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Review Article

Some Using Places of Marine-Derived Aspergillus niger in Biotechnology: A Mini Review

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ABSTRACT: Marine animals and plants, as well as inanimate habitats such as saltwater, sediments, hydrothermal vents, mud, and mudflats, are all home to marine fungi. Marine fungi are resistant to harsh marine conditions and may adapt. These marine microorganisms are significant because they can be easily cultivated and used repeatedly, as well as being safely stored in a laboratory setting. Thus, they are included in many biotechnological applications. In this review, we aimed to sum up the use of marine-derived *Aspergillus niger* fungus in biotechnology. The use of fungi in biotechnological applications such as bioremediation of heavy metals, biosynthesis of nanoparticle and nanofluid samples, production of valuable enzymes was explained in the section of biotechnological applications.

Key Words: marine fungi, eco-friendly applications, biotechnology

1 INTRODUCTION

The marine environment is under investigation increasingly for various biotechnical applications and as a new source of discoveries of science. The extreme circumstances of the sea environment are resisted by marine creatures [1]. Among the marine microorganisms, marine fungi are particularly noteworthy because they coexist in symbiotic partnerships with marine plants, vertebrates, and invertebrates and also which are isolated from the marine inanimate areas are an environmentally friendly alternative for biotechnology applications while delivering the highest value compounds under these extreme conditions [2].

In biotechnology, marine fungi are typically assessed based on their ability to produce novel secondary metabolites and are also the production source of enzymes, vitamins, polysaccharides, pigments and lipids. Nearest research has demonstrated the significance of marine fungi in the fields of biotechnology, including the synthesis of several extracellular enzymes which have applications across various domains, including food, beverage, detergent and medicine, and use in the bioremediation such as hydrocarbon degradation, removal of heavy metals, and increasing secondary metabolite production with nanoparticle applications, and biosurfactant production [3].

Tieghem (1867) designated *Aspergillus niger* as a species belonging to the *Nigri* section from the *Aspergillus* genus [4]. This

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asexually spore-bearing saprophyte inhabits aerobic settings, including 13% soil, 10% mutant environments, 24% marine environments, 27% endophytes, and 26% other environments [5,6]. *A. niger* fungus which was explored to produce citric acid for the first time, is also demonstrated as a natural source of many active compounds. *A. niger* produces many extracellular enzymes, including cellulase, pectinase, oxidase, catalase, dehydrogenase, and α-amylase, as well as a wide variety of useful proteins by developing technology [7,8]. In addition to production of secondary metabolites, which is a unit of biotechnological applications, this fungus is also used in other biotechnological applications such as bioremediation of heavy metals, biosynthesis of nanoparticle and nanofluid samples, production of valuable enzymes.

In this paper, we aimed to brief the use of marine-derived *Aspergillus niger* fungus in biotechnology. Utilization of the marine fungus *Aspergillus niger* in biotechnological applications is explained according to using purpose.

2 USE OF MARINE DERIVED *ASPERGILLUS NIGER*

2.1 Bioremediation of Heavy Metals

Toxic metals, one of the biggest problems brought by industrialization, harm both nature and people. Fungi can transport extracellular metals into the cell by increasing their growth capacity and binding them to the cell surface by improving their access through mycelium branching [9]. Thus, the fungi are an important option as natural bioremediation agents. On this subject, studies on marine fungi are very rare compared to terrestrial fungi. Marine-derived *Aspergillus niger* was studied as a bioremediation agent.

When the researches on chromium (Cr), which is among the metals of concern by the World Health Organization (WHO) due to its high toxicity and carcinogenic properties, are examined, the marine-derived *A. niger* has shown significant biosorbent competence by biomass and micellar pellets. At 25, 50 and 100 ppm concentrations of Cr (IV), there was no significant change in the dry weight of *A. niger* associated with *Eucheuma* sp. red algae, while 3.5, 7.76, and 18.1 mg of Cr were found in their pellets, respectively. Both results showed that the fungus tolerated a wide range of chromium and accumulated more than 25% of chromium [10]. Marine-associated *A. niger* displayed the greatest absorption of Cr (VI) with 117.33 mg/g biomass at pH 1.0 in the presence of 400 mg/L Cr at 50 °C, according to a kinetic investigation on the Cr (VI) sorption of dead biomass [11]. Mycelial pellets of the fungus *A. niger* isolated from the East China Sea were prepared and the ability of Cr (IV) to reduce to Cr (III) and its bio-adsorbent properties were investigated. It has been estimated that micelle

pellets produce substances that reduce Cr (IV) to Cr (III) and that these substances can diffuse into the solution containing chromium. By confirming that the fungal cell wall also adsorbs Cr (IV), marine-derived *A. niger* can be used as a natural biosorbent in wastewater treatment was predicted [12].

In studies on tolerance to copper (Cu) and lead (Pb) and their uptake capacity, *A. niger* which occurred from the sediment of the Langat River, Selangor, Malaysia, tolerated 1000 mg/L of Cu (II) and 5000 mg/L of Pb (II). In addition, the fungus removed 20.910 ± 0.581 mg/g Cu (II) at 200 mg/L of Cu (II) and 54.046 ± 0.328 mg/g Pb (II) at 250 mg/L of Pb (II) [13]. *A. niger* isolates from the Mandovi estuary in Goa, India, tolerated concentrations of 12.5 mM Pb (II) and 5 mM Cu (II). The isolate from mangroves showed a good absorption capacity of 32 - 41 mg/g of Pb^{2+} and 3.5 - 6.5 mg/g of Cu^{2+} of mycelium dry weight [14].

In other studies on heavy metals, the spores or mycelial pellets of *A. niger* fungus isolated from *Padina* sp. algae were used for aluminum recovery from bauxite ore by utilized as substrates [15].

2.2 Decolorization

Dyes, which are toxic to human cells and organs, are one of the most dangerous industrial wastes that are difficult to treat [16]. In addition, dye-containing wastewater threatens aquatic life by preventing the passage of daylight, reducing photosynthesis and causing chemical toxicity [17]. Among many dye removal procedures such as advanced oxidation processes, solvent extraction, adsorption, coagulation, ion exchange and membrane separation, the adsorption method is effectively used [17,18]. The discussion revolves around issues like clogging, efficient separation of liquid and solid after adsorption, and the potential for reusing the adsorbent [19]. The immobilization of the adsorbent biomass allows the solution of these problems to be reported. Fungi such as *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp. have been found to undergo self-immobilization forming stable mycelial pellets. Thus, fungal biomass is a good alternative for dye removal [20-23]. *Aspergillus niger* obtained from Gorgan Bay in the Caspian Sea performed the dye removal by its adsorption ability. One gram of fungal mycelium cell adsorbed the dye molecules in a temperature range of approximately 28-30 °C in 20 hours and achieved 97% colour removal [24].

Self-immobilizing mycelial pellets of halophilic fungus *A. niger* (ZJUBE-1 strain) acquired from the East China Sea were used as biosorbent and the removal of an azo dye, also known as Congo red, was investigated. As a result, *A. niger* mycelial pellets have the highest possible adsorption capacity of 263.2 mg/g of mycelium and also have effective

decolourization capabilities of > 98.5 reported [25].

2.3 Nanotechnological Applications

While utilizing marine microbes in the biosynthesis of nanoparticles (NPs) is being increased, studies on the use of marine microorganisms are still limited. Especially silver (Ag) salts and elemental silver as antimicrobial agents induced great expectations with the nanoparticle form in medical applications [26]. The synthesis of extracellular silver nanoparticles (AgNPs) of *A. niger* which was obtained from the sea of Bhavnagar coast, Gulf of Khambhat, West Coast of India, was carried out for the first time by treated with different concentrations of silver nitrate (0.25–1 mM). The utilization of laser optical speckles derived from spherical and 5-26 nm AgNPs holds potential for applications in sensors and biomedical optics [27]. AgNPs of the same fungus were biosynthesized at different pH ranges (pH:5, 8, 9, and 10). This biosynthesis, which usually takes 24 hours, took place within 3 minutes in the alkaline pH range. Antimicrobial activities of AgNPs synthesized at different pH ranges against *Bacillus megaterium*, *Shigella sonnei*, *Staphylococcus aureus* and *Proteus vulgaris* bacteria alone and in combination with gentamicin (antibiotic) were investigated. It was reported that NPs are promising antimicrobial agents, both alone and in combination, and organisms are more susceptible in an acidic pH environment [26]. Gold (Au) nanoparticles (GNPs) that affect the properties of light are utilized in potential applications of biomedicine, imaging, catalysis, and photonics. In another study on the same marine-derived fungus, it was informed that *A. niger* synthesized extracellular GNPs by its biomass exposed to Au (III) solution with different pH (7, 8, 9, 10). The weak localization of light (approximately 225 nm) in the synthesized gold nanofluids was observed. Consistent backscattering experiments were performed at two particle sizes (15 nm and 35 nm) in a water-based suspension [28]. Aspernigrin A, Aurasperone A and fonsecinone A among substances of naphtopyrones derivatives which were isolated from *Aspergillus niger* attained from a tunicate *Phallusia nigra* obtained from a coral reef in the Red Sea near Hurghada, Egypt showed the inhibitory effects. These components are evaluated as AChE enzyme inhibitors for Alzheimer's disease, and increased their inhibitory activity 16, 84 and 13 times, respectively, with AgNP synthesis [29].

2.4 Enzyme Production

Marine organisms produce therapeutic enzymes such as oncolytics, thrombolytics, and anticoagulants, as well as biocatalytic enzymes such as oxidoreductases, hydrolases, transferases, isomerases, ligases and lyases

[30,31]. One of the hydrolytic enzymes used to break down biopolymers and produce biochemicals from renewable biomass is cellulose [32]. Cellulose, which causes environmental problems when not used efficiently, is hydrolyzed by the cellulase enzyme and converted into glucose. The hydrolysis effect of cellulase is due to the synergistic effects of enzymes from exoglucanase, endoglucanase and β-glucosidase which are included in cellulase. Endoglucanase and exoglucanase mainly depolymerize cellulose, while β-glucosidase converts depolymerized substrates to glucose. Necessary to produce stable and effective enzymes that can withstand this salinity rate for the hydrolysis of cellulose (exp., marine algae [33]) that causes water pollution in a high salinity environment. In this regard, the enzymatic activity and thermodynamic characteristics of β-glucosidase obtained from *A. niger* which was obtained from the East China Sea at different salinities were investigated. Activity of β-glucosidase was elevated 1.46 times in 6% NaCl (w/v) solution salinity, 66 ^{C} temperature and pH 5.0 optimum conditions [34]. Optimization of cellulase production of the same fungal strain was investigated using different substrates (*Eichhornia crassipes*, corn cob, rice straw, wheat bran), and natural seawater (a mineral salt source). In 96 hours of incubation, biomass is maximized, while production of cellulase is 17.80 U/g based on substrate dry weight in 144 hours of incubation. An eco-friendly process was developed for cellulase production using response surface methodology [35]. *A. niger* which was acquired from sediment in the East China Sea, contained cellulase enzyme composed of high amounts of β-glucosidase with endoglucanase and exoglucanase which show low activity for cellulose hydrolysis. To increase the activity of the marine *A. niger* cellulase enzyme on cellulose hydrolysis, a vector occurring *Bacillus amyloliquefaciens* cellulase enzyme which has endoglucanase and exoglucanase with high activities was integrated into this marine fungus. The promoter glaA-signal peptide sequence-B. amyloliquefaciens cellulase-trpC terminator synthesised sequence served as the vector. The endoglucanase and exoglucanase activities in the original strain were 0.21 U/mL and 4.51 U/mL, respectively, while the activities in the vector-transformed strain were 0.89 U/mL and 15.12 U/mL, respectively. The β-glucosidase activity was 17.86 U/mL and 18.21 U/mL for the transformant and the host strain, respectively. While the cellulase activity expressed in the filter paper assay (FPA) was 4.47 U/mL, the cellulase activity of the original strain was 0.63 U/mL. In this study, the functions of endoglucanase and exoglucanase from the marine *A. niger* can be increased without changing the β-glucosidase activity for the hydrolysis of cellulose, which causes

pollution, especially in salty waters explained by gene expression [36]. In another study, the vector which is occurred promoter glaA-signal peptide sequence-*Piromyces rhizinflata* cellulase−trpC terminator, is integrated into the same marine *A. niger* strain. While endoglucanase and exoglucanase activities in the original strain were 0.21 U/mL and 4.51 U/mL respectively, after the expression they increased to 0.81 U/mL and 19.10 U/mL respectively. FPA increased from 0.63 U/mL to 4.69 U/mL. The cellulase obtained from the transformant strain showed both high activity and preserved its halostable which was in the original strain [37]. In another study, the same team investigated the function of exoglucanase from the same *Aspergillus niger* strain at a range of salt-free to 18% salinity. Exoglucanase exhibited the highest activity at 12% NaCl solution. Exoglucanase can be used for the hydrolysis of cellulose in settings of elevated salinity [38]. Salt-tolerant β-glucosidases (BGL1 and BGL2) were obtained in a study using *A. niger* (ZJUBE-1 strain) isolated from East China Sea mud as a homologous expression host. Pure BGL1 and BGL2 enzymes exhibited the highest activity at pH 3.0-4.0 and 3.5-4.5, respectively. BGL1 is resistant to metal ions, while BGL2 is sensitive to Cu2+, Fe3+ and Ag+ ions. In addition, the activity of BGL2 enhanced by 44% in the existence of 4 M NaCl was found [39]. Cellobiohydrolase is another enzyme found in cellulase. To create cellobiose, cellobiohydrolase splits the cellulose chain from its ends. β-glucosidase also reduces this cellobiose to glucose. In the study performed by Cai et al. on the same *Aspergillus niger* (ZJUBE-1 strain), they used *Agrobacterium tumefaciens* AGL-1 for transformation and expressed two genes (cel7a and cel7b) for cellobiohydrolase homologously. The industrial uses of the obtained cellobiose hydrolases (AnCel7A and AnCel7B) under acidic, high salinity and high-temperature conditions were evaluated. AnCel7B was more thermostable than AnCel7A, exhibiting half-lives of 90, 35, and 15 minutes at 80, 90, and 100 °C, respectively. In addition, AnCel7B exhibited halotolerance at 0.9 M NaCl optimal salt concentration, while AnCel7A showed acidophilic properties with optimal pH 2.5-4.5 values [40].

