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Editor : Prof. Dr. Selami SELVİ
Address : Balikesir University,
Altinoluk Vocational School, Edremit/Balıkesir, Turkiye
Phone : +90 266 396 1552
E-mail : sselvi@balikesir.edu.tr

Publisher : Prof. Dr. Izzet KARA
Address : Pamukkale University, Education Faculty,
Kinikli Campus, 20070 Denizli, Turkiye
Phone : +90 258 296 1036
Fax : +90 258 296 1200
E-mail : ikara@pau.edu.tr
ijsm.editor@gmail.com

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Table of Contents

International Journal of Secondary Metabolite
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Research Article

Efficiency of pre-sowing seeds by UV-C and X-ray exposure on the accumulation of antioxidants in inflorescence of plants of *Matricaria chamomilla L.* genotypes

Vladyslav Zhuk[1], **Daryna Sokolova**[1,*], **Alexandra Kravets**[1], **Volodymyr Sakada**[1], **Ludmila Glushenko**[2], **Mykola Kuchuk**[1]

[1]Institute of Cell Biology and Genetic Engineering, National Academy of Sciences of Ukraine, Kyiv
[2]Experimental Station of Medicinal Plants of The Institute of Agroecology and Nature Management, National Academy of Agrarian Sciences of Ukraine, Lubny

Abstract: Secondary metabolites of the medicinal plants are among the main active substances of the drugs used in medicine. An important place among them belongs to phenols and flavonoids, which are some constitutive components of the redox homeostasis maintaining system through the animal and plant organisms.

Radiation exposure is one of the most powerful factors leading to the oxidative stress, stimulating the formation of radioprotectors with antioxidant, anticancer, immunomodulatory, and anti-inflammatory effects. The data presented in the previous report indicated some differences in the pharmaceutical raw material yield stimulation of various genotypes under UV-C and X-ray exposure. This stage of the study is devoted to the investigation of the stimulating the yield of flavonoids and phenols as the markers of the secondary metabolism reorganization.

The differences in the dynamics of the flavonoids and phenols content in plants of eight genotypes of the chamomile in the control and under pre-sowing UV-C and X-ray radiation exposure of seeds were studied. Groups of the genotypes by the stimulating effect on the content of antioxidants were determined mainly under UV-C exposure, as well as groups with a significant increase in the content of antioxidants under X-ray exposure were identified. A highly significant correlation (R = 0.84) between the stimulation of the flavonoid synthesis under X-ray exposure and the level of these antioxidants in the control is shown. Above average (R = 0.64) insignificant correlation is observed between the flavonoids level under UV-C exposure and in the control variant.

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1. INTRODUCTION

Enzymatic and non-enzymatic antioxidants are constitutive components of the redox homeostasis maintenance system in the animal and plant organisms (Kretovich, 1986; Kudryashov, 2001; Croft, 1998). The antioxidant system also plays an active protective role under the biotic and abiotic stressors effect (Kudryashov, 2001; Winkel-Shirley, 2002; Treutter, 2006; Mittler, 2002).

*CONTACT: Daryna Sokolova dasokolova88@gmail.com Institute of Cell Biology and Genetic Engineering, National Academy of Sciences of Ukraine, Kyiv

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Radiation exposure is one of the most powerful factors of the oxidative stress and stimulates radioprotectors formation (Kudryashov, 2001; Mittler, 2002; Khattak & Simpson, 2008), which has some antioxidant, anticancer, immunomodulatory, and anti-inflammatory effects (Alothman *et al.*, 2009; Harrison & Were, 2007; Dai & Mumper, 2010; Moghaddam *et al.*, 2011; Kaur & Mondal, 2014).

Biotechnology uses radiation exposure for the reorientation of the plant metabolic processes to achieve the desired direction for the practice based on the systematic defense reactions. Formation of non-target effects such as the induction of protective and adaptive reactions in non-exposed organs of the organism («abscopal effect») and even in non-exposed organisms that are found in the same environment as exposed ones («by stander effect») (Sengul *et al*., 2009; Kuzin, 1970; Little, 2007; Kravets *et al*., 2009) enables the products of the plant protective reactions in non-exposed structures, which are some medicine material (Gould & Lister, 2006).

The data presented in the previous report indicated some differences in the pharmaceutical raw material yield stimulation of various genotypes under UV-C and X-ray exposure (Sokolova *et al*., 2021). The genotypes were differing not only with the medicinal raw material yield but also with the flowering dynamics under different types of radiation exposure. The next stage of the study is devoted to the investigation of the stimulating effective secondary metabolism of the chamomile under seeds pre-sowing UV-C and X-ray exposure. The yield of non-enzymatic antioxidants, such as flavonoids and phenols, was used as the marker of the secondary metabolism reorganization.

Despite the diversity of the functions of these compounds, which are involved in a number of blocks of primary plant metabolism such as photosynthesis, the formation of lignin and suberin of the cell walls, plant biochemistry classifies them as the products of secondary metabolism (Kretovich, 1986) playing role of some protective agents in the plant pathogenesis. Plant and animal biology considers them as some low-molecular antioxidants and the most essential components of the endogenous radiosensitivity background (Kudryashov, 2001). Currently they are widely used in medicine as the oncoprotectors for the treatment of inflammatory processes and diseases of the vascular system (Alotman *et al*., 2009; Aziz *et al.*, 2015; Cermak & Wolffram, 2006; Clark *et al*., 2015).

The study focused on the analysis of key issues of the biotechnology development:

- the assessment of the initial level of the antioxidants through the different genotypes;
- the intensity of their response to a particular type of radiation exposure;
- the correlation assessment of the indexes and the comparative evaluation of the target metabolite dynamics in the control and under the exposure.

2. MATERIAL and METHODS

The research was done using 8 genotypes of *Matricaria chamomilla*. Six certified varieties of the different origin were used: 1 – the generative generation of the mutant Perlyna Lisostepu (treated with the herbicide RaudAr in concentration 10); 2 – the variety Quedlinburg (Germany); 3 – the variety Goral (Slovenia); 4 – the variety Azulena (Russia); 5 – the variety Zlaty Lan (Poland); 6 – the variety Perlyna Lisostepu (Ukraine). Some non-varietal material, in fact the edaphic ecotypes, were included to the study: 7 – from manufacturer Gold Garden (Ukraine) further – the ecotype Gold Garden; 8 – from manufacturer Seed Era (Ukraine) further – the ecotype Seed Era. The experiment was repeated three times.

Dry seeds were exposed with the dose 10 Gy, dose rate 1.42×10^{-2} Gy/sec using the X-ray installation RUM-17 (Russia). UV-C exposure with the dose 10 kJ/m^2, dose rate 3.4 W/m^2 was

carried out using the installation OBM-150 M (Ukraine) with two lamps Philips Special TUV 30 W (the Netherlands).

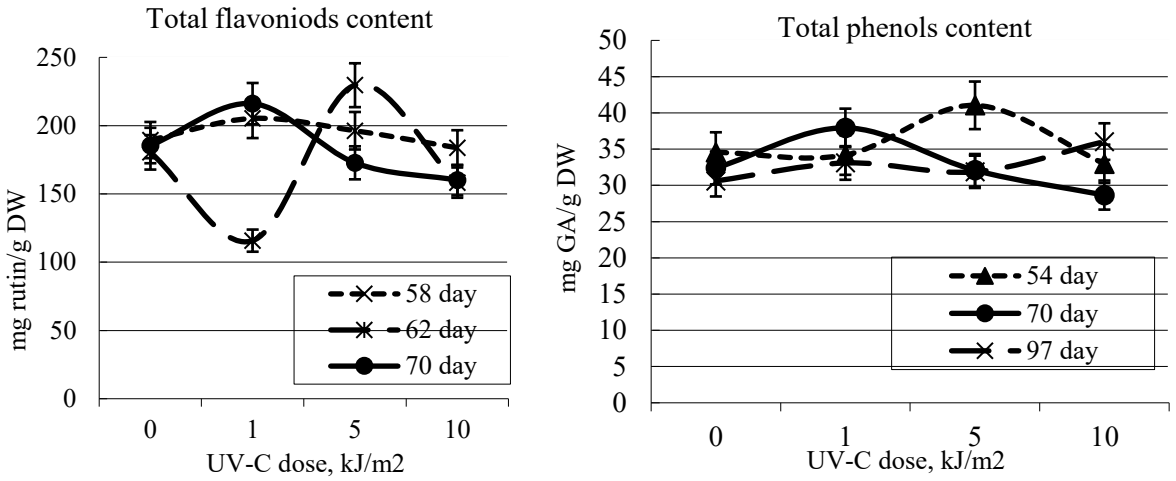
The research was conducted using some plant material, the methodology of obtaining the material was given in the previous report (Sokolova *et al.*, 2021). The determination of X-ray exposure dose was based on the investigation of the medicinal plants conducted before. The results were assigned with the patent (Shylina *et al*., 2018). The dose curve of UV-C exposure is in the text.

The extraction of flavonoids and phenols was performed according to the generally accepted methods (Croft, 1998). The dry flower mass (50 mg) was homogenized and macerated in 5 ml of 70% ethanol at 24° C for 72 h, then filtered, the amount of filtrate was adjusted to the initial volume by 70% ethanol and centrifuged. 0.5 ml of a 2% solution of aluminum chloride was added in 50% ethanol and 2 ml of 70% ethanol to 0.5 ml of the extract and mixed. The reference solution contained 0.5 ml of the extract, 1 drop of acetic acid and 2.5 ml of 70% ethanol. The determination of the flavonoid content was performed by forming a yellow colored complex of the flavonoid-aluminum. After 20 min of the incubation, the optical density of the solution was measured on a SF-46 spectrophotometer at a wavelength of 410 nm against a reference solution, and the concentration of total flavonoids content was determined according to the rutine calibration graph and expressed in mg of rutine per g of dry weight (DW). The total phenols content was determined from the same extract as flavonoids. 0.5 ml (1/10 of diluted by distilled water) Folin–Ciocalteu reagent and 1 ml of distilled water were added to 0.1 ml of the extract, mixed and kept at room temperature for 1 minute. After 1 min, 1.5 ml of 20% Na_2CO_3 water solution was added, mixed, and incubated in the dark for 2 hours at room temperature. The optical density of the blue solution was determined at a wavelength of 760 nm on a spectrophotometer against a sample containing 0.1 ml of 70% ethanol instead of extract and expressed in mg of gallic acid (GA) (according to the calibration graph) per g DW. Statistical analyzes were performed by standard methods.

3. RESULTS and DISCUSSION

The study of dose curve under UV-C pre-sowing exposure by the secondary metabolite yield in the inflorescence indicated some dependence on the dose not only through the antioxidants yield but also in the dynamics of the index change. For example, the highest flavonoid yield after the exposure with 5 kJ/m^2 was indicated on the 62th day after the sowing. For the phenols the highest index was indicated on the 54th day after the sowing (Figure 1). In the inflorescences of the plants from the exposed with 1 kJ/m^2 seeds the highest secondary metabolites yield was shown on the 70th day after the sowing. The index was lower than another one under 5 kJ/m^2 radiation exposure. The maximum yield of the flavonoids and phenols under 10 kJ/m^2 exposure was indicated on the 58th and 97th days after the sowing. In general, the dependence of the maximum accumulation of the antioxidants on the dose was non-monotonic, which was typical for the field of small doses.

Figure 1. Dose-response curve of the flavonoids and phenols content in the chamomile inflorescences. Perlyna Lisostepu variety.



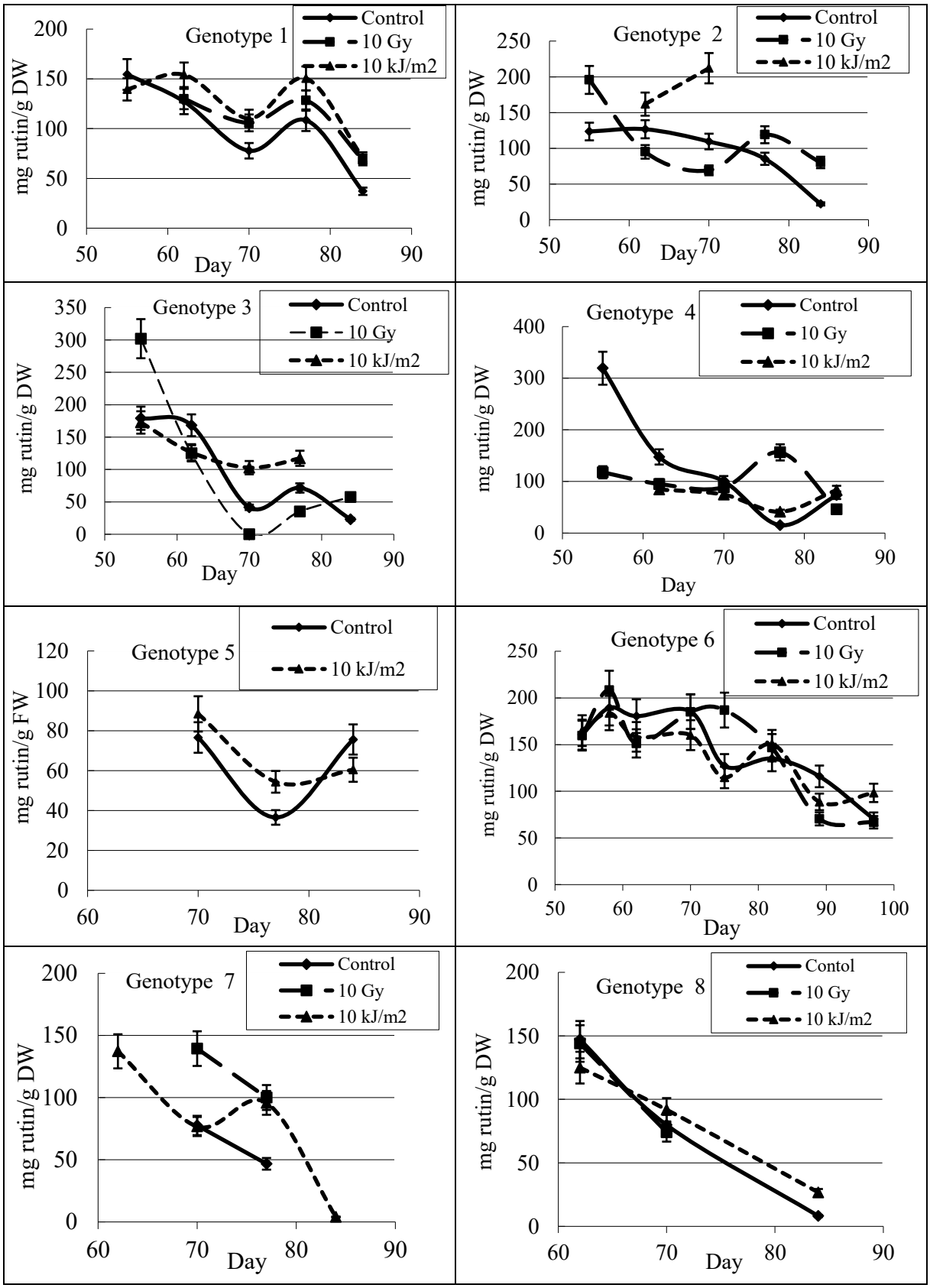
According to the estimation (Figure 2) there were major differences between the flavonoid yield in the dry mass of florescence of control variants through 8 chamomile genotypes. The highest initial yield of the antioxidant was indicated for Azulena and Goral varieties, the lowest one – for both Zlaty Lan variety and ecotype Golden Garden. Mostly through the control variants of all genotypes, except for the Zlaty Lan, the highest flavonoid yield was observed at the beginning of the flowering during the first selection of the medicinal raw material with the lowest harvest yield.

There were significant differences in the reaction of the different genotypes under the UV-C and X-ray exposure. The accumulation of flavonoids for almost all variants was non-monotonic both in control and under radiation exposure. Two maximum flavonoid yields were indicated in the inflorescences of the mutant Perlyna Lisostepu grown from the UV-C exposed seeds on the 62nd and 77th days from the sowing. The same maximum index for varieties Quedinburg and Goral was shown on the 55th day under the X-ray exposure and on the 70th day under the UV-C one. The maximum flavonoid yield under the X-ray exposure of the Azulena variety seeds was on the 77th day after the sowing. The same maximum index for the variety Zlaty Lan was indicated on the 70th day under the UV-C exposure.

The maximum flavonoid yield under the X-ray exposure of the Perlyna Lisostepu variety seeds was on the 58th, 70th and 77th days after the sowing. The maximum flavonoid yield under the X-ray exposure of the ecotype Golden Garden seeds was on the 70th day after the sowing and on the 62nd day under the UV-C exposure.

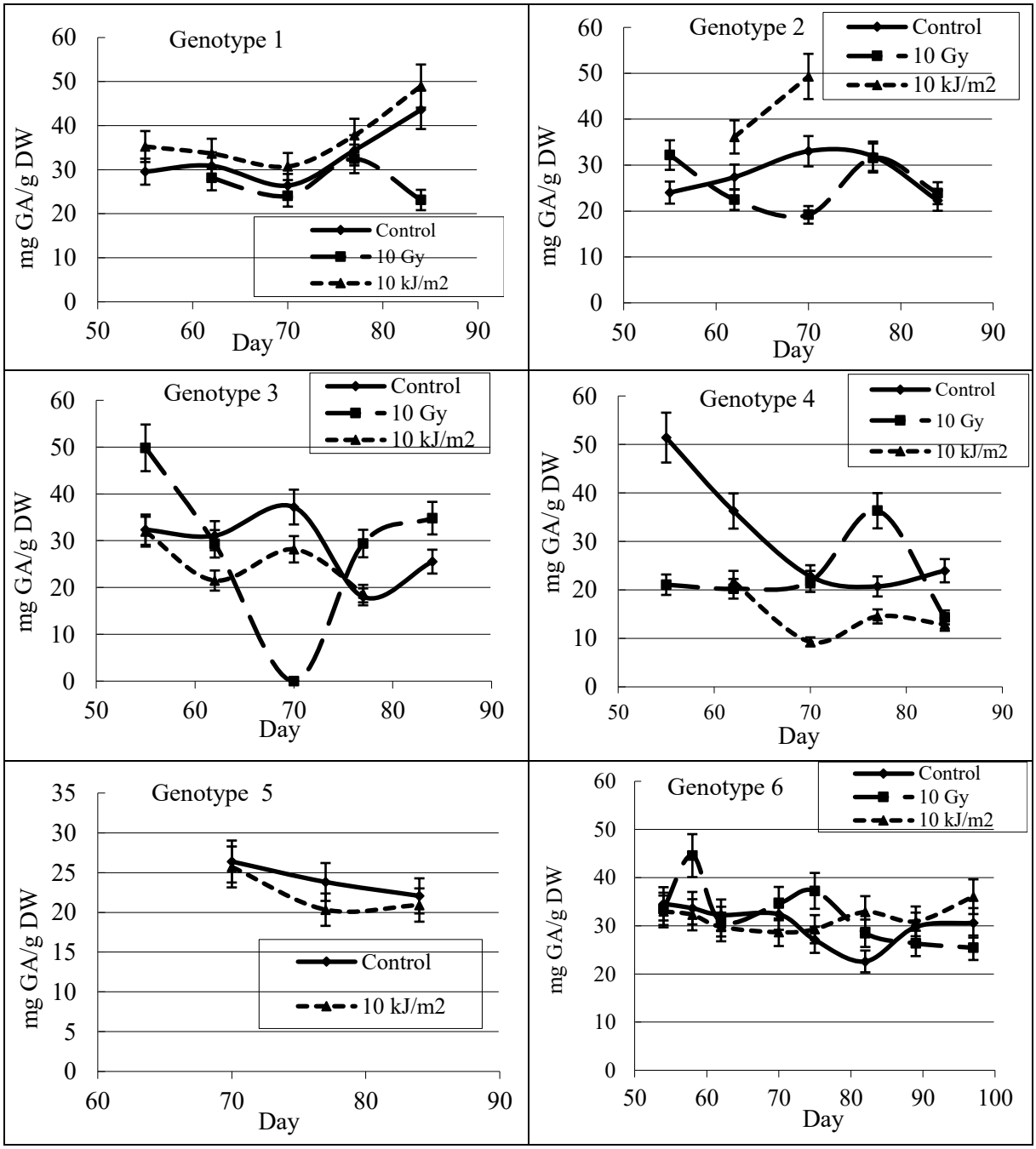
From the practical point of view, it was important that the increasing flavonoid yield through the exposed variants coincided with the maximum formation of the inflorescences. This effect was not indicated for the control.

