysis was performed using one-way ANOVA with post hoc Duncan test. P values<0,05 were considered statistically significant (GPx : nmol/min/mg protein; SOD: U/mg of protein; CAT: k/g of protein). Different letters in the same column are statistically significant.

DISCUSSION AND CONCLUSION

As oxidative stress causes DNA damage, it has a role in cancer pathogenesis (23). The antioxidant enzymes CAT and GPx can scavenge hydrogen peroxide, which is produced by SOD from superoxide radical. In some cases, hydrogen peroxide is converted to highly toxic hydroxyl radicals by Fenton reaction due to the low CAT level (24). For the normal cellular function, there should be an appropriate balance between Cu/Zn-superoxide dismutase (Cu/Zn-SOD), catalase, and selenium-glutathione peroxidase activities. CAT and SOD activity could demonstrate the positive or negative balance of the antioxidant defense system (25).

On the hepatic metastasis model, Heukamp et al. (26) found that vitamins A (retinol), C (ascorbic acid) and E (alpha-tocopherol)

increased GPx and SOD activities and decreased thiobarbituric acid-reactive substances (TBARS) levels. They concluded that these vitamins prevent oxidative stress in hepatocytes. In a study on young and aged rats, they found that the AA level decreased due to the increase in lipid peroxidation and MDA levels (27).

Although it is considered to be an antioxidant agent, at pharmacological doses (0.3-20 mM) AA, have been reported to be selectively toxic to cancer cells by producing ascorbate radicals, and inducing H₂O₂ formation (28). AA has also been reported in other studies to have cytotoxic effects on various types of cancer cells through a hydrogen peroxide generation (5, 29, 30, 31). Studies showed that specific protein transcription factors significantly decreased, and iron metabolism disrupted after AA treatment in colon cancer cells that are involved in cancer progression (32, 33). In

Ryszawy et al. (34) study, sodium ascorbate showed anti-tumor activity in glioblastoma multiforme cells. Gao et al. (35) demonstrated that AA could lead to liver cancer cells' death via intracellular reductive stress.

Increasing ROS production and oxidation, Se is known to induce apoptotic cell death in cancer cells (36, 37). Se, which is an essential element for many biological functions, has been shown to have cancer-protective effects in epidemiological and experimental animal studies. In addition to its protective effect against cancer, Se shows prooxidant activity and increased oxidative stress in cancer cells (38).

Wu et al. (39), demonstrated the therapeutic effect of selenite on peritoneal cancer in a mouse model. They indicated that selenite induced ROS production depending on its dose in the H22 cells and 4 mg Se/kg could be more effective than that of cisplatin in killing cancer cells. The mechanism of Se is related to endogenously formed selenium nanoparticles and its effect to hijack the Trx- and Grx-coupled GSH systems to produce ROS to kill cancer cells. Some organic Se components such as selenoamino acids, selenoproteins, and synthetic organic Se compounds have been reported to have cancer-protective effects (40, 41). Many preclinical, epidemiological studies and experimental animal models supported that Se compounds play a protective role against cancer. To date, studies have demonstrated the importance of the dose, which is administered, and the chemical structure of Se, which is used to determine the protective activity of Se components from cancer (42).

It is thought that there are many mechanisms in the anticancer activity of Se (43). But it was revealed that cell deaths that are related to Se applications were associated with ROS production (37, 38, 44, 45), and apoptotic cell death due to increased ROS production was observed through the caspase-independent apoptotic pathway (46). Estevez et al. (47) investigated the effects of chitosan-stabilized selenium nanoparticles, which were compared with other selenium-containing species on cell proliferation, apoptosis, and cell cycle pattern in HepG2 cells. They compared the effect of different type of Se compounds and concluded that cells which were exposed to Se(IV) showed strong induction of apoptosis and a significant population of cells when they are compared to control cells.

In our study, while the effects of AA on CAT activity were found to be dose-dependent, no changes were found in GPx and SOD activities. GPx activities increased, but CAT activities decreased in L-selenomethionine, and SeO_2

applied to HepG2 cells when they are compared to the control group. L-selenomethionine generated a significant increase in GPx activity than SeO_2. There were no statistical differences in SOD activity between L-selenomethionine and SeO_2 applied HepG2 cells. Compared to the control group: GPx activities increased in HepG2 cells treated with L-selenomethionine, SeO_2, and different doses of AA, but CAT activities decreased in all groups but increased in 0.313 mM AA applied cells compared to the control group.

The results of this study indicated that L-selenomethionine, SeO_2, and AA application increase GPx enzyme activities in HepG2 cells. L-selenomethionine and SeO_2 decrease CAT enzyme activities but do not chance SOD enzyme activities. SOD enzyme activities are increased in both 0.313 mM and 31.3 mM AA concentrations. Depending on its doses, AA effects CAT enzyme activities. 0.313 mM AA increases, and 31.3 mM decreases CAT enzyme activities in HepG2 cells.

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Fabrication of Superhydrophobic Coatings on Glass based on Poly(dimethylsiloxane) and Fumed Silica

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Abstract: This study reports the preparation of superhydrophobic covalently attached poly(dimethylsiloxane)(PDMS)/fumed silica composite coatings on glass via spin-coating and subsequent heating. The effect of PDMS molecular weight and fumed silica type (hydrophilic and three different hydrophobic) on the wettability and morphology were studied in detail, and the wettability and morphology of the coatings were characterized by contact angle measurements and scanning electron microscopy (SEM). Superhydrophobic coatings which have advancing contact angles of 172-174° and contact angle hysteresis (CAH) as low as 7-8° were obtained using 4% (w/v) PDMS modified hydrophobic fumed silica and all kinds of PDMS. The surfaces which were prepared by 4% (w/v) hydrophilic and dimethylsiloxy-modified fumed silica had high advancing contact angles of 163-168°, but the CAH on these surfaces were higher than the ones prepared using PDMS modified fumed silica. It is due to the incompatibility of the hydrophilic and dimethylsiloxy-modified fumed silica with the PDMS matrix also confirmed with the SEM results.

INTRODUCTION

There is a great interest in the fabrication of artificial superhydrophobic surfaces over the last 20 years after Barthlott et al. reported the self-cleaning property of a lotus leaf in 1997, which is known as a "lotus effect" (1-5). Superhydrophobic surfaces, which have water contact angles (CA) larger than 150°, sliding angles lower than 5° or contact angle hysteresis (CAH) lower than 10°, can be used in various applications such as oil-water separation, self-cleaning, anti-icing, anti-fouling, and drop transport (5-7). There are two crucial factors when designing hydrophobic surfaces; (1) using a low energy material as a coating and (2) creating a rough surface, which will increase the hydrophobic surface character according to the Cassie Baxter wetting situation (8). Therefore, superhydrophobic

surfaces can be prepared either by modifying rough surfaces using low energy materials or by making a roughness using low energy materials (2-7,9).

To date, researchers used different techniques to create various superhydrophobic surfaces such as sol-gel, lithography, templating method, colloidal assembling and aggregation, layer-by-layer assembly, plasma method, chemical vapor deposition, phase separation, electrospinning and electrospraying, dip-coating and so on (4,10-11). These techniques are quite complicated and expensive due to multiple steps, specific equipment, and costly reagents. Besides, they can be applied to specific substrates. Overcoming such difficulties, different kinds of polymers, together with various nanoparticles, were used to fabricate superhydrophobic surfaces on different substrates

where the nanoparticles were used to create surface texturing on the surfaces (12-18). Silica-based nanofillers are the most used ones in the literature to create superhydrophobic and self-cleaning surfaces (14, 19-26). Manoudis et al. prepared superhydrophobic composite films containing hydrophilic silica nanoparticles and poly(methyl methacrylate) (PMMA) and commercial poly(alkyl siloxane) (Rhodorsil 224) on various substrates (glass, silicon, wood, concrete, aluminum, silk, marble) via spray coating (14). Superhydrophobic films were fabricated on glass via a drop-coating method using Sylgard 184 silicone (containing curing agent and poly(dimethylsiloxane) (PDMS) prepolymer) and silica particles (19). Chang et al. successfully prepared the superhydrophobic wood surfaces via a dip-coating method using PDMS Syglard 184, curing agent, and hydrophobic silica particles (20). They also investigated the morphology, wetting behavior, and roughness of the coatings as a function of silica concentration (20). Superhydrophobic composite films containing hydrophobic fumed silica particles and different polymers (thermoplastic resin, PS, cross-linked epoxy resin, PDMS-urea copolymer, polyether-based polyurethane urea, PMMA, polycarbonate) were prepared on glass via spin-coating process or doctor blade coating method by Yılgor and coworkers (21-23). Cai et al. fabricated superhydrophobic fabrics via successive dip-coating of PDMS and silica suspensions and successive annealing steps (24). Also, bioinspired superhydrophobic cross-linked polyurethane-acrylate coatings containing hydrophobic fumed silica on glass were prepared via bar coating following UV curing by Fourmentin and coworkers (25). The superhydrophobic coating on cellulosic filter paper for oil-water separation was achieved via solution casting technique using PMMA copolymer and silica nanoparticles by Siriam et al. (26).

As noted above, several papers in the literature report the preparation of superhydrophobic coatings on various substrates such as glass, wood, aluminum, silicon wafer using PDMS, low surface energy material, and nanoparticles (14,19,20,24,27). Either these coatings are not covalently attached to the substrates (14), or they are prepared using curing agents (19,20,24, 27). In 2011, McCarthy and coworkers showed, for the first time, that linear polysiloxanes containing only Si-O-Si in their main chain react with inorganic surfaces containing hydroxyl groups upon heating to desired temperature (especially 100 °C for 24 h) (28). Furthermore, they reported that they obtained low hysteresis surfaces and concluded that they could control the thickness of the PDMS layers on silica by changing the molecular weight of the PDMS.

In the present study, we have prepared superhydrophobic PDMS/silica coatings which are covalently attached to glass via a simple spin-

coating process and subsequent heating. The wettability and surface morphology of PDMS/silica composites on glass were characterized by contact angle measurements (equilibrium, advancing and receding contact angle) and scanning electron microscopy (SEM), respectively. The effect of PDMS molecular weight, silica concentration, and silica type on coatings' morphology and contact angles were also systematically studied, and the results were compared with the findings reported in the literature.

EXPERIMENTAL DETAILS

Materials
Polydimethylsiloxane (PDMS) with three different weight average molecular weight (M_w) of 2000 (2k), 63000 (63k) and 117000 (117k) were obtained from Alfa Aesar (Massachusetts, USA). Amorphous hydrophobic fumed silicon (IV) oxide (~50 nm; PDMS modified, referred to as FS) was obtained from Alfa Aesar. Amorphous hydrophobic pyrogenic silica with different particle sizes and different surface modification (~40 nm; dimethylsiloxy modified; referred as H15 and ~30 nm; PDMS modified; referred as H18) and amorphous hydrophilic pyrogenic silica (~25 nm; referred as N20) were obtained from Wacker (Burghausen, Germany). Toluene was obtained from Sigma-Aldrich. Ethanol, sulfuric acid (H_2SO_4, 95-97%), hydrogen peroxide (H_2O_2, 30%), and ultra-pure water were obtained from Merck (Darmstadt, Germany). All reagents were used as received without further purification. Glass microscope slides were obtained from ISOLAB GmbH (Wertheim, Germany) and cut into 25 mm x 35 mm x 1 mm pieces. Deionized water (18.2 MΩ cm^{-1}) was obtained through a system including pretreatment, reverse osmosis, and ion exchange steps (Elga Option Q15).

Preparation of PDMS-silica Coatings
Glass microscope slides were cleaned by ethanol and then activated by piranha solution before the coating procedure. Glass slides were immersed into freshly prepared piranha solution, which is a 7:3 (v:v) mixture of concentrated H_2SO_4 and 30% H_2O_2. They were kept at 80 °C for 2 h and then left at room temperature overnight. Then the glass slides were washed with deionized water and dried at 125 °C for 30 min in an oven. Activated glass slides were used immediately for PDMS-silica composite film preparation.

The coating solution was prepared as follows; the desired amount of silica particles (to obtain x% w/v for the toluene volume, where x is 0, 0.4, 1.2, 2, 3, and 4) were added to 1 % w/ v PDMS toluene solution. The polymer solution was mixed with a magnetic stirrer for 1 h and then sonicated for 30 min at room temperature. 10 µL of the homogeneous polymer solution was dropped onto

the freshly activated glass slide and spin-coated at 2000 rpm for 60 s. Then the coatings were kept at 100 °C for 24 h in an oven (Figure 1).
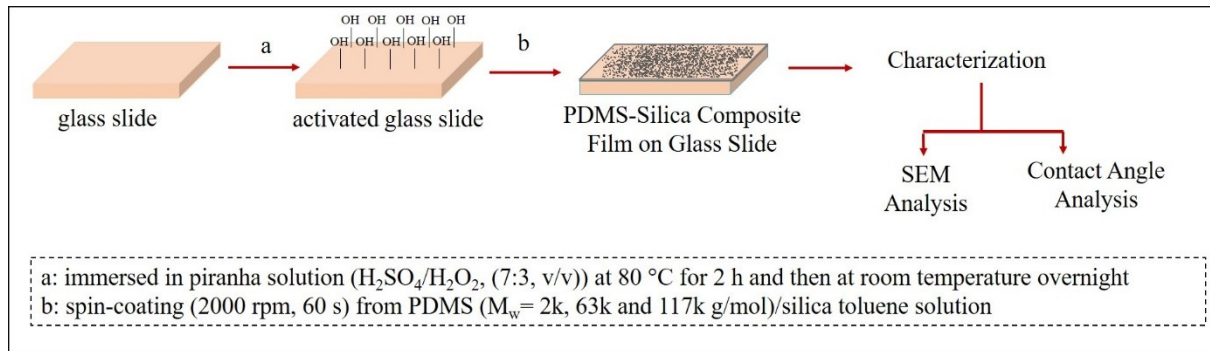

a: immersed in piranha solution (H_2SO_4/H_2O_2, (7:3, v/v)) at 80 °C for 2 h and then at room temperature overnight
b: spin-coating (2000 rpm, 60 s) from PDMS (M_w= 2k, 63k and 117k g/mol)/silica toluene solution

Figure 1: Schematic illustration of the PDMS-silica composite film preparation and characterization procedure.

Characterization of The Coatings

The surface morphology of the coatings was characterized by scanning electron microscopy (SEM, Philips XL 30 SFEG). PDMS-silica composite films were coated with a gold layer before SEM analysis to prevent charging effects. Contact angles were measured by a contact angle meter (KSV-CAM 200) with a PC controlled motorized syringe using ultra-pure water under air at room temperature. Static contact angles (Θ_e) were measured by forming 5 µL drops of water on samples, and the values recorded 2 s after the release of the needle. Advancing contact angles (Θ_a) were measured by increasing the volume of the water droplet on the surface from 3 to 8 µL, and receding contact angles (Θ_r) were measured by decreasing the volume of a water droplet from 10 to 3 µL by using the automatic dispenser through the needle. The needle was kept within the water droplet during the Θ_a and Θ_r measurements. The reported values were calculated by taking the averages of 5 different measurements made on the same sample.

Chemical stabilities of PDMS/silica composite coatings were tested by washing the samples with toluene for 5 minutes and dried at 100 °C for 24 h in the oven. SEM images were again taken, and contact angle (Θ_e, Θ_a, and Θ_r) values were again measured after washing with toluene, and the results were compared with the unwashed samples' results.

RESULTS AND DISCUSSION

In this study, covalently attached superhydrophobic PDMS/silica coatings were prepared on piranha-cleaned glass slides. The superhydrophobic coatings were prepared via a spin-coating of silica/PDMS dispersions and subsequent heating at 100 °C for 24 h. The covalent attachment was achieved via heating, as reported in the literature by McCarthy et al. (28). PDMS with three different molecular weights, hydrophilic, and three different hydrophobic fumed silica were used to prepare the coatings. The effect of hydrophobic fumed silica concentration on surface morphology and wettability were also studied.

Table 1 shows the effect of fumed silica concentration on the equilibrium (Θ_e), advancing (Θ_a) and receding (Θ_r) contact angle and contact angle hysteresis (CAH) values of composite films. The composites films were prepared by two different molecular weights (63k and 117k g/mol) of PDMS and five different concentration of hydrophobic fumed silica (0.4, 1.2, 2,3 and 4% w/v) where hydrophobicity is achieved by modification with PDMS (~50 nm; referred as, FS). As seen in Table 1, the Θ_e, Θ_a and Θ_r values of bare PDMS coating increase from 108±1°, 111±1° and 96±2° to 119±1°, 120 ±1°and 103±2° respectively as the molecular weight of PDMS increases from 63 k to 117 k. This trend is similar to the results reported in the literature [28]. The Θ_e, Θ_a, and Θ_r values of the coatings increased with an introduction of hydrophobic fumed silica (FS) as depicted in Table 1 regardless of PDMS molecular weight. As the concentration of hydrophobic fumed silica (FS) increased from 0.4 to 4 % w/v, and increase in Θ_e, Θ_a and Θ_r values were seen up to 169±2°, 173±1°, 166±1°, and 170±2°, 172 ±2°and 164±1° for the coatings prepared by 63k and 117k PDMS respectively (Table 1). The highest contact angle values and lowest CAH (7° for 63k PDMS and 8° for 117k PDMS) were obtained at 4 % w/v silica concentration for PDMS coatings prepared by different molecular weights. The highest Θ_e value (164°) of cross-linked silicone-based coatings prepared by spray coating using $CaCO_3/SiO_2$ particles on glass by Yang et al. (27) was lower than our results. Manoudis et al. reported that they obtained siloxane-based coatings via spraying on glass using 2% w/v hydrophilic silica particles with a Θ_a of 164° and CAH of 3°. The CAH hysteresis value (3°) is better than our values (7° for 63k PDMS and 8° for 117k PDMS), but the information on the chemical composition and the molecular weight of

the poly(alkyl siloxane) which was used to prepare the coating was not given in the paper (14).