Another hydrolytic enzyme, xylanase, is a hemicellulotic that disrupts the bond between lignin and xylan, which protects cellulose fibres [41]. Particularly in the bio-bleaching process, xylanases are preferred because they do not contain cellulase, are effective at alkaline pH, are stable at extreme temperatures, and are active in the presence of sulfated lignin. The fungus *Aspergillus niger* which was obtained from the detritus of rotting mangrove leaves from mangrove swamps on Chorao Island, Goa, India, was investigated as

a suitable source of alkaline xylanase for use in the biological bleaching of pulp. The culture filtrate showed optimum activity at 50 °C and a secondary activity at 90 °C for pH 3.5, while optimum activity was observed at 80 °C for pH 8.5. The crude enzyme containing 580 U/L xylanase without cellulase was thermostable for at least 4 hours at 55 °C and retained approximately 60% activity. In addition, b-xylosidase and a-L-arabinofuranosidase acted synergistically with xylanase in crude culture phytate. There were reports of their moderate activities [42].

L-asparaginase (L-asparagine amidohydrolase, LA) is an important oncolytic enzyme used in the treatment of pediatric acute lymphoblastic leukaemia. *Erwinia carotovora*, *Erwinia chrysanthemi* and *Escherichia coli* bacteria are the sources of trade LA products [43,44]. However, these bacterial sources also bring with them anaphylaxis, diabetes, leukopenia, neurological seizures, pancreatitis and coagulation abnormalities possibly leading to intracranial thrombosis or bleeding [45]. In the search for a new source of LA, the fungus *A. niger* (AKV-MKBU strain) was isolated from the *Avicennia marina* mangrove located on the Bhavnagar coast, Gulf of Khambhat, West Coast of India was found as the strongest producer with the LA activity 7.5642 U/mL [46]. Optimization of LA production was achieved by using models such as the response surface methodology (RSM) used in microorganism enzyme production and the artificial neural networks (ANNs) used for enzyme production optimization. LA production which was 15.78 U/mL at pH 4, was increased by 108.62%, while the ANN method was found to be superior to the RSM analysis [47]. In another optimization study, LA activity was increased by 22.0% with 9.2285 U/mL in the medium optimized with the Plackett-Burman design and 73.52% with 13.1252 U/mL in using the central composite design (CCD) of RSM [48]. The LA enzyme purified from the fungus is 90 kDa molecular weight, active at the pH 4-10, and stable in temperatures 20-40 °C. Purified LA demonstrated its potential antiproliferative activity against HepG2, A549, U87MG, JURKAT E6, and chronic myeloid leukemia cells obtained from bone marrow, showing IC_{50} values of 0.375, 0.399, 0.204, 0.22 and 0.2 U/mL, respectively [49].

3 CONCLUSION

Biotechnological disciplines encompass environmental, industrial, pharmacological, and clinical applications. Marine fungi, in particular, represent a significant resource that is assessed in various biotechnological domains. *Aspergillus* species are considered one of the most significant marine fungal species that have been studied. The review demonstrates the significance of

utilising marine-derived *Aspergillus niger* as a valuable alternative component in biotechnological applications. According to reports, hydrolytic enzymes derived from the fungus have shown increased activity. Therefore, it is imperative to consider *Aspergillus niger* fungus as a crucial candidate for future research on the synthesis of biocatalyst enzymes, which are essential for enhancing the sustainability and efficiency of industrial processes. The significance of marine *A. niger* in the field of grey biotechnology lies in its ability to effectively eliminate poisonous dyes and heavy metals from soil and water, hence addressing environmental pollution. This organism plays a crucial role as a natural symbiont, particularly in the elimination of hazardous heavy metals like chromium, copper, lead, and aluminium that tend to collect in water and soil contaminated with waste. Additionally, it is effective in removing dangerous dyestuffs that can negatively impact human cells and tissues. The marine *A. niger* fungi should be regarded as the foremost option in the realm of environmental biotechnology, specifically designed for the purpose of biological remediation, waste management, and purification applications. The *A. niger* fungus is assessed for its ability to produce bioactive chemicals for medicinal and therapeutic use within the field of red biotechnology. Moreover, the investigation of the therapeutic characteristics of nanoparticles derived from isolated substances and synthesised with elements such as Ag and Au is a new and promising field of research. This review serves as a guide to accessing biotechnological studies on marine *Aspergillus niger*. There is a need to enhance the assessment of marine-associated *Aspergillus niger* in the biotechnology domains indicated. Furthermore, it serves as a significant resource model for other biotechnological domains that have yet to be assessed. Further scientific investigations should incorporate marine *A. niger* to enhance the comprehensiveness of the research.

4 AUTHOR CONTRIBUTIONS

Hypotesis: Z.T.; Design: Z.T.; Literature review: Z.T.; Data Collection: Z.T.; Analysis and/or interpretation: Z.T.; Manuscript writing: Z.T.

5 CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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Antimicrobial Activity of *Paronychia kurdica* Boiss. subsp. *kurdica* var. *kurdica* cultivated in Medicinal and Aromatic Plants Garden of Inonu University Faculty of Pharmacy

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ABSTRACT: It was aimed to examine the antimicrobial activities of the aqueous-methanol extract of *Paronychia kurdica* Boiss. subsp. *kurdica* var. *kurdica* on Gram-positive and Gram-negative bacteria and fungal microorganisms. The plant taxon is collected from Medicinal and Aromatic Plants Garden of Inonu University Faculty of Pharmacy. The aerial parts were powdered and extracted with methanol. The samples were tested at a concentration of 800-1,56 µg/ml against the standard culture collections of Gram negative bacteria (*Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*), Gram positive bacteria (*Enterococcus faecalis, Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)) and *Candida* species fungal strains *(C. albicans* and *C. glabrata*). *P.kurdica* extract was found to be more effective against Gram-positive bacteria than Gram-negative bacteria. Its effect on our fungal strains was found to be similar to Gram-positive bacteria. Globally, this paper is the first antimicrobial activity report of *Paronychia kurdica* Boiss. subsp. *kurdica* var. *kurdica*.

Keywords: Antimicrobial activity, Malatya, *Paronychia kurdica* Boiss. subsp. *kurdica* var. *kurdica*

1 INTRODUCTION

In tropical countries, the rate of deaths from infection is approximately half compared to other causes of death. Infection-related diseases and deaths are increasing day by day in developed countries. This situation necessitates new searches for the prevention and treatment of infectious diseases. Secondary metabolites, which are biologically active chemical compounds such as flavonoids, alkaloids, terpenoids, tannins, etc. synthesized by plants, are extensively employed in the management of infectious illnesses. The fact that pathogenic microorganisms in humans inactivate drugs over time, failing in the treatment of infectious diseases, creates a need for antimicrobials in the form of a vicious cycle. Although many pathogenic microorganisms have developed resistance to drugs today, there are also many plants with antimicrobial effects, causing researchers to prefer plants in the search for antimicrobial agents. The therapeutic effects of

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plants, which have significant potential in the fight against microorganisms, arise from the synergistic effect of many substances in their composition, rather than a single active substance isolated from its content. Phyto compounds provide a more effective treatment by counteracting the resistance of microorganisms that are difficult to kill with a single antibiotic. Antimicrobial action is influenced by the kind and load of the target microorganism, the content of the food, the conditions during processing and storage, and the species, composition, and concentration of the plant. The chemical compositions of some medicinal plants that prevent microbial growth, either in combination with traditional antimicrobials or individually, may vary depending on geographical conditions, collection conditions, time of acquisition, and growth conditions. This situation directs researchers to investigate the inhibitory effect compositions of natural antimicrobial agents obtained from plant extracts [1-8].

The genus *Paronychia* Mill., which is in the Illecebraceae family in the Flora of Turkey, has been assigned to the Paronychioideae subfamily of the Caryophyllaceae family in recent years. The genus, represented by 117 species in the world and 45 taxa belonging to 32 species in Türkiye, has native range from temperate and subtropical America, Macaronesia, Mediterranean to Iran and northeastern tropical Africa. The subshrub *P. kurdica* Boiss. subsp. *kurdica* var. *kurdica* taxon is native to Iran, Iraq, Lebanon-Syria, Transcaucasia, and Türkiye. It is mainly found in temperate biomes [9-14].

P. kurdica, which has been used traditionally for many years in some rural regions of Turkey, has therapeutic effects such as hypoglycemic, diuretic, and cancer suppressant. Also, it is used in kidney stone treatment [15]. *P. kurdica* known under the name Haşişılselulet in Midyat district of Mardin locally used as infusion in the treatment of wounds and gallbladder [16], in the treatment of warts by eating with salt in Hizan district of Bitlis [17], and in Amasya, the aerial parts are made into mush and mixed with vinegar and garlic, and then soaked in a cloth against bloodshot eyes, placed on the head against headache [18].

Various usage forms of some species of *Paronychia* are recorded in traditional folk medicine in Turkey. In Eskişehir, the aerial parts of the *P. amani* Chaudhri species are crushed when fresh and externally used for the treatment of ingrown and inflamed wounds. In Mersin, the decoction prepared from the aerial parts of the *P. argentea* Lam. (1-2 glasses per day, 3 days) is used internally for kidney stones [18]. *P. mughlaei* Chaudhri known under the name dolamaotu in Muğla is used against boil and felon [19].

In phytochemical studies of *Paronychia* species, especially saturated fatty acids, sterols, essential oils, terpenes, tannins, saponins, phenolic compounds and flavonoids were found [15, 20-26]. In biological activity studies conducted on *Paronychia* species, antioxidant, antimicrobial, antiviral and anti-inflammatory effects were reported [15, 19, 23-24, 27].

In this research, it was aimed to investigate the antimicrobial activities of the aqueous-methanol extract of the *P. kurdica* subsp. *kurdica* var. *kurdica* taxon, which was discovered to be least researched, on Gram-positive and Gram-negative bacteria and fungal microorganisms.

2 MATERIAL AND METHOD

2.1 Plant Material

P. kurdica subsp. *kurdica* var. *kurdica* taxon cultivated in Medicinal and Aromatic Plants Garden of Inonu University Faculty of Pharmacy was collected by Dr. Narin Sadıkoğlu during the flowering period, in April 2021. The specimen is kept in the Herbarium of Inonu University Faculty of Pharmacy (INUE) with the voucher number NS/2021/030.

2.2 Microbial Strains

Gram positive bacterial strains; *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213 and Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300.

Gram negative bacterial strains; *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853.

Fungal strains*: Candida glabrata* ATCC 90030, *Candida albicans* ATCC 14053.

2.3 Extraction and Fractionation

The aerial parts were dried in the shade and at room temperature. The powder drug (47.6704 g) was extracted with methanol on a magnetic stirrer for 8 hours by shaking. The obtained extracts were concentrated with the help of rotavapor under low pressure (200 mbar) and 40 °C. This process was repeated 4 times and the efficiency was calculated.

The resulting dry extract was dissolved in 100 mL of 90% methanol and subjected to liquid-liquid extraction with 100 mL of hexane. This process was terminated with the end of the substance transition to the hexane phase, and thus lipophilic impurities were removed from the extract. The amount of 90% methanol extract obtained was recorded.

2.4 Antimicrobial Activity

By the recommendations of the Clinical Laboratory Standards Institute (CLSI), the samples were tested at different concentrations of 800 (for samples) and 1,56 µg/mL (for antibiotics) against standard culture collections of the most commonly isolated strains among society and

hospital-acquired infectious agents using a method of serial dilutions using sterile 96-well microplate readers (PLT microtiterplate ESP) [28].

To create the stock solution, 10 mg of the material was dissolved in 1000 µL of dimethyl sulfoxide. To the test wells, 100 µL of Müller-Hinton Broth (Merck 110293, USA) was added. We took 100 µL of our material's stock solution, performed serial dilutions from the first to the tenth well, and used the final two wells as control groups. A turbidity threshold of Mc Farland 0.5 was used to create 10 µL of bacterial suspensions, which were then added to all samples, including the control wells [29]. Thermo USA's PST 60HL orbital shaker was utilized for five minutes to combine our material with the microorganisms. The microplate lid was shut, and it was incubated for eighteen to twenty hours at 35 ºC. A sterile plastic loop was used to streak the culture from each well onto a Müller-Hinton Agar plate, and the plate was then incubated under the same conditions to monitor the development of bacteria. The substance's minimal inhibitory concentration (MIC) was established as a pre-dilution of growth [30-32]. Using Sabouraud Dextrose Broth and Agar (oxoid CM0147, CM0041, USA) under identical conditions, antifungal activity was assessed. Furthermore, under identical testing settings as our substances, reference drugs for every category of bacteria and fungus were examined in our investigation [33].

3 RESULT

3.1 Yield

The amount of crude methanol (100%) extract produced from the powdered plant is 4.6603 g (11.37 g/g), whereas 90% methanol extract taken from the crude extract after the lipophilic contaminants were eliminated and used for antimicrobial activity is 3.4391 g.