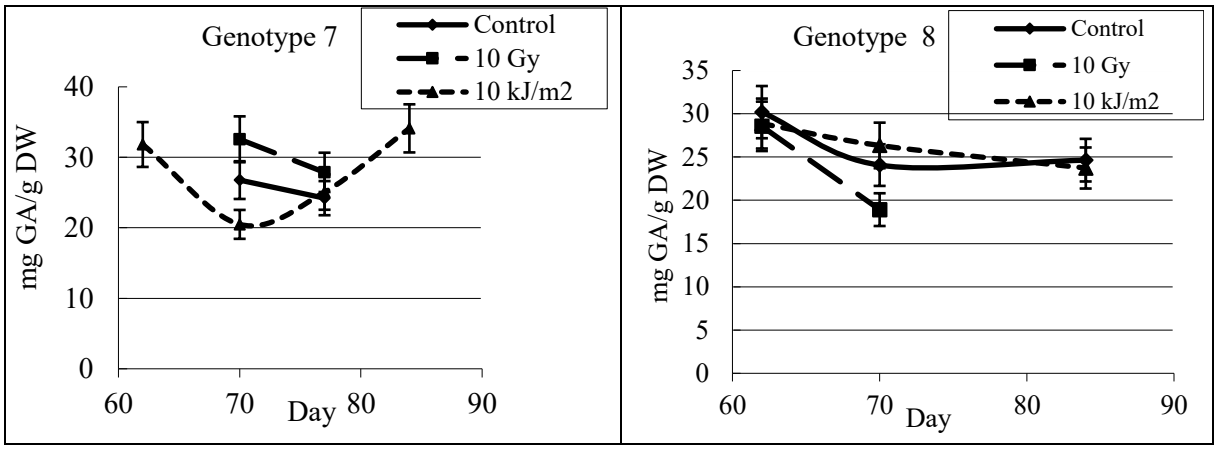
Figure 2. Total flavonoids content in the chamomile inflorescences of the different genotypes: 1 – the generative generation of the mutant Perluna lisostepu, 2 – the Quedlinburg, 3 – the Goral; 4 - the Azulena, 5 – the Zlaty Lan, 6 -the Perlyna Lisostepu, 7 - the Golden Garden, 8 - the Seed Era.



Experimental chamomile varieties were diverse with the yield of the phenols in the inflorescences. The maximum yield was indicated for Azulena, the lowest one – for Zlaty Lan (Figure 3). The pre-sowing UV-C exposure of the chamomile showed the increasing phenol yield in the inflorescences of the Quedlinburg variety and the mutant Perlyna Lisostepu and its decreasing yield for the Goral and Azulena varieties. The X-ray exposure led to increase in phenol yield in the inflorescences of the varieties Goral and Perlyna Lisostepu and the ecotype Golden Garden.

Figure 3. Total phenols content in the chamomile inflorescences of the different genotypes: 1 – the generative generation of the mutant Perlyna Lisostepu, 2 – the Quedlinburg, 3 – the Goral; 4- the Azulena, 5 – the Zlaty Lan, 6 – the Perlyna Lisostepu, 7 – the Golden Garden, 8 – the Seed Era.





In general, UV-C and X-ray exposure did not cause major differences of the phenol content in the medicinal material. Under the UV-C exposure there was some increasing phenol yield for the variety Quedlinburg and the mutant Perlyna Lisostepu. The varieties Goral and Azulena demonstrated decrease of the index. There was increase in the phenol yield for Goral variety under the pre-sowing X-ray exposure. One of the key issues in the assessment and practical implementation of the pre-sowing radiation exposure effects is to study the connection between antioxidant yield in the control material and under the stress factor effect. The estimation of the correlation between the flavonoid yield in the control and UV-C exposed variants is R = 0.63 – insignificant at this small sample. The correlation between the flavonoid yield in the control and X-ray exposed variants is R = 0.84 with the significance level 0.05.

The correlation between the phenol yield in the control and UV-C exposed variants is absent, R=0.22. The correlation between the phenol yield in the control and X-ray exposed variants is average R=0.59 - insignificant at this level of the degrees of freedom.

Summarizing the results, we have concluded that not all the genotypes responded to the various radiation exposure in the right direction for the practice. Some non-variety seeds, i.e. randomly chosen ecotypes, did not show the effective increasing marker metabolite. The most effective relative flavonoid increase were varieties: the Perlyna Lisostepu, the Quedlinburg, the Goral and the mutant Perlyna Lisostepu. The calculation of the Pearson's linear correlation is quite informative when selecting genotypes for some biotechnological research.

The applied research is based on numerous theoretical findings that have caused changes in the paradigm of modern radiobiology and have shifted research directions towards low doses (1-2 orders of magnitude below the LD_{50} for a species) and non-targeting effects. The application of radiation in the range of medium and high doses in biotechnology also stimulated protective mechanisms (Harrison & Were, 2007; Moghaddam *et al.*, 2011), but could lead to the loss of crops of medicinal raw materials.

4. CONCLUSION

The obtained results are still far from the practical application. Nevertheless, they contain the answers to a number of questions, the solution of which is necessary for the implementation in practice.

First, pre-sowing low-dose UV-C and X-ray radiation exposure of seeds leads to significant changes in primary and secondary metabolism. The marker of the first one is the differences in growth processes and in the yield of chamomile inflorescences shown earlier. The marker of the second one is the changes in flavonoid and phenol accumulation. Both changes are characterized with a non-targeted nature, in other words, they are observed in organs that were not directly exposed. Radiation exposure of dry seeds is technologically easier than the

exposure of seedlings, plants or their organs. The response to both types of the exposure is specific by variety and a simple principle of variety selection to stimulate secondary metabolism has been proposed.

Second, the study indicates the possibility to increase the medicines yield due to the simultaneous increasing yield of the medicinal raw material and the specific content of the target metabolite. This forms the basis to use the systemic effects of the ionizing and non-ionizing radiation exposure in the pharmacology.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Author 1: Investigation, Methodology, Visualization, Software, Formal Analysis. **Author 2**: Investigation, Methodology, Visualization, Software, Formal Analysis and Writing original draft. **Author 3**: Investigation, Methodology, Visualization, Supervision, Validation and Writing original draft. **Author 4**: Methodology. **Author 5**: Resources. **Author 6**: Supervision.

Orcid

Vladyslav Zhuk https://orcid.org/0000-0003-1966-7537
Daryna Sokolova https://orcid.org/0000-0002-4540-0177
Alexandra Kravets https://orcid.org/0000-0002-4979-5022
Volodymyr Sakada https://orcid.org/0000-0002-9142-3660
Ludmila Glushenko https://orcid.org/0000-0003-2329-5537
Mykola Kuchuk https://orcid.org/0000-0001-7365-7474

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Research Article

Synergistic interaction between propolis extract, essential oils, and antibiotics against *Staphylococcus epidermidis* and methicillin resistant *Staphylococcus aureus*

Omar Belmehdi[1], Abdelhakim Bouyahya[2], József Jekő[3], Zoltán Cziáky[3], Gokhan Zengin[4,*], Gyula Sotkó[5], Aicha El baaboua[1], Nadia Skali Senhaji[1], Jamal Abrini[1]

[1]Biotechnology and Applied Microbiology Team, Department of Biology, Faculty of Sciences, Abdelmalek Essaadi university, Tetouan, Morocco
[2]Laboratory of Human Pathologies Biology, Department of Biology, Faculty of Sciences, and Genomic Center of Human Pathologies, Faculty of Medicine and Pharmacy, Mohammed V University of Rabat, Morocco
[3]Agricultural and Molecular Research and Service Institute, University of Nyíregyháza, Nyíregyháza, Hungary
[4]Physiology and Biochemistry Research Laboratory, Department of Biology, Science Faculty, Selcuk University, Campus, Konya, Turkey
[5]Sotiva Seed Ltd., H-4440 Tiszavasvári, Petőfi str. 63/a, Hungary

Abstract: The development of multi-drug-resistant bacteria pushed the scientific community to look for new alternatives to solve the problem. Propolis is a beehive substance and one of the richest natural products in bioactive compounds with antibacterial activity. This study was aimed to investigate the possible synergistic interaction between propolis and antibacterial drugs, such as essential oils (EOs) and antibiotics, in order to find increased activity with decreased concentrations. Two ethanol extracts of propolis were used for the test, which were collected from the north of Morocco. The chemical composition was determined by UHPLC-MS. The synergistic effect of propolis extracts with EOs and antibiotics was tested using the checkerboard technique. The chemical analysis showed the presence of more that 100 compounds in propolis extracts, belonging mainly to flavonoids. The combination of propolis with the other antibacterial drugs showed different types of interactions with FIC index values varied from 0.18 to 1, but no antagonist effect was noticed. With FICI<0.5, the synergistic effect was obtained with essential oils as well as with antibiotics. These results indicate that propolis can be a promising source of molecules with medical interest to treat bacterial infection and/or to increase the action of antibiotics.

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Antibiotics,
Checkboard technique.

1. INTRODUCTION

Multi-drug-resistant bacteria became one the major problems of public health (Ventola, 2015). This fact is increasing, and the situation continues to be more complicated. The overuse and misuse of antibiotics, such as the use of broad-spectrum antibiotics without disease diagnostic, are the main reasons of this antibiotic resistance. To overcome the problem, researchers have been trying to find alternatives to compensate the less active antibiotics and/or to increase their

*CONTACT: Dr. Gokhan Zengin ✉ gokhanzengin@selcuk.edu.tr Physiology and Biochemistry Research Laboratory, Department of Biology, Science Faculty, Selcuk University, Campus, Konya, Turkey

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efficacy. The combination antibiotic therapy has been known to reduce the evolution of drug resistance (Bantar *et al*., 2004). Indeed, nature is considered an inexhaustible source of bioactive compounds with significant antibacterial action. However, the search for specific molecules from the nature is challenging because of the complexity of the natural products.

Propolis is one of the richest natural products in bioactive compounds. It is a resinous substance collected by honeybees from plants exudates (Ghisalberti, 1979). It is constituted by a mixture of plant secondary metabolites and bee secretions. A highly complex hive products, propolis has been used in traditional medicine for a long time to treat several health problems. The traditional use of propolis has been proven by scientific studies (Kuropatnicki et al., 2013). Thus, it was reported as potent antimicrobial, antiviral, antiparasitic, anti-diabetic, anti-inflammatory, anti-leishmanial, immunomodulatory, and anticancer agent (Krol *et al*., 1993; Orsatti *et al*., 2010; Rivero-Cruz *et al*., 2020; Kwon *et al*., 2020). Recently, the interest in propolis highly increased around the world, especially with the development of sophisticated techniques of separation and identification. In fact, the instable chemical composition of propolis, which varies according to the geographical origin, is what makes it a target for several researchers. This variability led recently to the discovery of several new compounds and made propolis inexhaustible source of new bioactive compounds (Huang *et al*., 2014; Šturm and Ulrih, 2019). These compounds belong to several chemical classes such as phenolics, flavonoids, terpenoids, alkaloids, etc.

Propolis have been highly studied for its antibacterial activity, but few studies have been reported about its combined effect with other antibacterial drugs (Krol *et al*., 1993). Thus, the synergistic interaction between antibacterial drugs is very interesting in the medical field. In fact, the more the effective dose is low the more the product is desired. In this regard, the aim of this study was to investigate the chemical composition and to evaluate the possible interaction between propolis and other antibacterial drugs such as essential oils and antibiotics.

2. MATERIAL and METHODS

2.1. Propolis Collection and Preparation

Propolis samples were collected from the north of Morocco at two geographically different sites; namely, Beni Arouss and M'diq. The samples were harvested from traditional hives. After collection, propolis was congealed, crushed, and extracted using ethanol as solvent. Ethanol was eliminated using rotary evaporator, which allows to obtain sec extract called ethanol extract of propolis (EEP). The extracts were conserved at low temperature in the dark.

2.2. Chemical Analysis: UHPLC/MS

Chromatographic separation was accomplished with a Dionex Ultimate 3000RS UHPLC instrument, equipped with Thermo Accucore C18 (100 mm x 2.1 mm i. d., 2.6 µm) analytical column for separation of compounds. Water (A) and methanol (B) containing 0.1% formic acid were employed as mobile phases, respectively. The total run time was 70 minutes, the elution profile and all exact analytical conditions have been published (Zengin *et al*., 2018).

2.3. Bacterial Strains

Three bacterial strains were used for the test, namely methicillin resistant *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 12228. These bacteria were stocked in glycerol containing medium under -80°C. Before, use they were transferred to an enrichment medium (Brain Heart broth) in order to optimize their growth. All the tests were carried out using bacterial culture in exponential phase.

2.4. Determination of Minimal Inhibitory Concentration (MIC)

In order to determine the MICs of propolis extracts, essential oils, and antibiotics, the microdilution method was adopted. Briefly, a series of decreased concentrations of each tested agent was prepared in a sterile 92-microplate, in which the tested bacterial strain, in its exponential phase, was added (the final concentration of each bacterium was 10^6 CFU/mL). The microplates were incubated at 37°C for 18h and then 10 µL of resazurin was added to each well. Afterward, the microplates were reincubated at the same temperature during 2h. Finally, the MICs were determined based on the resazurin coloration change. The purple coloration of resazurin changes to pink by the products of bacterial metabolism. In this regard, MIC is the lowest concentration of the antibacterial agents, in which no change of resazurin color is noticed (absence of growth) (Yousif et al., 2020).

2.5. Checkerboard Technique

To determine the interaction between propolis extracts and EOs (*Origanum compactum* and *Origanum elongatum*) and antibiotics (ampicillin, tetracycline, oxytetracycline, chloramphenicol, vancomycin, and neomycin TH) the checkerboard technique was used. This method was carried out in liquid medium using 92-microplate. A panel of EEP concentrations were combined with each antibacterial agent (essential oil and antibiotics). In the microplate the MIC of each agent was determined (EEPs, EOs, antibiotics) as well as the MIC of their combination. From the microplate the fractional inhibitory concentration index (FICI) was calculated applying this formula: Σ FICI = FIC (A) + FIC (B). With: FIC (A) = (MIC of A in combination) / (MIC of A alone), and FIC (B) = (MIC of B in combination) / (MIC of B alone).

The type of interaction was determined based on FICI values: FICI ≤0.5 means that the interaction is synergistic, 0.5< FICI ≤0.75 indicates the presence of a partial synergy, 0.76≤FICI<1 means an additive interaction, 1≤FICI≤4 FICI signifies that there is no interaction (not differential), and FICI>4 indicates an antagonism interaction (Denes & Hidri, 2009).

3. RESULTS and DISCUSSION

3.1. Chemical composition of EEP

The chemical profile of propolis extracts was determined based on their retention time and mass spectra (Figure 1). The results of the chemical analysis of the two extracts are represented in Table 1. More than 100 compounds were identified in the two propolis extracts. These molecules belong to numerous chemical groups such as flavonoids, phenolic acids, organic acids, alkaloids. etc. In fact, flavonoids represent the major part in term of compounds number, which represent more than 75%. There were slight differences between the two regions. This variability can be due to the difference in the vegetable source, bee races, date of collection, and other parameters (Bankova *et al*., 2014).

Alkaloids had not been identified in propolis until the last decade. In this present work, an alkaloid called trigonelline was identified in propolis extracts. This molecule has been known by its interesting biological activities (Zhou *et al*., 2012; Mohamadi *et al.*, 2018) . In addition, the chemical analysis showed also the presence of flavonoid glycosides, rare compounds in propolis with high pharmacological interest.

The chemical components containing in propolis extracts are the secondary metabolites of plants (Salatino *et al*., 2011). Thus, the chemical profile of propolis is highly diversified and depends on the plant species at the site of collection. In this study, two propolis samples were collected from two geographically different sites namely, Beni Arouss and M'diq. These sites exist in the north of Morocco. The north of Morocco is known by its diversified medicinal plants (Bouyahya, 2017), and the popularity of beekeeping. Indeed, propolis of this region could be rich in bioactive compounds, especially those from medicinal plants. In fact, this hypothesis

was proven in this study, since the chemical analysis showed the presence of several components known by their interesting biological activities. Among these important molecules, there are, as example, caffeic acid, ferulic acid, apigenin, kaempferide, quercetin, sakuranetin which are known to possess multiple biological properties such as antibacterial and antioxidant activities (Guz *et al*., 2001; Wu *et al*., 2008; Hirai *et al*., 2010).

3.2. MICs of EEPs, EOs and antibiotics

The minimal inhibitory concentration was determined in liquid medium using the microbroth method. The results are expressed in Table 2. As shown, the MICs of Beni Arouss and M'diq EEPs against *S. aureus* MRSA were 0.62 and 0.32 mg/mL, respectively. The MICs of essential oils of *O. compactum* and *O. elongatum* were 1 and 0.12%, respectively. While, the MICs of antibiotics were low and varied from 0.0025 to 0.02 mg/mL. Concerning *S. epidermidis*, the MICs of the two propolis extracts were 0.62 mg/mL for Beni Arouss and 1.25 mg/mL for M'diq extracts, and those of EOs were 0.5% for both species, and the antibiotic MIC values varied from 0.01 to 0.12 mg/mL. Finally, the MIC values against *S. aureus* 25923 were: 0.31 and 0.15 mg/mL, for Beni Arouss and M'diq extracts, 0.5 and 0.12% for OCEO and OEEO, respectively, and from 0.0025 to 0.04 for antibiotics.