Table 1. Equilibrium (Θ_e), advancing (Θ_a) and receding (Θ_r) contact angle and contact angle hysteresis (CAH) values of PDMS/SiO$_2$ composite films prepared by hydrophobic fumed silica (FS) and PDMS with different molecular weights. The PDMS (1% w/v) was prepared by dissolving PDMS (63k or 117k) in toluene.

PDMS (M_W (g/mol))	Silica/toluene (% w/v)	Θ_e (°)	Θ_a (°)	Θ_r (°)	CAH (°)
63k	0	108±1	111±1	96±2	15
63k	0.4	119±2	124±1	108±2	16
63k	1.2	161±1	168±2	150±1	18
63k	2	167±1	171±1	155±2	16
63k	3	169±1	172±2	164±1	8
63k	4	169±2	173±1	166±1	7
117k	0	119±1	120±1	103±1	17
117k	0.4	129±2	146±1	107±1	39
117k	1.2	166±2	167±1	142±1	25
117k	2	167±1	168±1	134±1	34
117k	3	169±2	172±1	159±2	13
117k	4	170±2	172±2	164±1	8

The Θ_e, Θ_a, Θ_r, and CAH values of bare PDMS and composite coatings before and after washing with toluene were shown in Table 2. The coatings were prepared using PDMS with different molecular weights (2k, 63k, and 117k) and 4 % w/v hydrophobic fumed silica (FS). As seen in Table 2, the Θ_e, Θ_a, Θ_r values of the bare 2k PDMS and 63k PDMS coatings were slightly decreased (1-2°) or increased (1-2°) after washing with toluene, but the decrease in Θ_e, Θ_a, Θ_r values of the coating prepared using 117 k PDMS were higher compared to the other coatings. The increase in all the contact angle values was seen as the molecular weight of the PDMS rises, as shown in Table 2, and the trend did not change after washing with toluene. Similar the bare PDMS coatings, there was a slight change (1-2°) in the Θ_e, Θ_a, Θ_r values of the composite coatings prepared using 4 % w/v silica (FS) after washing with toluene. The results in Table 2 clearly show that bare PDMS and composite coatings were covalently attached to the piranha cleaned glass slides, and the superhydrophobic character of the composite coatings did not alter after washing with toluene.

Figure 2 shows the SEM images of the coatings prepared by PDMS with different molecular weights PDMS/silica (FS) composite before and after washing with toluene. All the SEM pictures in Figure 2 a-c clearly show that the homogenous PDMS/silica (FS) composite coatings were obtained with a similar morphology regardless of the PDMS molecular weight. No change in the homogeneity of the composite coatings was observed after washing with toluene. (Figure 2 d-f).

After investigating the effect of silica concentration on coatings' wettability, 4 % w/v silica concentration and 63k PDMS were chosen to study the effect of silica size and type on coating morphology and wettability (Table 3 and Figure 3). The Θ_e, Θ_a, Θ_r, and CAH values of PDMS/silica composite films prepared using different types and size fumed silica were shown in Table 3. The Θ_e contact angles of the PDMS coating increased up to 159-172° with an addition of different types and sizes of fumed silica (Table 3). Although high Θ_a values were obtained for the coatings prepared by hydrophilic fumed silica (N2O) (168±2°) and dimethylsiloxy modified hydrophobic fumed silica (H15) (N2O) (163±2°), the surfaces were not superhydrophobic due to the high CAH hysteresis (24° and 16° respectively). Superhydrophobic composite coatings were obtained when two different sizes of PDMS modified fumed silica particles (FS; ~50 nm and H18; ~ 30nm) were used to prepare the coatings. The Θ_a, Θ_r, and CAH values of the coating prepared using FS and N18 were

measured as 173±1°, 166±1°, 7°, and 174±1°, 166±1°, 8° respectively. These differences can be due to the incompatibility of the hydrophilic (N20) and dimethylsiloxy modified hydrophobic (H15) fumed silica and compatibility of the PDMS modified hydrophobic (FS and H18) fumed silica with the PDMS matrix.

Table 2. Equilibrium (Θ_e), advancing (Θ_a) and receding (Θ_r) contact angle and contact angle hysteresis (CAH) values of PDMS/SiO$_2$ composite before and after washing with toluene. The composite films composed of hydrophobic fumed silica (FS) and PDMS with different molecular weights. The PDMS (1% w/v) was prepared by dissolving PDMS (2k, 63k, or 117k) in toluene.

PDMS (M_w (g/mol))	Silica/toluene (% w/v)	Washing	Θ_e (°)	Θ_a (°)	Θ_r (°)	CAH (°)
2k	-	No	94±1	95±2	81±1	14
2k	-	Toluene	92±2	99±2	82±1	17
2k	4	No	170±1	173±2	166±1	7
2k	4	Toluene	168±1	172±1	162±1	10
63k	-	No	108±1	111±1	96±2	15
63k	-	Toluene	109±1	110±1	96±1	14
63k	4	No	169±2	173±1	166±1	7
63k	4	Toluene	168±1	172±1	165±2	7
117k	-	No	119±1	120±1	103±1	17
117k	-	Toluene	110±2	117±1	98±1	19
117k	4	No	170±2	172±2	164±1	8
117k	4	Toluene	171±2	173±2	165±1	8
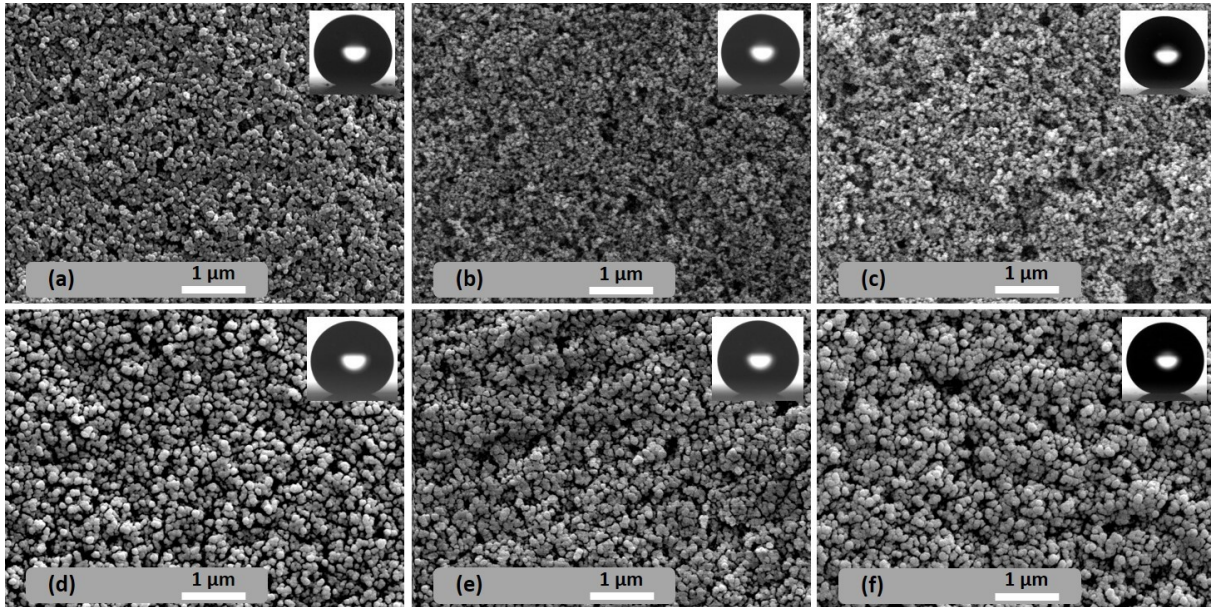
Figure 2. SEM pictures and water droplets of PDMS-silica composite films prepared with fumed silica (FS) and PDMS with different molecular weights : (a) 2k PDMS; (b) 63k PDMS; (c) 117k PDMS; (d) 2k PDMS, washed with toluene; (e) 63k PDMS, washed with toluene; and (f) 117k PDMS, washed with toluene (Silica concentration is 4 % w/v).

Table 3. Equilibrium (Θ_e), advancing (Θ_a) and receding (Θ_r) contact angle and contact angle hysteresis (CAH) values of PDMS/SiO$_2$ composite films with hydrophilic and three different hydrophobic fumed silica and PDMS (63k). The PDMS (1% w/v) was prepared by dissolving PDMS (63k) in toluene.

Silica type	Silica/toluene (% w/v)	Θ_e (°)	Θ_a (°)	Θ_r (°)	CAH (°)
-	-	108±1	111±1	96±2	15
FS	4	169±2	173±1	166±1	7
N2O	4	163±2	168±2	144±2	24
H15	4	159±1	163±1	147±1	16
H18	4	172±2	174±1	166±1	8

SEM pictures of the PDMS/fumed silica coatings which were prepared using 4% w/v PDMS modified hydrophobic fumed silica (FS and H18) and dimethylsiloxy modified fumed silica (H15) and hydrophilic fumed silica (N2O) are depicted in Figure 3 a, b, c, and d respectively. Figure 3 a and b shows that the coatings prepared using FS and N10 are homogenous and very small aggregates of the fumed silica were seen on the surfaces confirming the compatibility of the PDMS modified fumed silica with the PDMS matrix. Distinct big aggregates of fumed silica, which is embedded in the PDMS matrix and non-homogeneous coating formation were observed as seen in Figure 2 c and d when dimethylsiloxy modified fumed silica (H15) and hydrophilic fumed silica (N2O) were used conforming the non-compatibility of the particles with the PDMS matrix.
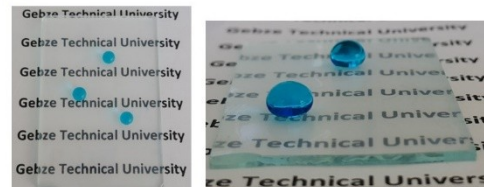
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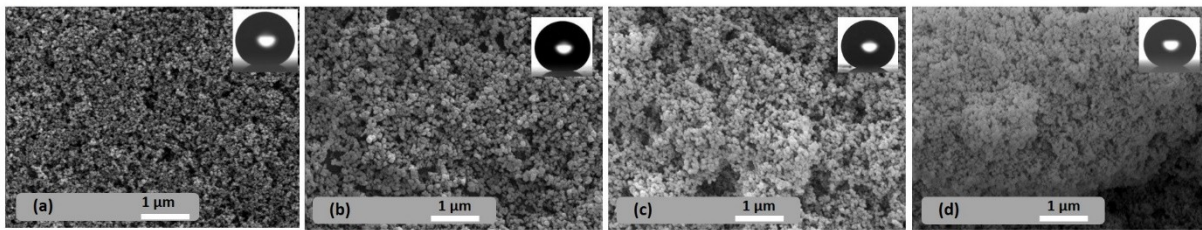
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Figure 3. I. SEM pictures and water droplets of PDMS-silica composite films prepared with 63k PDMS and three different hydrophobic and hydrophilic fumed silica particles: (a) FS (b) H18, (c) H15 and (c) N20 (Silica concentration is 4% w/v). II. Photographs of superhydrophobic PDMS-silica composite film (63k PDMS, 4% w/v FS) coated glass slide (water droplets were dyed with methylene blue).

CONCLUSIONS

Summarizing, in this paper, we have demonstrated the preparation of covalently attached superhydrophobic PDMS/silica composite coatings on activated glass slides via an easy and straightforward spin-coating process. SEM and contact angle measurements were used to characterize the morphology and the wettability of the surfaces. The superhydrophobic coatings were obtained when PDMS modified fumed silica (FS and H18) were used, and the concentration of the fumed silica was 4 % w/v whereas the superhydrophobic surfaces were not obtained when hydrophilic (N2O). Dimethylsiloxy-modified (H15) fumed silica were used due to the high CAH. To obtain a superhydrophobic surface using PDMS and fumed silica via a spin coating process, the type and the concentration of the fumed silica need to be optimized.

ACKNOWLEDGMENTS

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In situ Crosslinking System of Gelatin with Acrylated β-cyclodextrin Towards the Fabrication of Hydrogels for Sustained Drug Release

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Abstract: Cyclodextrins (CDs) are of interest in the fabrication of various polymeric platforms, especially the crosslinked networks that possess unique advantages in biomedical applications. When one benefits from the abilities of CDs to form inclusion complexes with hydrophobic drug molecules, hydrogel-based supported drug platforms employ CDs as nano-buckets in the network structure. In the current study, we report on the fabrication of gelatin-based hydrogels chemically crosslinked with acrylated β-cyclodextrin (β-CD) as sustained drug release platforms. A practical and straightforward synthesis of crosslinked networks was achieved by Michael's addition reaction of gelatin amino functionalities onto acryloyl groups of modified β-CD. Tunable synthesis of hydrogels incorporating a different amount of β-CD molecules was maintained by simple adjustment of the reaction stoichiometry between amine and acrylate groups. The resulting hydrogels were characterized by their surface morphology, equilibrium swelling ratios, and rheological properties. Thus obtained hydrogels were evaluated in terms of their drug loading capacities and sustained release efficiencies. The results demonstrated that the amount of drug loading and prolonged release is dependent on the amount of β-CD in the gel network. Thanks to their in situ gel-forming abilities, these hydrogels can be used as injectable formulations for various biomedical applications.

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INTRODUCTION

Hydrogels purvey some unique and advantageous properties that make them ideal candidates in several biomedical applications. (1–3) Significant research has been devoted to engineered hydrogels in practical clinical utilization, such as in controlled/sustained drug release platforms or tissue construction scaffolds. In tissue engineering, hydrogels are employed as environments for adhesion, encapsulation, growth, or directed spread of cells. (4–6) In therapeutic applications, hydrogels are exploited as slow drug releasing platforms in which the rate of release can be controlled via response toward external stimuli such as temperature or pH of the local environment. (7–9) While the eximious features of hydrogels are advantageous, the challenges associated with the practical introduction of the hydrogels into the body might limit their routine clinical implementation. Surgical implantation of the pre-formed hydrogels to defect sites is often associated with patient compliance and discomfort. In this context, non-invasive techniques such as employment of injectable hydrogel formulations to administrate regenerative factors or therapeutics to the body have been addressed. (10–12) In situ cross-linkable hydrogels that can form the network structure in vivo through physical or chemical interaction of gel, precursors could allow straightforward inclusion of drugs,(13) growth factors (14) or genes (15) to the target body tissues.

Gelatin is a biopolymer of animal-origin, derived from collagen by partial hydrolysis and denaturation. Due to essential characteristics such

as excellent biocompatibility, nontoxicity, high resemblance to extracellular matrix in a gel state, and low immunogenicity make gelatin widely used in the biomedical fields. (16–18) Gelatin contains peptide sequences that can bind to cell surface integrins, which is notable for cellular recognition. (19) Improved cell attachment and proliferation make gelatin-based scaffolds ideal for tissue regeneration, cell encapsulation, and 3D cell culture applications. (20–22) The utility of gelatin-based controlled-release systems covers a wide variety of drug and gene delivery applications. (23) Although gelatin can form temperature-induced physical gelation, a much-sought strategy is the fabrication of chemically crosslinked hydrogels to increase the stability of the gels. Toward this, several injectable gel-forming systems such as dextran-gelatin hydrogels via oxidized dextran and gelatin, (24) gelatin polymers with pendant norbornene (GelN) or tetrazine (GelT) groups,(25) and UV-curable acrylated gelatin hydrogels (26) have been reported.

Cyclodextrins (CDs) are torus-shaped molecules that are derived mainly degradation of starch. The structures of CDs possess an interesting molecular property with a hydrophobic inner cavity and hydrophilic outer structure. CDs can form inclusion complexes through the hydrophobic inner cavities with molecules that are physically compatible with the cavity size. The dynamic inclusion complexation abilities of CDs with low polarity organic compounds make them ideal materials in the fabrication of biomaterials augmenting the properties in various

applications.(27,28) To date, CDs have been employed in the fabrication of diverse hydrogel-based polymeric drug delivery systems, especially to modify the release kinetics. (29–32) While the overall hydrophilic nature of CDs increases the swelling state of the network structure, the hydrophobic inner core enables enhanced loading and prolonged/sustained release of hydrophobic therapeutic compounds.