3.2 Antimicrobial Effect

The antibacterial and antifungal activity of *P. kurdica* subsp. *kurdica* var. *kurdica* extract was determined in the range of MIC values of 50-400 µg/mL. The antimicrobial activity of our extract varies between bacterial and fungal species. *P. kurdica* extract was found to be more effective against Gram-positive bacteria (*S. aureus*, *E. faecalis*, *S. aureus* MRSA) than Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*). Its effect on our fungal strains (*C. glabrata*, *C. albicans*) was found to be similar to Gram-positive bacteria (Table 1).

4 DISCUSSION

It has been reported that *Paronychia argentea* extract, the most studied species, protects kidney endothelial cells against oxidative damage [26]. In addition, the protective activity of the methanol extract of this species against tobacco mosaic virus (TMV) infection in tomatoes has been proven

Table 1. The antimicrobial activity of *P.kurdica* subsp. *kurdica* var. *kurdica* extract varies between bacterial and fungal species.

	S.aureus	***E.faecalis***	***E.coli***	***P.aeruginosa***	***C.albicans***	***C.glabrata***	***A.baumannii***	***K.pneumoniae***	**MRSA**
P.kurdica	**50**	**50**	**200**	**400**	**100**	**100**	**200**	**200**	**200**
Control	**Growth**	**Growth**	**Growth**	**Growth**	**Growth**	**Growth**	**Growth**	**Growth**	**Growth**
Ampicillin	**1.56**	**1.56**	**3.12**					**1.56**	
Amikacin				**1.56**			**3.12**	**1.56**	
Ciprofloxacin			**1.56**					**1.56**	
Ciprofloxacin					**6.25**	**3.12**			
Vankomicin									**3.12**
Tigecycline							**1.56**		

agriculturally [25]. It has some therapeutic effects such as hypoglycemic, diuretic, cancer suppressant, and treatment of kidney stones and heartaches. It is used widely in traditional medicine, particularly in Middle Eastern countries such as Jordan, Israel and Palestine, in the treatment of urinary systems and diabetes [15, 21, 34-35].

Antimicrobial effect of *P. argentea* species grown both in the field (ex vitro) and in the laboratory (in vitro) with Cobalt (Co), copper (Cu) or lead (Pb) heavy metals in Jordan, against Gram-positive bacteria *Listeria monocytogen* and *S. aureus*, Gram-negative bacteria *Coronobacter sakazakii* and *Salmonella typhimurum* and fungus *Calvularia lunata* has been examined. As a result, it has been proven that *P. argentea* strain growing in medium with 0.3 mg/L of supplement Cu in extracts showing similar antimicrobial activity showed maximum inhibition on *S. aureus* (30.0 mm inhibition zone) and then on *C. lunata* (30.0 mm inhibition zone) [36].

The antibacterial activity of chloroform, ethanol and distilled water extracts of *P. argentea* species collected from Gaza Strip-Palestine, against *E. coli*, *K. pneumoniae*, *Morganella morganii*, Methicillin-sensitive *S. aureus* (MSSA) and Methicillin-resistant *S. aureus* (MRSA) bacteria has been investigated. It has been shown that for chloroform, ethanol, and aqueous extracts, the average diameter of the

inhibitory zones against the studied bacteria varied between 9–14 mm, 13–19 mm, and 13–20 mm, respectively. The aqueous extract has a value of 4.17 mg/mL against *K. pneumoniae* and *MRSA* species, while the mean minimum inhibitory concentration (MIC) values were between 4.17-33.33 mg/mL and 1.04-2.08 mg/mL for the chloroform and ethanol extracts, respectively [24].

The antimicrobial activities of aqueous and ethanol extracts of *P. argentea* plant collected from the West Bank region of Palestine was evaluated against *P. aeruginosa, P. aeruginosa* (clinical isolate), *E. coli, Proteus mirabilis* and *K. pneumoniae* by agar well diffusion method. It has been reported that the ethanol extracts examined showed higher MIC values, ranging from 1.56 to 50 mg/ml, compared to aqueous extracts [37].

Crude saponin extracts of the aerial part of *P. argentea* collected from near Marrakech, Morocco, was examined for antimicrobial activity against gram-negative bacteria *E. coli, K. pneumoniae*; gram-positive bacteria *Bacillus cereus, Micrococcus luteus, S. aureus,* and fungi *C. albicans, C. glabrata, C. krusei* and *C. parapsilosis*. The agar disc diffusion method was used to assess antibacterial activity, and the microdilution method was used to assess the minimum inhibitory concentration and antibiotic synergistic interaction. With an inhibitory zone of 11.10±0.35 mm, *M. luteus* was the most susceptible gram-positive bacterium to *P. argentea* extract. Moreover, the extract had minimal antibacterial activity on *E. coli* and no effect on *K. pneumoniae*. The extract showed an inhibition zone ranging from 9.40 to 13.07 mm on *Candida* strains. The MIC values of *P. argentea* extract were 8, 16 and 16 mg/mL for *M. luteus, B. cereus*, and *S. aureus*, respectively [38].

The antimicrobial activities of aqueous and methanol extracts of *P. mughlaei* species collected from Muğla were evaluated at concentrations of 1, 2.5, 5 and 10% by the agar well diffusion method, against Gram-negative bacteria *Aeromonas hydrophila, E. coli, K. pneumoniae, M. morganii, P. mirabilis, P. aeruginosa, S. typhimurium* and *Yersinia enterocolitica*, Gram-positive bacteria *B. brevis, B. cereus, B. subtilis, L. monocytogenes* and *S.s aureus* and fungi *C. albicans* and *Saccharomyces cerevisiae*. As a result, methanol extract showed weak antimicrobial activity only on *A. hydrophila, B. brevis, B. cereus, B. subtilis*, and water extract showed weak antimicrobial activity on *B. brevis* [19].

Aqueous extract of *P. kurdica* subsp. *kurdica*, collected from Bingöl-Elazığ area displayed beneficial effects on nipple papillomatosis in cattle, when administered

orally or subcutaneously injection, while its application as ointment is not very successful [15].

Antibiotic resistance has been accelerated in the past few years due to the extensive use of antibiotics to boost production in animal husbandry and agriculture, and to treat infectious diseases. The current state of affairs has rendered the creation of novel antibiotics necessary. Nevertheless, challenges encountered in the process of creating novel pharmaceuticals have prompted scientists to explore the potential of plants possessing inherent antibacterial characteristics. Our work focused on exploring the antibacterial characteristics of *Paronychia* species, which have not been previously investigated. The efficacy of our extract has been specifically demonstrated against Gram positive bacteria and fungal strains. However, as this is the first study conducted on this specific species, there hasn't been any previous research on the antibacterial qualities of other members of the same genus as our species.

Consequently, there is a substantial disparity in the MIC values between the ones we obtained in our study and the MIC values of the antibiotics currently used as reference medications (1.56 µg/ml). Nevertheless, the absence of any impediment that would restrict the use of plants with natural qualities, from which we derive extracts, effectively nullifies this drawback.

As a result, it seems that the genus has not been researched much, except for *P. argentea*, and more detailed studies need to be done on *P. kurdica* compared to other *Paronychia* species. The antimicrobial effect mechanism of the plant extract should be investigated by analyzing its components. It becomes necessary to find new antimicrobial agents due to both the side effects of antimicrobial agents and the resistance mechanism developed by microorganisms against them. Studies that determine the antimicrobial activities of plants in order to find new alternatives to treat diseases or reduce possible side effects, which are among today's popular research topics, are one of the best alternatives in terms of both cost effectiveness and easy availability of plants.

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6 AUTHOR CONTRIBUTIONS

Hypothesis: N.S.; Design: N.S.; Literature review: Z.T., N.S.; Data Collection: N.S., Z.T.; Analysis and/or interpretation: S.G., Z.T.; Manuscript writing: N.S., Z.T., S.G.

7 CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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Research Article

Investigation of the Adsorption of 6-(3-(4-(2-methoxyphenyl)piperazin-1-yl)propylamino)-1,3-dimethylpyrimidine-2,4(1*H*,3*H*)-dione (urapidil) on Graphene Oxide by Density Functional Theory Calculation Method

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ABSTRACT: Graphene oxide (GO) has become a very interesting structure in recent years due to its important results in biomedical applications of nano-bio researchers. Graphene oxide is a form of graphene decorated with oxygen-containing groups. When compared to graphene, GO is easily dispersible in water as well as any other solvents. It is easy to process and also make graphene too. Graphene-based materials are also widely used studied in biomedical applications in smart medicine and genetic engineering. In this work, the electronic properties of commercially available pyrimidine-2,4(1*H*,3*H*)-dione used in antihypertensive treatment and its adsorption on GO nanocage were calculated using density functional theory (DFT). Based on calculations, it is probable that the urapidil molecule's -NH group will interact with the GO surface's acid group. Most likely, proton exchange is the basis for the adsorption taking place in this section. The N-O interaction bond length was found to be 2.05115A^{o} in the computation done within the context of this option.

Keywords: *urapidil, ebrantil, graphene oxide, antihypertensive*

1 INTRODUCTION

GO was synthesized by oxidation of graphite to graphite oxide followed by exfoliation [1]. GO has a large surface area and contains many epoxy and hydroxyl groups on its surface [2-6]. GO are important for material science because of thermal, optical, electrical and magnetic properties [7-9]. GO chemistry is largely based on Brodie's work on graphite oxide in the 18th century [10]. Generally accepted models of GO structure are those based on hydroxyl, carboxylic acid and epoxide groups as the dominant functional groups (Figure 1). It also contains groups such as ketones, phenols, lactols and lactones in its structure [11]. There are many outstanding properties that define such materials in terms of reactivity. Oxygen-containing functional groups cause GO to function as a solid acid. The nanovoids present in GO give it unpaired spins, which helps in the activation of small molecules by the spin flip process. GO is an ideal material where covalent modification or non-covalent interactions occur [12]. Figure 1 shows potential active sites that can be added to the GO scaffold. The bonding between GO and organic materials can be covalent bond or

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non-covalent bond interaction. GO has been extensively researched to develop biosensors due to its functionalizable surface and sensitive electrical properties [13, 14]. Detection of important biomolecules such as nucleic acids, proteins, and growth factors has been successfully achieved using appropriately functionalized graphene derivatives [15]. In addition to biomolecules in buffered solutions, hormonal catechol amine molecules secreted from living neuroendocrine cells have been also detected using GO [16].

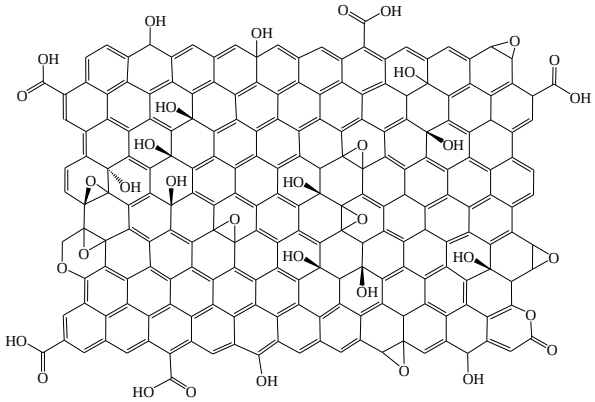


Figure 1. Proposed structure of GO [17].

Single malaria-infected red blood cells have also been detected by devices fabricated on microfluidic channel arrays and graphene films functionalized with receptor proteins [18].

Patients with hypertension are at an increased risk of morbidity and mortality from cardiovascular diseases (stroke and coronary events). The magnitude of this risk depends on the severity of hypertension. The goal of treatment is to lower blood pressure, thereby reducing the risk of stroke and coronary events. Drugs commonly used to treat hypertension include diuretics, β-receptor blocking drugs, angiotensin-converting enzyme (ACE) inhibitors, calcium antagonists, and α-adrenoceptor antagonists [19]. This pyrimidine derivative (Figure 2) is a widely used drug with antihypertensive effects in central and peripheral areas. This compound blocks the effects of the nervous system on the vascular muscular system.

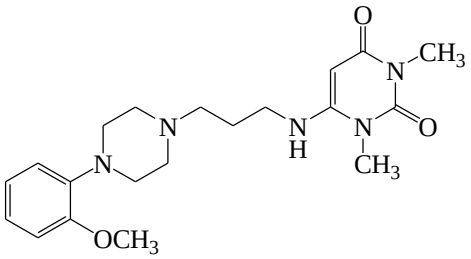


Figure 2. Pyrimidine derivative.

Quantum chemistry methods play an important role in obtaining extremely precise geometries for molecules and predicting various properties of molecules. Density functional theory (DFT) methods offer an alternative use of inexpensive computational methods in studying the properties of relatively large molecules [20]. DFT is widely used to study the electronic properties, molecular structure, chemical reactivity and hydrogen bonding of pharmaceutical compounds [21]. With DFT calculations, the

molecular orbitals and geometries of organic compounds are characterized by their activities. The properties of the components are related to the highest occupied molecular orbital energy (E_{HOMO}), the lowest unoccupied molecular orbital energy (E_{LUMO}) and the use of frontier orbital energy difference ($\Delta E = E_{LUMO} - E_{HOMO}$) [22]. In this study, the geometric and electronic structures of the 6-(3-(4-(2-methoxyphenyl) piperazin -1-yl)propylamino) -1,3- dimethylpyrimidine-2,4 (1*H*,3*H*) -dione (urapidil) , used as an antihypertensive drug, were examined by the DFT method. The adsorption of compound on graphene oxide was studied with the same method.