The minimal inhibitory concentration of an antibacterial agent is the lowest concentration that prevent the bacterial growth in its optimal conditions. Therefore, MIC indicates the efficacy of the antibacterial drug. The antibacterial activity of propolis against *Staphylococcus spp.* has been reported previously (Lu *et al*., 2005). This activity was shown to vary from a region to another, and from a season to another depending on the chemical profile of propolis *(*Hegazi *et al.*, 2000; Lu *et al*., 2005). In the present study, EEPs showed a strong antibacterial activity against *S. aureus* and *S. epidermidis*, noticed by low MIC values. This high efficacy of Moroccan propolis extracts could be explained by their high content in flavonoid and phenolic compounds. These latter were reported as the responsible for the antibacterial action (Sforcin *et al.*, 2000). Indeed, the antibacterial activity is complicated and could not be attributed to a single molecule since several synergism effects can take place between minor molecules (Krol *et al*., 1993).

3.3. Interaction Between Propolis, Essential Oils, and Antibiotics

In order to evaluate the combined effect of EEPs, EOs, and antibiotics the checkboard method was used. The results are represented in Table 3. As shown, different types of interactions were recorded such as synergistic, partial synergy, additive, while there was no antagonistic interaction, and in some cases no interaction was noticed.

The synergistic interaction against MRSA was noticed when the propolis of Beni Arouss was mixed with *O. compactum* EO, ampicillin, and vancomycin, with FIC indexes of 0.49, 0.49, and 0.44, respectively. While the M'diq extract showed synergistic interaction only with ampicillin against this strain with FIC index equal to 0.35. Against *S. epidermidis*, the extract of Beni Arouss interacted synergistically with *O. compactum* EO and neomycin, with FICI of 0.49 and 0.19, respectively. While the extract of M'diq acted synergistically against this bacterium when it was mixed with *O. compactum*, *O. elongatum*, and oxytetracycline, with FICI of 0.18, 0.49, and 0.31, respectively. There was no synergistic effect between the Beni Arouss extract and the tested products against S. aureus ATCC 25923, and only a partial synergy was recorded with chloramphenicol, neomycin, oxytetracycline, and *O. compactum*. On the other hand, the M'diq extract acted synergistically against this bacterial strain when it was mixed with ampicillin (FICI=0.29).

Propolis and essential oils are chemically complex substances, which contain a variety of bioactive molecules. In fact, a synergistic effect may exist between the components of the same sample. Giving as example propolis extract tested in this study, the chemical analysis showed

the presence of more than 100 compounds belonging to several chemical groups. Many of these molecules are known by their antibacterial activity such as galangin, kaempferide, caffeic acid, and others. In addition, the chemical characterization of the essential oils of *O. compactum* and *O. elongatum*, also showed a high complexity. In this regard, it is difficult to attribute specifically the synergistic effect to specific compounds. However, the recent insights of the mechanisms of action of propolis extracts and essential oils on bacteria could explain the synergistic effect of these natural products. In fact, by their amphipathic criteria, essential oils are known to affect the bacterial cell. (Ultee *et al*., 2002) reported that p-cymene, one of the main compounds of the studied EOs, caused swelling of cell membrane of *S. aureus*. Thus, the incorporation of p-cymene in lipid bilayer of *S. aureus* membrane could facilitate the transport of propolis compounds through the cytoplasmic membrane, and therefore increase the efficacy of this latter. In addition, other molecules exist in the studied EOs, namely carvacrol and thymol have been known to increase the permeability and depolarize bacterial cell (Lambert *et al*., 2001; Xu *et al*., 2008; Bouhdid *et al*., 2009). In this regard, the interaction of these compounds with other propolis molecules could explain the synergistic effect of propolis and essential oils.

Concerning the interaction between propolis and antibiotics, similar results were reported by (Fernandes Júnior et al., 2005) who showed that propolis interacts synergistically with chloramphenicol, vancomycin, tetracycline. In fact, antibiotics have been known to inhibit protein synthesis. The same authors did not notice any antagonistic effects between propolis and antibiotics, which is in concordance with the present work. The interaction differs as function of the two propolis extracts. This could be explained by the difference in the chemical composition as shown in the first part. The increase of the antibacterial activity of antibiotics could be explained by the fact that some propolis compounds like caffeic acid (CAPE) and quercetin, affect the membrane permeability by causing a disequilibrium at the level of bacterial membrane, which facilitate the entry of antibiotics into the bacterial cell. This could explain the high synergistic effect between EEPs and antibiotics.

Table 1. Chemical composition of ethanol extracts of propolis.

No.	Name	Formula	Rt	$[M + H]^+$	$[M - H]^-$	Fragment 1	Fragment 2	Fragment 3	Fragment 4	Fragment 5	Beni Arouss	M'diq
1	Trigonelline	$C_7H_7NO_2$	1.22	138.05550		110.0604	96.0453	94.0657	92.0501	65.0393	+	+
2	Esculetin (6,7-Dihydroxycoumarin)	$C_9H_6O_4$	14.71	179.03444		151.0391	135.0445	133.0287	123.0443	117.0335	+	+
3[1]	Chlorogenic acid (3-O-Caffeoylquinic acid)	$C_{16}H_{18}O_9$	14.89	355.10291		193.0499	163.0391	145.0285	135.0443	89.0390	+	+
4	Caffeic acid	$C_9H_8O_4$	15.08		179.03444	135.0438	107.0487				+	+
5	Dihydroxy-methoxycoumarin	$C_{10}H_8O_5$	17.13	209.04500		194.0212	181.0499	166.0261	153.0544	149.0235	+	+
6	Fraxetin (7,8-Dihydroxy-6-methoxycoumarin)	$C_{10}H_8O_5$	17.59	209.04500		194.0212	181.0500	163.0391	149.0235	135.0444	+	+
7	Eriodictyol-O-hexoside isomer 1	C21H22O11	18.38		449.10839	287.0563	151.0024	135.0439	125.0231	107.0125	-	+
8[1]	4-Coumaric acid	C9H8O3	18.44		163.03952	119.0487	93.0330				+	+
9	Caffeoylshikimic acid	C16H16O8	18.46		335.07670	179.0340	161.0233	135.0439	111.0434	93.0329	-	+
10[1]	Scopoletin (7-Hydroxy-6-methoxycoumarin)	C10H8O4	19.09	193.05009		178.0263	165.0547	149.0598	137.0599	133.0287	+	+
11	Eriodictyol-O-hexoside isomer 2	$C_{21}H_{22}O_{11}$	19.24		449.10839	287.0562	151.0023	135.0438	125.0230	107.0123	-	+
12	Luteolin-di-O-glucuronide	$C_{27}H_{26}O_{18}$	19.68		637.10409	461.0719	285.0405	151.0019			-	+
13[1]	Taxifolin (Dihydroquercetin)	$C_{15}H_{12}O_7$	19.83		303.05048	285.0406	217.0498	175.0389	153.0185	125.0229	+	+
14[1]	Ferulic acid	$C_{10}H_{10}O_4$	19.85		193.05009	178.0263	149.0595	137.0229	134.0360		-	+
15	Eriodictyol-O-hexoside isomer 3	$C_{21}H_{22}O_{11}$	20.74		449.10839	287.0565	151.0024	135.0438	125.0226	107.0121	-	+
16	Isoferulic acid	$C_{10}H_{10}O_4$	20.98		193.05009	178.0260	149.0595	137.0231	134.0360		-	+
17	Tetrahydroxyflavanone-O-rhamnosylhexoside	$C_{27}H_{32}O_{15}$	21.06		595.16630	459.1136	287.0562	175.0025	151.0024	135.0438	+	+
18	Myricetin-3'-O-glucoside (Cannabiscitrin)	$C_{21}H_{20}O_{13}$	21.33		479.08257	317.0301	316.0224	287.0195	271.0250	178.9975	+	+

19	Scoparone (6,7-Dimethoxycoumarin)	$C_{11}H_{10}O_4$	21.69	207.06574		192.0420	191.0343	179.0707	163.0393	151.0756	+	+
20	Verbascoside or isomer	$C_{29}H_{36}O_{15}$	22.32		623.19760	461.1658	315.1087	179.0339	161.0232	133.0282	+	+
21	Dihydrokaempferol (3,4',5,7-Tetrahydroxyflavanone)	$C_{15}H_{12}O_6$	22.42		287.05556	269.0457	259.0610	243.0660	177.0545	125.0229	+	+
22	Padmatin (7-Methoxy-3,3',4',5-tetrahydroxyflavanone) or isomer	$C_{16}H_{14}O_7$	22.60	319.08178		301.0710	286.0482	273.0758	153.0183	137.0599	+	+
23	Luteolin-7-O-glucuronide	$C_{21}H_{18}O_{12}$	22.74		461.07201	285.0405	217.0496	199.0389	175.0390	133.0280		+
24	Luteolin-7-O-glucoside (Cynaroside)	$C_{21}H_{20}O_{11}$	22.81		447.09274	327.0513	285.0406	284.0329	256.0386	151.0025	-	+
25	Luteolin-O-rhamnosylhexoside	$C_{27}H_{30}O_{15}$	22.85		593.15065	327.0515	285.0404	284.0327	133.0284	107.0124	+	+
26	Isorhamnetin-O-rhamnosylhexoside	$C_{28}H_{32}O_{16}$	23.02		623.16121	315.0513	314.0436	300.0276	299.0201	271.0249	+	+
27	Methoxy-tetrahydroxy(iso)flavone-O-glucuronide	$C_{22}H_{20}O_{13}$	23.02		491.08257	315.0512	300.0277	272.0322	113.0227		-	+
28	Hyperoside (Quercetin-3-O-galactoside)	$C_{21}H_{20}O_{12}$	23.18		463.08765	301.0354	300.0277	271.0248	255.0296	178.9978	+	+
29	Trimethoxycoumarin	$C_{12}H_{12}O_5$	23.39	237.07630		222.0524	207.0290	193.0499	191.0341	176.0469	+	+
30[1]	Isoquercitrin (Quercetin-3-O-glucoside)	$C_{21}H_{20}O_{12}$	23.41		463.08765	301.0355	300.0276	271.0247	255.0296	178.9976	+	+
31	Eriodictyol-O-hexoside isomer 4	$C_{21}H_{22}O_{11}$	23.64		449.10839	287.0562	151.0024	135.0439	125.0228	107.0123	-	+
32	Vanillin acetate	$C_{10}H_{10}O_4$	23.71	195.06574		153.0548	125.0601	111.0444	93.0342	65.0393	+	_
33	Padmatin (7-Methoxy-3,3',4',5-tetrahydroxyflavanone) or isomer	$C_{16}H_{14}O_7$	23.76	319.08178		301.0708	286.0470	273.0758	153.0184	137.0599	+	+
34	Reinutrin (Quercetin-3-O-xyloside)	$C_{20}H_{18}O_{11}$	23.74		433.07709	301.0359	300.0278	271.0249	255.0299	178.9981	+	-
35	Avicularin (Quercetin-3-O-arabinofuranoside)	$C_{20}H_{18}O_{11}$	24.01		433.07709	301.0356	300.0277	271.0250	255.0295	178.9974	+	+

36	Apigenin-O-rhamnosylhexoside	$C_{27}H_{30}O_{14}$	24.36		577.15574	269.0455	268.0376	117.0327			-	+
37	Methoxy-trihydroxy(iso)flavone-O-rhamnosylhexoside	$C_{28}H_{32}O_{15}$	24.54	609.18195		463.1240	301.0709	286.0475	129.0550	85.0290	-	+
38[1]	Myricetin (3,3',4',5,5',7-Hexahydroxyflavone)	$C_{15}H_{10}O_8$	24.68		317.02974	271.0238	178.9975	165.0179	151.0024	137.0231	+	+
39	Guaijaverin (Quercetin-3-O-arabinoside)	$C_{20}H_{18}O_{11}$	24.74		433.07709	301.0354	300.0277	271.0249	255.0304	178.9976	+	+
40	Dimethoxy-trihydroxy(iso)flavone-O-glucuronide	$C_{23}H_{22}O_{13}$	24.75		505.09822	329.0666	314.0435	299.0199	271.0250	113.0230	+	+
41	Chrysoeriol-7-O-glucuronide	$C_{22}H_{20}O_{12}$	24.76		475.08766	299.0562	284.0328	256.0385			-	+
42[1]	Quercitrin (Quercetin-3-O-rhamnoside)	$C_{21}H_{20}O_{11}$	24.95		447.09274	301.0355	300.0277	271.0249	255.0298	178.9976	+	+
43	Isorhamnetin-O-hexoside isomer 1	$C_{22}H_{22}O_{12}$	25.22		477.10330	315.0515	314.0435	285.0406	271.0248	243.0295	+	–
44[1]	Eriodictyol (3′,4′,5,7-Tetrahydroxyflavanone)	$C_{15}H_{12}O_6$	25.39		287.05556	269.0469	151.0024	135.0439	125.0230	107.0124	+	+
45	Isorhamnetin-O-hexoside isomer 2	$C_{22}H_{22}O_{12}$	25.40		477.10330	315.0513	314.0434	285.0406	271.0248	243.0295	+	–
46	Methoxy-tetrahydroxy(iso)flavone-O-hexoside	$C_{22}H_{22}O_{12}$	25.69		477.10330	315.0510	314.0435	299.0198	271.0246	243.0298	+	–
47	N-trans-Feruloyltyramine	$C_{18}H_{19}NO_4$	25.52	314.13924		194.0811	177.0548	149.0599	135.0443	121.0651	-	+
48	Cedeodarin (6-Methyl-3,3',4',5,7-pentahydroxyflavanone) or isomer	$C_{16}H_{14}O_7$	26.72	319.08178		301.0707	273.0760	245.0811	167.0341	163.0391	+	+
49	N1,N5,N10-Tricoumaroylspermidine cis/trans isomer 1	$C_{34}H_{37}N_3O_6$	26.74		582.26042	462.2028	342.1457	316.1657	145.0283	119.0487	+	+
44	Acetyltaxifolin	$C_{17}H_{14}O_8$	27.07		345.06105	327.0508	303.0510	285.0406	151.0024	125.0229	-	+

50	Quercetin-O-coumaroylhexoside	$C_{30}H_{26}O_{14}$	27.30		609.12444	463.0886	301.0354	300.0276	271.0249	255.0295	+	–
51[1]	Quercetin (3,3',4',5,7-Pentahydroxyflavone)	$C_{15}H_{10}O_7$	27.49		301.03483	273.0404	178.9975	151.0024	121.0280	107.0124	+	+
52	N1,N5,N10-Tricoumaroylspermidine cis/trans isomer 2	$C_{34}H_{37}N_3O_6$	27.74		582.26042	462.2033	342.1458	316.1663	145.0283	119.0487	+	+
53[1]	Luteolin (3',4',5,7-Tetrahydroxyflavone)	$C_{15}H_{10}O_6$	28.36		285.03991	217.0501	175.0388	151.0024	133.0281	107.0124	+	+
54	N1,N5,N10-Tricoumaroylspermidine cis/trans isomer 3	$C_{34}H_{37}N_3O_6$	28.60		582.26042	462.2031	342.1450	316.1657	145.0284	119.0487	+	+
55	Quercetin-3-O-methyl ether	$C_{16}H_{12}O_7$	28.74		315.05048	300.0276	271.0249	255.0296	243.0296	227.0346	+	+
56	Kaempferol-O-coumaroylhexoside	$C_{30}H_{26}O_{13}$	28.78		593.12952	447.0938	285.0405	284.0327	255.0295	119.0489	–	+
57	Kaempferol-O-coumaroylhexoside isomer 1	$C_{30}H_{26}O_{13}$	28.79		593.12952	447.0936	285.0405	284.0327	255.0296	119.0485	+	-
58	O-Acetylpadmatin or isomer	$C_{18}H_{16}O_8$	28.91		359.07670	341.1380	317.0663	299.0560	289.0724	284.0327	-	+
59	Dimethoxy-tetrahydroxy(iso)flavone	$C_{17}H_{14}O_8$	29.00		345.06105	330.0380	315.0147	287.0199	271.0247	259.0246	+	+
60	Kaempferol-O-coumaroylhexoside isomer 2	$C_{30}H_{26}O_{13}$	29.19		593.12952	447.0934	285.0405	284.0328	255.0296	119.0489	+	-
61	N1,N5,N10-Tricoumaroylspermidine cis/trans isomer 4	$C_{34}H_{37}N_3O_6$	29.45		582.26042	462.2036	342.1460	316.1659	145.0282	119.0488	+	+
62[1]	Kaempferol (3,4',5,7-Tetrahydroxyflavone)	$C_{15}H_{10}O_6$	29.84		285.03991	257.0453	229.0495	169.0648	151.0022	107.0123	+	+
63[1]	Apigenin (4',5,7-Trihydroxyflavone)	$C_{15}H_{10}O_5$	30.20		269.04500	227.0340	225.0550	151.0024	149.0232	117.0330	+	+
64[1]	Isorhamnetin (3'-Methoxy-3,4',5,7-tetrahydroxyflavone)	$C16H_{12}O_7$	30.33		315.05048	300.0276	283.0254	271.0246	164.0102	151.0023	+	+
65	Chrysoeriol (3'-Methoxy-4',5,7-trihydroxyflavone)	$C_{16}H_{12}O_6$	30.48		299.05556	284.0327	256.0373	227.0351	151.0020	107.0128	+	+