In this contribution, we report on the synthesis of gelatin-based hydrogels incorporating β-cyclodextrin residues towards sustained drug release applications. Briefly, hydrogels were prepared via reacting gelatin amino groups with acrylate modified β-CD in aqueous buffer solutions (Figure 1). The method demonstrated a facile and efficient crosslinking process via aza-Michael addition of complementary functional precursors, which can be employed as an injectable gel-forming system. A series of hydrogels displaying tunable physical properties were obtained by changing the stoichiometric balance between gelatin amino groups and acrylate modified β-CD crosslinker. Drug loading and sustained release studies of fabricated hydrogels were investigated by using poorly water-soluble anti-inflammatory drug diclofenac sodium. The results indicated that the amount of drug loading and release profiles could be controlled by changing the β-CD crosslinker ratio. It is believed that the benign gelation methodology outlined here can be utilized in the design and synthesis of bulk and injectable hydrogels for tissue engineering, drug delivery, and various other biomaterial applications.
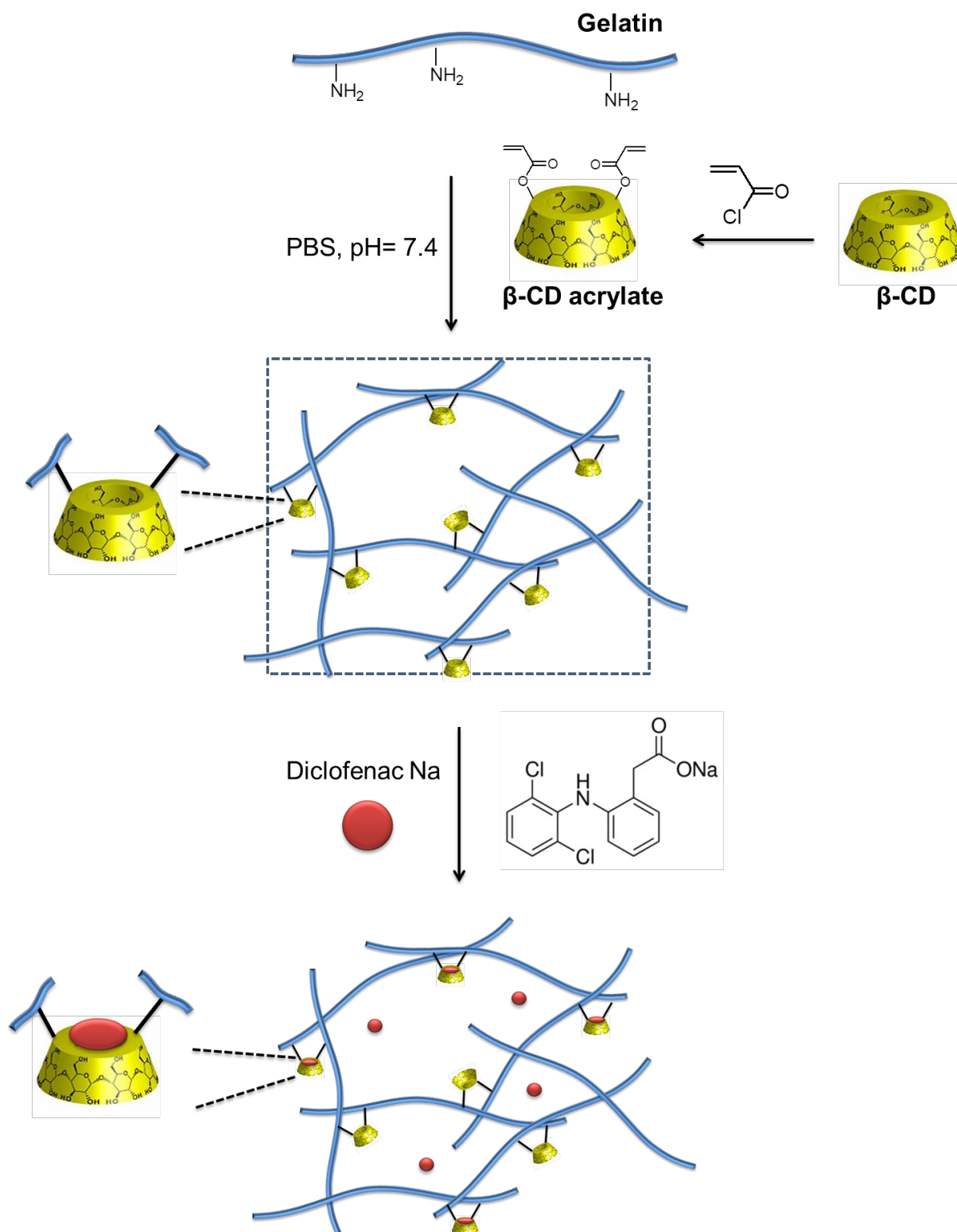
Figure 1. Schematic representation of the gelatin-based hydrogel synthesis with a β-CD-based crosslinker and the diclofenac Na loading into the hydrogel augmented by the inclusion complex formation.

EXPERIMENTAL SECTION

Materials and characterization

β-cyclodextrin, acryloyl chloride, diclofenac sodium salt, 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU), gelatin from porcine skin (type A, 300 g bloom, average M_n: 50.000-100.000 g/mol) were obtained from Aldrich Chemical Co. All solvents and inorganic materials were obtained from Merck Co. and used as received unless otherwise noted. Synthesis of acryloyl-modified β-cyclodextrin (β-CD-acrylate, the average degree of acetylation ~ 5.9

per molecule. Detailed synthesis procedure is given in Supporting Information) was carried out based on the reported procedure. (33) Characterization of β-CD acrylate was performed using ^{1}H NMR spectroscopy (Varian 400 MHz). Fourier-transform infrared spectroscopy analyses were conducted on a Nicolet 380 spectrometer. Scanning electron microscopy analysis was carried out using an ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument with 10 kV accelerating voltage. The rheological measurements were performed using an Anton Paar MCR 302 rheometer between 0.05-100 rad/s at 37 °C. UV-vis studies were carried out using a Varian Cary 50 Scan UV/Vis spectrophotometer.

Methods
Synthesis of gelatin-based β-CD incorporated hydrogels
In a vial, gelatin (100 mg) was placed and dissolved in 1 mL PBS buffer (phosphate-buffered saline, pH of 7.4) at 37 °C. In a separate vial, a pre-determined amount of β-CD-acrylate and DBU (0.1 molar equivalents to acrylate groups) were placed and dissolved in 200 μL of PBS solution. The two-ingredient solutions were thoroughly mixed and sonicated briefly. The mixture solution was heated at 37 °C for 6 h for a crosslinking reaction. After that, the resultant hydrogel was washed with distilled water several times to remove unreacted species. Dried hydrogels were obtained after the freeze-drying of water-swollen samples.

Equilibrium swelling ratios (ESRs)
Equilibrium swelling of hydrogels in distilled water was determined at 25 °C. 20 mg hydrogel was conditioned in distilled water, and the mass increase of sample as a function of time was recorded until a constant weight was attained. The percent swelling was determined using empirical equation 1:

$$ESR\ (\%) = (W_{wet} - W_{dry})\ /\ W_{dry} \times 100$$
$$(Eq.\ 1)$$

The swelling tests were performed in triplicate for each sample, and average data with standard deviation was used for obtaining swelling curves.

Rheological characterization
The rheological properties of hydrogels were studied by dynamic oscillatory experiments at a constant 1 % strain. Loss (G") and storage (G') moduli of disk-shaped water-equilibrated hydrogels were measured at 37 °C between 0.05 and 100 rad/s angular frequency. 8 mm diameter parallel plate was used for measurements and the plate distance was set to 2.0 mm.

Drug loading and release studies
Diclofenac-Na was loaded into the hydrogels by employing solution absorption. Briefly, 50 mg water-swollen gel sample was soaked in 0.5 wt.% drug solution in PBS (pH: 7.4). The hydrogel was incubated at 37 °C for 24 h, protected from light. Total drug loading was determined from the concentration difference between initial and final soaking solutions using a UV-Vis spectrophotometer (276 nm absorption maxima of diclofenac-Na in PBS solution).

Drug release profiles of hydrogels were investigated by immersing gel samples in 3 mL of PBS solution at 37 °C. Aliquots of the release medium (1 mL) were taken out and replaced with the new solution at pre-determined intervals. The total drug content in the collected medium was determined spectrophotometrically at 276 nm. The experiments were performed triplicate, and the release profiles were expressed in terms of cumulative release.

RESULTS AND DISCUSSION

Synthesis and characterization of gelatin-based β-CD containing hydrogels
Hydrogels composed of a gelatin matrix and distinct β-CD units were fabricated via amine-acrylate Michael addition of gelatin amino functionalities onto acryloyl groups of modified β-CD (Figure 2). Though it may vary depending on the source, the amino acid composition of gelatin mostly consists of glycine, proline, and hydroxyproline. Amine-containing lysine residues also constitute the structure of both bovine and porcine skin gelatin. (34) In chemical derivatization and modification of gelatin, amino groups of lysine have been exploited in reactions with isocyanates, (35) aldehydes, (36) vinyl sulfones(37), and genipin. (38) Towards the synthesis of gelatin-based hydrogels, reactive amino groups of amino acid chain strands were utilized in crosslinking reaction with activated carbonyls (39) as well as a modification with photo-curable acrylate functionalities. (26)
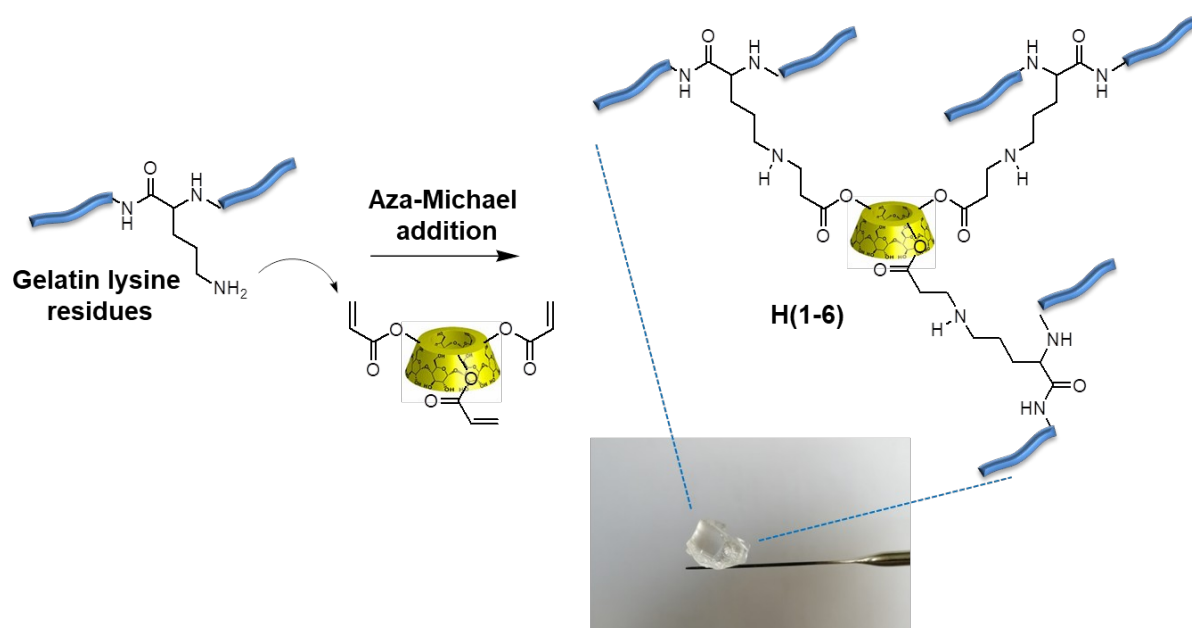
Figure 2. Crosslinking mechanism via multi-addition of gelatin amino groups onto acrylate functionalities of β-CD-acrylate. Representative picture of transparent hydrogel H-4.

A series of hydrogels composed of different β-CD-acrylate crosslinker ratio were synthesized in aqueous PBS buffer environment at 37 °C reaction temperature. Due to the multiple additions of amine groups onto acryloyl functionalities, relatively fast network formation was established within minutes. To ensure maximum available crosslinking, gelation reactions were continued for 6 h. Although the efficiency of the crosslinking process was prominent in uncatalyzed conditions (Table 1, Entry 1 and 2), gel formation was further promoted by using organo-catalysts DBU (Table 1, Entry 3-6). In aqueous conditions, β-CD is an efficient catalyst for aza-Michael addition of various amines onto electron-deficient carbonyls. (40) On the other hand, DBU possesses superior promotion of nitrogen nucleophile reactivity towards α,β-unsaturated carbonyl compounds. (41)

A strong hydrogenation reaction was confirmed by FT-IR characterization of reaction ingredients and resulting hydrogels (Figure 3). The spectrum of β-CD-acrylate shows characteristic vibrations at 1720 cm^{-1} and 1664 cm^{-1} due to the stretchings of C=O and C=C, respectively. In the FT-IR spectrum of gelatin, typical amide peaks are resolved at 1639 cm^{-1} and 1535 cm^{-1}. The spectrum of hydrogel H-4 is characterized by the presence of the typical vibration peaks of both gelatin and β-CD-acrylate, demonstrating the structural integration of the hydrogelation components into the matrix network.
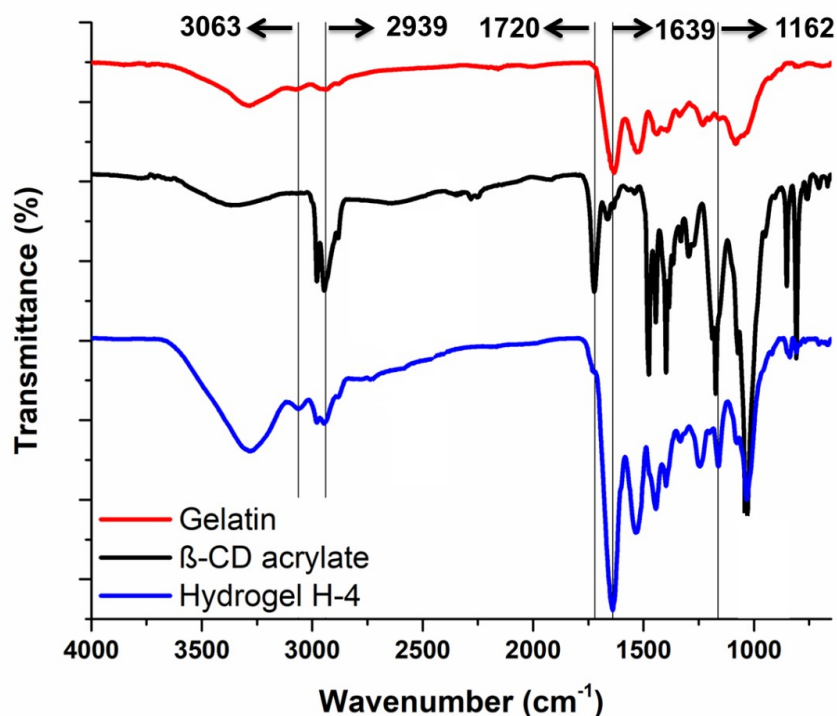
Figure 3. FT-IR structural analysis of gelatin, β-CD-acrylate, and hydrogel H-4.

The properties of fabricated hydrogels are summarized in Table 1. As expected, increased β-CD-acrylate feed in gel formulation resulted in higher gel conversions. The catalytic introduction of DBU in the gelation process has led to enhanced crosslinking efficiencies in different β-CD-acrylate feeds. Obtained hydrogels are clear transparent samples in the water-swollen state (Figure 2). The morphological characterization of hydrogels using SEM analysis on freeze-dried samples revealed interconnected porous structures with varying pore sizes (Figure 4).

Table 1. Properties of hydrogels with varying gelatin / β-CD-Ac ratio.