2 MATERIAL AND METHOD

2.1 Materials

The adsorption of urapidil molecule on the GO nanocage surface was investigated by DFT calculations. The calculations are made on Gaussian09 program using the B3LYP/DGTZVP basis set [23-25]. To accurately the interaction, the adsorption energies (ΔEad) were calculated as follows:

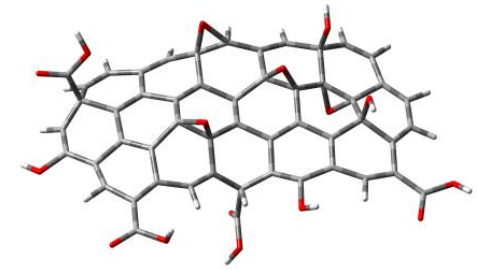
$$\Delta Ead = \Delta E(complex) - \Delta E(GO) - \Delta E(pyrimidine\ derivative) \quad (1)$$

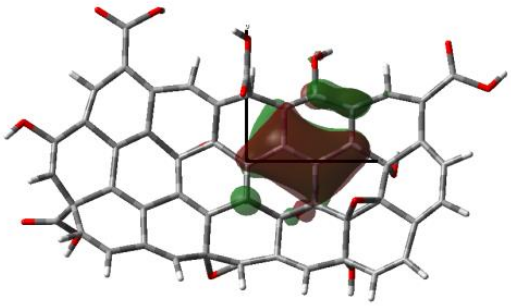
Quantum chemical parameters ΔE_{HOMO}, ΔE_{LUMO} and ΔE_{gap} were calculated and discussed for all types of interactions.

In addition, electronegativity "χ", chemical softness "S", ionization potential "I", dipole moment "μ", chemical hardness "η" and electron affinity "A" [26, 27] calculations were performed for GO and pyrimidine derivative.

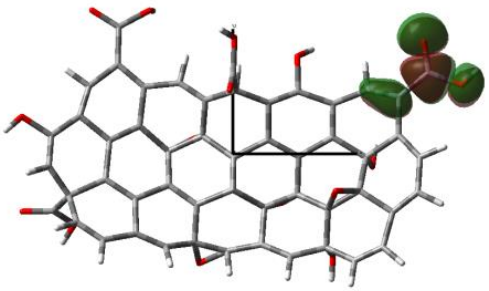
3 RESULTS AND DISCUSSION

Full geometry optimizations of the GO nanocage and urapidil were performed using DFT based on Beck and Lee–Yang–Parr [28] non-local correlation functional (B3LYP) DGTZVP basis sets in the Gaussian09 program [23] (Fig. 3a,b).



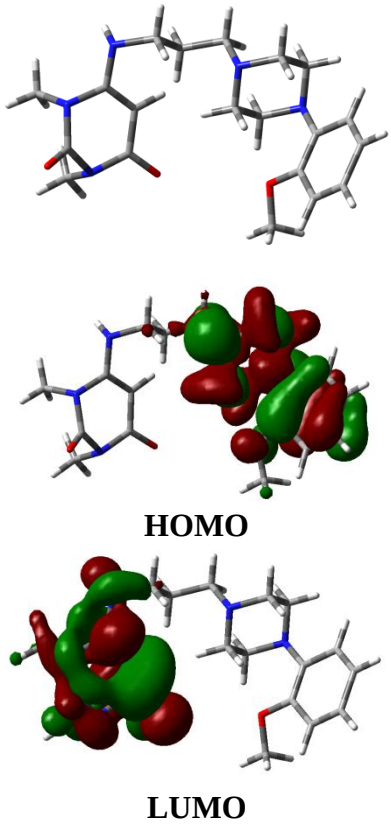


HOMO



LUMO

Figure 3a. GO nanocage HOMO and LUMO profile.



HOMO

LUMO

Figure 3b. Pyrimidine derivative HOMO and LUMO profile.

When the electronic structure of the GO nanocage is examined, it is seen that electrons are concentrated in the epoxy regions. It is seen that the electrophilic character is concentrated in the carboxylic acid regions. This shows that the aliphatic nitrogen atom on the pyrimidine derivative will attach to the carboxylic acid region of GO. The adsorption of pyrimidine derivative on the GO surface can be analyzed by theoretical calculations. HOMO and LUMO values are the most important parameters used to predict the adsorption activity of pyrimidine derivative on the GO surface. In addition, parameters such as ionization potential, electron affinity, chemical softness, dipole moment, chemical hardness and electronegativity can be obtained through quantum calculations.

The absolute electronegativity (χ) of the compounds is calculated with the following equation (2), depending on the ionization potential and electron affinity [29].

$$\chi = \left(\frac{I+A}{2}\right) \quad (2)$$

The chemical hardness of the compound is calculated based on its ionization potential and electron affinity (equality) (3).

$$\eta = \frac{1}{2}\left(\frac{\partial^2 E}{\partial N^2}\right)_{\vartheta(r)} = \frac{1}{2}\left(\frac{\partial \mu}{\partial N}\right) = \frac{I-A}{2} \quad (3)$$

Another important parameter that shows the reactivity of compounds is chemical softness. Chemical hardness and softness are also connected to each other as in the equation below.

$$S = \frac{1}{\eta} \quad (4)$$

Obtained HOMO, LUMO, I, A, χ, η and S values as a result of the calculations are given in table 1.

Table 1. The quantum chemical parameters for GO and pyrimidine derivative (e*V*).

	E_{HOMO}	E_{LUMO}	ΔE	I	A
GO	-7.169	-0.975	6.194	7.169	0.975
Ura	-4.941	-1.899	3.042	4.941	1.899
	η	S	χ	*μ(D)*	
GO	6.194	0.161	4.072	6.472	
Ura	1.521	0.657	3.420	4.923	

Nonlinear optical properties for GO and pyrimidine derivative were calculated. As a result of these calculations, total dipole moment μ_{tot}, average polarizability (α_{tot}) and average first hyperpolarizability (β_{tot}) were calculated.

$$\mu tot = \mu_X^2 + \mu_Y^2 + \mu_Z^2 \quad (5)$$

$$\alpha tot = (\alpha_{xx} + \alpha_{yy} + \alpha_{zz})/3 \quad (6)$$

$$\beta tot = [(\beta_{xxx} + \beta_{xyy} + \beta_{xzz})^2 + (\beta_{yxx} + \beta_{yxx} + \beta_{yzz})^2 + (\beta_{zzz} + \beta_{zxx} + \beta_{zyy})^2]^{\frac{1}{2}} \quad (7)$$

The values of the resulting calculations are given in Table 2. The high dipole moment, molecular polarizability and hyper polarizability values of the compound are directly proportional to its good nonlinear optical (NLO) properties. According to the values obtained as a result of the calculations, it was determined that the compounds studied had very good NLO properties.

The molecular electrostatic potential maps (MEPs) were calculated using DFT and DGTZVP base set in the Gaussian09 program for GO and pyrimidine derivative. In MEPs, which provide important information about the charge distribution of the molecules, the charge distributions are given in different colors. In the MEPs given in Figure 4a,b the areas represented in red represent the regions where negativity is concentrated in the molecule (nucleophile), and the regions in blue color represent the areas in the molecule where positivity is concentrated (electrophile). The electrostatic potential increases during red> orange> yellow> green> blue. In these our compounds the highest potential is on oxygen atoms.

Table 2. NLO properties of GO, pyrimidine derivative and complex structure.

Parameters (a.u)	GO	pyrimidine derivative	Complex
β_{xxx}	589.8926	-105.3978	-803.5864
β_{xyy}	155.7559	32.8724	-144.4748
β_{xzz}	-60.7116	24.9881	-32.0193
β_{yyy}	-269.3956	2.4770	1059.2316
β_{yxx}	-228.1507	36.0261	136.2827
β_{yzz}	78.0111	-7.3493	16.9900
β_{zzz}	-51.6876	-18.0063	-36.8272
β_{xxz}	69.4933	-56.0057	256.9811
β_{zyy}	7.4148	-26.0346	168.5767
β_{tot} (esu) 10^{-33}	803.607	115.0637	1606,79
α_{xx}	17.9609	-153.5790	-441.7985
α_{yy}	-1.5240	-156.9748	-553.2645
α_{zz}	-16.4370	-180.6349	-577.7118
α_{tot} (esu) 10^{-33}	-0.00003	-163.7296	-524.2582
μ_x	4.3060	-0.7441	-9.8410
μ_y	-4.6709	3.5837	8.2756
μ_z	1.2362	-3.2930	5.0619
μ_{tot} (esu) 10^{-33}	6.4720	4.9234	13.8186

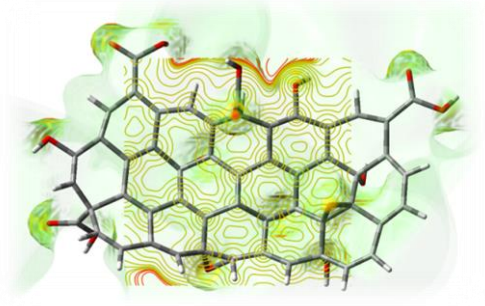


Figure 4a. MEP of GO.

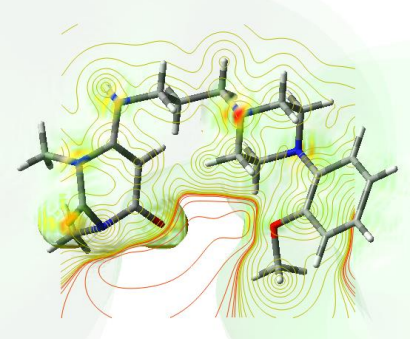


Figure 4b. MEP of pyrimidine derivative.

Calculations show that the -NH group in the pyrimidine derivative is likely to interact with the acid group on the GO surface. The adsorption occurring in this part is most likely based on proton exchange. In the calculation made within the framework of this possibility, it was determined that the N-O interaction bond length was 2.0511A^0 (Figure 5).

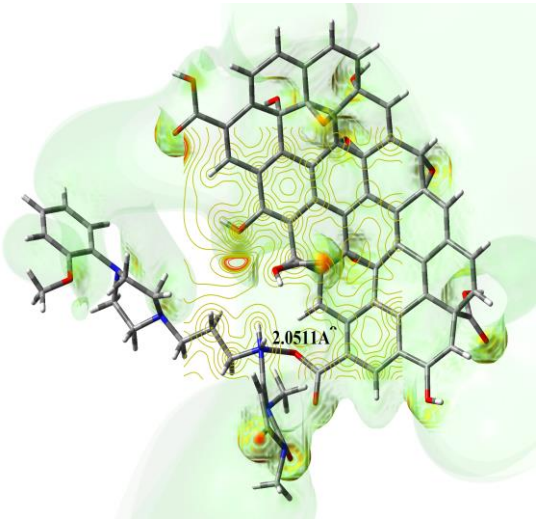


Figure 5. Complex of pyrimidine derivative/GO.

The electronic energy of the pyrimidine derivative /GO complex was calculated by the following equation.

$$\Delta Ead = \Delta E(complex) - \Delta E(GO) - \Delta E(pyrimidine\ derivative)$$

$$\Delta Ead = (-164{,}50) - \Delta E(-117{,}49) - \Delta E(-46{,}99)$$

$$\Delta Ead = -0.02\ eV$$

Accordingly, the two main mechanisms involved in the adsorption of pyrimidine derivative to the carboxylic acid site on the GO nanocage surface are orbital and charge-induced interactions (electrostatic effect). In particular, the hydrogen atom bonded to the nitrogen atom interacts with the oxygen in the carboxylic acid group, inducing intermolecular electrostatic interactions. Consequently, complex is the most stable from its NH side due to the interaction between pyrimidine derivative and the GO nanocage.

Figure 6 shows the density of the state spectra for pyrimidine derivative and complex. The decrease in the Eg value of the GO- pyrimidine derivative compared to the GO nanocage is due to this opposite electric peak after the adsorption process of pyrimidine derivative. Furthermore, a closer examination of the DOS spectrum reveals that the HOMO and especially the LUMO levels are shifted to the higher energy region after adsorption of pyrimidine derivative.

Based on the computation findings, it is possible to conclude that the pyrimidine derivative molecule's -NH group will create a strong bond through proton transfer with the

acid group on the GO surface during adsorption.

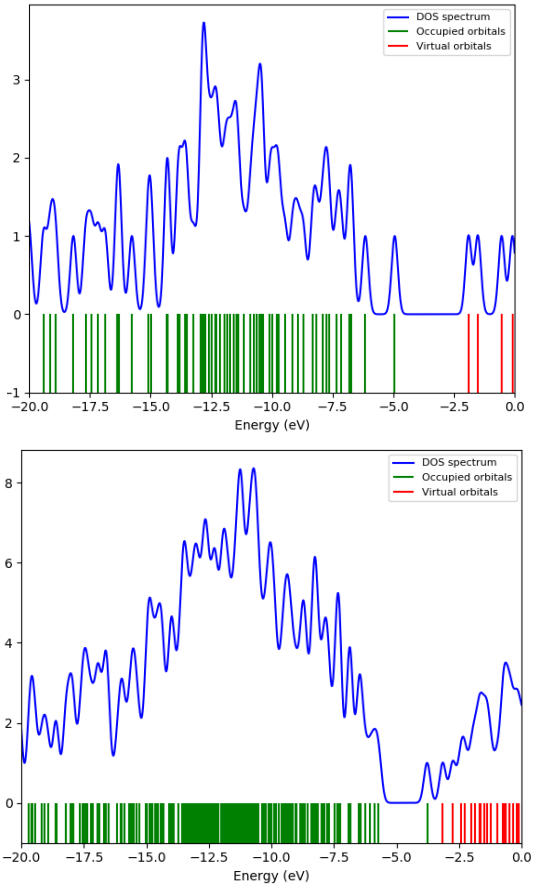


Figure 6. DOS plots of pristine pyrimidine derivative and complex, respectively.