66	Isokaempferide (3-Methoxy-4',5,7-trihydroxyflavone)	$C_{16}H_{12}O_6$	30.87	301.07122		286.0474	285.0399	258.0524	212.0466	121.0283	+	+
67	Dimethoxy-trihydroxy(iso)flavone isomer 1	$C_{17}H_{14}O_7$	31.06		329.06613	314.0433	299.0197	285.0406	271.0248	243.0294	+	+
68	Hydroxy-methoxy(iso)flavone	$C_{16}H_{12}O_4$	31.11	269.08138		254.0574	226.0626	167.0337			+	+
69	Trihydroxy-trimethoxy(iso)flavone isomer 1	$C_{18}H_{16}O_8$	31.68		359.07670	344.0537	329.0302	314.0071	301.0355	286.0120	+	+
70	Rhamnetin (7-Methoxy-3,3',4',5-tetrahydroxyflavone)	$C_{16}H_{12}O_7$	32.31		315.05048	300.0277	193.0133	165.0181	121.0280	97.0280	+	+
71	Pinocembrin (5,7-Dihydroxyflavanone)	$C_{15}H_{12}O_4$	32.69		255.06573	227.0706	213.0551	151.0024	107.0123	83.0122	+	+
72	Dimethoxy-trihydroxy(iso)flavone isomer 2	$C_{17}H_{14}O_7$	32.71		329.06613	314.0434	313.0355	299.0197	285.0405	271.0248	+	+
73	Isosakuranetin (5,7-Dihydroxy-4'-methoxyflavanone)	$C_{16}H_{14}O_5$	32.72		285.07630	270.0535	243.0660	164.0103	151.0024	136.0153	+	–
74	Acetyltrihydroxy(iso)flavanone	$C_{17}H_{14}O_6$	33.07		313.07122	271.0611	253.0503	225.0553	151.0024		–	+
75	Trihydroxy-trimethoxy(iso)flavone isomer 2	$C_{18}H_{16}O_8$	33.09		359.07670	344.0537	329.0303	314.0066	301.0355	286.0124	+	+
76	Dihydroxy-trimethoxy(iso)flavone isomer 1	$C_{18}H_{16}O_7$	33.11	345.09743		330.0734	329.0655	315.0501	299.0552	287.0552	+	+
77	Dimethoxy-trihydroxy(iso)flavone isomer 3	$C_{17}H_{14}O_7$	33.26		329.06613	314.0433	299.0197	285.0415	271.0248	243.0300	+	+
78	Dihydroxy-methoxy(iso)flavone isomer 1	$C_{16}H_{12}O_5$	33.37	285.07630		270.0524	269.0445	257.0813	242.0575	229.0859	–	+
79[1]	Chrysin (5,7-Dihydroxyflavone)	$C_{15}H_{10}O_4$	33.77	255.06573		209.0593	153.0183	129.0339	103.0546	67.0185	+	+
80	Caffeic acid phenethyl ester	$C_{17}H_{16}O_4$	34.07		283.09703	179.0339	178.0254	161.0231	135.0438	133.0281	+	+
81	Acacetin (5,7-Dihydroxy-4'-methoxyflavone)	$C_{16}H_{12}O_5$	34.39	285.07630		270.0523	242.0573	153.0181	133.0652		+	+

82	Trihydroxy(iso)flavone	$C_{15}H_{10}O_5$	34.65	271.06065		253.0504	215.0704	197.0597	165.0187	153.0185	_	+
83	Dihydroxy-methoxy(iso)flavone isomer 2	$C_{16}H_{12}O_5$	35.00	285.07630		270.0525	269.0445	242.0573	167.0340		+	+
84	Dihydroxy-trimethoxy(iso)flavone isomer 2	$C_{18}H_{16}O_7$	35.13	345.09743		330.0734	329.0669	315.0498	301.0705	287.0549	+	+
85	Dihydroxy-dimethoxy(iso)flavone	$C_{17}H_{14}O_6$	35.43		313.07122	298.0483	283.0249	269.0450	255.0297	227.0338	+	+
86	Dihydroxy-tetramethoxy(iso)flavone	$C_{19}H_{18}O_8$	35.45		373.09235	358.0694	343.0458	328.0219	315.0516	313.0355	+	+
87	Dihydroxy-trimethoxy(iso)flavone isomer 3	$C_{18}H_{16}O_7$	35.50	345.09743		330.0735	329.0657	315.0499	301.0712	287.0549	+	+
88	Isoimperatorin	$C_{16}H_{14}O_4$	36.20	271.09704		203.0341	175.0390	159.0442	147.0442	131.0495	+	+
89	Hydroxy-tetramethoxy(iso)flavone	$C_{19}H_{18}O_7$	37.02	359.11308		344.0893	343.0815	329.0659	315.0863	301.0709	+	+
90	Pinostrobin (5-Hydroxy-7-methoxyflavanone)	$C_{16}H_{14}O_4$	37.10	271.09704		229.0859	173.0598	167.0341	131.0495	103.0548	–	+
91	Unidentified compound 1	$C_{20}H_{30}O_3$	37.54		317.21167	299.1992	273.1853	247.1693	189.0912	173.0596	+	+
92	Tectochrysin (5-Hydroxy-7-methoxyflavone)	$C_{16}H_{12}O_4$	38.02	269.08138		254.0574	226.0626	167.0340			+	+
93	Unidentified compound 2	$C_{20}H_{34}O_3$	38.53		321.24297	303.2330					+	_
94	Hydroxy-trimethoxy(iso)flavone	$C_{18}H_{16}O_6$	39.26		329.10252	314.0786	313.0709	299.0552	285.0763	271.0600	+	-
95	Unidentified carboxylic acid	$C_{20}H_{32}O_3$	39.80		319.22732	275.2383	259.2067				+	-
96	Apigenin-4',7-dimethyl ether (4',7-Dimethoxy-5-hydroxyflavone)	$C_{17}H_{14}O_5$	38.67	299.09195		284.0679	256.0730	167.0341	133.0650		+	+
97	Unidentified compound 2	$C_{20}H_{32}O_3$	38.84		319.22732						_	+
98	Hexadecanedioic acid	$C_{16}H_{30}O4$	40.72		285.20659	267.1964	241.2167	223.2062			+	+
99	Unidentified caffeic acid derivative	$C_{29}H_{36}O_6$	41.65		479.24336	317.2112	299.2015	179.0339	135.0438		+	+

100	Unidentified compound 3	$C_{22}H_{36}O_4$	42.12		363.25353	321.2447	303.2329	59.0122			+	+
101	Unidentified compound 4	$C_{20}H_{36}O_3$	42.64		323.25862	305.2492	279.2694	263.2379	247.2067		+	-
102	Linoleamide	$C_{18}H_{33}NO$	44.40	280.26404		263.2371	245.2264	109.1016	95.0861	81.0705	+	+
103	Palmitic amide (Hexadecanamide)	$C_{16}H_{33}NO$	45.38	256.26404		144.1388	130.1224	116.1072	102.0918	88.0763	+	+
104	Oleamide	$C_{18}H_{35}NO$	45.68	282.27969		265.2526	247.2422	135.1171	83.0861	69.0706	+	+
105	Ginkgoic acid or isomer	$C_{22}H_{34}O_3$	47.64		345.24298	301.2536	175.1117	133.0645	119.0486	106.0410	+	+

[1] *Confirmed by standard*
- Absent
+ present

Table 2. MIC values of propolis extracts, EOs and antibiotics.

	EEPBA	EEPM	OCEO	OEEO	VA	OT	TE	C	AM	N
S. aureus ATCC 43000 MRSA	0.62	0.31	1%	0.12%	0.005	0.005	0.0025	0.01	0.02	ND
S. epidermis ATCC 12228	0.62	1.25	0.5%	0.5%	ND	0.04	0.01	0.12	ND	0.08
S. aureus ATCC 25923	0.31	0.15	0.5%	0.12%	0.0025	0.01	0.005	0.01	0.012	0.04

MIC values of essential oils are expressed in % (v/v)
MIC values of EEPs and antibiotics are expressed in mg/mL
EEPBA: Ethanol extract of propolis of Beni Arouss
EEPM: Ethanol extract of propolis of M'diq
OEEO: *Origanum elongatum* essential oil
OCEO*: Origanum compactum* essential oil

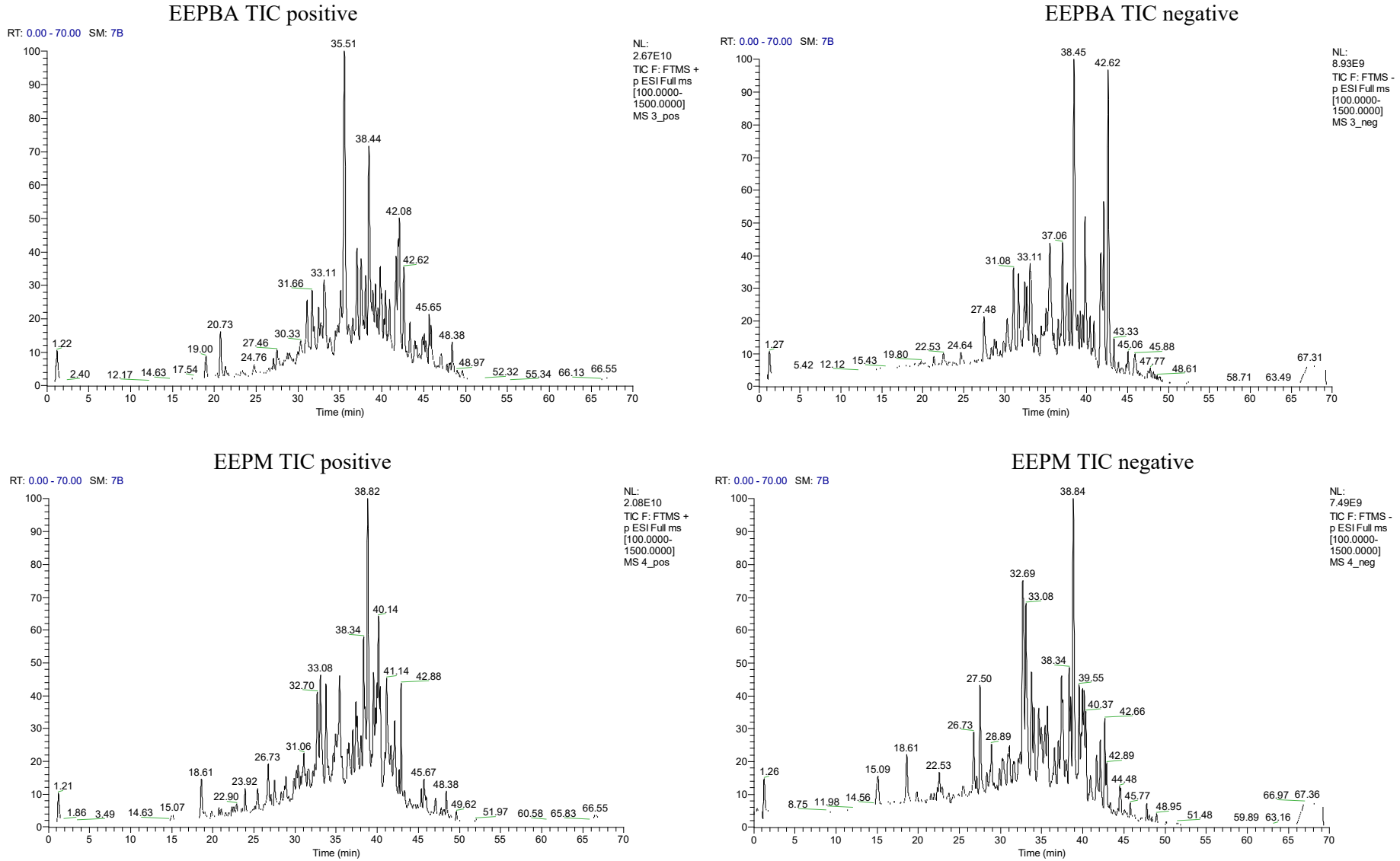
Table 3. Combined effect of EEPs, EOs, and antibiotics.

Strain	Combination	MIC of EOs and antibiotics		MIC of EEPs		FICi	Interpretation
		Alone	in combination	Alone	in combination		
S. aureus ATCC 43000 MRSA	EEPBA + *OCEO*	1	0.25	0.62	0.15	0.49	**Synergy**
	EEPBA + *OEEO*	0.12	0.06	0.62	0.15	0.74	Partial synergy
	EEPBA + C	0.01	0.005	0.62	0.312	1.00	No interaction
	EEPBA + TE	0.0025	0.0012	0.62	0.15	0.72	Partial synergy
	EEPBA + OT	0.005	0.0025	0.62	0.04	0.56	Partial synergy
	EEPBA + AM	0.02	0.005	0.62	0.15	0.49	**Synergy**
	EEPBA + VA	0.005	0.001	0.62	0.15	0.44	**Synergy**
	EEPM + OC	1	0.5	0.31	0.15	0.98	Additive
	EEPM + *OE*	0.12	0.06	0.31	0.15	0.98	Additive
	EEPM + C	0.01	0.005	0.31	0.15	0.98	Additive
	EEPM + TE	0.0025	0.0012	0.31	0.07	0.71	Partial synergy
	EEPM + OT	0.005	0.0025	0.31	0.07	0.73	Partial synergy
	EEPM + AM	0.02	0.0025	0.31	0.07	0.35	**Synergy**
S. epidermis ATCC 12228	EEPBA + *OCEO*	0.5	0.125	0.62	0.15	0.49	**Synergy**
	EEPBA + *OEEO*	0.5	0.25	0.62	0.04	0.56	Partial synergy
	EEPBA + C	0.12	0.06	0.62	0.31	1.00	No interaction
	EEPBA + TE	0.01	0.005	0.62	0.31	1.00	No interaction
	EEPBA + OT	0.04	0.02	0.62	0.15	0.74	Partial synergy
	EEPBA + N	0.08	0.01	0.62	0.04	0.19	**Synergy**
	EEPM + *OCEO*	0.5	0.06	1.25	0.075	0.18	**Synergy**
	EEPM + *OE*	0.5	0.12	1.25	0.31	0.49	**Synergy**
	EEPM + TE	0.01	0.005	1.25	0.62	1.00	Additive
	EEPM + OT	0.04	0.01	1.25	0.07	0.31	**Synergy**
	EEPM + N	0.08	0.0025	1.25	0.625	0.53	Partial synergy

S. aureus ATCC 25923	EEPBA + OCEO	0.5	0.12	0.31	0.15	0.72	Partial synergy
	EEPBA + OEEO	0.125	0.06	0.31	0.15	0.96	Additive
	EEPBA + C	0.01	0.005	0.31	0.07	0.73	Partial synergy
	EEPBA + TE	0.005	0.0025	0.31	0.15	0.98	Additive
	EEPBA + OT	0.01	0.005	0.31	0.04	0.63	Partial synergy
	EEPBA + N	0.04	0.02	0.31	0.07	0.73	Partial synergy
	EEPBA + AM	0.012	0.006	0.31	0.15	0.98	Additive
	EEPBA + VA	0.0025	0.00125	0.31	0.15	0.98	Additive
	EEPM + OCEO	0.5	0.25	0.15	0.02	0.63	Partial synergy
	EEPM + OEEO	0.12	0.06	0.15	0.07	0.97	Additive
	EEPM + C	0.01	0.005	0.15	0.07	0.97	Additive
	EEPM + TE	0.005	0.0025	0.15	0.02	0.63	Partial synergy
	EEPM +OT	0.01	0.0024	0.15	0.07	0.71	Partial synergy
	EEPM + N	0.04	0.02	0.15	0.04	0.77	Additive
	EEPM + AM	0.012	0.0003	0.15	0.04	0.29	**Synergy**
	EEPM + VA	0.0025	0.00125	0.15	0.04	0.77	Additive

EEPBA: Ethanol extract of propolis of Beni Arouss; EEPM: Ethanol extract of propolis of M'diq; AM: Ampicillin; C: Chloramphenicol; VA: Vancomycin; N: Neomycin; TE; Tetracycline; OT; Oxytetracycline; OEEO: Origanum elongatum essential oil; OCEO: Origanum compactum essential oil. FICI ≤0.5 = synergistic interaction, 0.5<FICI ≤0.75 = Partial synergy, 0.76 ≤FICI<1 = additive interaction, FICI between 1 and 4 = no interaction (not differential), FICI > 4 = antagonism (Denes & Hidri, 2009)

Figure 1. Total ion chromatograms of EEPs. *EEPBA: Ethanol extract of propolis of Beni Arouss EEPM: Ethanol extract of propolis of M'diq*.



4. CONCLUSION

Combination antibiotic therapy is an effective way to reduce the emergence of bacterial resistance and to fight infections. Thus, natural products are known by their diversified bioactive components with antibacterial action. In this study, the combination of propolis extracts with essential oils and antibiotics was investigated. The results showed some synergistic interaction between these antibacterial products against methicillin resistant *S. aureus* and *S. epidermidis*. The chemical analysis showed the presence of several compounds known by their antibacterial activity in the tested propolis extracts. In this regard, the synergistic effect could be the result of the interaction of major or minor molecules contained in propolis with antibiotics and essential oils compounds. It can be concluded from this study that propolis extract is a promising source of bioactive antibacterial compounds that can be used in combination therapy against infectious diseases.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Omar Belmehdi: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Abdelhakim Bouyahya:** Investigation, Resources, and Writing-original draft. **József Jekő:** Methodology, Supervision. **Zoltán Cziáky:** Methodology, Supervision**. Gokhan Zengin:** Methodology, Supervision. **Gyula Sotkó:** Methodology, Supervision**. Aicha El baaboua:** Investigation, Resources, Visualization, Software, Formal Analysis. **Nadia Skali Senhaji:** Investigation, Resources, Visualization, Software, Formal Analysis**. Jamal Abrini:** nvestigation, Resources, Visualization, Software, Formal Analysis

Orcid

Omar Belmehdi https://orcid.org/0000-0002-7356-1648
Abdelhakim Bouyahya https://orcid.org/0000-0001-9317-1631
József Jekő https://orcid.org/ 0000-0001-5594-6870
Zoltán Cziáky https://orcid.org/ 0000-0002-1846-3236
Gokhan Zengin https://orcid.org/ 0000-0001-6548-7823
Gyula Sotkó https://orcid.org/ 0000-0001-9894-7216
Aicha El baaboua https://orcid.org/ 0000-0002-3048-5390
Nadia Skali Senhaji https://orcid.org/ 0000-0002-7356-1648
Jamal Abrini https://orcid.org/ 0000-0002-5103-9365

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Research Article

Chemical compositions and antioxidant activities of four different mushroom species collected from Turkey

Arzuhan Sihoglu Tepe [1,*]

[1]Kilis 7 Aralik University, Vocational High School of Heath Services, Department of Pharmacy Services, Kilis, Turkey

Abstract: Many researchers agree that edible mushrooms have beneficial effects on human health. The aim of this study is to investigate the chemical composition and antioxidant activities of methanol extracts obtained from *Coprinus comatus*, *Hydnum repandum*, *Agaricus impudicus* ve *Sarcodon imbricatus* collected from Yuvacık-İzmit, Kastamonu, and Uzungöl-Trabzon. Spectrophotometric analysis showed that the total phenolic (64.69 mg GAEs/g) and flavonoid (1.73 mg QEs/g) content of *S. imbricatus* was higher than that of the others. In line with its superiority in phytochemical composition analyzes, *S. imbricatus* extract showed the highest activity in β-carotene bleaching, DPPH free radical scavenging and reducing power assays (836.0, 89.0 and 267.0 mg TEs/g extract, respectively). Metal chelating test resulted in the superiority of *A. impudicus* (1282.0 mg TEs/g). Relative antioxidant capacity index (RACI) value of *S. imbricatus* was quite high compared to other extracts (0.90). Apart from the metal chelating assay, there was a high correlation between the antioxidant activities of the extracts and their RACI values. Pearson correlation analysis showed that the correlation between the phenolic/flavonoid contents of the extracts and their antioxidant activities was over 0.9. The antioxidant activity of *A. impudicus* was brought to the literature for the first time with this study. When the data obtained from the current study were evaluated as a whole, it was concluded that *S. imbricatus* could be a rich source for new and alternative antioxidant compounds.