Entry	Hydrogel [a]	Feed	Gel Conv. (%)	ESR (×100%)	Drug Load (mg/g dry gel)
		Gelatin (mg) : β-CD-acrylate (mg)			
1	H-1	100.0 : 5.0	67	-	-
2	H-2	100.0 : 10.0	71	-	-
3	H-3	100.0 : 5.0	73	9.4 (± 2.7)	11.9 (± 4.4)
4	H-4	100.0 : 10.0	77	6.7 (± 2.1)	18.7 (± 5.8)
5	H-5	100.0 : 15.0	82	5.8 (± 1.8)	27.1 (± 5.3)
6	H-6	100.0 : 20.0	87	3.9 (± 1.3)	31.7 (± 7.4)

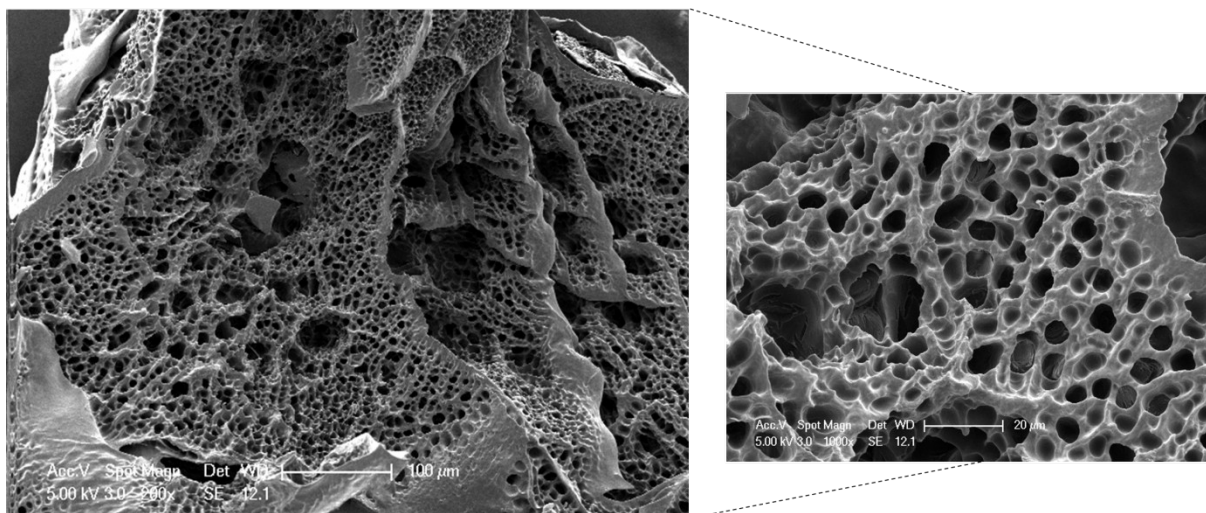[a] H-1 and H-2: No organo-catalyst; H (3-6): DBU catalyst.

Figure 4. Representative SEM images of freeze-dried hydrogel H-4 with two different magnification (Scale bars: 100 μm for larger image and 20 μm for smaller image).

Equilibrium water uptake ratio of a hydrogel is an essential physical property in addressing gel mechanical properties, surface and network properties, and diffusion of solutes. (1) Though various parameters (i.e., pH, temperature) can contribute to ascertain the water uptake, maximum swelling is mostly related to the nature of polymer matrix and crosslinking degree. The swelling properties of the fabricated hydrogels were investigated in aqueous PBS conditions (pH: 7.4, 25 °C). All the hydrogels displayed pronounced swelling degrees (~ 940-390 % to the mass of gel sample)

because of the hydrophilic groups present on the gelatin backbone and cyclodextrin crosslinker (Figure 5). Equilibrium water uptakes were reached in relatively short periods in applied experimental conditions. It was observed that hydrogels exhibit maximum water uptake dependency on the feed ratio of the gelatin matrix and β-CD-acrylate crosslinker. Relatively higher maximum swelling degrees were observed in case of a lower crosslinker ratio in the feed. This can be attributed to higher free gel volumes and voids of network possessed by lower crosslinking degree.
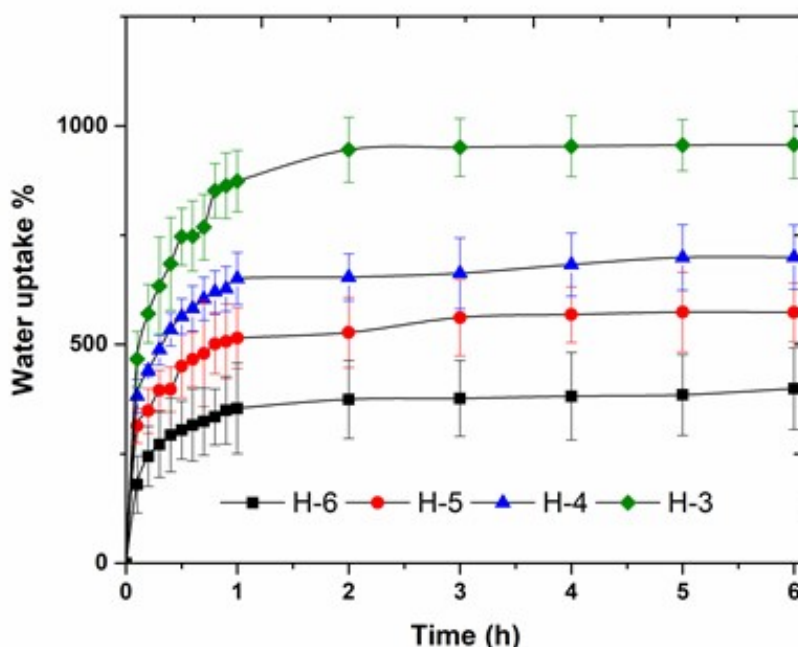

Figure 5. Equilibrium swelling profiles of gelatin-based β-CD containing hydrogels in PBS buffer.

Hydrogel viscoelastic properties were examined via dynamic frequency scan analysis of prepared hydrogels at 37 °C. The tests of hydrogels having different crosslinker ratios were performed at a

constant 1.0 % strain between 0.05-100 rad/s. As it can be seen in Figure 6, all samples exhibited permanent elastic character in the test frequency interval where storage moduli (G′) were higher than

loss moduli (G"). According to the results, G' and G" values of the samples show relatively low oscillation frequency dependency maintaining highly homogenous crosslinking of gel networks. (42) An increase in the ratio of βCD-based crosslinker has

resulted in higher moduli, which could be due to the increased network structure. The damping factors (tan δ, G"/G') of hydrogels were less than 1 in all cases representing the higher elastic character of networks over viscous behavior. (43)
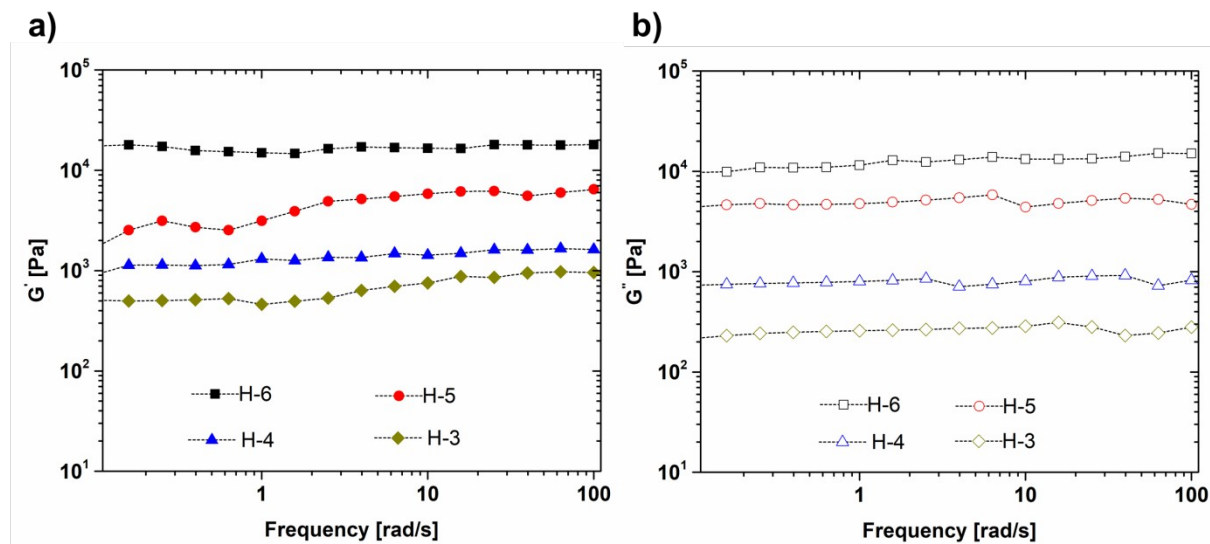

Figure 6. Dynamic frequency scan analysis of hydrogels: a) Storage and b) loss moduli.

Drug loading and release studies

Loading and sustained/controlled release of drugs, biomolecules, and other therapeutically relevant materials using hydrogels procure great potentials in realizing effective administration systems. The typical strategy for loading and release of drugs is based on the diffusion phenomena governed by weak interactions of the hydrogel matrix with subject molecules. In hydrogel networks that accommodate cyclodextrins as molecular buckets, inclusion complexation between hydrophobic molecules and cyclodextrins ensures another means of drug loading. Diclofenac-Na, widely used as a non-steroidal anti-inflammatory drug for various complaints and diseases, was employed as a model hydrophobic molecule to study the drug loading and release studies of fabricated hydrogels. It has been established that diclofenac-Na can form a molecular inclusion complex with β-cyclodextrin. (44) Hydrogels prepared as disk-shaped samples were loaded with the drug by solution absorption at 37 °C. 0.5 wt.% soaking solution was used for 24 h incubation, which is the required time to reach equilibrium as determined by UV-spectrophotometry. The drug loading efficiencies of hydrogels determined from the difference between initial and final soaking concentrations were given in Table 1. According to the results, the loaded drug amount increased from 11.9 (mg/g) to 31.7 (mg/g) for different hydrogels. A direct relation between crosslinker amount and drug levels was accounted for, where increased β-CD-acrylate crosslinker in the hydrogel resulted in considerably higher drug loading. The enhanced drug loading could be due to the higher available binding sites of cyclodextrin

cavities to complex with diclofenac-Na molecules. The inclusion complexation might have increased the solubility of slightly hydrophobic drug molecules within the hydrophilic gel network.

In vitro drug release profiles of the hydrogels containing different β-CD-acrylate crosslinker ratios were investigated in PBS buffer (pH: 7.4) at 37 °C. Initially, drug-loaded hydrogels taken from soaking solutions were gently washed with PBS buffer and then placed in the release medium. Aliquots of the release medium were taken out and replaced with the fresh solution at pre-determined intervals to resemble the physiological conditions. Time-dependent release profiles determined via UV-spectrophotometry were given in Figure 7. Overall, hydrogel H-3 having the lowest β-CD-acrylate, released its content faster than other hydrogels. An initial accelerated release was observed for all four hydrogels, which could be attributed to the burst removal of drug molecules present in the aqueous phase and adsorbed on the network matrix. (45) As expected, H-6 with the highest crosslinker content showed the lowest initial burst release and followed by H-4 and H-5. Incorporation of higher β-CD-acrylate caused a higher crosslinking degree of the network as well as enhanced drug/cyclodextrin complexation interactions, yielding slow diffusion of diclofenac-Na from the hydrogels. After the initial burst releases, all gelatin-based hydrogels exhibited sustained release profiles over a couple of days. In a specific time, relatively slower and lower total drug release from higher cyclodextrin content hydrogel could be ascribed to enhanced molecular inclusion interactions.

In an overall structure-property relationship of fabricated hydrogels, it was observed that the amount of β-CD content in the hydrogels maintained enhanced drug/matrix interactions. Hydrogel physical properties can also be manipulated by adjusting the crosslinking degrees, which of them are simply controlled by the stoichiometric ratios of

gelatin and β-CD-acrylate in gel formulations. The efficient covalent crosslinking reaction allows in situ gel formation that might improve the versatility of material to injectable drug release systems. One can suggest that the demonstrated approach could be utilized in the design and synthesis of hydrogel-based controlled/sustained drug delivery systems.
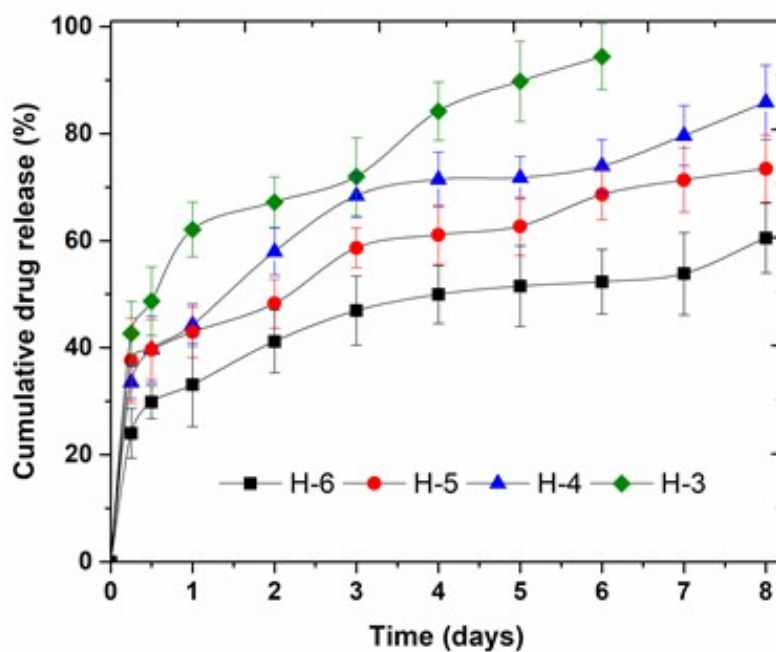
Figure 7. Cumulative release profiles of diclofenac-Na from gelatin-based β-CD containing hydrogels at 37 °C in PBS buffer (pH 7.4). Data with mean ±SD (n=3).

CONCLUSION

A series of gelatin-based hydrogels incorporating β-CD units were fabricated under benign reaction conditions. Chemical crosslinking was established by utilizing unmodified pristine porcine gelatin via aza-Michael addition reaction. Practical and straightforward synthesis methodology allowed tunable control of the β-CD-based crosslinker amount in gel networks. It was shown that hydrogel physical properties could be tuned by adjusting the stoichiometry between amine and acrylate groups of precursor materials. The potential sustained drug release application of hydrogels was investigated by employing diclofenac-Na as a model hydrophobic molecule capable of inclusion complex formation with β-CD. The results suggested that the amount of β-CD content in the hydrogels maintained enhanced drug/matrix interactions leading to higher drug loading into the gel network. Lower initial burst releases and more sustained drug release for days was also attained in case of higher β-CD incorporation. It is believed that the efficient covalent crosslinking reaction demonstrated here could be utilized in the fabrication of biologically relevant gelatin and cyclodextrin based biomaterials.

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SUPPORTING INFORMATION
In situ Crosslinking System of Gelatin with Acrylated β-cyclodextrin Towards the Fabrication of Hydrogels for Sustained Drug Release

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Synthesis of Acryloyl-modified β-cyclodextrin (β-CD-acrylate): Synthesis of the β-CD-acrylate was conducted by following a previously reported procedure.(1) β-CD (1.0 g, 0.88 mmol) previously dried by lyophilization, and triethylamine (0.5 mL, 6.16 mmol) were dissolved in 10 mL dry dimethylformamide. The mixture was cooled to 0 °C in an ice bath and acryloyl chloride (0.85 mL, 6.16 mmol) was added dropwise. The reaction was continued for 16 h at room temperature. After the reaction was complete, a white precipitate was removed by filtration. The purified product was obtained by precipitation of the mixture into cold acetone and filtration (Yield: 41%). The structure of the compound is consistent with the reported literature data (Figure S1). Degree of acetylation based on peak integrals of H1 protons at 5.03 ppm and vinyl protons at 5.82 - 6.40 ppm revealed average degree of acetylation ~ 5.9 per molecule.
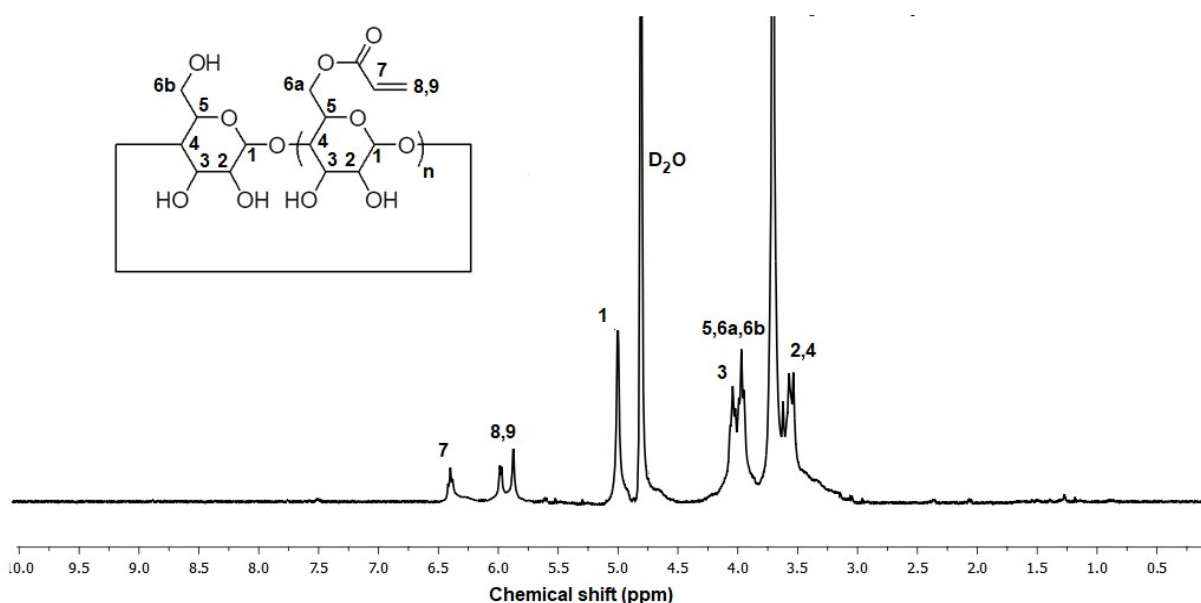
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RESEARCH ARTICLE

A comprehensive study on electrospinning of poly (vinyl alcohol): effects of the TCD, applied voltage, flow rate, and solution concentration

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Abstract: In the present work, the electrospun fibers of poly (vinyl alcohol) (PVA) are fabricated from its solution in water under ambient conditions, and the effect of altered working parameters used such as tip-to-collector distance (TCD), applied voltage, flow rate and solution concentration on the average diameter and frequency distribution of them is deeply discussed. The mean electrospun PVA fiber diameters and its distributions are estimated by a combination of the Scanning electron microscope (SEM) and the ImageJ analyzer program, which is extensively utilized in respective sciences. The achieved results from the experiments indicate that higher both TCD and applied voltage used decrease the average electrospun PVA fiber diameter. Conversely, when the flow rate or solution concentration is increased, diameters of achieved fibers are soared. It is anticipated that this comprehensive study will be beneficial to academia and industry working on potential PVA fiber applications.