4 ACKNOWLEDGEMENTS

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5 CONFLICT OF INTEREST

Authors declare that there is no a conflict of interest with any person, institute, company, etc.

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Research Article

Determination of Guanidinoacetic Acid in Urine by High-Performance Liquid Chromatography-Fluorescence Detection Method

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ABSTRACT: Creatine deficiency syndromes are newly recognized inherited metabolic disorders. Creatine deficiency is classified into three groups according to the synthesis steps: guanidinoacetate N-methyl transferase (GAMT) deficiency, arginine-glycine amidinotransferase (AGAT) deficiency, and creatine transporter disorder. The common biochemical finding in all three diseases is cerebral creatine deficiency. Low urinary guanidinoacetate (GAA) levels are observed in AGAT deficiency and high urinary GAA levels are observed in GAMT deficiency. In this study, we developed a new, simple, inexpensive, and rapid chromatographic analysis method for the quantitative determination of guanidinoacetate in urine samples. Guanidinoacetate was chromatographically separated with a buffer containing 50 mM formic acid and methanol, 1.0 mL/min flow, C18 (150 mm 4.6 mm, 5.0 µm) analytical column and fluorescence detector at λex: 390 nm, λem: 470 nm.

Keywords: *Guanidinoacetic Acid, Creatine deficiency, High-performance liquid chromatography-fluorescence detection (HPLC-FLD)*

1 INTRODUCTION

Creatine (α-N-methyl-guanidino-acetic acid) is a nitrogen-containing organic acid and is involved in the storage, utilization, and transport of energy, especially in organs with high energy requirements such as the brain and muscles. In humans, dietary creatine provides half of the daily requirement, and the remaining 50% is synthesized endogenously. In mammals, creatine is synthesized in a two-reaction pathway. In the first step, guanidino-acetate (GAA) and ornithine are formed from arginine and glycine by the enzyme arginine-glycine amidino transferase (AGAT). In the second step, GAA is converted to creatine by a methylation reaction mediated by the enzyme guanidinoacetate methyl transferase (GAMT). In this conversion, S-adenosyl methionine (SAM) is used as the methyl donor [1,5].

1.1 Arginine-Glycine Amidino Transferase Deficiency (AGAT)

The enzyme arginine-glycine amidino transferase catalyzes the first step in creatine biosynthesis. In enzyme deficiency, the production of GAA, the precursor molecule in

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creatine synthesis, is impaired. Arginine-glycine amidino transferase deficiency (OMIM#612718) is an autosomal recessive disorder caused by mutations in GAMT [6-8]. AGAT deficiency should be considered in all patients with muscle weakness/myopathy in addition to developmental delay, behavioral problems, and cognitive or mental retardation, but these findings are not specific and can be seen in many neurometabolic disorders. Low GAA levels in urine samples are important in the diagnosis [7,8].

1.2 Guanidino-Acetate Methyl Transferase Deficiency

The enzyme guanidino-acetate methyl transferase constitutes the second step in creatine synthesis and enables the formation of creatine from guanidino-acetate with the help of SAM. GAMT deficiency (OMIM #612736) is an autosomal recessive creatine synthesis defect [9,10]. The disease develops due to mutations in the GAMT gene. The most important biochemical features of GAMT deficiency are cerebral creatine deficiency and elevated GAA. Cerebral creatine deficiency can also be seen in AGAT deficiency and creatine transporter defects, but elevated GAA is specific for GAMT deficiency. Elevated GAA can be detected in urine, blood, and blood-cerebrospinal fluid (CSF) [9,10]. Elevated GAA levels in body fluids are not associated with disease severity. Urine GAA screening is known to be an effective screening method in individuals with intellectual disability [10]. In cases of GAMT and AGAT deficiency, early diagnosis and treatment with creatine monohydrate leads to clinical improvement.

Simultaneous measurement of GAA and creatinine in biological samples is difficult and often requires advanced instrumental analysis techniques. The analytical methods used for analysis were reported as high-pressure liquid chromatography (HPLC) [11], liquid chromatography sequential mass spectrometry (LC/MS/MS) [12], gas chromatography-mass spectrometry (GC/MS) [13], capillary zone electrophoresis ultraviolet detector (CZE-UV) [14]. This study aimed to develop a new, simple, inexpensive, and rapid chromatographic analysis method for the quantitative determination of GAA in urine samples. GAA and creatinine were chromatographically separated simultaneously with 50 mM formic acid and methanol buffer, 1.0 mL/min flow, C18 (150 mm 4.6 mm, 5.0 µm) analytical column, and fluorescence detector at λex: 390 nm, λem: 470 nm.

2 MATERIAL AND METHOD

2.1 Chemicals and Synthetic Urine

GAA and creatinine were obtained from Sigma/Aldrich (St. Louis, MO, USA). Potassium hydroxide, trichloroacetic acid, ascorbic acid, Tris-HCI, phosphoric acid, and formic acid (98-100% extra pure) were purchased from Merck (Darmstadt, Germany).

Methanol and ninhydrin (puriss. p.a.) were purchased from Riedel-de Han (Seelze, Germany). The synthetic urine used in the study was purchased from Artificial Urine, Pickering Laboratories (Mountain View, California, USA). Synthetic urine is a ready-to-use and stable solution with a pH of 6.5, similar in composition to real human urine, and used in standard laboratory studies. The solutions used in this study were prepared using ultrapure water obtained from the Elga Purelab brand Option-7-15 model pure water system.

2.2 HPLC

HPLC Nexera-i LC-2040C 3D Plus high-performance liquid chromatography (Shimadzu, Kyoto, Japan) was used for GAA and creatinine analysis. A photo diode array PDA) detector was used in the system. LabSolutions software was used for instrument control, data acquisition, and data analysis. Chromatographic studies and analytical separation were performed on a Vertical GES C18 HPLC column (Tailand) (150 mm 4.6 mm, 5.0 µm) at 25°C.

2.3 Running Buffer and Optimum Conditions

The optimized mobile phase for simultaneous analysis of GAA and creatinine was formed from a gradient of 50 mM formic acid and methanol from 5% to 90% methanol over 19 min. The flow rate was set to 1.0 mL/min. [1]. The injection volume was 10 µL and the fluorescence detector was measured at λex: 390 nm, λem: 470 nm. The chromatogram obtained from this working buffer and optimized conditions is shown in Figure 1.

2.4 Sample Preparation

To precipitate the protein in urine samples, 100 µL of 30% trichloroacetic acid (TCA) was added to 20-fold diluted urine, centrifuged at 10000 rpm for 5 minutes, and filtered with a 25 mm 0.2 µm nylon filter. For GAA and creatinine analysis, 400 µL of urine sample was taken from the filtered sample, 300 µL of 1.3 M KOH, and 150 µL of 0.9%

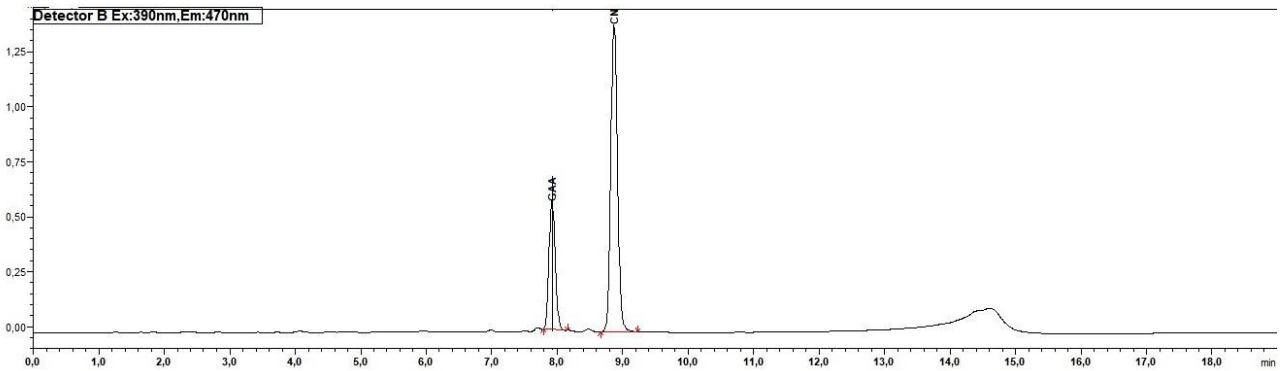

Figure 1. Chromatogram of peaks of 10 nmol/mL standard GAA and 10000 nmol/mL creatinine in optimized buffer solution medium.

ninhydrin was added to derivatize, the solution was mixed thoroughly and incubated for 15 minutes at room temperature [11]. Then 100 µL of 5% ascorbic acid and 100 µL of 5 M phosphoric acid were added and the solution was thoroughly mixed and incubated at 90 ◦C for 30 minutes. After cooling to room temperature, 10 µL of the sample was injected into the HPLC device.

3 RESULT

3.1 Validation

Validation of the proposed method was performed according to Eurachem guidelines [15].

3.1.1 Linear Range, the minimum limit of detection (LOD), and the minimum limit of quantification (LOQ)

The calibration line was plotted against the corrected peak areas (A/t) over the concentration range of 1, 5, 10, 25, 50, and 100 nmol/mL guanidinoacetate for GAA in pure water and 20-fold diluted urine. The regression parameters obtained for the calibration equation at these limits are given in Table 1. Three times the signal value of the noise detected from three different regions in the chromatogram was considered as LOD and this value was found to be 1.0 nmol/mL. Quantitatively, LOQ value was found to be 3.51 nmol/mL, 10 times the noise signal (Table 1).

3.1.2 Matrix Effect

The matrix effect was analyzed by calculating the ratio of the slopes of the matrix-matched calibration curve and the pure solvent calibration curve. We used 20-fold diluted urine for matrix-matched calibration and ultrapure water for pure solvent calibration. The matrix effect was found to be 8%. Since this value was not between -10% and 10%, the matrix effect was not significant (Table 1).

Table 1. Regression parameters, limit of observability, and limit of detection.

Regression parameters	In urine	In Aqueous Solution
Regression coefficient (R^2)	0,996	0,997
Linear range (nmol/mL)	1,0-100	1,0-100
Regression equation	y=165,5x + 138636	
Number of observations	6	6
Limit of dedection (nmol/mL)	1,0	
Limit of quantification (nmol/mL)	3,51	
Recovery (%)	91-101	

3.1.3 Repeatability

The precision of the method was checked by 7 successive injections of 5, 25, 50, and 100 nmol/mL GAA standard solution. The precision values for migration times and corrected peak areas (A/t) are given in Table 2 as intra-day and inter-day precision values as % RSD.

Table 2. Repeatability and recovery values obtained from synthetic urine samples under optimized conditions.

Added amount nmol/mL	Recovery %	Time (min)	Adjusted Area
5	91	7,92	5,58
25	98	7,93	5,67
50	101	7,91	5,84
100	97	7,92	5,72

3.1.4 Accuracy of the method

The accuracy of the method was determined by adding three different concentrations of 5, 50, and 100 nmol/mL GAA standards to the urine sample and taking 7 different measurements for each of them. Recovery values were between 91 and 101% (Table 2).

4 DISCUSSION

In conclusion, the determination of GAA levels in urine is of vital importance in creatine synthesis disorders. In this study, it was observed that it could be easily determined in 7.92 minutes in 50 mM formic acid-methanol buffer using a C18 (150 mm 4.6 mm, 5.0 µm) analytical column in a HPLC system with fluorescence detector. It was found to be a highly sensitive method in terms of the results obtained. It has been demonstrated that GAA can be used in routine analysis and clinical research.

5 AUTHOR CONTRIBUTIONS

Hypotesis: M.S.C., E.I.; Design: M.S.C., E.I.; Literature review: M.S.C., E.I.; Data Collection: E.I.; Analysis: E.I.; Manuscript writing: M.S.C., E.I.

6 CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Review Article

Herbal Supplements Used in the Treatment of Attention Deficit and Hyperactivity Disorder

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ABSTRACT: Attention-deficit/hyperactivity disorder (ADHD) is a multifactorial neurodevelopmental disorder with cognitive, motor, sensory, emotional and behavioural problems. Treatment of the disorder varies according to age. Behavioural therapy is mainly recommended for cases aged 4-5 years. If no results are obtained or if symptoms persist, medication may be added. The use of medical treatment, particularly in childhood, is viewed with suspicion by families because of some undesirable side effects and the risk of addiction and abuse. In this article, we would like to discuss some medicinal plants that are used in ADHD patients without or in addition to medical treatment, and their safety and efficacy.

Keywords: *herbal supplements, attention-deficit/hyperactivity disorder, plants, natural products*

1 INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental and neuropsychiatric disorder characterised by primary core symptoms including distractibility, inability to sustain attention, irritability, impulsivity and hyperactivity [1,2].

It is a disorder that affects the quality of children's daily lives, disrupts their development and continues into adulthood, and therefore needs to be properly diagnosed and treated. The aetiology of ADHD is multifactorial. It is defined in relation to various genetic, biological, environmental and psychosocial factors. It affects around 3-7% of the child population. It is three times more common in men than in women. People with ADHD usually show symptoms before the age of 7 and these symptoms last for at least 6 months [3-5].

For a diagnosis of ADHD, problems must occur in at least 2 different areas. ADHD has three clinical manifestations: hyperactivity subtype, attention deficit subtype and combined manifestation. Children with ADHD have difficulty focusing and sustaining attention. Behaviours such as difficulty waiting in line, wanting to give an answer before the question is finished, inability to postpone their requests, interrupting the words of speakers, rushing, and as a result of these behaviours, negatively affecting the child's functionality, suggest impulsivity problems [6,7].