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1. INTRODUCTION

In recent years, searches for the addition of new supplements to existing food supplements have started to intensify among both researchers and end consumers. In this quest, mushrooms have gained great importance due to their nutritional properties and powerful bioactive chemicals (Mujić *et al*., 2011; Bowe, 2013; Falandysz & Borovička, 2013; Zhang *et al*., 2016; Jayachandran *et al*., 2017). For hundreds of years, mushrooms have been used by humans both to meet their nutritional needs and to treat various diseases (Gaglarirmak, 2011; Gustafson, 2016; Chatterjee *et al*., 2017). Edible mushrooms are considered among the indispensable elements of diets due to their rich nutritional contents, as well as being delicious enough to be consumed by many people (Csóka *et al*., 2017; Luo *et al*., 2017; Sharma & Gautam, 2017;

*CONTACT: Arzuhan ŞIHOGLU TEPE ✉ arzuhan.tepe@kilis.edu.tr Kilis 7 Aralik University, Vocational High School of Heath Services, Department of Pharmacy Services, Kilis, Turkey

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Politowicz *et al*., 2018). In recent studies, it has been reported that especially phenolic compounds contribute significantly to the biological activities of mushrooms (Sevindik *et al*., 2020).

During metabolic reactions in organisms, the amount of free radicals increases. Organism uses endogenous defense mechanisms against this increase. However, insufficient endogenous antioxidant enzyme systems cause free radicals to damage biological molecules. It has been reported that free radicals underlie many metabolic, chronic or neurological disorders in organisms (Khansari *et al*., 2009; Salminen *et al*., 2012; Cencioni *et al*., 2013; Nowak, 2013; Polidori & Scholtes, 2016; Tan *et al*., 2018). When the results obtained from both epidemiological and clinical studies are evaluated as a whole, one of the most effective ways to deal with this problem is to support the organism with external antioxidants. Many researchers agree that there is a decrease in the incidence of diseases caused by free radicals in those who are fed diets rich in antioxidants (Cherubini *et al*., 2005; Fletcher, 2010; Sevindik *et al*., 2020).

In many studies, it has been documented that edible mushrooms have many useful effects on human health. It is known that mushrooms are foods rich in antioxidant compounds and thus both help to scavenge free radicals and increase the activities of endogenous antioxidant enzymes (Liu *et al*., 2004; Asatiani *et al*., 2007a, b; Oyetayo, 2009). Researchers suggest that some edible mushrooms contribute to the reduction of DNA damage caused by oxidative stress on various cell lines (Shi *et al*., 2002; Miyaji *et al*., 2004). In addition, some mushroom compounds are known to inspire the development of various bioactive molecules in the pharmacology industry (Orangi *et al*., 2016). In addition to their antioxidant activities, mushrooms also accumulate various minerals depending on the mineral substance concentration in the soil, and therefore are among the important sources for meeting the mineral needs of organisms (Kosanić *et al*., 2016).

The aim of this study is to investigate the chemical composition and *in vitro* antioxidant activities of the methanol extracts of *Coprinus comatus* (O.F. Müll.) Pers., *Hydnum repandum* L., *Agaricus impudicus* (Rea) Pilát and *Sarcodon imbricatus* (L.) P. Karst, collected from Yuvacık-İzmit, Kastamonu, and Uzungöl-Trabzon locations. As a result of the literature search, no study was found on the chemical composition and antioxidant activity of *A. impudicus*. In addition, the data obtained in the β-carotene bleaching test of *H. repandum* and metal chelating tests of *S. imbricatus* were also brought to the literature for the first time with the present study. Therefore, it is thought that the presented data would fill an important gap in the literature.

2. MATERIAL and METHODS

2.1. Mushrooms and The Extraction Process

The fruit bodies of the edible mushrooms analyzed in the present study were collected in July 2019 from the localities detailed in Table 1. Dried fruit bodies (5 g) out of direct sunlight and in an environment with good air flow were extracted with 100 ml of methanol at 25 °C at 150 rpm by stirring for 24 hours. The obtained extract was purified from its residues by passing it through filter paper. The same plant material was extracted with methanol two more times using the above-mentioned method, and the extracts were combined with the first methanol extract. The methanol in the combined extracts was removed under low vacuum with the aid of a rotary evaporator. Extract yields are given in Table 2. Extracts were stored in the dark at +4 °C until tests were performed.

2.2. Determination of Total Phenolics of Extracts

The total amount of phenolic compounds of the extracts was determined by using Folin-Ciocalteu Reagent (FCR) (Sarikurkcu *et al*., 2008). Sample solutions containing 1 mg of extract

were made up to 46 ml with distilled water. 1 ml of FCR was added to this mixture and 3 ml of 2% Na_2CO_3 solution was added 3 minutes later. The mixture was shaken at room temperature for 2 hours. Then the absorbance of the samples at 760 nm was read. The total amounts of phenolic compounds in the extracts were determined using the following equation (1) obtained from the standard gallic acid plot, and the results were given in gallic acid equivalents (mg GAEs/g extract):

$$A = 34.16 \text{ (mg gallic acid)} - 0.04 \ (R^2: 0.999) \quad (1)$$

Table 1. Mushrooms analyzed in the current study.

Mushrooms	Family	Habitat	Substrate	Herbarium no	Locality
C. comatus	Agaricaceae	Roadside	Soil	A&HA. 051	Yuvacık-İzmit
H repandum	Hydnaceae	Beech forest	Soil	A&HA. 090	Yuvacık-İzmit
A impudicus	Agaricaceae	Near fir forest	Soil	Akata 7234	Kastamonu
S. imbricatus	Bankeraceae	Spruce forest	Soil	A & Y. 965	Uzungöl-Trabzon

2.3. Determination of Total Flavonoids Extracts

The total amount of flavonoid compounds in the extracts were determined using the $AlCl_3$ method (Dewanto *et al*., 2002; Sarikurkcu *et al*., 2008). 1 ml of $AlCl_3$ solution prepared in 2% methanol was mixed with the samples containing the same volume of extract. The absorbance was read against the blank after 10 minutes of incubation at 415 nm at room temperature. The blank was prepared to contain 1 ml of extract solution and 1 ml of methanol or water. The total amounts of flavonoid compounds in the extracts were determined using the following equation (2) obtained from the standard quercetin plot, and the results were given in quercetin equivalents (mg QEs/g extract):

$$A = 29.54 \text{ (mg quercetin)} - 0.027 \ (R^2: 0.996) \quad (2)$$

2.4. Determination of Total Antioxidant Activity by β-carotene Bleaching Test

The antioxidant activity was determined by the β-carotene-linoleic acid test system based on the measurement of the inhibition of conjugated diene hydroperoxides and volatile organic compounds resulting from linoleic acid oxidation (Dapkevicius *et al*., 1998; Dewanto *et al*., 2002; Sarikurkcu *et al*., 2008). The β-carotene solution was prepared by dissolving 1 mg of β-carotene in 2 ml of chloroform. 50 µg of linoleic acid and 400 mg of Tween 20 were added to this solution. After chloroform was removed by rotary evaporator, it was mixed with 200 ml of oxygenated distilled water. 2.5 ml of this emulsion was added to 0.5 ml of the extracts. For control, 0.5 ml of methanol was added to the test tube instead of the extract. As soon as the emulsion was added to the test tubes, the initial absorbance was measured at 490 nm using a spectrophotometer. Tubes were incubated at 50 ºC. Incubation was continued until the color of the β-carotene disappeared (120 minutes). The β-carotene bleaching ratio (R) was calculated according to equation (3) and the results were given as trolox equivalent (mg TEs/g extract):

$$R = \text{In } (a/b)/t \quad (3)$$

where, ln: natural logarithm, a = initial absorbance and b = absorbance after 120 min incubation.

Antioxidant activity (AA) was calculated according to equation (4):

$$AA = [(R_{control}-R_{sample})/R_{control}] \times 100 \quad (4)$$

2.5. Determination of Free Radical Scavenging Activities of Extracts

Free radical scavenging activities of the extracts were determined using 1.1-diphenyl-2-picrylhydrazil (DPPH) free radical (Cuendet *et al*., 1997; Dapkevicius *et al*., 1998; Dewanto *et al*., 2002; Sarikurkcu *et al*., 2008). 1 ml of 0.4 mM methanol solution of DPPH was mixed with 1 ml of the extracts and their absorbance was measured at 517 nm after 30 minutes of incubation at room temperature. The absorbance values of the samples were evaluated against the blank (1 ml methanol). Results were given as trolox equivalent (mg TEs/g extract).

2.6. Determination of Reducing Power Potentials of Extracts

The reducing power potential tests of the extracts were carried out according to the method of Oyaizu (1986). 2.5 ml of the extracts were added to the test tubes. 2.5 ml of 0.2 M phosphate buffer (pH: 6.6), and 2.5 ml of 1% potassium ferricyanide were added to each tube and the mixture was incubated at 50 ºC for 20 minutes. Then, 2.5 ml of 10% trichloroacetic acid was added to the reaction mixture and 2.5 ml of sample was taken from the solution. After adding 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ on the sample, absorbance values were determined at 700 nm. As a control, methanol was used instead of the sample. Results were given in trolox equivalents (mg TEs/g extract).

2.7. Determination of Chelating Capacity of Extracts

The chelating capacity of the extracts on Fe^{2+} were determined according to the method specified by Dinis *et al.* (1994). 2 mM 0.05 ml $FeCl_2$ solution was added to the test tubes containing 2 ml of the extract solution. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After mixing, the mixture was incubated for 10 minutes at room temperature and then absorbance was measured at 562 nm. Results were given in trolox equivalents (mg TEs/g extract).

2.8. Statistical Analyzes

All tests were performed in triplicate and results are given as the mean and standard deviation of triplicate experiments. The level of significance between the results was determined by Tukey's test by choosing 95% confidence interval (α=0.05) with the help of ANOVA analysis of variance using SPSS v22 program. Correlation analyzes between tests were performed using the Pearson correlation test. Another statistical analysis, called the relative antioxidant capacity index (RACI), was applied to rank the extracts in terms of their antioxidant activity potentials, taking into account the cumulative effects of phenolics and flavonoids. This test was carried out to ensure that the results obtained from different antioxidant activity tests were comparable with each other (Sun & Tanumihardjo, 2007).

3. RESULTS / FINDINGS

Information on the family, habitat, substrate, herbarium number and localities of the mushroom species evaluated in the current study are given in Table 1. While *C. comatus* was collected from the roadside in Yuvacik-İzmit, other mushroom species were obtained from areas with high vegetation density such as beech, fir or spruce forests. All of the mushroom samples evaluated in the current study are grown in the soil.

Extraction yields obtained as a result of the extraction process are given in Table 2. According to the data in the table, a minimum extraction yield of 26.23% was obtained as a result of the extraction of mushroom species with methanol (*H. repandum*). Although the extract yields of *C. comatus*, *A. impudicus* and *S. imbricatus* were over 30%, it was determined that *C. comatus* had the highest yield with 36.84%.

3.1. Chemical Compositions of Mushroom Extracts

Total phenolic and flavonoid contents of methanol extracts obtained from *C. comatus*, *H. repandum*, *A. impudicus* and *S. imbricatus* are given in Table 2. It was determined that the phenolic contents of the samples were higher than the flavonoid contents. According to the data in the table, the methanol extract richest in phenolic compounds belongs to *S. imbricatus* [64.69 mg GAEs/g extract]. It was followed by *C. comatus*, *A. impudicus* and *H. repandum*, respectively (total phenolic contents were 21.6, 9.82 and 5.29 mg GAEs/g extract, respectively).

Table 2. Extract yields, total flavonoid and phenolic contents of methanol extracts of mushrooms.[1]

Mushrooms	Extract yield (%)	Total phenolics (mg GAEs/g extract)	Total flavonoids (mg QEs/g extract)
C. comatus	36.84	21.6±0.52^{b}	0.15±0.02^{b}
H. repandum	26.23	5.29±0.04^{c}	0.21±0.02^{b}
A. impudicus	33.16	9.82±0.68^{c}	0.004±0.001^{c}
S. imbricatus	35.11	64.69±3.25^{a}	1.73±0.05^{a}

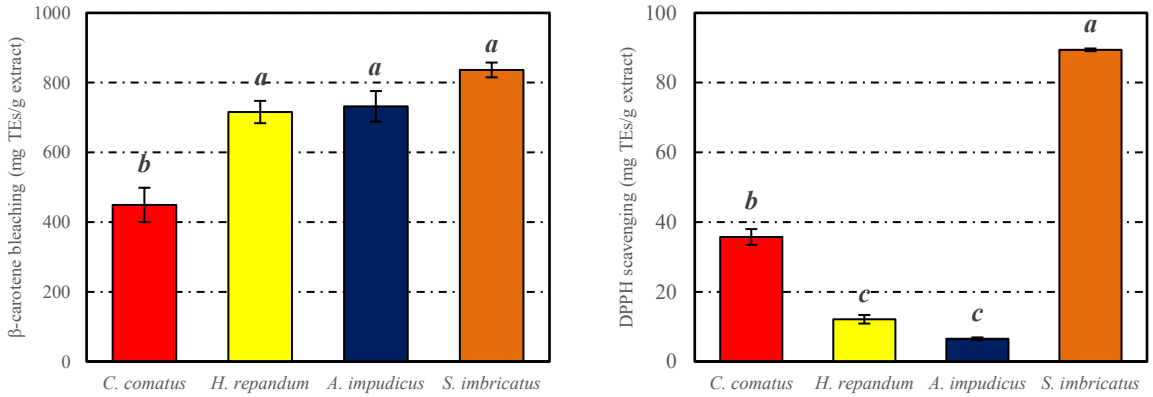
[1]Different superscripts in the same column indicate that the data is statistically significantly different from each other.

As understood from the total flavonoid content data in Table 2, *S. imbricatus* was the richest mushroom extract with 1.73 mg QEs/g. However, it was determined that the total flavonoid content of other mushroom extracts remained below 1.0 mg QEs/g. It was determined that *A. impudicus* was the poorest extract in terms of this compound class, with a flavonoid value of 0.004 mg QEs/g.

3.2. Antioxidant Activities of Mushroom Extracts

Mushroom extracts were subjected to four different test systems called β-carotene bleaching, DPPH free radical scavenging, reducing power and chelating effect in order to evaluate their antioxidant activities in a multi-dimensional manner. The results obtained from the β-carotene bleaching and DPPH free radical scavenging tests are given in Figure 1, while the data on the reducing powers and chelating effects of the extracts are presented in Figure 2.

Figure 1. Total antioxidant and radical scavenging activities of mushrooms.



According to the data in Figure 1, the extract that showed the highest activity in protecting linoleic acid against oxidation stress in the β-carotene bleaching test belonged to *S. imbricatus*. The activity value of this extract obtained from the test system in question was 836.0 mg TEs/g. *S. imbricatus* was followed by *A. impudicus* (732.0 mg TEs/g) and *H. repandum* (716.0 mg TEs/g) extracts, whose activity values were quite close to each other, respectively. *C. comatus*

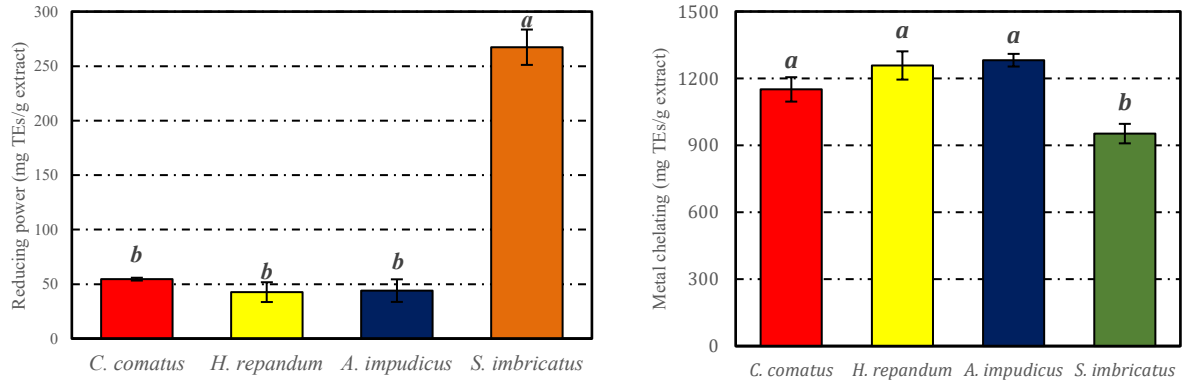
exhibited the lowest activity in this test system (449.0 mg TEs/g). Statistical analyzes showed that there was no significant difference between the activities of *H. repandum* and *A. impudicus*. However, the total antioxidant activities of the other extracts differed statistically significantly.

Data on DPPH radical scavenging activities of mushroom extracts are also presented in Figure 1. As can be seen from the figure in question, the extract exhibiting the most effective scavenging activity on the DPPH free radical was *S. imbricatus* (89.0 mg TEs/g), as in the β-carotene bleaching test. This value is almost three times higher than the free radical scavenging activity of *C. comatus* (36.0 mg TEs/g), the closest follower of the aforementioned mushroom species. In this test system, *H. repandum* and *A. impudicus* showed weak DPPH scavenging activity (12.0 and 7.0 mg TEs/g, respectively). The data obtained from the Tukey's test revealed that the DPPH radical scavenging activities of *H. repandum* and *A. impudicus* were not significantly different from each other, but other mushroom extracts showed statistically different activities.

According to Figure 2, which includes data on the reducing powers of the extracts, *S. imbricatus* again showed the highest activity (267.0 mg TEs/g), as in the β-carotene bleaching and DPPH free radical scavenging tests. However, the reducing powers of *C. comatus*, *H. repandum* and *A. impudicus* extracts were found to be too low and close to each other compared to *S. imbricatus* (55.0, 43.0 and 44.0 mg TEs/g, respectively). While the reducing power of *S. imbricatus* was statistically significantly different from the other three mushroom species, no statistical difference was found between the reducing powers of *C. comatus*, *H. repandum* and *A. impudicus*.

The final test system in which the antioxidant activities of the mushroom extracts evaluated in the present study was metal chelating assay. According to the data in Figure 2, the mushroom extracts showed a different potential than the activity profiles they exhibited in the first three test systems. In this test system, it was determined that the extract with the highest chelating capacity of metal ions belonged to *A. impudicus* (1282.0 mg TEs/g). However, the chelating capacity of *H. repandum* was found to be too close to that of *A. impudicus* (1258.0 mg TEs/g). The chelating activities of these two mushroom species were followed by *C. comatus* (1151.0 mg TEs/g) and *S. imbricatus* (953.0 mg TEs/g), respectively. Statistically, no difference was found between the metal chelating activities of *H. repandum* and *A. impudicus*. Moreover, it was determined that the results obtained from these two species and the metal chelating data of *C. comatus* were statistically partially similar. The metal chelating capacity of *C. comatus* was also found to be partially similar to that of *S. imbricatus*.

Figure 2. Reducing power and chelating activities of mushrooms.