INTRODUCTION

To date, with the increasing global interest, considerable research efforts have been generated aiming at the production of fibers of both natural or synthetic polymers and inorganic materials for a remarkable range of application areas such as chemistry, medicine, material science, nanotechnology, textile, and so on (1-8). In this respect, the most well-known fiber production methods mentioned in the literature are mechanical drawing, self-assembly, hydrothermal processing, electrospinning, melt blowing, phase separation, template synthesis, extraction, vapor-phase polymerization, and solvent casting (7, 9). Among them, especially, electrospinning process, which can be applied as the spinning of the wet or dry solution, melt at elevated temperatures, gel, and emulsion has emerged as an efficient, affordable, and most extensively utilized method. A typical relatively simple electrospinning instrument setup consists of a metallic nozzle, syringe pump, voltage supply, and collector (screen or roller) apparatus for making fibers with diameters ranging from few nanometers to a few hundred nanometers. Generally, the setup is designed for feeding ranging from $\mu L.h^{-1}$ to $mL.min^{-1}$ and making 0–40 kV voltage value over distances of 0–40 cm between the nozzle and collector. During the experiments, after the voltage is imposed, the polymer droplet that emerged on the nozzle is transformed into a conical shape (known as Taylor cone) via potential difference formed. The continuously elongated polymeric jet that is ejected from the pendant droplet by overcoming the surface tension of the spinning solution reaches the collector while the solvent is evaporated. Many types of electrospinning parameters such as TCD distance, applied voltage, flow rate and needle diameter (process parameters), the solvent used, concentration, viscosity and conductivity of the solution, molecular weight, solubility, glass-transition temperature,

chain entanglement density of polymer (molecular parameters), relative humidity, pressure and temperature of surrounding (ambient parameters) can strongly influence the various properties and structure of the final product (10). Producing the long lengths fibers, providing the higher surface area to volume ratio and superior mechanical properties, and allowing the functionalization and tunable surface morphologies are some of the advantages that it provides. Moreover, the electrospinning process offers many advantages due to its ease controllability, economic competitiveness, and versatility in broad range potential fields such as biosensor technology, enzyme immobilization, optoelectronics, filtration, tissue engineering, drug delivery, wound dressings, self-cleaning, environmental remediation, and etc (11).

PVA is biodegradable and biocompatible, semicrystalline and water-soluble industrial polymer, and is available as entirely (91-99%), moderately (92-96%) or partially (87-89%) hydrolyzed forms produced by sequential polymerization of vinyl acetate monomer and hydrolysis of achieved poly (vinyl acetate) (PVAc). Depending on its hydrolysis degree, the mechanical, chemical, and physical properties of PVA, such as rigidity, tensile strength, flexibility, solubility, crystallinity, and biodegradability are in a wide range (9, 12). As an FDA-approved material, PVA based products have been in use for advanced widespread industrial, commercial, food, and medical applications, including textile, paper, antimicrobial packaging, tissue engineering, wound dressing, contact lenses, and drug delivery on account of its excellent features (13-15). Hence, PVA has piqued the interest of industry and academia for many years due to the information mentioned above. Furthermore, PVA aqueous solution can be easily prepared and electrospun; thus, the present comprehensive study will be promising for the scientists working on different PVA fiber applications.

This study aims to demonstrate the influence of a variety of electrospinning parameters on the average diameter and frequency distribution of electrospun PVA fibers to create a detailed database for scientists and industry workers. The morphological analysis of PVA fibers was studied through scanning electron microscopy and accurate image processing.

EXPERIMENTAL SECTION

Materials
During the experiments, the utilized commercially available fully hydrolyzed Poly (vinyl alcohol) (PVA, M_w =60,000 g.mol^{-1}) was purchased from Merck (Darmstadt, Germany). To prepare the PVA electrospinning solutions, water (H$_2$O, ultrapure grade) procured from Merck (Steinheim, Germany)

was used. Rectangular microscope cover glasses used as fiber accumulated substrate (3×1 inch) were supplied from ISOLAB (Istanbul, Turkey). No necessary purification and distillation procedures were implemented for all chemicals and solvents used.

Preparation of electrospinning solutions
Commercially available, fully hydrolyzed PVA was dissolved in distilled H$_2$O by vigorously stirring on a magnetic stirrer at 60 °C for 3 h to obtain 11, 13, and 15% (w/w) solutions to be used in the electrospinning process for determining the effect of solution concentration.

Electrospinning procedure of PVA
To fabricate the electrospun fibers of PVA achieved previously from its solutions at different concentrations, a transparent chamber of electrospinning setup equipped with a syringe pump (NE-500, New Era Pump Systems Inc., Turkey), a high voltage power source (Electrosis, PW1010, Turkey). A 5 mL plastic syringe with metal needle and rectangular microscope cover glasses, which are cleaned two times with chromic acid solution-distilled water cycle, having 3×1 inch dimensions stuck aluminum plate collector was utilized. To conduct the detailed investigation on the effect of different parameters on electrospinning, 15, 20 and 25 kV are applied on PVA solutions at 11, 13 and 15% concentrations when they are fed by 0.6, 1.0 and 1.4 mL.h^{-1} rates fixed to 7, 10 and 13 cm TCDs, separately. During the experiments, relative humidity and temperature were 41% and 24.1 °C and almost constant. After conducting the electrospinning experiments, electrospun fiber coated glasses were kept in a desiccator to remove the residual H$_2$O for 72 h at room temperature to be used for SEM analysis. Electrospinning time was 5 min for all experiments.

Characterizations
Surface morphologies and characteristics of each PVA fibers obtained by using different electrospinning parameters were examined with the help of scanning electron microscopy (SEM, at 10.0 kV, JEOL JSM-6335F, Tokyo, Japan, after coating with Pt (Platin) by sputter coater device (Polaron SC7620, East Sussex, United Kingdom). The average diameters and histograms of fibers were determined to utilize Image J processing software application (National Institutes of Health; USA).

RESULTS AND DISCUSSION

The electrospinning variables influencing the full range of features of the fibers have motivated the investigators in many types of research for use in diverse applications (16, 17). In our study, electrospinning parameters mentioned elsewhere in the literature were changed; a variety of optimum spinning conditions were utilized to achieve the

smooth and uniform PVA fibers without the free beads. Some SEM images selected were presented in Figures (a)-(e) to provide seeing the micrometer scale, unimodal, cylindrical, and beadless structure and smooth surface of the achieved PVA fibers to readers. As can be easily seen from the ×2,500 and 10,000 zoomed SEM images, while the flow rate and solution concentration fixed, if the applied voltage or TCD is increased to higher values, it is visibly evident that average diameter of the obtained fibers decreases to lower, as expected. Moreover, the obtained PVA fiber structures were almost the same in these conditions.

The primary purpose of this study was to provide researchers with a comprehensive overview of how several electrospinning parameters would affect the average fiber diameters of PVA fibers and their distribution. In this respect, the detailed Table 1 and some selected histograms (Figures 2(a)–(e) were presented.

The effect of polymer solution concentration on the diameter of electrospun fibers has been studied in the literature by several groups. Indeed, the uniaxial stretching of a charged jet, which can strongly be affected by the solution concentration mentioned in the Introduction part, is the essential phenomenon in the electrospinning technique. Based on the literature results, it is well-known that the increasing solution concentration caused to an increase in solution viscosity and formation of uniform fibers after the critical chain entanglement value, and increasing average diameter of fibers as well (18, 19). Firstly the effect of solution concentration on the average diameter of electrospun PVA fibers was investigated, and PVA solution concentration was varied from 11 to 15% (w/w). After the solution concentration was increased from 11 to 13 or 15%, the mean diameter of PVA fibers gradually was increased. The obtained results were in good agreement with the literature, as tabulated in Table 1 (20).

As stated in previous information, feeding or flow rate is another parameter that affected the resulted

average diameter of fibers in the electrospinning process. Higher flow rate produces fibers with relatively larger diameter or vice versa due to the formation of a higher accumulated amount of suspended droplet on the spinneret that can lead to insufficient time to elongation of polymer chains (21). In our study, the flow rate was varied in the range of 0.6 and 1.4 mL.h^{-1}. One can see in Table 1, after the used flow rate was increased, the mean diameter of electrospun PVA fibers was increased, supporting the above-stated results achieved from concentration changes.

The reverse influences solution concentration and flow rate effects were obtained by altering both TCD and applied voltage parameters. The prevention of bead formation and adjusting the fiber diameter can be taken control by selecting the optimal TCD providing adequate time to complete solvent evaporation from the fibers before reaching the collector (19). As can be seen in Figures 1(a)-(e) and 2(a)-(e), and Table 1 as well, while the electrospinning TCD changed from 7 to 13 cm, the mean PVA fiber diameter decreased probably due to the reason mentioned above. On the other hand, the applied voltage that is known as driving force to jet initiation during electrospinning was the other examined parameter for solution electrospinning of PVA. In this respect, in our present study, PVA solution having different either concentrations and feed rates or varied TCD was electrospun with 15, 20, or 25 kV applied voltage. The average diameter of all the electrospun fibers achieved as uniform without bead formation decreased gradually with the increasing applied voltage from 15 to 25 kV, supporting the trend of mean diameter change when the TCD increased from 7 to 13 cm. These applied voltage changing results combined with the TCD alteration results were attributable to producing ultrafine or nanofibers resulting from higher electrostatic force to stretch the jet during electrospinning. Also, the observed results were in good agreement with the literature, as expected (22).
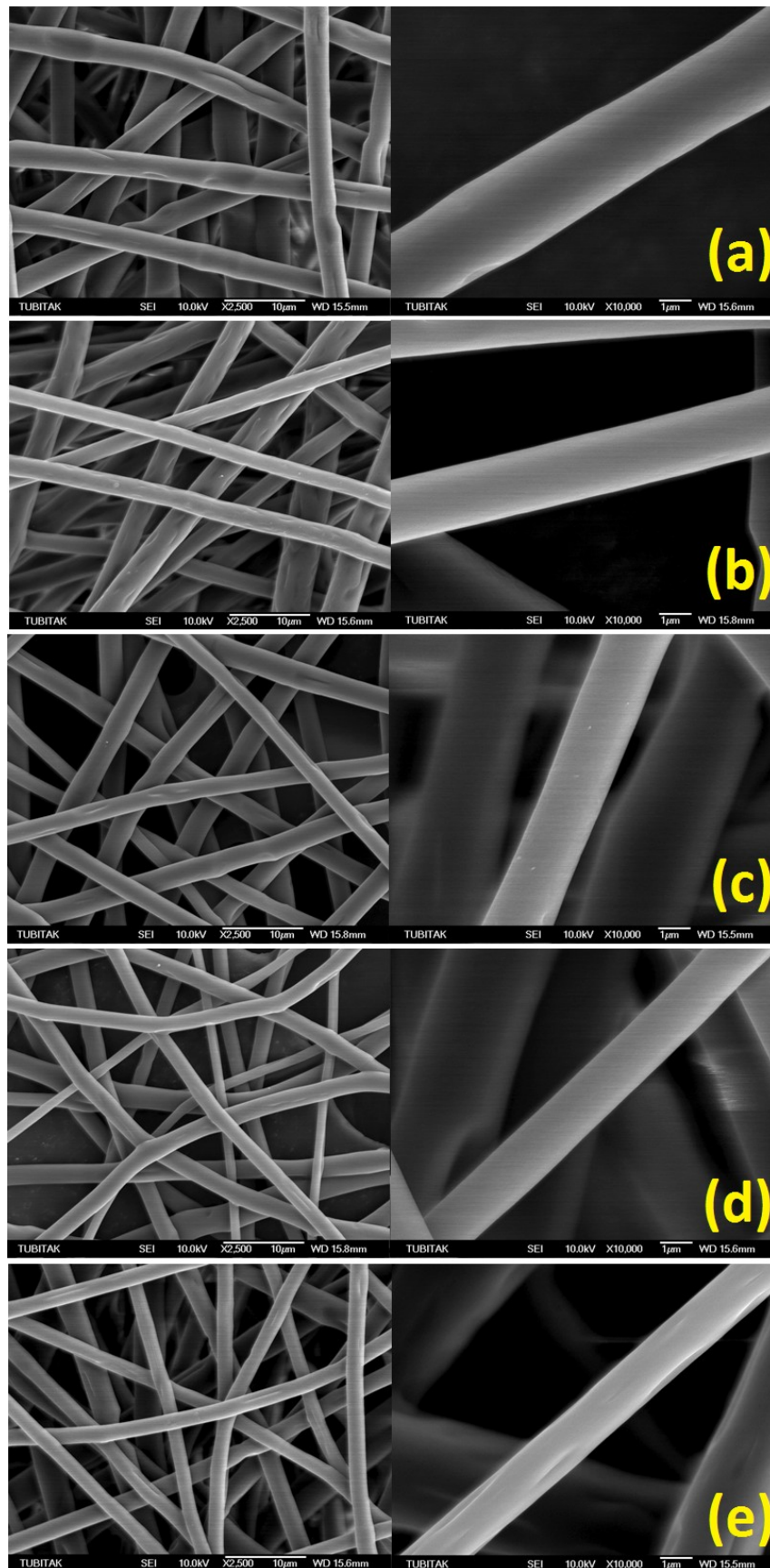
Figure 1. The SEM images (left, ×2,500) and their magnified micrographs (right, ×10,000) of electrospun PVA fibers obtained from 1 mL .h^{-1} flow rate, 13% (w/w) solution concentration, (a) 15 kV (7 cm TCD) (b) 20 kV (7 cm TCD) (c) 25 kV (7 cm TCD) (d) 25 kV (10 cm TCD) (e) 25 kV (13 cm TCD) applied voltages.
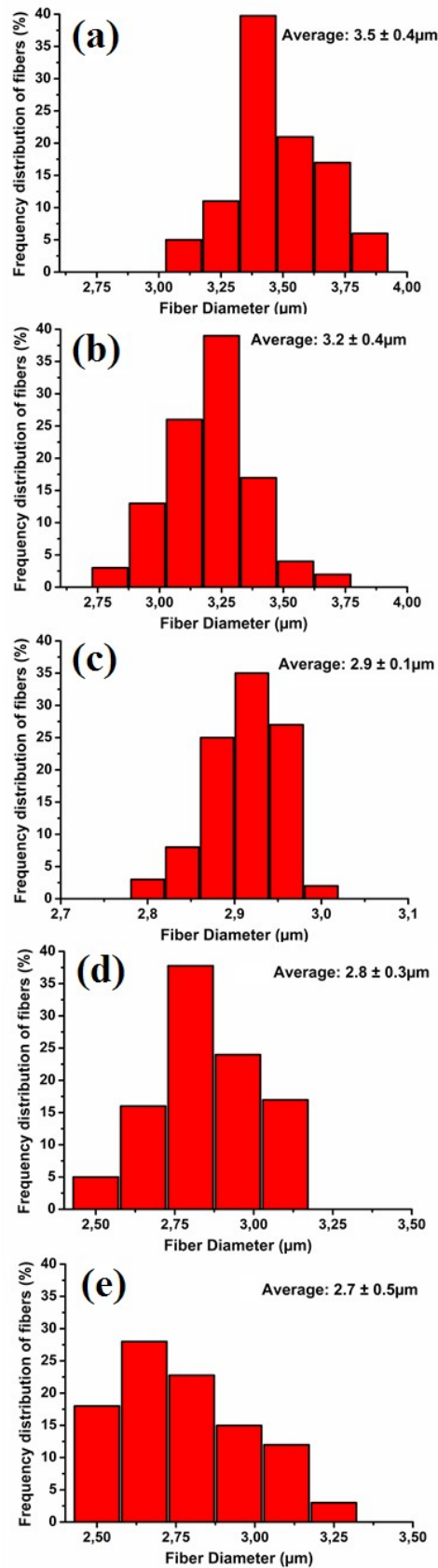
Figure 2. Frequency distribution and average diameter histograms of electrospun PVA fibers obtained from 1 mL .h^{-1} flow rate, 13% (w/w) solution concentration, (a) 15 kV (7 cm TCD) (b) 20 kV (7 cm TCD) (c) 25 kV (7 cm TCD) (d) 25 kV (10 cm TCD) (e) 25 kV (13 cm TCD) applied voltages.