Some studies have shown that dietary

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supplements (such as minerals, vitamins, omega-3 fatty acids) given to children with ADHD can reduce the symptoms of the condition. Zinc, which is used as a cofactor in the production of norepinephrine and dopamine, and iron and copper deficiencies are thought to be related to the aetiology or symptoms of ADHD. It is thought that the consumption of foods with a high glycaemic index may be associated with inattentive and hyperactive behaviour, and that behavioural problems may be caused by additives such as preservatives and food colorings [8,9].

Non-drug methods are recommended as first-line treatment for children with ADHD. In cases where these methods do not work, medication is tried. Medications used in treatment are thought to work by reversing neurotransmitter dysfunction in the prefrontal cortex and reducing ADHD symptoms. Medications used to treat people with ADHD include psychostimulants (dextroamphetamine, methamphetamine, amphetamine salts, methylphenidate, methylphenidate transdermal system, dexmethylphenidate, lisdexamfetamine), selective norepinephrine reuptake inhibitors (atomoxetine), alpha-2 adrenergic agents (guanfacine, clonidine), antidepressants, and dopaminergic agents [10].

Current treatment options mainly include a pharmaceutical component, a behavioural component and a psychosocial component, separately or in combination. Concerns about the adverse effects of pharmacotherapy have stimulated research into alternative treatment strategies, including the use of dietary supplements, and the role of nutrition and dietary supplements in the aetiopathophysiology and treatment of ADHD has become a focus of clinical interest and research. In recent years, the use of herbs as adjuvant therapy to conventional pharmacological treatments in the control of ADHD symptoms has been evaluated. In this article, we aim to discuss some of the herbs used in ADHD patients without or in addition to medical treatment, and their safety and efficacy.

2 Herbal Supplements Used

2.1 *Ginkgo biloba* L.

Ginkgo biloba is the oldest tree species, having lived on earth for around 200 million years. The therapeutic use of *Ginkgo biloba* leaves and seeds was first mentioned in Chinese sources 5000 years ago. The health benefits of *Ginkgo biloba* are thought to be due to the polyphenols, flavonoids and ginkgolides it contains. While ginkgolides inhibit platelet activating factor in the metabolism, the antioxidants in its structure protect cells from protein and lipid oxidation by quenching free radicals such as hydroxyl, superoxide and peroxyl radicals in the environment. Clinical studies show that standardised Ginkgo extract can be used to treat poor circulation, heart

disease, eye disease, tinnitus, chronic brain disease, short-term memory loss, brain trauma, depression, dementia and age-related conditions. The primary clinical use of ginkgo is in the treatment of peripheral vascular disease such as brain failure. Ginkgo leaf extract is effective in improving memory and treating multiple sclerosis. It may also help prevent and treat Alzheimer's disease and other types of dementia [11,12].

Various doses of *Ginkgo biloba* were shown to be effective in reducing ADHD symptoms by Uebel-Von Sandersleben et al. (2014), Shakibaei et al. (2015) and Salehi et al. (2010). While mild to moderate side effects were observed in the study by Salehi and Shakibaei, no side effects were observed in the study by Uebel-Von Sandersleben et al. (2014) [13-15]. Again, Chutko et al. (2019) reported that 240 mg of *Ginkgo biloba* daily was effective in reducing symptoms in adult patients with ADHD after 8 weeks of treatment [16].

2.2 *Valeriana officinalis* L.

Valerian (*Valeriana officinalis* L.), a member of the Valeriaceae family, is a flowering plant native to Europe and Asia, used mainly for the treatment of insomnia and anxiety disorders. Valerian species have been reported to have many therapeutic properties such as hypnotic, sedative, anxiolytic, anticonvulsant and antidepressant. In traditional medicine, various herbal formulations of this plant have been recommended for the treatment of hypertension, angina, palpitations, asthma, hepatic colic and menstrual cramps [17]. Razlog et al. (2011) reported that this herb was effective in reducing symptoms in patients with ADHD [18].

2.3 *Bacopa monnieri* L.

It is a perennial herb found in the wetlands of India, Nepal, Sri Lanka, China, Florida, Hawaii and the southern states of the USA. It is of great importance in traditional medicine due to its active compounds such as alkaloids (brahmin and herpestine), saponins (d-mannitol and hersaponin, acid A and monnierin), flavonoids, betulinic acid, stigmastrol, beta-sitosterol and bacopa saponins. In addition to its antidepressant, anti-inflammatory and antimicrobial effects, it is also the most popular neurotonic and memory enhancer. It has also been reported to aid physical processes associated with relaxation and heightened mental awareness, and extracts of this plant have been shown to improve cognitive function in animals. Ethanolic extracts of this plant have been found to increase antioxidant enzyme activity in various brain regions of rats [19,20]. Dave et al. (2014) reported that this plant was effective in improving learning and psychiatric problems and reducing symptoms of ADHD. In this study, a daily dose of 225 mg was given for 6 months [21]. In another study conducted by

Kean et al. (2022), the use of different doses did not result in regression of ADHD symptoms at the end of 14 weeks, while cognitive flexibility, sleep quality and mood improved [22].

2.4 *Matricaria chamomilla* L.

It is native to Eastern Europe and Asia. However, in recent centuries it has spread to central Europe. It was brought to America and Australia with cereals. The largest producers are Germany, Hungary, Russia, Belgium, France, Spain, Greece and Turkey. It is used for coughs and bronchitis, whooping cough, skin inflammations, itching, acne, shingles, skin cracks and tears, muscle contractions of nervous origin, sleep disorders in children, nervous abdominal pain in children, anxiety, as a calming, relaxing, stress-relieving, anti-inflammatory and antispasmodic, antibacterial, antifungal, antiviral, wound healing accelerator, pain reliever and skin care aid [23,24]. In the study of Niederhofer (2009), it was reported that this plant was mildly effective in regressing ADHD symptoms [25].

2.5 *Ginseng* L.

Ginseng has been an important herb in traditional Chinese medicine for thousands of years. The roots and rhizomes of Korean *ginseng* (*Panax ginseng* C.A. Meyer) and American *ginseng* (*Panax quinquefolius* L.) are the main sources of ginseng. *Ginseng* products are generally used to maintain homeostasis and protect the body from physical, chemical and biological influences. The tonic and adaptogenic effects of *ginseng* are thought to reduce the negative effects of the ageing process, support the body by strengthening bodily functions against disease, and increase physical performance and overall fitness in healthy individuals. The predicted clinical and physiological activities of *ginseng* are summarised as increasing endurance against stress, regulating cardiovascular activities, facilitating and improving the learning process, enhancing memory, regulating neuroendocrine system activities, regulating carbohydrate, fat and protein metabolism [26,27]. Lyon et al. (2001) reported that an extract of Panax quinquefolius L led to a reduction in the symptoms of ADHD [28]. In the study by Ko et al. (2014), a significant reduction in attention and hyperactivity scores was observed at the end of 8 weeks in patients given 40 ml of Korean red *ginseng* twice Daily [29]. Lee et al. (2011) reported in their observational study that Korean red *ginseng* extract 1000 mg twice daily for 8 weeks improved attention deficit symptoms [30].

2.6 *Pine Bark Extract* [Pycnogenol (French Maritime Pine Bark Extract)]

It is a plant extract with a flavonoid/polyphenol structure obtained from *Pinus pinaster (Pinus maritima)*, which grows on the south-west coast of France. It is known that pine bark was used in ancient times for

inflammatory diseases, wound healing, haemorrhage prevention, cough syrup and treatment of toothache. Its powerful antioxidant and anti-inflammatory properties have been demonstrated in in vitro, in vivo and clinical studies. Pycnogenol is used orally throughout the world as a dietary supplement to support the treatment of many physiological disorders such as dysmenorrhoea, muscle and spasm pain, attention deficit, hyperactivity, allergies and respiratory disorders, to protect against cardiovascular disease, to control blood sugar levels in diabetics and to improve complications [31,32]. It was observed that catecholamine levels reached normal levels and hyperactivity decreased in children treated with Pycnogenol for attention deficit and hyperactivity. A statistically significant decrease in dopamine levels was observed in 57 children (47 boys, 10 girls, aged 6-14 years) with attention deficit and hyperactivity after administration of 1 mg/kg Pycnogenol for 1 month [33]. Iravani and Zolfagharin (2014) reported that Pycnogenol reduced hyperactivity and improved antioxidant status in children [34]. Trebaticka et al. (2006) reported that 1 mg/kg/day of pycnogenol improved ADHD symptoms after 1 month of treatment [35]. Chovanova et al. (2006) reported that 8-oxo-7,8-dihydroguanine (8-oxoG) levels were increased in children with attention deficit hyperactivity disorder compared to controls. The significant reduction in 8-oxoG levels in children taking pycnogenol supplements suggests that pycnogenol may be an indicator that pycnogenol protects DNA from damage [36].

2.7 *Melissa officinalis* L.

It is a Mediterranean plant from the Labitae family and has a lemon scent. Lemon balm is a perennial herbaceous plant that grows to a height of 60-100 cm. It is a plant that has long been known and used for its pleasant odour. In vitro, in vivo and clinical studies on *M. officinalis*, which has been used for medicinal purposes for many years, have shown that its essential oil and extracts of different polarities have various biological activities. It has been reported to have antiviral, antibacterial, antifungal, antioxidant, antiulcer, antispasmodic, hypolipidaemic, sedative, cytotoxic and protective effects against Alzheimer's disease [37,38]. A study by Katz et al. (2010) reported positive results, particularly in patients with attention deficit. No major side effects related to the plant were reported in this study [39].

2.8 *Crocus sativus* L.

It is a precious plant of the Iridaceae family, known as saffron and used as a spice throughout the world and in Anatolia. The stigmas of C. sativus, the powdered spice made from the dried stigmas, and the plant itself are known as saffron. The dried stigmas of saffron flowers have medicinal properties such as antidepressant, antioxidant, anticarcinogenic,

aphrodisiac, antispasmodic, anti-inflammatory and cholesterol and blood sugar regulating [40,41]. Baziar et al. (2019) compared the efficacy of safron and methylphenidate (MPH) in improving ADHD symptoms. Patients were randomised by weight to treatment with MPH or safron capsules. Symptoms were assessed at baseline, week 3 and week 6 using the Attention Deficit/Hyperactivity Disorder Rating Scale for Teachers and Parents IV (ADHD-RS-IV). The authors found no significant difference in ADHD-RS-IV scores between the two groups at baseline or at the end of the study [42]. Blasco-Fontecilla et al. (2022) reported that in patients given safron and methylphenidate, safron was effective for hyperactivity symptoms and methylphenidate was effective for attention deficit symptoms [43]. Again, Khaksarian et al. (2021) reported lower test scores in patients with ADHD who received safron with methylphenidate [44].

2.9 *Scutellaria baicalensis* L.

The plant, native to China, Korea, Mongolia and parts of Russia and Siberia, is a flowering plant in the Lamiaceae family traditionally used in Chinese medicine. The extract from its roots consists of compounds such as baicalein, baicalin and beta-sitosterol. More than 100 flavonoids have been isolated from the roots, the part used in treatment. Baicalein, baicalein, baicalein, wogonoside, wogonin, scutellarein are the main flavonoids found in the plant and have been extensively studied for many bioactivities. Bioactivity studies have shown that extracts or pure substances isolated from the plant have anti-inflammatory, antibacterial, antiviral, antioxidant, anti-diabetic, anticancer, anti-Alzheimer, neuroprotective and hepatoprotective effects [45,46]. Zhou et al. (2017) reported that baicalin and MPH were able to normalise exercise capacity and impulsivity in animal experiments [47].

2.10 *Passiflora ıncarnata* L.

Passion flower (passiflora incarnata or passion flower) is an herbal supplement historically used to treat anxiety, insomnia, and hysteria. This plant, which was grown specifically in Europe in the last century, is used extensively in complementary medicine. The plant is available in forms used as tea, liquid extract and tincture [48]. In the study conducted by Akhondzadeh et al. (2005) on 34 children with ADHD, no difference was reported between the group receiving passion flower twice a day and the group receiving methylphenidate. In the same study, the potential for side effects was observed to be less in the group receiving passion flower [49].

2.11 *Hypericum perforatum* L.

It is one of the 500 species of the Hypericum family. Although the Mediterranean region is a very rich region for Hypericum species, Asia and America also show significant biodiversity for many endemic Hypericum species. It is especially

widely used in the treatment of patients with mild to moderate depression. This plant, which contains many active ingredients, is also reported to have antibacterial, antinociceptive, neuroprotective, healing dermal activity in some skin disorders, especially wounds and burns, and activity in reducing menopausal symptoms [50,51]. Niederhofer (2010) reported that it is beneficial in improving symptoms in children with ADHD [52]. On the other hand, Weber et al. reported in their study that *H. Perforatum* did not have an effect on reducing ADHD symptoms [53]. Therefore, more studies are needed on the use of this herb in ADHD symptoms.

3 CONCLUSION

In summary, all studies included in this article focused on plants with proven potential against inflammatory processes, positioning them as promising candidates for the treatment of ADHD, especially in patients who may not respond well to conventional medications. The use of plants for medicinal purposes has a long history in human history. However, it is important to note that although natural remedies are generally considered safe, they have not yet achieved status as a standard source of treatment for ADHD. Additionally, there is no established standard animal or in vitro experimental model for using plant-derived compounds to improve ADHD symptoms. Further studies are needed with the plants presented here.

4 AUTHOR CONTRIBUTIONS

Hypotesis: Ö.Ç.; Design: Ö.Ç.; Literature review: Ö.Ç.; Data Collection: Ö.Ç.; Analysis and/or interpretation: Ö.Ç.; Manuscript writing: Ö.Ç.