3.3. Antioxidant Activity/RACI Correlations of Mushroom Extracts

As a result of the RACI calculations carried out in order to make the data obtained from different antioxidant activity tests and expressed in different units mathematically comparable with each other (Figure 3), when the data obtained from the four test systems are evaluated as a whole, it is seen that the antioxidant activity potential of *S. imbricatus* was found to be significantly higher than the other species (RACI value: 0.90). This mushroom species was followed by *H. repandum* and *A. impudicus* with the same RACI value (RACI value: - 0.24). *C. comatus* was in the last place in terms of antioxidant activity (RACI value: -0.42).

Figure 3. RACI values of methanol extracts of mushrooms.

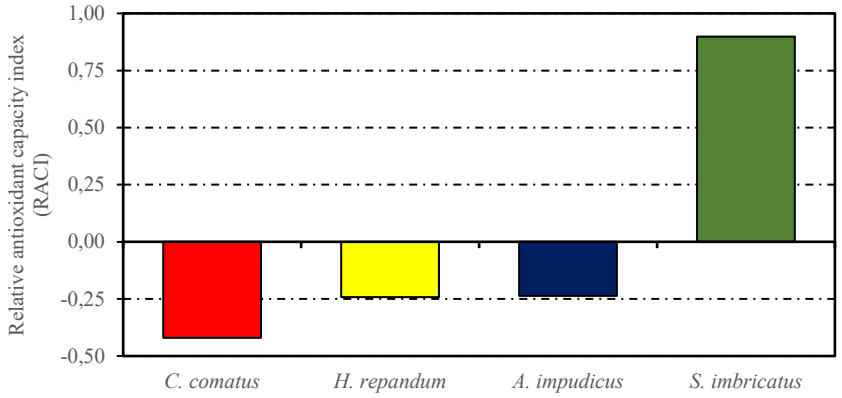
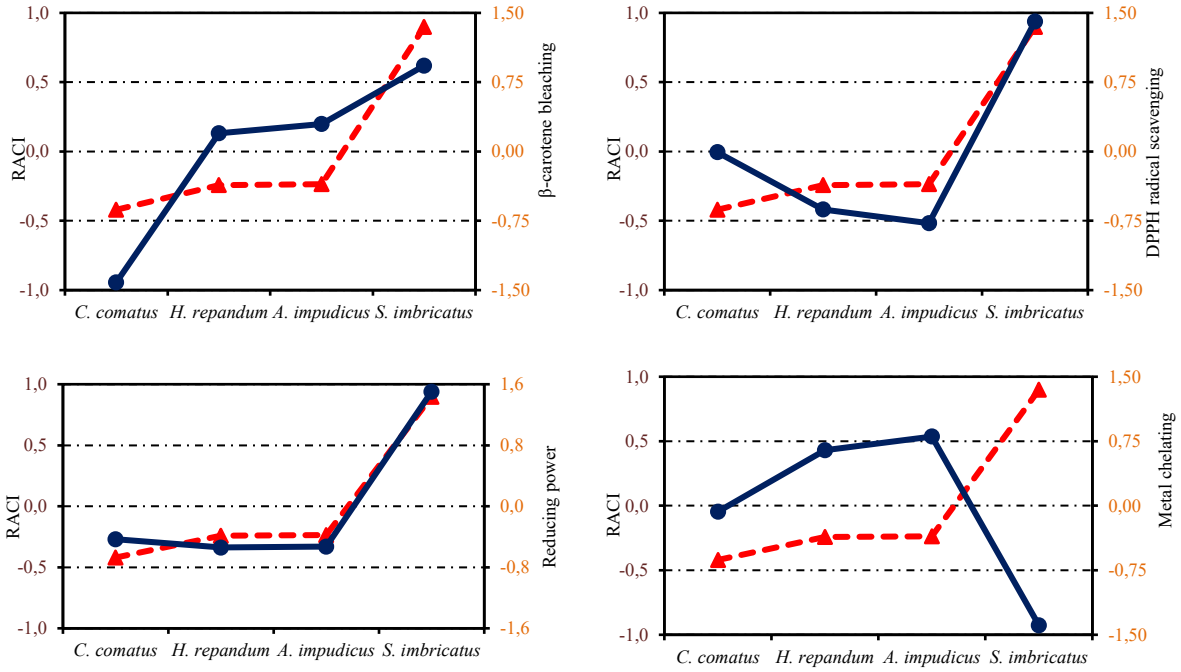


Figure 4 shows the correlation between the RACI values and the data obtained from the antioxidant activity tests of each of the extracts. It was determined that there was a high correlation between the data obtained from β-carotene bleaching, DPPH free radical scavenging, and reducing power tests and the RACI values of the extracts. However, it was found that there was an inverse correlation between the activity data of the extracts and the RACI values in the metal chelating test. This situation is thought to be a rational basis for the fact that the activity profile of the extracts in the metal chelating test is different from the activity profiles exhibited in other tests.

Figure 4. Correlation between RACI values and antioxidant activities of mushroom extracts.



3.4. Pearson Correlations Between Parameters

The data obtained as a result of the Pearson correlation analysis, which was carried out in order to statistically determine to what extent phenolics and flavonoids contribute to the antioxidant activities of the extracts and to reveal the correlation between the applied test systems, are given in Table 3. According to the data in the table, it was determined that there was a high correlation between the total phenolic and flavonoid compound amounts of the extracts and their DPPH free radical scavenging capacity and reducing power (correlation coefficients above 0.9). It was found that the correlation between the groups of compounds in question and the activities of the extracts in the β-carotene bleaching test is lower than the ones given above (correlation coefficients 0.401 and 0.573, respectively). It was also found that the correlation between the RACI values of the extracts and their reducing power was significant (correlation coefficient: 0.976).

Table 3. Pearson correlations between parameters.

Tests	β-carotene bleaching	DPPH radical scavenging	Reducing power	Metal chelating
DPPH radical scavenging	0.320	-	-	-
Reducing power	0.577	0.952	-	-
Metal chelating	-0.165	-0.920	-0.844	-
RACI	0.698	0.886	0.976	-0.807
Toal phenolics	0.401	0.986	0.976	-0.906
Total flavonoids	0.573	0.952	0.990	-0.868

4. DISCUSSION and CONCLUSION

4.1. Chemical Compositions of Mushroom Extracts

As a result of the literature search, no study was found investigating the chemical composition of *A. impudicus*. Therefore, the data presented for this mushroom species in the present study is the first record for the literature. On the other hand, there are some studies in the literature to elucidate the total phenolic/flavonoid contents and chemical compositions of *C. comatus*, *H. repandum* and *S. imbricatus*. In a study by Seklic *et al*. (2016), total phenolic and flavonoid contents of *C. comatus* were reported as 11.96 mg GAEs/g and 10.0 mg RUEs/g, respectively, while in another study by Sadi *et al*. (2015), these values were 76.32 and 3.67 µg/g, respectively. According to Kalaw & Albinto (2014), the total phenolic compound content of this species is 17.82 mg GAEs/g. There are also data on the quantitative chemical composition of *C. comatus* in the literature. Studies have reported that this mushroom species contains quinic (Karaman *et al*., 2019), vanillic, gallic, gentisic and cinnamic acids (Tesanovic *et al*., 2017). There is also a report in the literature regarding the total phenolic and flavonoid content of *H. repandum*. In this study by Ozen *et al*. (2011), the total phenolic and flavonoid contents of the mentioned species were reported as 3.67 mg pyrocatechol/g and 0.102 mg QEs/g, respectively. In addition, a study by Vasdekis *et al*. (2018) reported that *H. repandum* contains piceatannol. It is also reported in the literature that the total phenolic and flavonoid content of *S. imbricatus* is 1.50-13.20 and 1.46-5.45 mg/g, respectively (Barros *et al*., 2007a,b; Shomali *et al*., 2019) and contains gallic acid (0.75 mg/g) and myrcetin (2.89 mg/g) (Shomali *et al*., 2019). It is seen that the total phenolic/flavonoid compound data obtained from the present study differ from the literature data. It is a fact accepted by many researchers that the chemical compositions of both mushroom and plant samples are affected by environmental conditions such as climate, soil structure, pH, altitude, humidity, locality, collection time, etc. (Boira *et al*., 1998; Yatin *et al*., 2000). Therefore, it is thought that this difference arises due to environmental conditions.

4.2. Chemical Compositions of Mushroom Extracts

There is no data on the antioxidant activity of *A. impudicus* in the literature. In addition, the data obtained in the β-carotene bleaching of *H. repandum* and metal chelating tests of *S. imbricatus* were also brought to the literature for the first time with the present study.

There are some findings in the literature regarding the antioxidant activities of *C. comatus* obtained through the test methods used in the current study (Asatiani *et al*., 2007a,b; Liv *et al*., 2010; Akata *et al*., 2012; Kalaw & Albinto, 2014; Sadi *et al*., 2015; Seklic *et al*., 2016; Tesanovic *et al*., 2017; Cao *et al*., 2019). According to the data in these studies, the mushroom species in question showed 72.06% activity at a concentration of 2.0 mg/ml in β-carotene assay (Asatiani *et al*., 2007a, b). In DPPH free radical scavenging test, the mushroom species in question exhibited moderate or high activity according to some researchers (Li *et al*., 2010; Akata *et al*., 2012; Kalaw & Albinto, 2014; Sadi *et al*., 2015; Tesanovic *et al*., 2017; Seklic *et al*., 2016). According to Cao *et al.* (2019), polysaccharides contribute significantly to the DPPH free radical scavenging activity of *C. comatus*. In a study conducted by Akata *et al.* (2019), it was reported that the activity values of *C. comatus* in reducing power and metal chelating tests were 12.40-28.83 mg TEs/g and 7.76 mg EDTAEs/g, respectively. Another supporting finding that the species has metal chelating ability was reported by Sadi *et al.* (2015) (0.842 mg/ml).

The only available source in the literature regarding DPPH, reducing power, and metal chelating capacity of *H. repandum* is the study by Ozen *et al.* (2011). According to the results of this study, the related mushroom species exhibited 53.10%, 0.307 absorbance and 80.36% activity values in DPPH, reducing power and metal chelating tests at 50 µg/ml concentration, respectively.

According to a study carried out by Barros *et al.* (2017b), the activity values of *S. imbricatus*, another mushroom species analyzed in the present study, in β-carotene bleaching, DPPPH radical scavenging and reducing power tests, were 3.97, 1.44 and 2.79 mg/ml, respectively. In a study conducted by Barros *et al.* (2017c), it was reported that cooking and processing processes reduced the effectiveness of *S. imbricatus* in the β-carotene bleaching test. In other studies on the DPPH radical scavenging activity of *S. imbricatus*, it has been reported that methanol extract exhibited remarkable efficacy (Marcotullio *et al*., 2008; Luo *et al*., 2017; Shomali *et al*., 2019).

When the literature data given above are evaluated as a whole, it is understood that the data obtained from the present study generally share the same common denominator with those reported by other researchers, although some differences have been detected with some of the literature data. In particular, *S. imbricatus* is thought to be a potential source for new and alternative antioxidant compounds in the food industry. However, it was concluded that more detailed techniques such as fractionation accompanied by biological activity should be applied to detect the chemical compounds responsible for the activity.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

Authorship Contribution Statement

Arzuhan Sihoglu Tepe: Investigation, Methodology, Resources, Visualization, Software, Formal Analysis, Validation, and Writing -original draft.

Orcid

Arzuhan SIHOGLU TEPE https://orcid.org/0000-0001-8290-9880

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Research Article

Macro-Micro Element Variation in Traditionally Grown Einkorn (*Triticum monococcum* L. subsp. *monococcum*) and Emmer Wheat (*Triticum dicoccon* Schrank)

Nusret Zencirci[1,*], **Fatma Pehlivan Karakas**[1], **Bulent Ordu**[2]

[1]Bolu Abant Izzet Baysal University, Art and Science Faculty, Biology Department, 14200, Gölköy, Bolu, Turkey
[2]Bolu Abant Izzet Baysal University, Faculty of Economics and Administrative Sciences, 14200, Gölköy, Bolu, Turkey

Abstract: Einkorn (*Triticum monococcum* L. subsp. *monococcum)* and emmer wheat (*T. dicoccon* (Schrank) Schubl.), disease and insect resistant, macro and microelement rich, and high quality are still grown in Turkey highlands (1000-1400 m). In this study, plant and soil samples were sampled for macro and micro elements and quality where appropriate. Nine traditional einkorn and emmer wheat farms soils were sampled in Bolu, Kastamonu, Karabük, Sinop, and Samsun provinces in the Western Black Sea Region, Turkey. Plants were sampled during July-August 2016. Methods applied according to the literature. Except for crude ash and copper in grains and the total salt and EC in soils, characters significantly differed. Grain wise, Population-4 had the highest P, K, Cu, Fe, and Mn; Kunduru-1149 had the highest 1000-grain weight, crude protein, energy, raw cellulose, and hectolitre; Population-8 had the highest energy, crude oil, carbohydrate, and starch. Soil wise, Population-5 had the highest Cu, Ca, and Mg; Population-8 had the highest N, Fe, and Mn; Population-2 had the highest N and Zn, $CaCO_3$, active lime, EC, K_2O, organic matter, P_2O_5, and salt. Correlations among grains were highly significant for N-Cu, P-Mn, P-K, carbohydrate-starch, energy-starch, and energy-carbohydrate. Correlations among soil samples were highly significant between Fe-Mn, total N-Mn, $CaCO_3$-active lime, saturation-salt, organic matter-P_2O, $CaCO_3$-K_2O, and active lime-K_2O. In conclusion, there was a wide variation in traditionally grown einkorn and emmer grain and soils in the Western Black Sea Region.

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1. INTRODUCTION

Wheat, a major food grain, is increasingly required especially in developing countries because of the food need of fast-growing human population. It is the type of grain with the highest protein content among the cereals and provides more than 20 % of the daily calorie needed by people living on Earth (Peng *et al.*, 2011a; Peng *et al*., 2011b). It is also rich in micronutrients (Cummins & Roberts-Thomson, 2009). Among wheat species, einkorn (*Triticum monococcum* subsp. *monococcum*) and emmer (*T. dicoccon* (Schrank) Schubl.) are two primary wheat

*CONTACT: Nusret Zencirci ✉ nzencirci@yahoo.com Bolu Abant IzzetBaysal University, Art and Science Faculty, Biology Department, 14200, Gölköy, Bolu, Turkey

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species, of which einkorn is the first cultivated one (Karagöz, 1996; Nesbitt, 1995; Giuliani *et al*., 2009).

Previous studies revealed that the gene centre of these wheat species is South East Anatolia (Diyarbakır-Karacadağ region), Turkey (Heun *et al*., 1997; Dubcovsky & Dvorak, 2007; Shewry, 2009; Özkan *et al*., 2010) and they still have been naturally grown in the area called Fertile Crescent (Zohary & Hopf, 2000; Özkan *et al*., 2010). The first domesticated plants in the Fertile Crescent were einkorn, emmer, barley, peas, lentils, chickpeas, sourdough, and flax (Zohary & Hopf, 2000). Einkorn is diploid (2n=2x=14, AA genome), and emmer is tetraploid (2n=4x=28, AABB genome) (Szabo & Hammer, 1996).

In Turkey, einkorn (Iza/Siyez) and emmer (kaplıca / çatalsiyez / gernik / kavılca) are still grown in limited areas of poor mountainous areas of the Western Black Sea Region, which starts from the western edge of Kızılırmak delta, extends to the east of Adapazarı and Bilecik, and reaches 2000 m above sea level on the West (Kan *et al*., 2015). Sloppy mountains make mechanized agriculture difficult and animal and manpower in the region is a must. Region has variously dark, humus-rich acidic washed soils. Every season is rainy. The annual temperature difference is low. These primary-primitive species are widely used as animal feed and organic / good bulgur production due to its favoured flavour. While einkorn is used for bulgur production and, to some extent, as animal feed around Kastamonu and Bolu provinces, emmer is generally for animal feeding in Sinop, Samsun, and Karabük. It is known that the local people used to make bread from emmer as well.

Einkorn and emmer are resistant to frost, drought, and diseases. Their hulled structure protects them especially in the storage and in the field (Nesbitt & Samuel, 1996). They are rich in raw fibre, antioxidants, minerals, and protein, with low glycaemic index and cholesterol. They are reported to have some protective effects against diabetes, cancer, and cardiovascular diseases. Einkorn and emmer contain more zinc, iron, and copper (Suchowilska *et al*., 2012) and selenium than bread wheat (Lachman *et al*., 2011). They are also rich in starch, fibre, minerals, and phytochemicals (Pekcan & Köksal, 2006; Arzani *et al*., 2017).

In this study, we collected five einkorn and four emmer populations from Bolu, Kastamonu, Karabük, Samsun, and Sinop provinces in July-August 2016. The registered check cultivars for grain comparison included were Bezostaja-1 bread (*Triticum aestivum* L.) and Kunduru-1149 durum (*Triticum durum* Desf.). Both wheat grain and soil samples had been analysed. In the soil samples we collected, macro nitrogen (N), magnesium (Mg), calcium (Ca), potassium (K), micro iron (Fe), manganese (Mn), zinc (Zn), sodium (Na), and copper (Cu) elements and water saturation, total salt, saturation with water pH, $CaCO_3$ (calcium carbonate), active lime, K_2O (potassium oxide), P_2O_5 (phosphorus pentoxide), electrical conductivity (EC), and organic matter were determined. In wheat grain samples N, P, K, Fe, Zn, Mn, Cu, 1000 grain weight, crude protein, energy, crude cellulose, crude oil, hectolitre, carbohydrate, starch, sugar, and raw ash were analysed.

2. MATERIAL and METHODS

The study material was five einkorn and four emmer wheats from five provinces of the Western Black Sea Region (Bolu, Kastamonu, Karabük, Samsun, and Sinop), which were collected in July-August 2016. Bezostaja-1 bread and Kunduru-1149 durum wheat were the controls. We recorded altitude, latitude, longitude, soil structure, and geographical characteristics (Table 1) of the collection sites. Environment expectedly affects yield, quality, and nutrition of einkorn (Pomeranz, 1971).

In grain samples, some macro (N, P, and K) and micro (Fe, Mn, Zn, and Cu) elements, energy, carbohydrate, protein, crude protein, starch, fat, fibre, sugar, crude ash, 1000 grain weight, % protein, hectolitre analyses were carried out in Konya Laboratory and Storage

Company. Total nitrogen, saturation, salt, EC, pH, lime, active lime, phosphorus, potassium, calcium, magnesium, iron, manganese, zinc, copper, sodium, and organic matter in the soil samples were determined in Bolu Soil and Leaf Analysis Laboratory (Anonymous, 1993; 1998; 2013; 2016).

Table 1. Populations, their species, origins, altitudes (m), latitudes, longitudes, soil structure, and some soil characteristics (Bezostaja-1 and Kunduru-1149 were controls).