Table 1. The data of experiments carried out at each variable of tip-to-collector distance, solution concentration, applied voltage, and solution flow rate.

Experiment No	Tip-to-Collector Distance, x= 7, 10 and 13 (cm)			Average Fiber Diameter ±std dev. (µm)		
	Solution Conc. (%w/w)	Applied Voltage (kV)	Solution Flow Rate (mL.h⁻¹)	x=7cm	x=10cm	x=13cm
1	11	15	0.6	3.1±0.3	3.0±0.1	2.9±0.7
2	11	15	1.0	3.1±0.5	3.0±0.5	3.0±0.1
3	11	15	1.4	3.1±0.9	3.1±0.4	3.0±0.9
4	11	20	0.6	2.9±0.4	2.7±0.1	2.6±0.8
5	11	20	1.0	2.9±0.9	2.9±0.2	2.8±0.8
6	11	20	1.4	3.0±1.0	3.0±0.6	2.9±0.9
7	11	25	0.6	2.7±0.5	2.6±0.9	2.4±0.2
8	11	25	1.0	2.8±0.1	2.7±0.7	2.6±0.7
9	11	25	1.4	2.8±0.5	2.9±0.5	2.9±0.1
10	13	15	0.6	3.4±0.3	3.2±0.3	3.1±0.1
11	13	15	1.0	3.5±0.4	3.4±0.6	3.2±0.4
12	13	15	1.4	3.6±0.3	3.4±0.2	3.3±0.3
13	13	20	0.6	3.0±0.4	2.9±0.3	2.8±0.3
14	13	20	1.0	3.2±0.4	3.1±0.5	2.9±0.4
15	13	20	1.4	3.3±0.4	3.2±0.3	3.1±0.6
16	13	25	0.6	2.8±0.2	2.7±0.3	2.5±0.3
17	13	25	1.0	2.9±0.1	2.8±0.3	2.7±0.5
18	13	25	1.4	3.0±0.5	2.9±0.1	2.8±0.7
19	15	15	0.6	3.6±0.5	3.5±0.4	3.3±0.5
20	15	15	1.0	3.7±0.4	3.6±0.3	3.5±0.3
21	15	15	1.4	3.8±0.3	3.6±0.7	3.6±0.1
22	15	20	0.6	3.4±0.5	3.2±0.5	3.1±0.3
23	15	20	1.0	3.4±0.8	3.3±0.7	3.2±0.1
24	15	20	1.4	3.6±0.4	3.6±0.8	3.5±0.9
25	15	25	0.6	3.1±0.3	2.9±0.3	2.8±0.1
26	15	25	1.0	3.2±0.5	3.1±0.7	3.1±0.2
27	15	25	1.4	3.2±0.8	3.2±0.2	3.1±0.9

CONCLUSION

In this study, the influence of electrospinning parameters such as polymer solution concentration, solution flow rate, tip-to-collector distance, and applied voltage was investigated deeply for the electrospun PVA fiber morphology, frequency distribution, and average diameter by conducting 27 separate experiments. Fiber properties such as fiber mean diameter, morphology, frequency distribution, etc. can be fully controlled with an appropriate combination of process, solution, and environmental parameters in the electrospinning process for the desired functions. The effect of these different variables on the resulted PVA fibers was tried to be explained logically by correlating with the previously published electrospinning literature papers. Depending on the attained results from the present comprehensive study, PVA fibers thus offer many attributions to scientists working on PVA fiber applications.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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RESEARCH ARTICLE

Antioxidant and Anticholinesterase Properties of *Sideritis perfoliata* subsp*. athoa* (Papan. & Kokkini) Baden and *Sideritis trojana* Bornm. Teas from Mount Ida-Turkey and Their Phenolic Characterization by LC-MS/MS

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Abstract: The phenolic profile, antioxidant, and anticholinesterase activities of teas prepared from two *Sideritis* (L.) species: *Sideritis perfoliata* subsp. *athoa (Papan. & Kokkini*) Baden and *Sideritis trojana* Bornm., collected from Mount Ida (Kaz Dağı) Turkey, were determined. The teas were prepared by infusion and decoction methods. The quantitative amounts of the phenolic contents were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The significant phytochemicals were found to be fumaric acid for infusion and decoction samples of *S. trojana* and infusion of *S. perfoliata* subsp*. athoa*, chlorogenic acid for decoction of *S. perfoliata* subsp*. athoa.* The tea samples prepared by the decoction method were found to be rich in phenolics. Apart from the fact that the decoction sample of *S. trojana* exhibited the best antioxidant effect in 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene bleaching and cupric (Cu^{2+}) ion reducing power assay (CUPRAC) methods among the tested samples at all concentrations, and showed significant inhibition effect at 200 µg/mL against acetylcholinesterase and butyrylcholinesterase enzymes (59.74%, 64.99%, respectively).

INTRODUCTION

For centuries, herbal teas have been used as herbal remedies to treat infections, ailments, and diseases. For the usage as a health-promoting beverage, the evaluation of the functional and bioactive composition of the tea, such as polyphenols, carotenoids, vitamin C, vitamin E, as well as essential major, minor, and trace elements are essential. Some studies have been reported their antioxidative, antimicrobial, anticarcinogenic, antihypertensive, antimutagenic, and antiangiogenic activities. These activities have shown that they are related to different bioactive components included in herbal teas (1–4).

The antioxidant activity is based on the availability of electrons to neutralize any free radicals. Phenolic compounds are known to be antioxidant compounds. The antioxidant activity of plants is attributed mainly to their phenolic compounds (1,3-4). Alzheimer's disease (AD) is the most common form of dementia. In addition to synthetic drugs, the treatment of this disease has gained significant importance with natural treatments. Therefore, finding natural sources to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which AD begins as a deficiency in the production of the neurotransmitter acetylcholine inhibitors, has been the subject of many studies recently.

The genus *Sideritis* (L.), a member of the Lamiaceae plant family, is distributed mainly in the Mediterranean area over 150 annual and perennial species (5,6). In Turkey, as well as in the world, *Sideritis* species is very widely consumed as herbal tea and has been used in folk medicine for their activities such as anti-inflammatory, antirheumatic, antispasmodic, and antimicrobial (7-11). In Turkey, the genus *Sideritis* is divided into three sections (Hesiodia, Burgsdorfia, and Empedoclia), and Turkey is the gene center of the Empedoclia section (5). They represented 46 species (52 taxa) with a very high endemism ratio (almost 80%) (9). Most of the studies carried on *Sideritis* species are focused on their bioactive constituents; essential oils, diterpenoids, glycosides- and activities of their extracts, and isolated pure compounds (1,3,6-13). Although they have been used as a tea for a long time, the studies of aqueous extracts of the genus *Sideritis* have recently become more popular. Traditionally, *Sideritis* teas are prepared via the infusion method using flowering branches of the plant. The studies showed that the main chemicals of the infusions are composed of water-soluble polyphenolic compounds, many of which have related to their potent antioxidant properties, also minerals and vitamins. The phytochemical composition and several activities of some *Sideritis* tea; *Sideritis scardica, S. raeseri, S. syriaca*, and S. *clandestina* subsp. *clandestina* have been reported previously (1,3,15–17).

Mount Ida, which is one of the wealthiest mountains concerning rareness and endemic species, is located between Balikesir and Canakkale boundaries in Turkey. *Sideritis perfoliata* subsp. *athoa* (Papan. & Kokkini) Baden., and *S. trojana* Bornm. are grown at Mount Ida. *S. trojana* is an endemic species, widely known as "Sarıkız çayı" and *S. perfoliata* subsp*. athoa* is known as "Kedi kuyruğu çayı". The teas prepared from both species are widely consumed with a view to its distinctive flavor and possible pharmacological effects such as common cold, including fever, flu, and bronchitis, and to relieve gastric disorders (18). Both species have been investigated for their essential oil, diterpenoids, and glycoside composition, and several activities in some studies (Table 1). There is no study on the detailed phenolic composition and anticholinesterase activity of teas prepared from *S. trojana* and *S. perfoliata* subsp*. athoa*.

The objective of the present study is to determine phenolic composition as well as antioxidant and anticholinesterase activity of the *S. trojana* and *S. perfoliata* subsp*. athoa* herbal teas. Two methods were used to prepare tea samples: infusion and decoction. The determined phenolic contents, divided of mainly three groups: flavonoids and derivatives, phenolic acids, and dicarboxylic acid, were screened by LC-MS/MS. Antioxidant properties were determined based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene linoleic acid, and cupric (Cu^{2+}) ion reducing power assay (CUPRAC). The anticholinesterase activity was also evaluated. This study is the first one on the phenolic composition and activity of the *S. trojana* and *S. perfoliata* subsp*. athoa* herbal teas.

Table 1. Previous studies on *S. trojana* and *S. perfoliata* subsp. *athoa*.

	S. trojana	**S. perfoliata subsp. athoa**
Major comp. of Essential oil (EO)	β-pinene α-pinene (19) Valeranone α-bisabolol β-caryophyllene (20)	Myrcene β-pinene Ar-curcumene (25)
Diterpenoids	7-*epi*-Candicandiol Siderol Sideridiol Isocandol B Candol A acetate *Ent*-7α-acetoxy-kaur-15-ene *Ent*-7α-acetoxy-15β,16β-epoxy-kaurane (7-Acetyl-sideroxol) *Ent*-2α-hydroxy-8(14),15-pimaradiene (21)	Linearol Foliol Sidol *Ent*-3β,7α-dihydroxy-kaur-16-ene *Ent*-7α,18-dihydroxy-beyer-15-ene 7-epi-Candicandiol *Ent*-3β-hydroxy-kaur-16-ene Athonolone *Ent*-3α,18-dihydroxy-kaur-16-ene (26, 27)
Phenolics, Flavonoids and derivatives	Melittoside 10-*O*-(*E*)-feruloylmelittoside 10-*O*-(*E*)-p-coumaroylmelittoside Stachysoside E Stachysoside G Verbascoside	

	Isoacteoside Lamalboside Leonoside A Isolavandulifolioside Isoscutellarein 7-*O*-[6‴-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside 4′-*O*-Methylisoscutellarein 7-*O*-[6‴-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside 3′-Hydroxy-4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl-β-allopyranosyl-(1→2)]-β-glucopyranoside Di-*O*-methylcrenatin (22)	
Activity	Antistress (23) Antimicrobial activity of EO (20) Antioxidant and antidiabetic activity (24)	*Candida albicans* Inhibitory (28) Insecticidal (29)

MATERIALS AND METHODS

General

LC-MS/MS experiments were performed by a Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometry, equipped with a Synergy Max C18 column (250 x 2 mm i.d., 5-µm particle size). The compounds used as standards in LC-MS/MS analyses and the experimental details are given in the supplementary data. For the antioxidant and anticholinesterase activities, absorbances (UV and visible range of 230 nm to 750 nm) were measured using a multiplate reader (Beckman Coulter DTX 880 Multimode Detector).

Plant Material

Sideritis perfoliata subsp.*athoa (Papan. &Kokkini)* Baden. were collected from natural habitats in B1, Balikesir-Edremit, Mount Ida (Kazdağı, Kapıdağ), around the fire tower, rocky areas, 1350 m altitude, July 2013, Turkey (herbarium number TD 3805).

Sideritis trojana Bornm., originated in Balikesir-Edremit, Mount Ida, near Sarıkız hill, limestone rocks, 1750 m altitude, July 2013, Turkey (herbarium number TD 3818). The species were identified by Prof. Dr. Tuncay Dirmenci (Balıkesir University), voucher specimens have been deposited at Balikesir University Necatibey Education Faculty Herbarium. The plant samples were allowed to dry in the shade.

Preparation of decoction and infusion samples

Herbal teas were prepared as following the traditional preparation method: infusion and decoction.
Infusion: Two grams of the dried aerial parts of the plant were added in a beaker which contained 100 mL of distilled boiled water and allowed to stay for 15 minutes. Then it was filtered through Whatman No.1 filters (Sigma-Aldrich). The filtrate (25 mL) was diluted with 25 mL of distilled water.

Decoction: Two grams of the dried aerial parts of the plant were added to 100 mL of distilled water in a beaker and brought to boiling. After the boiling was stopped, the mixture was left for 15 min and filtered. The filtrate (25 mL) was diluted with 25 mL of distilled water.

Infusion samples were named as **SPAI** (infusion sample of *S. perfoliata* subsp. *athoa* (Papan. & Kokkini) Baden.) and **STI** (infusion sample of *S. trojana* Bornm.), decoction was **SPAD** (decoction sample of *S. perfoliata* subsp. *athoa* (Papan. & Kokkini) Baden.) and **STD** (decoction sample of *S. trojana* Bornm).

Antioxidant Activities

The antioxidant activity was evaluated using in vitro the free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH·) (DPPH assay) (30), *β*-carotene-linoleic acid model system (31) and CUPRAC (32). BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) were used as standards in DDPH and *β*-carotene-linoleic acid assay. In CUPRAC assay ethanol was used as a negative control; whereas curcumin was used as a positive control. A detailed experimental procedure for the activity studies were given in the supplementary data.

Anticholinesterase activity

For the measurement of anticholinesterase activity, inhibition of AChE and BChE were measured by the slightly modified spectrophotometric method developed by Ellman, Courtney, Andres, and Featherston (33). A detailed procedure was given in supplementary material.

LC-MS/MS experiments

In the LC-MS/MS experiments the mobile phase was composed of water (A, 0.1 % formic acid) in methanol (B, 0.1 % formic acid), the gradient program of which was 0-1.00 minute 55 % A and 45

% B, 1.01-20.00 minutes 100 % B and finally 20.01-23.00 55 % A and 45 % B. The flow rate of the mobile phase was 0.25 mL/min, and the column temperature was set to 30 ˚C. The injection volume was 10 µL.

The mobile phase was composed to be a gradient of acidified methanol and water system because of its fragmented ion stability. The optimum ESI (Electrospray Ionization) parameters were identical to those described in the recent study (13-14). Detailed information, validation of experiments and uncertainty evaluation is given in supplementary data.

Preparation of test solution for LC-MS/MS
A one mL of each sample was added 4 mL of the ethanol-water mixture (50:50 v/v). A portion of 1 mL of this stock solution was transferred into a 5 mL

of another volumetric flask, and 50 mL of curcumin solution was added as internal standard and diluted to the volume with methanol and mixed. The solution was filtered through a 0.45 µm Millipore Millex-HV filter and the final solution (1 mL) was transferred into a capped auto sampler vial and 10 mL of sample was injected to LC for each run. The samples in the auto sampler were kept at 15 °C during the experiment.

Statistical Analysis
In both antioxidant and anticholinesterase activity tests, the experimental data were calculated as the mean ± standard deviation and analyzed. Variance ANOVA was studied, including one-way analysis. Significant differences between means were recorded by Duncan's multiple range tests. $p < 0.05$ was regarded as significant, and $p < 0.01$ was very significant.

RESULTS AND DISCUSSION

Table 2. Phenolic composition of *Sideritis trojana* and *S. perfoliata* subsp. *athoa*

Flavonoids and derivatives				
	SPAI	**SPAD**	**STI**	**STD**
Rutin (1)	1.64±0.11	-	1.70±0.11	-
Quercitrin (2)	-	15.17±0.97	-	-
Apigenin (3)	-	-	12.17±0.98	16.61±1.34
Penduletin (4)	16.73±1.7	58.53±5.93	2.92±0.3	59.30±6.01
Quercetagetin-3,6-dimethylether (5)	110.87±20.76	-	-	27.32±5.12
Luteolin-7-*O*-glucoside (6)	7.34±0.37	22.26±2.27	-	1.84±0.19
Luteolin-5-*O*-glucoside (7)	-	4.60±0.3	-	-
Pelargonin (8)	7.30±0.37		46.21±2.35	47.86±4.87
Total (mg/kg)	**143.88**	**100.56**	**63.00**	**152.93**
Phenolic acids				
p-Coumaric acid (9)	2.65±0.41	-	-	-
Caffeic acid (10)	34.65±6.86	28.36±5.61	22.89±4.53	15.53±3.07
t-Ferulic acid (11)	201.26±14.06	-	107.32±7.5	-
Chlorogenic acid (12)	205.28±28.43	**210.89±29.2**	18.20±2.52	184.86±25.6
Rosmarinic acid (13)	3.94±0.3	3.73±0.29	4.19±0.32	4.57±0.35
Gallic acid (14)	4.65±0.32	4.55±0.32	4.63±0.32	4.54±0.31
Syringic acid (15)	-	2.92±0.2	**-**	28.80±1.94
Total (mg/kg)	**452.43**	**250.945**	**157.23**	**218.30**
Dicarboxylic acid				
Fumaric acid (16)	**281.06±19.49**	206.49±14.32	**310.62±21.54**	**462.14±32.05**
Total (mg/kg)	**281.06**	206.49	**310.62**	**462.14**
Total (mg/kg)	**877.37**	**557.50**	**530.85**	**853.37**

Major compounds of the extracts showed as **bold**.