5 CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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Research Article

Synthesis of Membrane Structures Containing Nanocomposites for Immunoglobulin G (IgG) Adsorption

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ABSTRACT: The discovery of markers of many diseases, such as cancer, inflammation, diabetes, cardiovascular diseases and other autoimmune diseases, can be made through the characterization of serum protein or peptide. Proteomics, the comprehensive study of proteins on a large scale, is poised to significantly enhance our comprehension of gene functionality in the post-genomic age. It entails the quantitative and physical delineation of all proteins within a specific cell line, tissue, bodily fluids, or organism. Our study is aimed to adsorb antibodies from serum with GO-HEMA nanocomposite materials. In this context, GO-HEMA membranes were synthesized via the photopolymerization method, and subsequently, it was observed through scanning electron microscopy (SEM) that the particles exhibited a cylindrical structure. Fourier Transform Infrared Spectroscopy (FTIR) was employed to characterize the inclusion of graphene oxide into the HEMA membrane structure. During IgG binding to GO-HEMA membranes, pH, concentration, and time parameters were examined. As a result, the optimum binding conditions were determined to be pH 5.0, a concentration of 0.5 mg/mL, and an adsorption time of 30 minutes. This study is suitable for highly selective IgG adsorption.

Keywords: *proteomics, antibody removal, composite materials, membrane systems, graphene*

1 INTRODUCTION

Nanotechnology's advancements impact diverse fields such as physics, chemistry, materials science, medicine, and biotechnology. Biosensing, utilizing nanotech, relies on advanced materials and nanostructures. Serum and plasma are vital for disease marker detection, reflecting abnormal conditions in organs or tissues through the serum proteome [1]. Proteomic techniques have emerged to identify marker proteins in serum, crucial for early disease detection, monitoring, and treatment. Recently, various commercial products have been created to eliminate albumin and immunoglobulins from plasma, aiding in protein analysis [2].

Since its discovery, graphene has sparked considerable excitement. Furthermore, graphene oxide (GO) shows advantageous properties for biosensing due to its excellent abilities in biocompatibility, solubility, and selectivity [3]. Among these nanocarbons, the low-cost GO and reduced graphene oxide (rGO) have received more attention due to their large electrochemically

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active surface area, oxygenic functional groups in complexing with metal ions/metal nanoparticles (NPs) (redox probes), and immobilization of active antibodies, and good electrical conductivity [4]. Materials derived from GO are widely applied in electronics, membrane engineering, and medicine due to their remarkable electrical, thermal, mechanical, and optical properties. The hydrophilic nature of GO, along with its easy modification using various molecules through abundant oxygen functionalities, highlights its significant potential in drug delivery and biosensing applications [5]. As an illustration, tumor-targeting agents or GO-based materials functionalized with phospholipid polyethylene glycol (PEG) derivatives are effective anticancer agents [6].

Proteomics entails the quantitative and physical mapping of the entire complement of proteins within a specific cell line, tissue, bodily fluids, or organism [7]. The prevailing experimental methodologies in proteomics predominantly encompass two-dimensional protein electrophoresis (2-DE) for protein separation and mass spectrometry for subsequent protein identification [8]. The potential identification of disease markers across various conditions such as inflammation, diabetes, infection, cancer, cardiovascular diseases, Alzheimer's, and autoimmune diseases has spurred interest in proteomic studies. Consequently, there is increasing focus on comprehensively characterizing individual serum proteins and peptides, leading to numerous efforts to broadly understand the protein composition of human serum [9]. The serum harbors a plethora of over one million distinct proteins and peptides, characterized by a broad dynamic concentration range (i.e., 60-80 mg/mL^{-1}), presenting numerous opportunities and potentially advantageous attributes for proteomic scrutiny [10]. Nonetheless, the substantial protein content in serum poses significant challenges for proteomic analysis due to the remarkably dynamic concentration range of serum proteins, spanning from highly abundant proteins (tens of mg/mL to ~2 mg/mL), such as albumin, immunoglobulins (IgG and IgA), to antitrypsin [11].

Transferrin and haptoglobin have the capability to bind to proteins of exceedingly low abundance, such as the vasoconstrictor peptide endothelin -1. An indispensable factor exacerbating the analytical challenge of characterizing the serum proteome is the fact that approximately 90% of the protein content is constituted by merely 10 proteins [12,13]. Within the remaining 10%, a mere 12 proteins encompass 90% of the residual total. Consequently, only 1% of the complete serum protein content consists of proteins deemed to be in low abundance, thus sparking substantial interest in proteomic inquiries focused on identifying potential biomarkers [14]. The

primary challenge in serum proteome analysis is reducing sample complexity to minimize interference on biomarkers. Blood, serum, and plasma proteomic analysis is hindered by highly abundant proteins like albumin, immunoglobulins (IgG and IgA), antitrypsin, haptoglobin, and transferrin, masking proteins present in lower concentrations [15,16]. The removal of these proteins, which constitute approximately 90% of the total protein, increases the relative concentration of other proteins in small quantities and facilitates their detection [17]. Due to its extensive protein composition, plasma plays a pivotal role in disease diagnosis [18]. Early detection depends on spotting proteins that change with biological processes or diseases, vital for treatment response and detecting cancer recurrence. Yet, plasma analysis is complex due to varied protein concentrations [19]. Yet another challenge encountered in serum protein analysis pertains to the depletion of elevated concentrations of IgG [20]. The elimination of IgG is commonly achieved by immobilizing protein A or protein G onto affinity resins, which possess a binding affinity for the Fc region of IgG. However, specific antibodies can also be employed for this purpose [21,22].

Affinity chromatography is a method based on the specific and reversible adsorption of the target biomolecule onto ligands immobilized on an insoluble support matrix [23]. Biological interactions between ligands and target molecules involve electrostatic, hydrophobic, Van der Waals, and/or hydrogen bonds. Target molecule selection can be achieved by using a competitive ligand or adjusting the pH, ionic strength, or polarity (mobile phase) of the medium [24]. Moreover, the removal of IgG from human plasma is employed in the management of immune disorders. Depletion of plasma proteins can be done using different strategies, but the ultimate goal is to separate high amounts of proteins from low amounts of proteins [25]. The identification of cost-effective and highly efficient methods for the depletion of abundant proteins from blood, serum, and recently plasma has emerged as one of the foremost concerns and holds significant importance [2]. Currently, various removal technologies are available for the removal of albumin and antibodies (IgG) from serum, including ultracentrifugal filtration, dye affinity, immunoaffinity, immobilized metal affinity chromatography (IMAC), and suppression methods [2, 26].

Removal of IgG is usually achieved by protein A/G affinity adsorbents that are attached to the Fc region of IgG, but specific antibodies may also be used. Monoclonal antibodies targeting protein A/G and IgG are obtainable from commercial vendors [27]. Nonetheless, IgG is found in serum at concentrations ranging from 8 to 16 mg mL^{-1},

necessitating substantial quantities of antibodies and/or protein A/G for quantitative removal [28]. The high specificity of bioligands provides excellent selectivity [29]. Despite their high selectivity, adsorbents with protein A/G or antibodies have drawbacks: (i) Cost is often prohibitive. (ii) Immobilizing them in the correct orientation is challenging. (iii) Ligands may leak from the stationary phase, which is unacceptable in clinical practice where high removal of IgG from serum is necessary [30]. Among the materials and methods used for removal, it is seen in the literature that composite systems using nano-membrane systems together are not included [31]. Natural surface properties of nanomaterials (e.g., high surface/volume ratio) combined with membrane system dynamics: easy preparation, high selectivity and stability in harsh conditions, nanomaterials are expected to surpass conventional methods [32]. The main aim of the proteomic analysis of serum and plasma is to obtain the most reliable information for diagnosis and treatment [33].

The aim of this particular analysis is to remove antibodies present in blood, serum, and plasma and to develop new generation nanomembrane composite systems. In pursuit of this objective, an innovative and biocompatible GO-HEMA-based membrane composite system has been developed for the removal of IgG.

2 MATERIAL AND METHOD

2.1 Materials

Graphene oxide (GO), IgG, 2-Hydroxyethyl methacrylate (HEMA), Ethylene glycol dimethacrylate (EGDMA), as an initiator 2,2-Dimethoxy-2-phenylacetophenone (DMPA), Graphene powder were taken from Sigma Aldrich. All the other chemicals utilized in this study, such as Tetrahydrofuran (THF), Sodium hydride (NaH), vaseline, parafilm, Ethanol (C_2H_6O), Potassium permanganate ($KMnO_4$), Hydrogen peroxide (H_2O_2), Sulfuric acid (H_2SO_4), Phosphoric acid (H_3PO_4), Hydrochloric acid (HCl), Nitrogen gas, Potassium persulfate (KPS), Polyvinylalcohol (PVA) are of analytical purity.

FTIR spectra of monomers and polymers were obtained using the FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). Scanning electron microscope (SEM, Philips XL-30S FEG) and Zeta potential measuring device (Malvern) were used. Support was received from Ege University in the implementation of other devices and experiments. Centrifuges (Centrion Scientific Benchtop Centrifuges) were used for washing and settling processes. Precision balance (KERN&Sohn GmbH), pH meter (ISTEK, NeoMet), magnetic stirrer (Dragon lab, MX-F), sonicator for homogeneous mixing of solutions (Lab Companion, UC-10), UV lamp, oven

(Memmert) was used.

2.2 Nanographene Oxide Synthesis

Graphene oxide will be prepared from graphene powder by the improved Hummers method [34]. In short, 2 g of graphite powder, 12 g of potassium permanganate, and 261 mL of acid (235 mL H_2SO_4 + 26 mL H_3PO_4) will be mixed at 50°C for 12 hours in a balloon reactor. This mixture will then be poured onto the frozen mixture containing 260 mL of pure water and 2 mL of 30% H_2O_2. Then the resulting final solution will be centrifuged for 4 hours at 4000 rpm. The precipitated substance will be removed and washed first with 10% HCl solution and then with ethanol and dried overnight at 80°C.

2.3 Synthesis of Nano-Membrane Composite Structures

The polymerization mixture (10 mL) will be prepared by adding and dissolving 2500 µL HEMA and 100 µL EGDMA (as cross-linking agent), 100 mg graphene oxide and DMPA (as initiator). The mixture will be incubated in the bath of sonic for 5 minutes. And it will be cleaned with nitrogen for about 5 minutes and degassed.

The mixture will then be poured into cylindrical glass molds (diameter 10 mm) and the synthesis of composite structures will be carried out by UV-initiated photopolymerization. For this purpose, the mixture shall be exposed to UV light with a wavelength of 1 $mWcm^{-1}$ UV light 365 nm.

After polymerization, the synthesized nano-membrane composite structures will be cut into circular parts using a perforator. It will be washed with distilled water several times to remove reacting monomers, initiators, and other debris and left for orbital mixing for 24 hours. After the washing process, the nano-membrane composite structures will be dried overnight at 37°C [35].

2.4 Optimization of Adsorption Conditions of IgG

Optimization studies were conducted to achieve high levels of IgG binding. For this purpose, graphene oxide (GO) nanoparticle synthesis was performed using the Hummers method [34]. The synthesis of the GO-HEMA nanocomposite structure was carried out

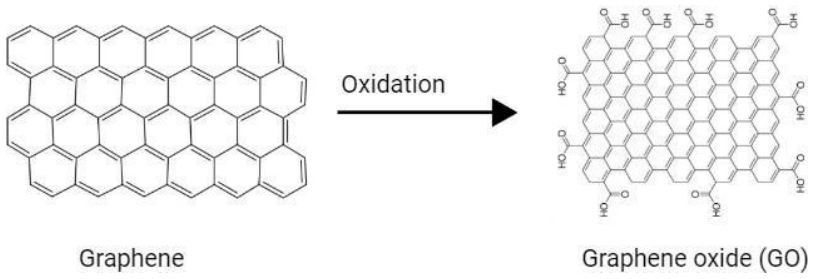

Figure 1. From Graphene oxidation to Graphene Oxide.

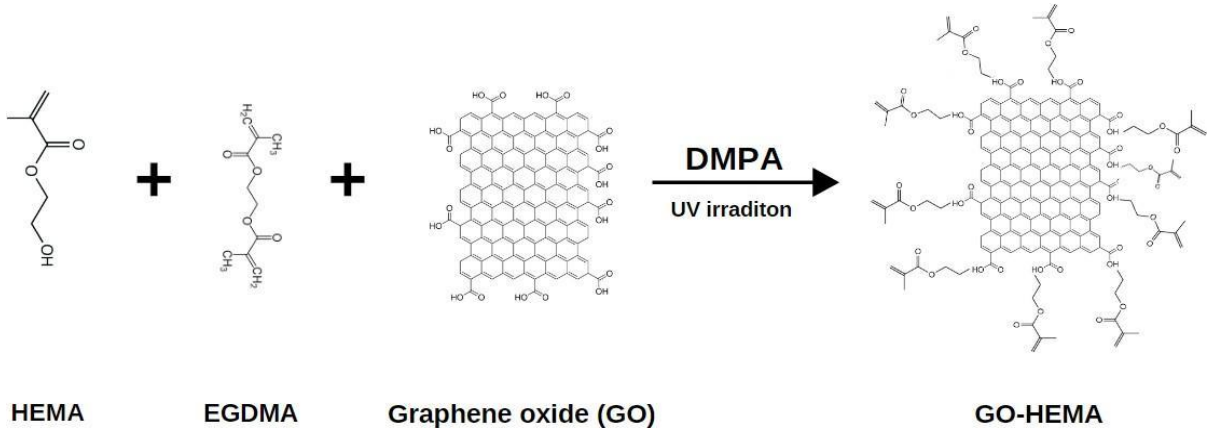

Figure 2. EGDMA with 2-hydroxyethyl methacrylate (HEMA) has formed a membrane structure under UV and is related to the functionalization of GO with 2-hydroxyethyl methacrylate (HEMA).

using the photopolymerization technique. The effect of pH (5.0, 6.0, 7.0, 8.0), time (30 min., 60 min., 90 min., 120 min.), and initial IgG concentration (0.05, 0.1, 0.25, 0.5, 0.75, 1 mg/mL) on adsorption was examined for the GO-HEMA nanocomposite. All experiments were conducted at 20°C [36].