Populations	Species	Origin	Altitude (m)	Latitude	Longitude	Soil structure	Forest/ Not forest	Flat / Rugged
Population-1	Emmer	Karabük, Safranbolu	811	41º25' 15"	32º48' 4"	Standard	Half forest	Rugged
Population-2	Einkorn	Kastamonu, (İhsangazi, Akkaya Village)	787	41º 13' 0"	33º 28' 58"	Clay soil	Not forest	Flat
Population-3	Einkorn	Kastamonu, İhsangazi, Çatalyazı Village	1258	41º 9' 37"	33º 37' 17"	Standard	Half forest	Rugged
Population-4	Einkorn	Kastamonu, İhsangazi, Enbiya Village	820	41º 12' 52"	33º 30' 52"	Standard	Half forest	Flat
Population-5	Emmer	Samsun, Lâdik, Çamlıköy	1055	40º 52' 39"	35º 45' 27"	Hard soil	Some forest	Rugged
Population-6	Emmer	Sinop, Durağan, Kirencik Village	1295	41º 25' 37"	35º 21' 23"	Hard, stony soil	Some forest	Rugged
Population-7	Einkorn	Bolu, Seben	911	40º 21' 38"	31º 30' 50"	Clay soil	Some forest	Flat
Population-8	Emmer	Sinop, Durağan, Gölgerişi Village	1116	41º 26' 14"	35º 23' 1"	Standard	Some forest	Rugged
Population-9	Einkorn	Bolu, Seben, Musosoflar Village	920	40º 21' 38"	31º 30' 47"	Clay soil	Some forest	Rugged
Bezostaja-1	Bread wheat							
Kunduru-1149	Durum wheat							

Nitrogen and crude protein in grain samples were analysed by ISO 1871 method (*Rossi et al*., 2004), crude oil and crude cellulose by AACC 32-25 method (Anonymous, 2010), crude ash by AACC 08-01 basic method (Anonymous, 2010), Hectolitre by TS EN ISO 7971-3 method (Anonymous, 2009), 1000 grain weight by TS 2974 method (Anonymous, 2016), starch (%) by TKB method (Anonymous, 2013), sugar (%) by NMKL 148 method (Anonymous, 1993), carbohydrate and energy by calculation method (Emmanuel *et al*., 2014), and Cu, Fe, Mn, Zn, K, and P by NMKL 161 (Anonymous, 1998).Total nitrogen in soil (total N%) was

determined by Kjeldahl method (Horwitz, 1955; Jackson, 1958; Chapman & Pratt, 1961; Black, 1965; Hanway 1968; Ülgen & Ateşalp, 1972), water saturation by pure water saturation method (Richards, 1954; Bower & Wilcox,1965; Özbek & Aydeniz, 1967), saturation with water Ph by Potentiometric measuring method using pH-meter, total salt % by the method of determining the salinity of the soil by measuring its resistance to electrical power (Hindistan & İnceoğlu, 1962; Peech, 1965; Mclean, 1973;), CaCo3 (lime) % by Scheibler calcimetry method (Çağlar, 1949), active lime% by Drouineau-Galet Method (Drouıneau, 1942), K_2O (Potassium oxide) by measuring fleym fotometre (Chapman & Pratt, 1961); Jackson, 1958; Doll & Lucas, 1973; Pratt, 1965), P_2O_5 (Phosphorus pentoxide) by Olsen method (Olsen *et al*., 1954), electrical conductivity (EC) ms/cm by measuring electrical conductivity in saturation extract method (Richards, 1954; Öztan & Ülgen, 1961; Öztan & Munsuz, 1961; Ayyıldız *et al*., 1983;), Na, K, Ca, and Mg by 1 N NH4OAc method (Carson, 1980), Zn, Fe, Mn, and Cu by DTPA 0.005 M extraction method (Berger & Troug, 1939; Viets, 1962; Follet & Linsay, 1970), organic matter by Modified the Walkley-Black Method (Allison, 1965; Walkley, 1946). Analysis of variance (ANOVA) and Least Significant Difference (LSD) for grain and soil characteristics, correlations among all character pairs of grain quality, soil macro-micro elements, and other soil characteristics, and dendrogram for all grain and all soil characteristics were run by SPSS version 25.

3. RESULTS / FINDINGS

The nutrition and macro-micro elements of wheat grains and soils collected in Bolu, Kastamonu, Samsun, Sinop, and Karabük provinces in the Western Black Sea Region were presented as follows:

3.1. Macro-Micro Element Diversity in Wheat Grains

Three replication randomized block design was applied for macro (N, P, and K) and microelements (Cu, Fe, Zn, and Mn) in wheat grains (Table 2).

Table 2. Macro-micro element means squares of wheat grains.

Source of Variation	*DF*	N (mg/kg)	P (kg/kg)	K (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)
Entries	10	0.46*	1216.26*	747.75*	1.10	296.68*	369.49*	218.26*
Error	22	0.02	0.055	0.625	106711.38	0.64	4.01	1.08

DF: Degrees of freedom; * significant at 0.05.

Macro and microelement mean squares statistically differentiated in grains (Table 2). The differences were significant for N, P, K, Fe, Zn, and Mn (p<0.05), but insignificant for Cu (Table 3). The error which is lower for P, K, Fe, Zn, and Mn increased the significance of F test.

Kunduru-1149 had the highest N (3.02 mg/kg). Emmer grain from Sinop Durağan (Population-6), while it had the lowest N (1.68 mg/kg). Einkorn grain from Kastamonu İhsangazi (Population-4) had the highest P (5687.00 mg/kg), while Bolu Seben (Population-7) einkorn grain had the lowest (3.472.67 mg/kg). The einkorn from Kastamonu İhsangazi (Population-4) had the highest K (565.00 mg/ kg), while Bezostaja-1 had the lowest (3.842.00 mg/kg).

Kunduru-1149 had the highest Cu (6.42 mg/kg), while emmer grain from Sinop Durağan (Population-6) had the lowest (4.16 mg/kg). Einkorn from Kastamonu İhsangazi (Population-4) had the highest Fe (59.32 mg/kg); on the other hand, emmer from Sinop Durağan

(Population-8) had the lowest (27.30 mg/kg). Kastamonu İhsangazi (Population-2) einkorn wheat had the highest Zn (74.68), while Bezostaja-1 had the lowest (35.62 mg/kg). Einkorn from Kastamonu İhsangazi (Population-4) had the highest Mn (53.75 mg/kg), while emmer from Sinop Durağan Population-6 had the lowest (24.48 mg/kg). When we look into the relevant literature, in a study (Allison, 1965) the concentrations of five macro and fifteen microelements in the whole grain of spring lines of emmer, einkorn, spelt and two common wheat cultivars, all grown under identical environmental conditions, were determined by ICP-SFMS analysis. *Triticum* species studied differed significantly for P, Zn, Fe, Mn, and Cu concentrations as in our study. The grain of all hulled wheats, compared with common wheat, contained significantly more Zn (from 34% to 54%), which was lower than einkorn of ours; Fe (from 31% to 33%) was lower than our emmer samples. In most cases, there were no relationships between the concentrations of the analysed elements, except for significant positive correlations between the levels of Fe, Zn, and Mn, in einkorn and emmer. The classical linear discriminant analysis enabled us to distinguish between the three *Triticum* species studied for the concentrations of all analysed elements in their grain. A significant discrimination indicates that the concentrations of the investigated elements are a species-specific character. A strong correlation between Zn, Fe, and Mn could have important implications for wheat quality breeding. Analysed 150 bread wheat lines of diverse origin and 25 lines of durum, spelt, einkorn, and emmer wheat micronutrient concentration varied in grain. Substantial variation existed among the grain Fe and Zn of 75 lines as in some of our samples.

Table 3. Entry ranking for wheat macro-micro elements by LSD.

Entries	N (mg/kg)	P (mg/kg)	K (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)
Population-1 Karabük—Safranbolu	2.11^{c*}	4324.33^{e}	4721.67^{c}	4.64^{a}	35.81^{e}	48.13^{e}	34.16^{c}
Population-2 Kastamonu-İhsangazi	2.20^{b}	4217.33^{f}	4240.67^{f}	4.79^{a}	30.67^{f}	74.68^{a}	44.75^{b}
Population-3 Kastamonu-İhsangazi	1.93^{c}	4341.33^{d}	4774.67^{c}	4.74^{a}	29.30^{f}	55.05^{c}	36.38^{c}
Population-4 Kastamonu-İhsangazi	2.13^{c}	5687.00^{a}	5651.00^{a}	4.86^{a}	59.32^{a}	62.05^{b}	53.75^{a}
Population-5 Samsun-Ladik	1.97^{c}	3774.33^{i}	4344.00^{e}	4.67^{a}	30.34^{f}	42.65^{g}	29.64^{e}
Population-6 Sinop-Durağan	1.68^{d}	3926.33^{h}	4452.33^{d}	4.16^{a}	27.55^{g}	38.15^{h}	24.48^{f}
Population-7 Bolu-Seben	2.50^{b}	3472.67^{j}	4176.00^{g}	5.37^{a}	46.30^{b}	53.75^{c}	31.07^{d}
Population-8 Sinop-Durağan	1.55^{d}	4420.33^{c}	5123.67^{b}	4.52^{a}	27.30^{g}	45.72^{f}	32.41^{d}
Population-9 Bolu-Seben	2.19^{b}	3328.33^{k}	4267.67^{f}	5.33^{a}	42.87^{c}	51.44^{d}	28.07^{e}
Bezostaja-1	2.02^{c}	4081.33^{g}	3842.00^{h}	4.59^{a}	30.11^{f}	35.62^{i}	34.41^{c}
Kunduru-1149	3.02^{a}	4647.00^{b}	4496.00^{d}	6.42^{a}	38.22^{d}	46.31^{e}	43.57^{b}
LSD 0.05	0.32	16.68	56.24	734.82	1.81	4.50	2.35

* The same letter in the same column describes no difference otherwise there is a difference.

3.2. Quality Characteristics of Grain Samples

Thousand kernel weight (1000 KW), crude protein %, energy, raw ash%, crude cellulose %, raw oil %; hectolitre (kg/hl), starch %, and total sugar % in grain samples collected from Bolu, Kastamonu, Samsun, Sinop, and Karabük in the Western Black Sea Region are presented as follows:

ANOVA designated significance (p<0.05) for thousand kernel weight (TKW), crude protein, energy, crude cellulose, raw oil, hectolitre, carbohydrate, starch, and total sugar, while raw ash was designated as insignificant (Table 4). Higher MS error for raw ash was the most likely reason for insignificance. Kunduru-1149 had the heaviest TKW (50.55g), while Kastamonu İhsangazi (Population-2) einkorn had the lightest (21.55g). Kunduru-1149 had the highest protein (16.50 %); on the other hand, Sinop Durağan (Population-8) emmer grain had the lowest (8.75 %). Sinop Durağan emmer (Population-8) had the highest energy (319.00 kcal/100 gr), while Kastamonu İhsangazi (Population-2) einkorn had the lowest (279.67). Bolu Seben (Population-9) had the highest raw ash (2.34 g), while Bolu Seben (Population-7) einkorn wheat had the lowest (1.42). Kunduru-1149 had the highest crude cellulose (3.80 %), while Kastamonu İhsangazi (Population-4) einkorn wheat had the lowest (1.28 %). Sinop Durağan emmer (Population-8) had the highest raw oil (2.04 %), while Bezostaja-1 had the lowest (0.42 %). Bolu Seben (Population-9) einkorn wheat had the heaviest hectolitre (80.33 kg/hl), while Karabuk Safranbolu (Population-1) had the lightest (71.97 kg/hl). Sinop Durağan (Population-8) emmer wheat had the highest carbohydrate (64.63 %), while Kastamonu İhsangazi (Population-2) einkorn had the lowest (54.50 kg/hl). Sinop Durağan (Population-8) emmer had the highest starch (63.28 %), while Kastamonu İhsangazi (Population-2) einkorn wheat sample had the lowest (53.63 %). Kastamonu İhsangazi (Population-4) einkorn had the highest total sugar (1.69 %), while Bezostaja-1 had the lowest (0.42 %) (Table 5). Old varieties and landraces of wheat may play an important role for food security since they both provide genes readily available to breeders and they perform sustainably higher yield in marginal lands. Today in many countries including Italy, Turkey, Germany, and the USA cereal sector requires seed companies that breed specifically adapted cultivars for organic and biodynamic farms and study macro and microelements in these varieties. In such a context, the dendrogram (Figure 1), based on the averages of macro-micro N (mg/kg), P (mg/kg), K (mg/kg), Fe (mg/kg), Cu (mg/kg), Zn (mg/kg), and Mn (mg/kg) elements as well as 1000 KW (g), crude protein (%), energy (kcal/100 gr), raw ash %, crude cellulose %, raw oil %, hectolitre %, carbohydrate %, starch %, and total sugar %, separated eleven wheat grain samples into two main groups.

Thirty-three wheat (*Triticum aestivum* L.) samples were assessed for their physical and chemical analysis, protein quality and quantity, and metabolizable energy and contrary to our findings none of the values, or combination of values, from the physical and chemical analyses showed significant correlation with metabolizable energy values.

Table 4. Grain quality mean squares.

Source of variation	*DF*	1000 KW (gr)	Crude protein %	Energy (kcal/100 gr)	Raw ash %	Crude cellulose %	Raw oil %	Hektoliter (kg/hl)	Carbohydrate %	Starch %	Total sugar %
Entries	10	273.98*	13.07*	346.09*	140.00^{ns}	1.47*	0.55*	29.53*	30.39*	25.92*	0.33*
Error	22	1.03	0.47	50.33	1563.82	0.01	0.01	4.58	2.23	2.54	0.0043

DF: Degrees of freedom; * Significant at 0.05

Table 5. Entry ranking for wheat grain quality diversity by LSD.

Entries	1000 KW (gr)	Crude protein %	Energy (kcal/100 gr)	Raw ash %	Crude cellulose %	Raw oil %	Hectoliter (kg/hl)	Carbohydrate %	Starch %	Total sugar %
Population-1 Karabük-Safranbolu	27.87^{f*}	12.07^{b}	307.00^{a}	1.77^{d}	3.04^{b}	1.62^{b}	71.97^{b}	61.24^{b}	60.32^{a}	0.58^{d}
Population-2 Kastamonu-İhsangazi	21.55^{h}	12.47^{b}	279.67^{b}	1.72^{f}	2.99^{b}	1.25^{c}	77.42^{a}	54.50^{c}	53.63^{c}	0.83^{b}
Population-3 Kastamonu-İhsangazi	33.57^{d}	11.13^{c}	283.00^{b}	1.70^{f}	2.44^{c}	1.43^{b}	80.07^{a}	56.41^{c}	55.46^{c}	0.91^{b}
Population-4 Kastamonu-İhsangazi	23.30^{g}	12.25^{b}	305.67^{a}	2.01^{b}	1.28^{f}	1.45^{b}	78.02^{a}	60.54^{b}	59.13^{b}	1.69^{a}
Population-5 Samsun-Ladik	29.18^{f}	11.25^{b}	302.57^{a}	1.64^{f}	2.27^{c}	1.71^{b}	70.67^{b}	60.45^{b}	59.95^{a}	0.66^{c}
Population-6 Sinop-Durağan	32.13^{e}	9.68^{c}	300.67^{a}	1.73^{e}	2.10^{d}	1.94^{a}	75.37^{b}	60.93^{b}	60.12^{a}	0.82^{b}
Population-7 Bolu-Seben	27.20^{f}	14.27^{b}	296.33^{a}	1.42^{h}	2.40^{c}	1.27^{c}	79.15^{a}	57.23^{c}	56.24^{b}	0.96^{b}
Population-8 Sinop-Durağan	37.87^{c}	8.75^{d}	311.00^{a}	1.51^{g}	1.70^{e}	2.04^{a}	75.10^{b}	64.63^{a}	63.28^{a}	0.77^{c}
Population-9 Bolu-Seben	25.50^{g}	12.72^{b}	285.33^{b}	2.34^{a}	2.13^{d}	1.50^{b}	80.33^{a}	55.47^{c}	54.68^{c}	0.65^{c}
Bezostaja-1	48.10^{b}	11.61^{b}	288.67^{b}	1.56^{g}	2.97^{b}	0.42^{d}	75.10^{b}	59.20^{b}	59.12^{b}	0.42^{e}
Kunduru-1149	50.55^{a}	16.50^{a}	303.00^{a}	1.82^{c}	3.80^{a}	1.58^{b}	77.57^{a}	55.16^{c}	56.37^{b}	0.54^{d}
LSD 0.05	2.29	1.54	15.96	88.96	0.284	0.283	4.82	3.36	3.59	0.15

* The same letter in the same column describes no difference otherwise there is a difference.

3.3. Soil Macro-Micro Elements

Mean squares in ANOVA were significant for macro-micro elements of N, P_2O_5, K_2O, K, Na, Fe, Cu, Ca, Zn, Mn, and Mg (Table 6). The highest N (0.74 mg/kg) was in Kastamonu İhsangazi (Population-2) soil sample, while the lowest was in Bolu Seben (Population-7) (0.13 mg/kg). Kastamonu İhsangazi (Population-2) had the highest P_2O_5 (54.90 mg/kg), while Karabük Safranbolu (Population-1) soil sample had the lowest (0.03 mg/kg) (Table 7). Kastamonu İhsangazi (Population-2) had the highest K_2O (258.22 mg/kg); Sinop Durağan (Population-6) soil sample had the lowest 65.87(mg/kg). Kastamonu İhsangazi (Population-4) had the highest K (371.27 mg/kg), whereas Sinop Durağan (Population-6) had the lowest (59.43 mg/kg). Karabük Safranbolu (Population-1) had the highest Na (7.92mg/kg), while Kastamonu İhsangazi (Population-2), Samsun Ladik (Population-5), Sinop Durağan (Population-6), Bolu

Seben (Population-7), Sinop Durağan (Population-8), and Bolu Seben (Population-9) had the lowest (0.01 to 0.00 mg/kg).

Large Fe differences existed among soil samples. Sinop Durağan (Population-8) had the highest (53.15 mg/kg), while Bolu Seben (Population-9) had the lowest (2.20). For Cu, Samsun Ladik (Population-5) soil sample had the highest value with 13.98 mg/kg result, while Bolu Seben (Population-7) had the lowest value with 3.35 mg/kg result of soil sample. Samsun Ladik (Population-5) had the highest Ca (5739.67 mg/kg), while Sinop Durağan (Population-8) had the lowest (3108.37 mg/kg). Kastamonu İhsangazi (Population-2) had the highest Zn (4.32 mg/kg), while Kastamonu İhsangazi (Population-3), Bolu Seben (Population-7), and Bolu Seben (Population-9) had the lowest (0.01 mg/kg). Sinop Durağan (Population-8) had the highest Mn (95.35 mg/kg); Bolu Seben (Population-7) had the lowest (22.23 mg/kg). Samsun Ladik (Population5) had the highest Mg (395.57 mg/kg), while Sinop Durağan (Population-6) had the lowest (88.17 mg/kg).

Table 6. Mean squares for soil structure factors of $CaCO_3$ %, active lime %, EC, K_2O, organic matter %, P_2O_5, water saturation, and saturation with water pH.