Phenolic Profile
The characterization of the phenolic components of decoction and infusion samples of *S. perfoliata* subsp. *athoa* and *S. trojana* was achieved by LC/MS

analysis. The results were given in Table 2. In the tea samples, around 10 to 12 phenolic compounds were determined. *S. perfoliata* subsp. *athoa* was found to be rich in phenolic acids, whereas *S.*

trojana was rich in dicarboxylic acid. SPAI was found to be the richest in terms quantity (877.37 mg/kg) and numbers of determined components (12 phenolic compounds).

Fumaric acid was the main component for the infusion sample of *Sideritis perfoliata* subsp. *athoa*, infusion sample of *S. trojana* (STI) and decoction sample of *S. trojana* (STD) (281.06, 310.62, 462.14 mg/kg, respectively). For decoction sample of *S. perfoliata* subsp. *athoa* (SPAD), chlorogenic acid was determined as the main component (210.89 mg/kg). The three main components for tea samples were determined as follows: fumaric acid, chlorogenic acid, t-ferulic acid for SPAI; chlorogenic acid, fumaric acid, penduletin for SPAD; fumaric acid, t-ferulic acid, pelargonin for STI and fumaric acid, chlorogenic acid, penduletin for STD. It was previously shown that *Sideritis* extracts, especially methanolic, were rich in phenylpropanoids like verbascoside and martinoside; flavones like apigenin and penduletin; and flavone glucosides like isoscutellarein and hypolaetin (12,13,34–36). In the aqueous extract, in addition to these compounds, mainly chlorogenic acid, ferulic acid, cinnamic acid, and caffeic acid, were determined (3,15,37,35).The results are consistent with previous studies.

Antioxidant and Anticholinesterase Activities
Since a higher correlation between phenolics and antioxidant activity has been demonstrated by several studies in aromatic and medicinal plants (39–42), there is a growing interest in phenolic compounds and flavonoids, which are the most widely occurring chemicals in plants having strong antioxidant properties. Due to the toxic effect of synthetic derivatives humans prefer taking these compounds supplied by nutritional sources such as fruits, vegetables, and herbal tea, which have high phenolic content and good antioxidant capacity.

Antioxidant activities of teas were determined according to three methods: DPPH, β-carotene, and CUPRAC. The results are given in Table 3. For DPPH and β-carotene, the activity tests were carried on four different concentrations: at 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL. In the DPPH method, for all concentrations, SPAI, SPAD, and STD showed very high radical scavenging activity (up to 60%), while STI had relatively lower activity. STI has showed the best inhibition activity at a concentration of 100 µg/mL with 42.34%, while BHA was 62.39% and BHT was 80.82%.

Considering that these three tea samples are rich in fumaric acid and chlorogenic acid, it can be said that these compounds are responsible for the significant antioxidant activity of the teas. Most studies on phenolic contents of herbal teas showed that the antioxidant activity of the teas could be attributed to the presence of phenolic compounds. Notably, the antioxidant capacities of the teas having chlorogenic

acid were found to be reasonably high (3,43,44). Although it is not a phenolic compound, fumaric acid is a compound commonly detected in plant extracts, and plants carrying this compound at a high amount have been found to exhibit high antimicrobial and antioxidant activity (14,45). Furthermore, in the β-carotene-linoleic acid method, the SPAI has had the best activity value, like the standard BHA. In the CUPRAC, $TEAC_{CUPRAC}$ values of the teas were calculated by using curcumin as a reference. $TEAC_{CUPRAC}$ of curcumin was found as 0.9 mmol TR g^{-1}(in Trolox mM equivalents of 1 mM antioxidant solution which studied). The tea samples prepared by the decoction method were showed higher activity (for SPAD 2.21 mmol TR g^{-1} and for STD 2.23 mmol TR g^{-1}), while the lowest activity was showed by STI (0.43 mmol TR g^{-1}), which was the lowest tea sample in terms of total phenolic content (Table 3).

The anticholinesterase activities of the tea samples were determined at a concentration of 200 µg/mL, and inhibition % values were calculated against AChE and BChE activities. The results are given in Table 4. AChE and BChE were inhibited by galantamine, which was used as a standard, at a rate of 86% and 77%, respectively. Among the tea samples, the best inhibition values against the AChE enzyme were shown by STD of 59.74% and SPAI of 58.30%. For the BChE enzyme, the results were similar. STD and SPAI had the best inhibition values; 64.99% and 53.60%, respectively. STI has the lowest inhibition against both enzymes. Although the antioxidant activities of the tea samples resulted from their high phenolic acid contents, Orhan et al., (2007) (46) reported that there was no correlation between AChE and BChE enzyme inhibition with phenolic contents. They had reported that some of these compounds are not inhibitor for AChE and BChE. Rather than phenolic acids, flavonoid derivatives such as quercetin, genistein, luteolin-7-*O*-rutinoside were found to be more effective inhibitors. If the flavonoid content of the tea samples were compared, it was found that STD and SPAI samples with higher flavonoid content (in total 152.93 and 143.88 mg/kg, respectively) were showed higher enzyme inhibition. STI, with the lowest amount of flavonoid content, has the lowest inhibition. These results are consistent with the literature.

Table 3. Activity results of SPA and ST.					
		10 µg/mL	**25 µg/mL**	**50 µg/mL**	**100 µg/mL**
DPPH (Inhibition %)	SPAD	68.92±0.70	68.98±0.68	62.78±0.65	65.83±0.30
	STD	69.38±0.93	68.47±0.40	65.92±0.90	62.98±1.20
	SPAI	66.25±0.72	67.31±2.70	64.73±2.54	62.33±1.24
	STI	4.88±0.84	4.99±1.18	8.85±2.63	42.34±3.26
	BHA	22.75±2.15	30.97±4.14	48.17±3.94	62.39±2.99
	BHT	73.09±2.62	77.68±0.74	78.79±0.76	80.82±1.56
β-carotene-linoleic acid (Inhibition %)	SPAD	53.44±1.55	61.27±1.12	59.25±0.67	47.82±0.23
	STD	70.47±1.55	69.88±2.13	66.34±2.15	54.83±2.55
	SPAI	39.96±2.6	85.87±0.46	70.49±3.77	76.95±0.54
	STI	5.77±1.45	68.38±6.47	54.72±1.73	62.24±2.58
	BHA	81.90±1.95	85.54±1.73	85.98±2.42	79.54±4.13
	BHT	82.56±5.03	72.38±11.8	77.12±2.93	71.02±1.01
CUPRAC		**mmol TR/g**			
	SPAD	2.21			
	STD	2.53			
	SPAI	1.43			
	STI	0.43			
	Curcumin	0.90			

Table 4 Anticholinesterase Activity of *S. trojana* and *S. perfoliata* subsp. *athoa*

Tea Samples	Anticholinesterase Activity	
	AChE	**BChE**
SPAI	58.30 ± 0.48	53.60 ± 0.33
SPAD	46.82 ± 0.74	32.40 ± 0.22
STI	30.06 ± 0.67	22.78 ± 0.04
STD	59.74 ± 0.80	64.99 ± 0.36
Galantamine	86.73 ±1.25	77.13 ± 1.48

% inhibition values at 200 µg/mL concentration of tea samples.
Galantamine was used as a standard.

CONCLUSION

In conclusion, *Sideritis perfoliata* subsp. *athoa* and *S. trojana* were found as a crucial antioxidant source in different *in vitro* tests like DPPH, β-carotene linoleic acid, and CUPRAC assays. Also, they have the best inhibition results on two enzymes, which have essential roles in Alzheimer's Disease. The quantities of some phenolics of the infusion and decoction of *S. perfoliata* subsp. *athoa* and *S. trojana* were successfully characterized by LC-MS/MS, and chlorogenic acid, t-ferulic acid, penduletin, and fumaric acid were determined as the most abundant chemicals for tea samples. The antioxidant and anticholinesterase activities of the *S. perfoliata* subsp. *athoa* and *S. trojana* were probably their phenolic and flavonoid composition. Furthermore, the activity could be attributed to the possible synergistic interaction between phenolic and non-phenolic components in its chemical composition. According to the results, it can be said that *S. trojana* and *S. perfoliata* subsp. *athoa* can be used as a supportive food for an antioxidant source for daily take.

Supplementary data
Method validation parameters of LC-MS/MS measurements, standards chromatogram of secondary metabolites, structures of the determined phenolics and procedures of biological activity assays can be found in the Supplementary Information section of this document.

Conflict of interest
The author declares no competing financial interest.

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SUPPLEMENTARY DATA

Antioxidant and Anticholinesterase Properties of *Sideritis perfoliata* subsp*. athoa* (Papan. & Kokkini) Baden and *Sideritis trojana* Bornm. Teas from Mount Ida-Turkey and Their Phenolic Characterization by LC-MS/MS

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Table S1. Method Validation Parameters of LC-MS/MS Measurements

Compounds	Linear regression equation	R^2	LOD (mg/L)	LOQ (mg/L)	RSD (%)
Kaempferol	y=0.2040x+0.0573	0.9925	0.002	0.008	5.47
Kaempferon-3-O-Rutinoside	y=0.1080x+0.0135	0.9977	0.014	0.045	8.15
Salvigenin	y=0.0355x+0.8620	0.9912	0.036	0.119	5.21
Penduletin	y=0.1630x+0.0262	0.9965	0.089	0.297	9.47
Isorhamnetin	y=0.0739x+0.5100	0.9608	0.088	0.294	3.67
Quercetin	y=0.1150x+0.0078	0.9938	0.001	0.002	0.11
Quercetagetin-3,6-dimethylether	y=0.0181x+0.0202	0.9924	0.022	0.074	0.1
Isoquercetin	y=0.0115x+0.0215	0.9959	0.199	0.665	9.42
Quercitrin	y=0.0290x+0.0058	0.9918	0.001	0.002	4.28
Luteolin	y=0.2120x+0.0699	0.9937	0.062	0.207	0.16
Luteolin-7-glucoside	y=0.1350x+0.0246	0.9957	0.022	0.072	8.56
Luteolin-5-glucoside	y=0.2300x+0.0413	0.9926	0.01	0.034	1.12
Apigenin	y=0.1780x+0.0850	0.9961	0.15	0.501	4.01
Rutin	y=0.0232x+0.0008	0.9969	0.01	0.034	7.9
p-Coumaric acid	y=0.2670x+0.1810	0.9774	0.006	0.021	6.39
Caffeic Acid	y=0.3300x+0.0036	0.9924	0.028	0.093	8.04
t-ferulic acid	y=0.0655x+0.0266	0.9925	0.047	0.158	5.21
Chlorogenic Acid	y=0.2620x+0.0674	0.998	0.445	1.483	5.45
Rosmarinic acid	y=0.1960x+0.0043	0.9982	0.022	0.072	3.73
Fumaric Acid	y=0.0569x+0.0177	0.9912	0.003	0.01	5.44
Pyrogallol	y=0.0438x+0.0073	0.9803	0.001	0.002	5.47
Ellagic acid	y=0.0244x+0.0048	0.9951	0.02	0.068	0.11
Vanillin	y=0.0982x+0.0158	0.9982	0.019	0.064	6.57
Syringic acid	y=0.0305x+0.0079	0.9973	0.022	0.073	8.39
Salicylic acid	y=0.0255x+0.1780	0.9701	0.211	0.704	0.21
p-OH benzoic acid	y=0.1230x+0.0280	0.9939	0.002	0.007	4.78
IS* Curcumin*			369.3	176.9	20