3 RESULT

3.1 FTIR Analysis

The GO-HEMA membrane FTIR spectrum is shown in Figure 5.

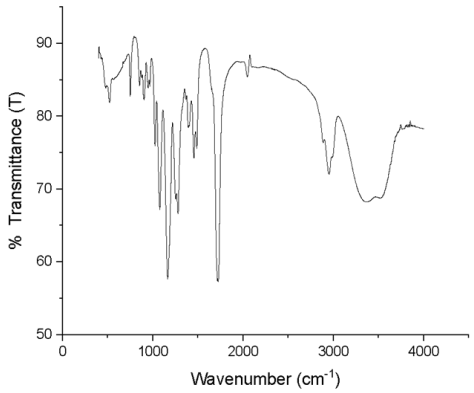

Figure 3. FTIR spectrum of membrane structures containing graphene oxide.

Figure 3 depicts the FTIR spectra of the GO/HEMA nanocomposite. The strong peak at 3441 cm^{-1} corresponds to the OH functional group; the peak at 1735 cm^{-1} is attributed to the C=O group, which is functionalized to produce the carboxylic group (COOH); and the peaks at 1156 cm^{-1}, 1459 cm^{-1}, and 1405 cm^{-1} are associated with the alcoholic C-OH and the C=C aromatic bond on the GO surface. Peaks of HEMA are observed at 2923 cm^{-1}, 2852 cm^{-1}, and 1365 cm^{-1}, corresponding to the bending vibrations of the C-H bonds of methylene and methyl groups. The peaks at 900 cm^{-1}, 945 cm^{-1}, 850 cm^{-1}, and 748 cm^{-1} are related to bending vibrations outside the aromatic plane or bending vibrations outside the CH surface. Additionally, the tensile vibrations of the -OH groups of GO-HEMA membranes in the range of 3300-3700 cm^{-1} indicate the incorporation of graphene oxide into the HEMA membrane structure.

3.2 SEM Analysis

Scanning electron microscope images showed the morphological structure of HEMA Membrane, GO-HEMA Membrane

nanopolymers in Figure 4.

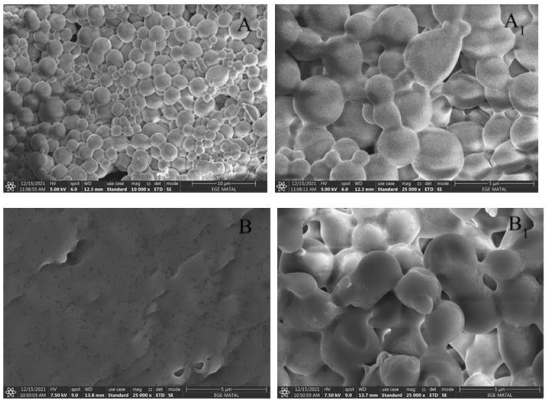

Figure 4. SEM images of HEMA-EGDMA membrane nanopolymer. (A, A_1: HEMA Membrane; B, B_1: GO-HEMA Membrane).

It is shown from its horizontal and vertical sections that the particles have a cylindrical structure. It can be said that these structures can be grafted with GO and turned into nanocomposites.

3.3 IgG Adsorption/Desorption to GO HEMA Membrane

3.3.1 Investigating the Effect of Concentration and Time on IgG Adsorption

IgG adsorption experiments were performed on the synthesized GO-HEMA membrane systems. IgG adsorption is highly influenced by initial IgG concentration, incubation period, ambient pH, temperature, and ionic strength. Therefore, the initial concentration of IgG was changed from 0.1 mg/mL to 2.0 mg/mL; The impact of incubation duration on IgG adsorption was investigated across various adsorption periods (30-120 minutes).

In typical IgG adsorption experiments, HEMA membranes (diameter: 0.75 cm) were mixed with a total of 1.0 mL of IgG solution at different concentrations under an orbital mixing at 400 rpm and at room temperature. Upon reaching the equilibrium time for optimal IgG adsorption, the HEMA membranes were recovered from the solutions via uncomplicated physical filtration. The adsorbed amount of IgG (Q) was quantified spectrophotometrically at 280 nm, deriving from the discrepancy between the initial and residual IgG concentrations in the adsorption solution. The Q values were determined utilizing the equation presented herein (Equation 1).

$$Q = \frac{(Ci - Cf) x V}{weight\ of\ membrane\ (g)} \quad \text{(Equation 1)}$$

In this context, within the adsorption solution (mg/mL), Q represents the quantity of IgG adsorbed into the HEMA membrane systems (mg/g), where Ci symbolizes the initial IgG concentration, Cf denotes the final IgG concentration, and V indicates the total volume of the adsorption solution (mL), while m represents the mass of the membrane. Since the HEMA membrane has a cylindrical structure, it is calculated with the cylindrical volume formula.

As shown in Figure 5, graphene oxide HEMA membrane structures are determined at best in 0.5 mg/mL concentration at 30 minutes. In the graph of Figure 3, IgG at 0.5 mg/mL

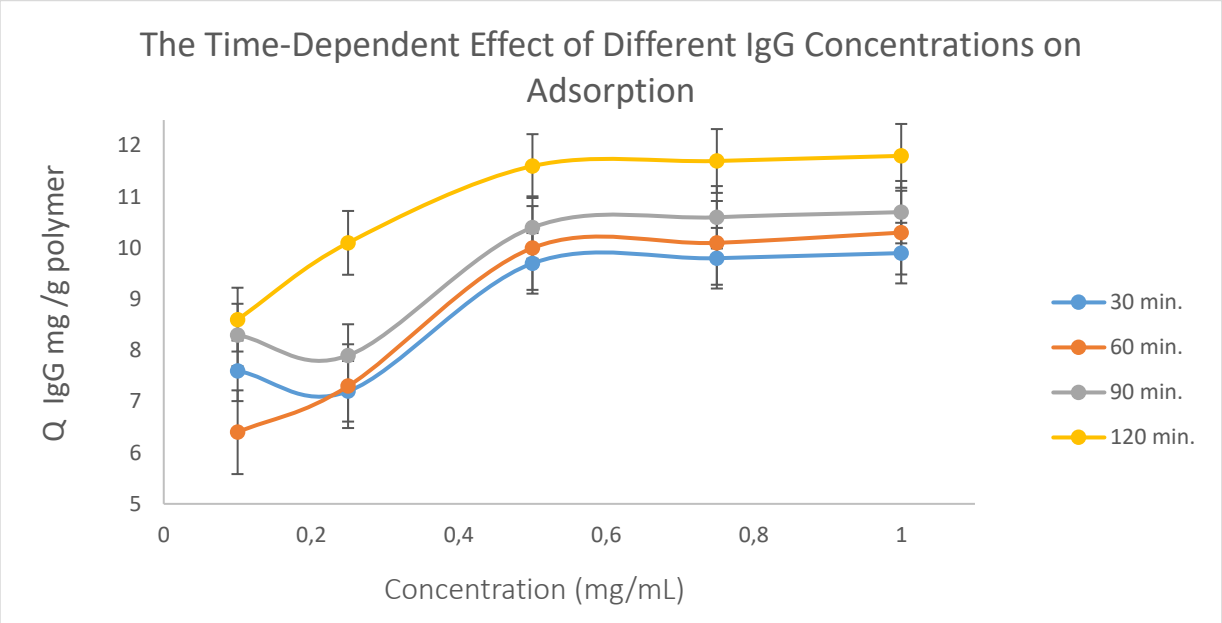

Figure 5. The Q equation was calculated by taking the absorbance measurements of the amount of IgG adsorbed to the GO-HEMA membrane structures and the standard graph was drawn.

concentration was bound to (GO-HEMA) membrane through secondary interactions, such as hydrogen bonding, hydrophobic interactions, etc.

For the calculation of the amount of adsorbed substance, the mass of the GO-HEMA membrane structures was calculated from the volume of the cylinder. At 30 and 45 minutes, the values showed similar results. In trials at 60 and 120 minutes, it was observed that IgG was more absorbed into the GO-HEMA membrane surface. It can be said that IgG can be bound to the nanocomposite materials synthesized with the obtained data.

3.3.2 Investigating the effect of pH on IgG adsorption

The pH-dependent impact on IgG binding to GO-HEMA membranes was explored through a systematic investigation of pH values ranging from 5.0 to 8.0 To this end, buffered solutions were meticulously prepared, comprising a 0.1 M acetate buffer for pH 5.0 and 0.1 M PBS for pH 6.0, pH 7.0, and pH 8.0, with rigorous pH level verification. Subsequently, 0.5 mg/mL IgG binding assays were meticulously conducted under these varied pH conditions.

The optimum pH value for IgG binding was determined as 5. After this value, decreases in bonding may be seen due to the deterioration of the IgG structure or the decrease in the area to be bound on the membrane surface.

Based on the data acquired from the optimization studies, optimum binding was determined as pH 5.0, 0.5 mg/mL concentration, and 30 min adsorption time. Figure 5 and Figure 6 demonstrate that a

protein

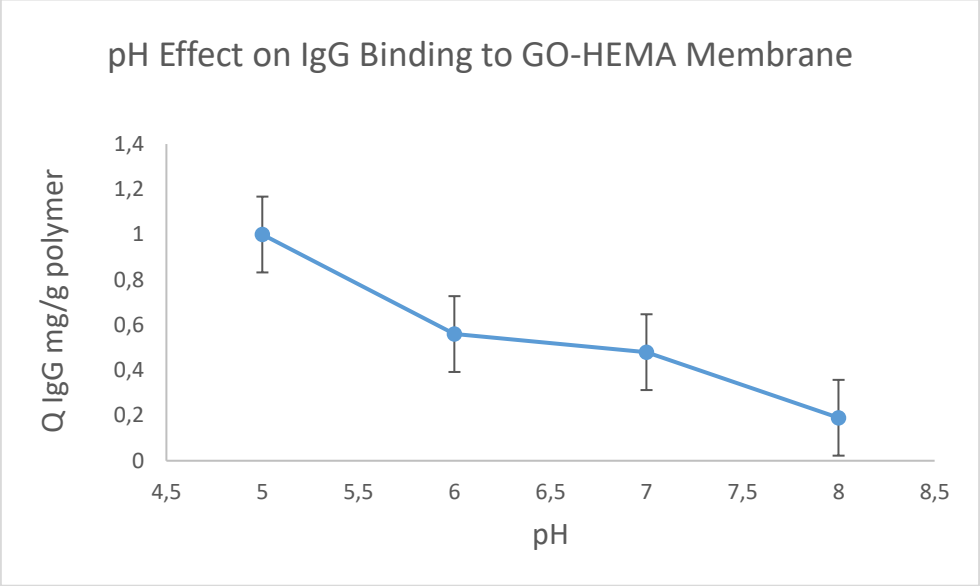

Figure 6. pH effect on IgG binding to GO-HEMA membrane (Room temperature, 30 min).

concentration of 0.5 mg/mL IgG is adequate for binding to GO-HEMA membrane structures.

4 DISCUSSION

Previous studies assert that plasma harbors a multitude of proteins, thus rendering it pivotal in disease diagnosis. They further elaborate that the removal of these proteins, constituting approximately 90% of the total protein content, amplifies the relative concentration of other proteins present in minor quantities, thereby facilitating their detection. Because the characterization of thousands of individual serum proteins/peptides can lead to the discovery of markers of many diseases, such as inflammation, infection, diabetes, cancer, cardiovascular disease, Alzheimer's, and other autoimmune diseases, human serum is attracting increasing interest in its proteomics studies with many attempts to characterize its components broadly. Treatment methods and drug systems for these diseases on a large scale are quite numerous. With the advancement of technology, the production of composite nanomaterials and their use in diseases are becoming widespread. Based on the most basic, it would be more accurate to separate proteins from blood, serum and plasma and use them in diagnosis and treatment. In our own study, we aimed to use inexpensive and highly efficient techniques for the removal of proteins and preferred nanocomposite materials. In addition to this, results show that the developed sorbent system has a great potential for use for the removal of IgG from human blood in proteomic studies.

In a study conducted by Demir et al. in 2017, Concanavalin A-poly (2-hydroxyethyl methacrylate-ethylene dimethacrylate) hydrogel membranes were employed for IgG adsorption, yielding a recorded adsorption

capacity of 3.52 mg/g [37]. Additionally, in a study by Bayramoğlu and Arıca in 2009, poly(HEMA/EGDMA) microspheres were utilized, revealing optimal IgG adsorption between pH 5.0 and 6.0 [38]. In our study, using GO-HEMA composite membrane at pH 5.0, an adsorption capacity of 11.8 mg/g was observed, demonstrating sufficient adsorption. The preference for the synthesized polymeric material in proteomic studies may stem from its cost-effectiveness and adequate adsorption capability.

In conclusion, a cost-effective and easily synthesizable nanocomposite polymeric structure demonstrating high specificity to IgG was synthesized for IgG detection. The use of non-toxic nanomaterials synthesized via photopolymerization technique and optimized polymer is anticipated to provide a precise detection and analysis method at the molecular level. This suggests the potential to offer an innovative diagnostic method in the biomedical field, providing high sensitivity and accuracy in detecting structural alterations in IgG.

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