* Used as internal standard
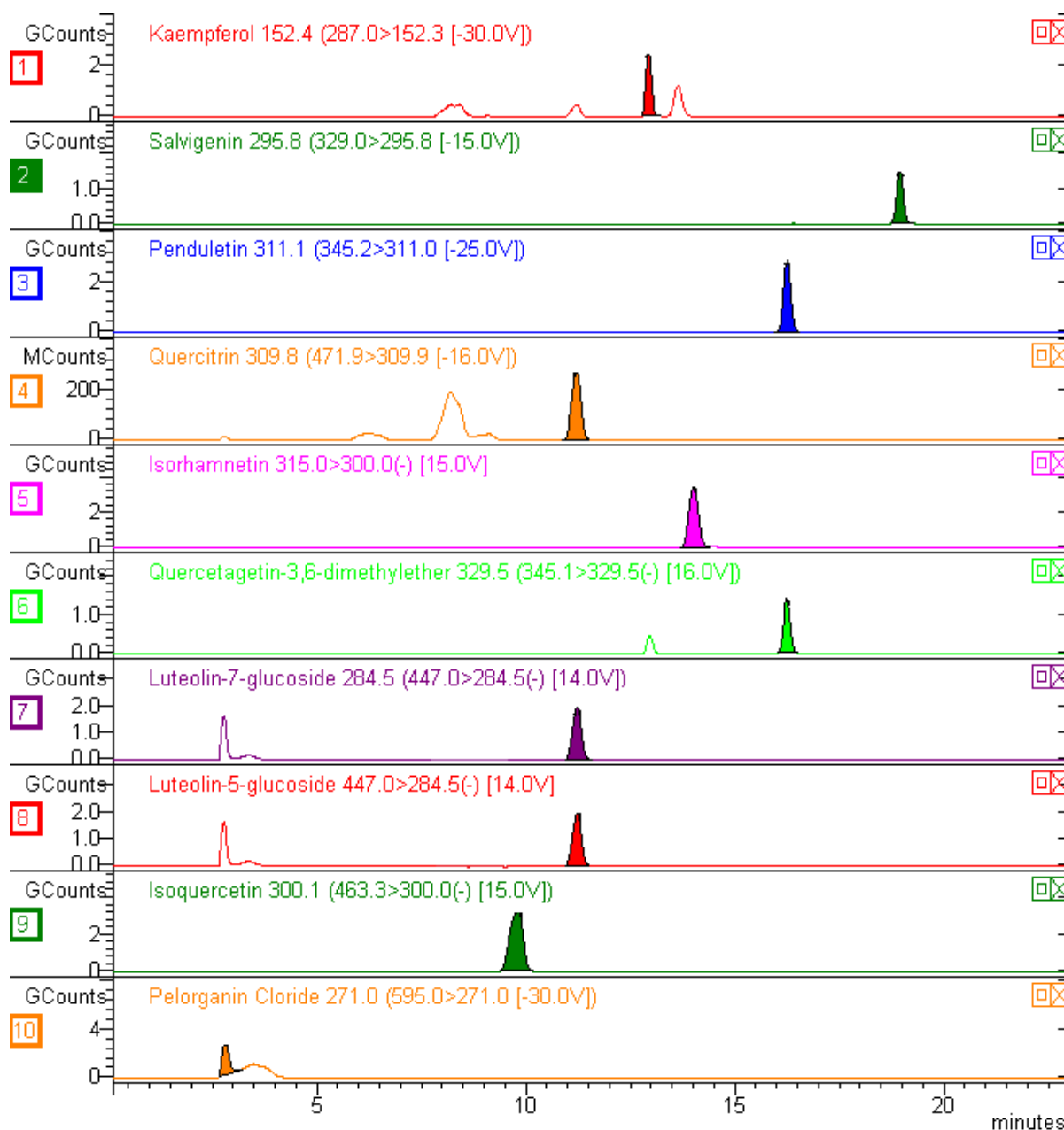
Figure S1 : Standards Chromatogram of Secondary Metabolites (Flavonoids) by LC-MS/MS (5 mg/L)
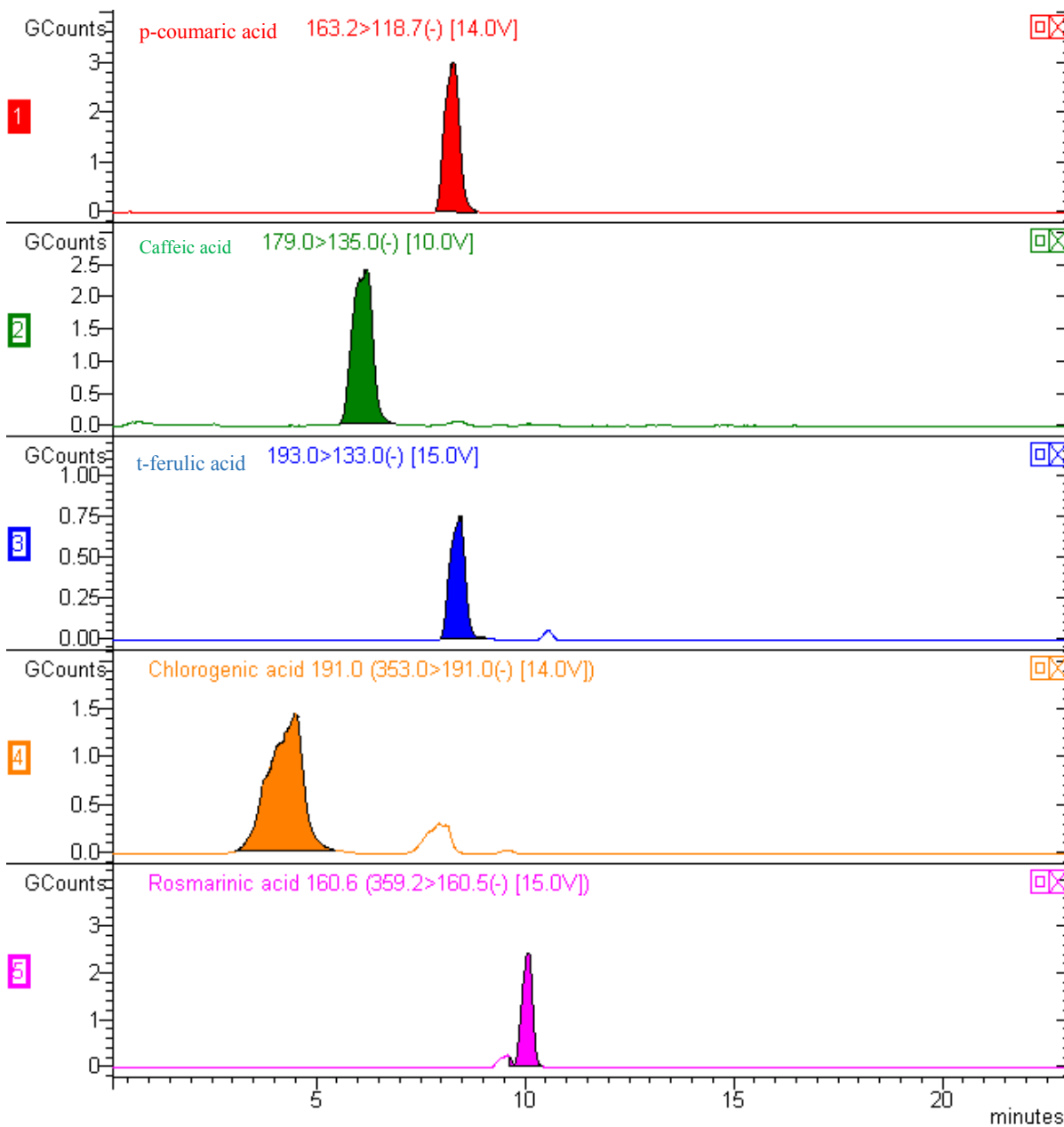
Figure S2: Standards Chromatogram of Secondary Metabolites (Phenolics and Others) by LC-MS/MS (5 mg/L)
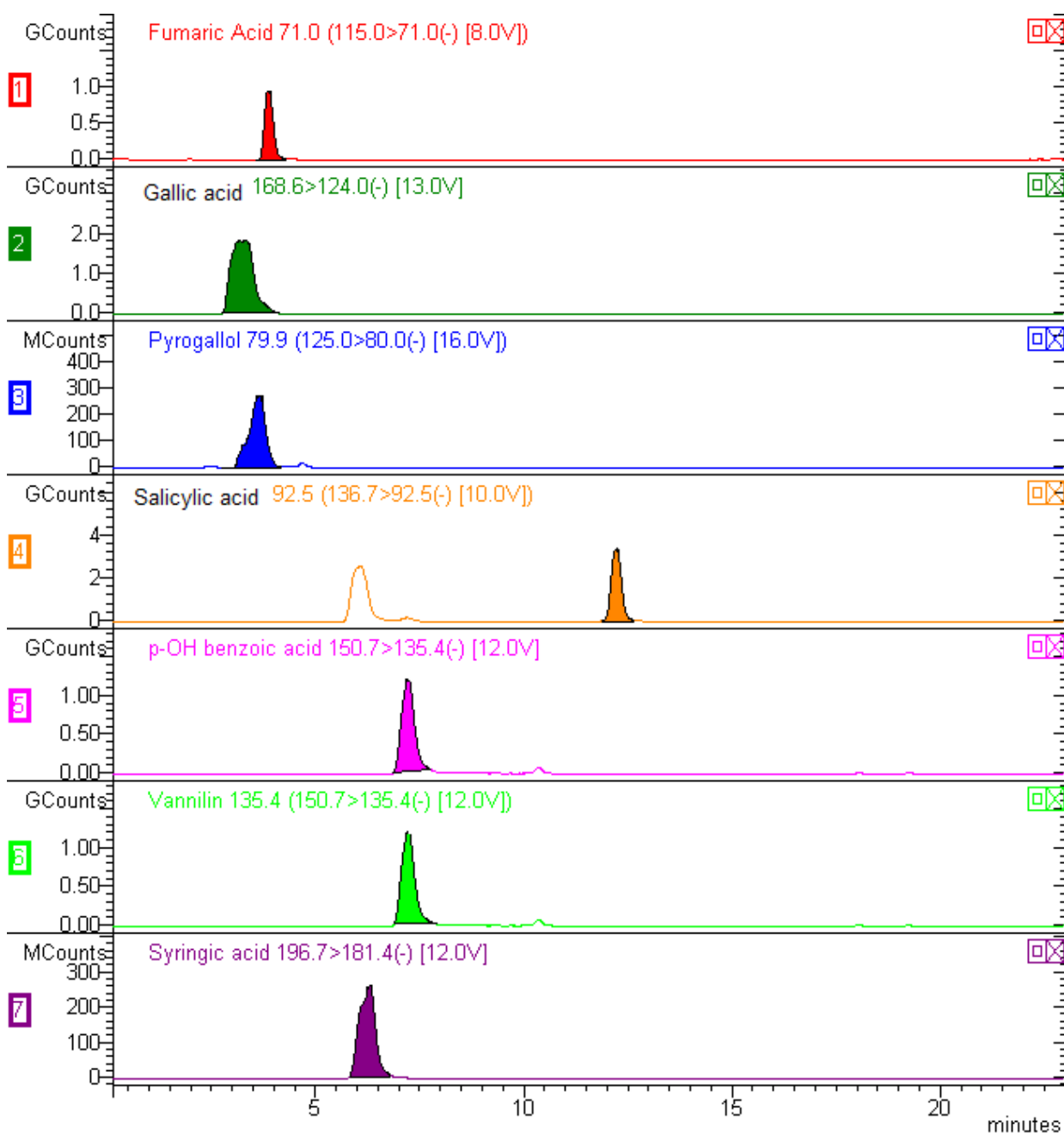
Figure S2 (Continued)
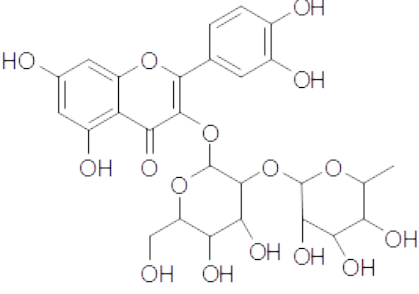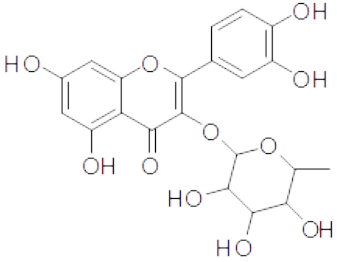
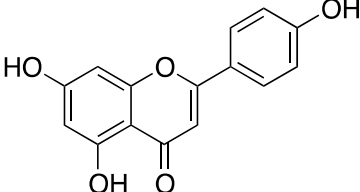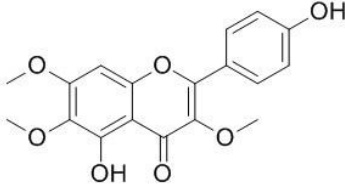**Table S2. Structures of the determined phenolics**
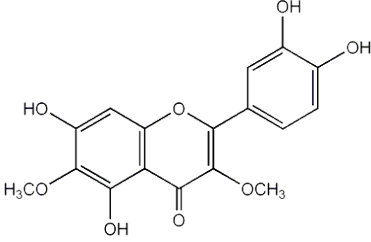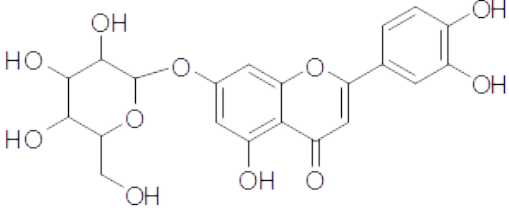
1	Rutin	
2	Quercitrin	
3	Apigenin	
4	Penduletin	
5	Quercetagetin-3,6-dimethylether	
6	Luteolin-7-*O*-Glucoside	

Table S2 (continued)
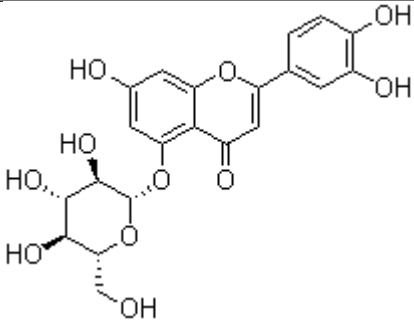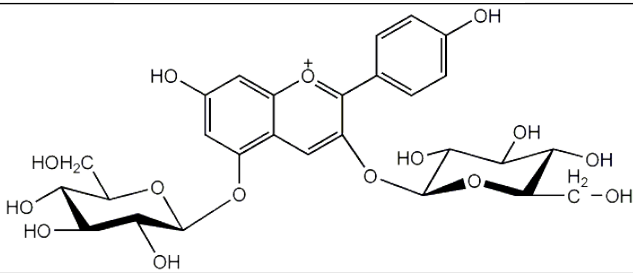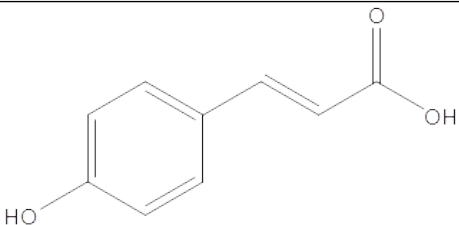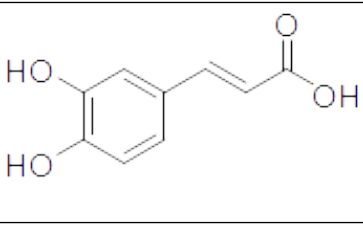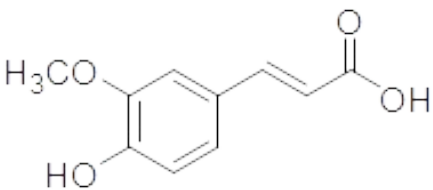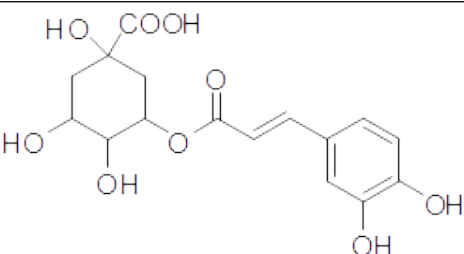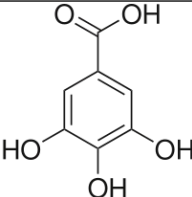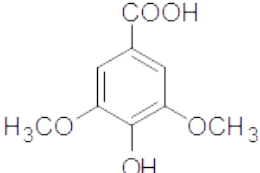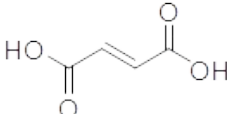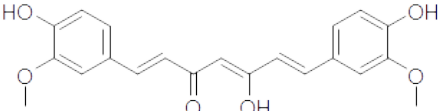
7	Luteolin-5-O-Glucoside	
8	Pelargonin	
9	p-coumaric acid	
10	Caffeic acid	
11	t-ferulic acid	
12	Chlorogenic acid	

Table S2 (continued)		
14	Gallic acid	
15	Syringic acid	
16	Fumaric acid	
IS	Curcumin-IS	

MATERIAL AND METHODS

Chemicals

The following compounds were used as standards in LC-MS/MS analyses: fumaric acid (99%, Sigma-Aldrich), pyrogallol (98%, Sigma-Aldrich), rutin (94%, Sigma-Aldrich), chlorogenic acid (95%, Sigma-Aldrich), gallic acid (99%, Merck), syringic acid (95%, Sigma-Aldrich), t-ferulic acid (99%, Sigma-Aldrich), caffeic acid (98%, Sigma-Aldrich), pelargonin chloride (98%, Sigma-Aldrich), quercitrin (97%, Sigma-Aldrich), salicylic acid (99%, Sigma-Aldrich), p-coumaric acid (98%, Sigma-Aldrich), luteolin-7-*O*-glu (99%, AppliChem), rosmarinic acid (96%, Sigma-Aldrich), pyrogallol (98%, Sigma-Aldrich), apigenin (95%, Sigma-Aldrich), kaempferol (96%, Sigma-Aldrich), and isorhamnetin (98%, ExtraSynthese, Genay-France).

LC-MS/MS experiments

Due to its fragmented ion stability, the best mobile phase solution was determined to be a gradient of acidified methanol and water system. The optimum ESI parameters were determined as 2.40 mTorr CID gas pressure, 5000.00 V ESI needle voltage, 600.00 V ESI shield voltage, 300.00 °C drying gas temperature, 50.00 °C API housing temperature, 55 psi Nebulizer gas pressure, and 40.00 psi drying gas pressure.

Procedures of Biological Activity Assays
Determination of the Anticholinesterase Activity

Inhibitory activities of acetyl- and butyryl-cholinesterase were measured by slightly modified spectrophotometric method, developed by Ellman, Courtney, Andres and Featherston (1). Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates of the reaction and DTNB method was used for the measurement of the anticholinesterase activity. Hundred and fifty microlitres of 100 mM sodium phosphate buffer (pH 8.0), test tea samples at different concentrations. Solution of AChE or BChE were mixed and incubated for 15 min at 25 °C, and 0.5 mM DTNB was added. The reaction was then initiated by the addition of acetylthiocholine iodide (0.71 mM) or butyrylthiocholinechloride (0.2 mM). The hydrolysis of these substrates were monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, at a wavelength of 412 nm. Methanol was used as a solvent to dissolve the controls. Percentage of inhibition of AChE or BChE was determined by a comparison of the rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH 8.0) using the formula:

$$\text{Inhibition \%} = [(E-S)/E] \times 100$$

where E is the activity of enzyme without test sample. and S is the activity of enzyme with test sample. Galanthamine was used as a reference compound.

Determination of the Antioxidant Activity with the β-Carotene Bleaching Method (2)

$β$-Carotene (0.5 mg) in 1 mL of chloroform was added to 25 µL of linoleic acid, and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, was added through vigorous shaking. A mixture of four thousand microliters was transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero-time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50 °C. A blank, devoid of *β*-carotene, was prepared for background subtraction. BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) were used as standards. The bleaching rate (R) of *β*-carotene was calculated using the following equation: R = ln (a/b) / t, where: ln=natural log, a=absorbance at time (0 min), and b=absorbance at time t (120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using the equation below:

$$AA = [(Rcontrol - Rsample) / Rcontrol] \times 100$$

Determination of Antioxidant Activity with the DPPH Free Radical Scavenging Method (3)

The free radical scavenging activity of the methanol extract was determined spectrophotometrically by the DPPH (1.1-diphenyl-2-picrylhydrazyl) assay. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 160 µL of this solution was added to 40 µL of sample solutions in methanol at different concentrations. After 30 min, the absorbance was measured at 517 nm. The lower absorbance of the reaction mixture indicates the higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

The CUPRAC Experiment (4)

The CUPRAC method was performed as described. with some minor modification. since experiments were performed 96-well plates.

Briefly, 1 mM dimethylformamide (DMF), 10 mM $CuCl_2$, 7.5 mM neocuproine, 1 M NH_4CH_3COO (pH 7.0) solution, and distilled water were mixed in a volume ratio of 1:1:1:0.6. Then, 180 µL of the mixture was dispersed into the wells. 25 µL diluted compounds (dilution ratio 1:20) in EtOH. After waiting for 30 minutes, the absorbance at 450 nm was measured against a reagent blank by Beckman Coulter DTX 880 Multimode Detection System. Ethanol was used as a negative control; whereas curcumin was used as a positive control.

TEAC $_{CUPRAC}$ of curcumin was found as 0.9 mmol TR g^{-1}. by using calculation formula.

TEAC $_{CUPRAC}$ values of compounds were calculated by using references. TEAC of plant extracts (mmol TR g^{-1}) = (Absorbance/ ε_{TR}) (205/25) (20/1) (2/0.02). Here, absorbance comes from the instrument; ε_{TR} = 16700 (1); 205 is total reaction volume; 25 is compound volume added to the reaction; 20/1 is the dilution factor; 2 is the solvent volume (mL) in which plant extracts; 0.02 is weight of plant extract as grams.

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4. Apak R. Güclü K. Özyürek M. Celik S E. Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay. Microchimica Acta. 2008;160(4):413-9.