Source of Variation	*DF*	$CaCO_3$ %	Active lime %	EC ms/cm	Organic matter %	Water saturation %	Saturation with water pH (%)	Total salt %
Entries	10	984.16*	246.69*	0.0133	2.36*	966.76*	0.72*	0.0001
Enter	22	0.11	0.21	0.0071	0.18	10.81	0.01	0.002

DF: Degrees of freedom; significance at 0.05.

A comparison of five macro and fifteen microelement concentrations in the whole grain of four emmer, einkorn, spelt, and two common *Triticum* species showed that *Triticum* species differed significantly for P, Zn, Fe, Mn, and Cu. The grain of all hulled wheats, compared with common wheat, contained significantly higher Zn (from 34 % to 54 %), Fe (from 31 % to 33%) and Cu (from 3 %. We obtained similar results as well found that compost application increased the soil total N and the available K, Fe, Zn, and Mn concentrations, whereas the available Cu decreased and the available soil P was not affected (Allison, 1965). The effect of three types of tillage (disc, DP; sweep, SW; and mouldboard, MP) and five N application rates (0, 45, 90, 135, and 180 kg ha-1) on macronutrients in soil and wheat (*Triticum aestivum* L.) tissues grown in a winter wheat–summer fallow rotation were studied The experiment included three types of tillage (disc, DP; sweep, SW; and mouldboard; MP) and five N application rates (0, 45, 90, 135, and 180 kg ha-1). Soil and tissue samples were analysed for the concentration of total N, S, and C, Mehlich III extractable P, K, Mg, Ca in the soil, and the total concentration of the same nutrients in wheat tissue. Soil N concentration was significantly greater under DP (1.10 g kg-1) than under MP (1.03 g kg-1). The P concentration in upper 20 cm soil depth increased with increased N rates. Comparison of experiment plots to a nearby-undisturbed pasture revealed a decline of P (32 %), SOC (34 %), Mg (77 %), and Ca (86 %) in the top 10 cm soil depth. Bread in Yemen is the staple food, produced in different kinds from local and imported wheat, most of which is not subjected to micro-elemental analysis. Fortunately, Fe, Cu, Mn, Zn, Co, Cd, and Pb micro-elements in samples of wheat grains produced locally from different cultivated regions in Yemen as well as those imported from the USA and Austria were generally within the permissible levels except for cadmium (Allison, 1965).

Table 7. Soil character distinction by LSD.

Entries	$CaCO_3$ %	Active lime %	E.C ms/cm	Organic matter %	Water saturation %	Saturation with water pH	Total salt %
Population-1 Karabük-Safranbolu	0.80^{h*}	0.40^{h}	0.46^{a}	1.33^{c}	127.40^{a}	7.24^{b}	0.06^{a}
Population-2 Kastamonu-İhsangazi	59.23^{a}	29.63^{a}	0.59^{a}	3.78^{a}	69.17^{e}	7.23^{b}	0.04^{a}
Population-3 Kastamonu-İhsangazi	16.63^{d}	8.43^{d}	0.60^{a}	2.62^{b}	96.69^{b}	7.51^{a}	0.04^{a}
Population-4 Kastamonu-İhsangazi	5.21^{f}	2.63^{f}	0.53^{a}	2.58^{b}	91.47^{b}	7.60^{a}	0.04^{a}
Population-5 Samsun-Ladik	2.98^{g}	1.53^{g}	0.60^{a}	1.72^{b}	72.60^{d}	7.36^{b}	0.03^{a}
Population-6 Sinop-Durağan	1.21^{h}	0.63^{g}	0.42^{a}	2.55^{b}	59.43^{f}	7.17^{b}	0.03^{a}
Population-7 Bolu-Seben	36.17^{b}	18.20^{b}	0.44^{a}	0.65^{c}	84.77^{c}	7.61^{a}	0.03^{a}
Population-8 Sinop-Durağan	0.04^{i}	0.01^{h}	0.47^{a}	2.37^{b}	77.00^{d}	6.22^{d}	0.03^{a}
Population-9 Bolu-Seben	19.23^{c}	9.63^{c}	0.47^{a}	0.93^{c}	79.23^{c}	7.55^{a}	0.03^{a}
Bezostaja-1	14.07^{e}	7.05^{e}	0.46^{a}	1.81^{b}	75.50^{d}	6.48^{c}	0.04^{a}
Kunduru-1149	14.22^{e}	7.15^{e}	0.49^{a}	1.87^{b}	77.72^{c}	6.57^{c}	0.04^{a}
LSD 0.05	0.75	1.05	0.19	0.96	7.40	0.24	0.12

* The same letter in the same column describes no difference otherwise there is a difference.

3.4. Soil Structure

Analysis of variance (Table 6) showed that $CaCO_3$%, active lime %, K_2O, organic matter %, P_2O_5, water saturation, and saturation with water pH were statistically significant (0.05), while EC and total salt % were not.

Large differences existed for lime amount among soil samples (Table 7). Kastamonu İhsangazi (Population-2) had the highest lime amount (59.23 %), while Sinop Durağan (Population-8) had the lowest (0.04). Active lime also differed greatly: Kastamonu İhsangazi (Population-2) soil had the highest (29.63 %), while Sinop Durağan (Population-8) had the lowest (0.01 %). Kastamonu İhsangazi (Population-3) and Samsun Ladik (Population-5) soil samples had the highest E.C. (0.60ms/cm), while Sinop Durağan (Population-6) soil sample had the lowest (0.42ms/cm). Kastamonu İhsangazi (Population-2) had the highest organic matter (3.78 %); Bolu Seben (Population-7) soil sample had the lowest (0.65 %). Karabük Safranbolu (Population-1) had the highest water saturation (127.40 %); whereas Sinop Durağan (Population-6) soil sample had the lowest (59.43 %). Kastamonu İhsangazi (Population-4) had the highest water pH (7.60); on the other hand, Sinop Durağan (Population-8) soil sample had the lowest (6.22). Karabük Safranbolu (Population-1) had the highest total salt (0.06 %); whereas Samsun Ladik (Population-5), Sinop Durağan (Population-6), Bolu Seben (Population-7), Sinop Durağan (Population-8) and Bolu Seben (Population-9) soil samples had the lowest (0.03 %).

3.5. Wheat Grain and Soil Structure Correlations

Probability theory and correlation in statistics indicate the strength and direction of the relationship between two independent variables. In widespread statistical use, the correlation shows how far the variables are away from independence. We, therefore, evaluated the results obtained from wheat grain and soil samples in the study with the correlation analysis methods as well. In this way, there existed some relationships among macro-micro elements and other properties presented as follows:

Correlations among eleven wheat entries for macro-micro elements provided a highly significant relationship for N-Cu (0.926), P-Mn (0.838), and P-K (0.788) and a significant relationship for Zn-Mn (0.634), Fe-Mn (0.532), and K-Mn (0.512). No relationships existed for N-Mn (0.400), N-Zn (0.219), N-Fe (0.449), N-P (0.049), P-Zn (0.218), P-Fe (0.389), P-Cu (0.026), K-Zn (0.275), K-Fe (0.424), Cu-Mn (0.318), Cu-Zn (0.145), Cu-Fe (0.409), and Fe-Zn (0.380). Relationships between K-N (-0.229) and Cu-K (-0.116) were, on the other hand, negative (Table 8).

Table 8. Correlations among macro-micro elements for wheat grain.

Characters	N (mg/kg)	P (mg/kg)	K (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Zn (mg/kg)
Mn (mg/kg)	0.400	0.838**	0.512	0.318	0.532	0.634*
Zn (mg/kg)	0.219	0.218	0.275	0.145	0.380	-
Fe (mg/kg)	0.449	0.389	0.424	0.409	-	
Cu (mg/kg)	0.926**	0.026	-0.116	-		
K (mg/kg)	-0.229	0.788**	-			
P (mg/kg)	0.049	-				

* Significant at 0.05, ** significant at 0.01.

In order to reduce the amount of trace metals such as cadmium in human food, a study attempted to predict the trace metal composition of cereal grains from well-chosen topsoil variables (Anonymous, 2016) They compared metal contents in grain and in topsoil samples. 198 samples of grains of winter wheat were collected from paired topsoil and crop surveys in the northern half of France. Canonical correlation analysis including multiple linear regression was used to study relationships between soil and grain data. Their findings showed an excellent regression model for grain Cd with a small number of topsoil variables, thus allowing an accurate prediction for winter wheat grains. Practically, farmers can use the prediction model to predict Cd and other micro elements such as Zn, Fe, etc. In another study Anonymous, 2013), (1) the effects of genotype, environment, and their interactions on the oil content (OC), protein content (PC), and grain yield (GY) of 25 varieties of winter wheat, (2) the correlations among these traits in different environments, and (3) the effects of different climatic variables and their interactions with wheat genotypes were studied for the examined traits. A significant positive correlation between wheat bran OC and GY existed, while highly significant negative correlations between PC and GY existed in three out of six environments.

3.6. Quality Character Correlations Among Wheat Grains

Correlation constants (r) among eleven wheat grains indicated the highest positive significant relationship between carbohydrate-starch (0.975**). Moreover, positive highly significant relationships were prevalent between energy-starch % (0.817**), energy-carbohydrate (0.762**), and crude cellulose-crude protein (0.609**). That is, the increase of one lead to the increase of the other. Insignificant relationships between raw oil-energy (0.565), 1000 KW-crude cellulose (0.504), raw ash-hectolitre (0.413), carbohydrate-raw oil (0.403), starch % - raw

oil (0.378), crude protein-hectolitre (0.353), total sugar-hectolitre (0.351), 1000 KW-crude protein (0.177), 1000 KW-energy (0.163), total sugar-raw oil (0.160), total sugar-energy (0.154), carbohydrate-total sugar (0.121), crude protein-raw ash (0.109), 1000 KW-carbohydrate (0.047), and raw oil-raw ash (0.024) existed. We also obtained negative insignificant correlation between total sugar-starch % (-0.009), crude protein-total sugar (-0.069), crude cellulose-hectolitre (-0.097), 1000 KW-hectolitre (-0.102), crude protein-energy (-0.137), raw ash-total sugar (-0.139), raw ash-crude cellulose (-0.160), raw oil-hectolitre (-0.194), 1000 KW-starch% (-0.234), 1000 KW-raw oil (-0.235), 1000 KW-raw ash (-0.245), energy-crude cellulose (-0.248), crude protein-raw oil (-0.304), raw ash-carbohydrate (-0.340), crude cellulose-raw oil (-0.346), energy-raw ash (-0.346), crude cellulose-starch % (-0.379), raw ash-starch % (-0.380), crude cellulose-carbohydrate (-0.531), 1000 KW-total sugar (-0.544), energy-hectolitre (-0.545), crude protein-starch% (-0.605), hectolitre-carbohydrate (-0.626), crude cellulose-total sugar (-0.687), hectolitre-starch % (-0.691), and crude protein-carbohydrate (-0.706). That is, the increase in one cause decrease in the other (Table 9). As known, correlation coefficient (r) changes between +1 and -1 and shows non-linear relationships between two traits under the study. Data provided were significant between only some traits such as carbohydrate-starch (0.975^{**}), energy-starch % (0.817^{**}), energy-carbohydrate (0.762^{**}), and crude cellulose-crude protein (0.609^{**}). In a semi-arid environment with agronomical and quality traits in durum wheat (*Triticum durum*) germplasm revealed a significant genotypical variance for all traits measured. The environment (year) variance was significant for all morphological and quality parameters. Genotype x environment interaction was significant for all traits except for biological yield and 1000 grain weight. Analysis of Pearson's correlation showed that most agronomical traits are inversely correlated with quality parameters.

Table 9. Quality character correlations among wheat grains.

Characters	1000 KW (gr)	Crude protein %	Energy (kcal/100 gr)	Raw ash %	Crude cellulose %	Raw oil %	Hectolitre (kg/hl)	Carbohydrate %	Starch %
Total sugar %	-0.544	-0.069	0.154	-0.139	-0.687	0.160	0.351	0.121	-0.009
Starch %	-0.234	-0.605	0.817**	-0.380	-0.379	0.378	-0.691	0.975**	-
Carbohydrate %	0.047	-0.706	0.762**	-0.340	-0.531	0.403	-0.626	-	
Hectolitre (kg/hl)	-0.102	0.353	-0.545	0.413	-0.097	-0.194	-		
Raw oil %	-0.235	-0.304	0.565	0.024	-0.346	-			
Crude cellulose %	0.504	0.609*	-0.248	-0.160	-				
Raw ash %	-0.245	0.109	-0.346	-					
Energy (kcal/100 gr)	0.163	-0.137	-						
Crude protein %	0.177	-							

* Significant at 0.05, ** significant at 0.01.

3.7. Correlations Among Soil Characteristics

3.7.1. *Correlations among soil macro-micro elements*

Correlation constants (r) among 11 soil samples for macro-micro element contents showed highly significant relationship between P_2O_5-Zn (0.910), Fe-Mn (0.802), Total N %-P_2O_5 (0,783), and total N %-Mn (0.736). The significant relationships existed between total N %-Zn (0.725), Cu-Mg (0.704), and Cu-Ca (0.608). Significant relationships were prevalent between Na-Ca (0.554), P_2O_5-Mn (0.494), Zn-Mn (0.458), Total N%-Cu (0.433), P_2O_5-Fe (0.414), Fe-Zn (0.405), Total N%-Fe (0.402), K-Fe (0.390), Ca-Mg (0.355), K-Mn (0.338), Fe-Mg (0.282), K-Mg (0.251), K-Zn (0.241), Total N%-K (0.144), Cu-Mn (0.117), P_2O_5-Cu (0.113), K-Cu (0.113), K-Ca (0.072), Na-Cu (0.062), Na-K (0.054), Cu-Zn (0.054), Total N%-Na (0.019), and Na-Mn (0.018). The interaction between these values is not significant. We obtained negative results between P_2O_5-K (-0.006), Mn-Mg (-0.006), Fe-Cu (-0.024), Total N%-Mg (-0.033), P_2O_5-Mg (-0.117), Zn-Mg (-0.137), Total N%-Ca (-0.149), Na-Mg (-0.216), Na-Zn (-0.289), Na-Fe (-0.299), Ca-Mn (-0.321), P_2O_5-Na (-0.401), P_2O_5-Ca (-0.479), Ca-Zn (-0.497), and Fe-Ca (-0.533). The results we get are negative and there is a negative relationship between them. The increase in one cause the other to decline (Table 10).

A comparison of macro- and microelement concentrations in the whole grain of four *Triticum* species revealed that five macro- and fifteen microelements in the whole grain of emmer, einkorn, spelt, and two common wheat species differed for P, Mg, Zn, Fe, Mn, Na, Cu, Sr, Rb, and Mo (Pekcan & Köksal, 2006). The grain of all hulled wheats, compared with common wheat, contained significantly more Zn (34-54 %), Fe (31-33 %), and Cu (3-28 %). In most cases, there were no relationships between the concentrations of the analysed elements, except for significant positive correlations between the levels of Fe, Zn, and Mn, in *T. monococcum* L. subsp. *monococcum* and *T. dicoccum* Schrank. A strong correlation between Zn, Fe, and Mn could have implied positive views for wheat quality breeding. The metallomic profile related to micro Zn, Fe, Cu, Mn, Ni and Cr and macro Ca, Mg and K and toxic trace elements (Cd and Pb) was obtained by ICP-AES analysis in a large set of tetraploid wheat genotypes (*Triticum turgidum* subsp. *durum* Desf.), which were grown in two different experimental fields. The significantly higher content of Mg (among the macronutrients) and the highest levels of Mn, Fe and Zn (among the micronutrients) existed for wild accessions with respect to durum cultivars. Moreover, the former genotypes were also the ones with the lowest level of accumulation of the trace toxic elements, Cd. According to the performed statistical analyses, the wild accessions also appeared to be less influenced by different environmental conditions. This is in accord with literature data, indicating the superiority of "old" with respect to modern wheat cultivars for mineral content.

3.8. Correlations Among Soil Characteristics

3.8.1. *Correlations among soil macro-micro elements*

Correlation constants (r) among 11 soil samples for macro-micro element contents showed highly significant relationships between P_2O_5-Zn (0.910^{**}), Fe-Mn (0.802^{**}), Total N %-P_2O_5 (0.783^{**}), and total N %-Mn (0.736^{**}). The significant linear relationships existed between total N % - Zn (0.725^{*}), Cu-Mg (0.704^{*}), and Cu-Ca (0.608). Higher but insignificant relationships were prevalent between Na-Ca (0.554), P_2O_5-Mn (0.494), Zn-Mn (0.458), Total N %-Cu (0.433), P_2O_5-Fe (0.414), Fe-Zn (0.405), and total N %-Fe (0.402). We obtained insignificant negative results between P_2O_5-K (-0.006), Mn-Mg (-0.006), Fe-Cu (-0.024), total N %-Mg (-0.033), P_2O_5-Mg (-0.117), Zn-Mg (-0.137), total N %-Ca (-0.149), Na-Mg (-0.216), Na-Zn (-0.289), Na-Fe (-0.299), Ca-Mn (-0.321), P_2O_5-Na (-0.401), P_2O_5-Ca (-0.479), Ca-Zn (-0.497), and Fe-Ca (-0.533) (Table 10).

Table 10. Correlations among soil macro-micro elements.

Characters	Total N %	P_2O_5 (kg/da)	Na (mg/kg)	K (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Ca (mg/kg)	Zn (mg/kg)	Mn (mg/kg)
Mg(mg/kg)	-0.033	-0.117	-0.216	0.251	0.282	0.704*	0.355	-0.137	-.006
Mn (mg/kg)	0.736**	0.494	0.018	0.338	0.802**	0.117	-0.321	0.458	-
Zn (mg/kg)	0.725*	0.910**	-0.289	0.241	0.405	0.054	-0.497	-	
Ca (mg/kg)	-0.149	-0.479	0.554	0.072	-0.533	0.608*	-		
Cu (mg/kg)	0.433	0.113	0.062	0.113	-0.024	-			
Fe (mg/kg)	0.402	0.414	-0.299	0.390	-				
K (mg/kg)	0.144	-0.006	0.054	-					
Na (mg/kg)	0.019	-0.401	-						
P_2O_5 (kg/da)	0.783**	-							

* Significant at 0.05, ** significant at 0.01.

A research (Emmanuel *et al*., 2014) to study the distribution of available macronutrients (N, P, K and S) and micronutrients (Fe, Mn, Zn, Cu) and their relationship with some physico-chemical properties of soil of different blocks showed that values of the organic C, alkaline KMnO4, extractable N, Olsen's P, neutral ammonium acetate, extractable K and $CaCl_2$, and extractable S in the district ranged between 0.13-1.64 %, 125.44-338.68 kg N ha-1, 7.34-76.70 kg P_2O_5 ha-1 , 66.08-271.04 kg K_2O ha-1 and 2.41-42.5 S ppm, respectively. The values of DTPA extractable Fe, Mn, Zn and Cu ranged between 12.42-54.06, 0.96-22.06, 0.26-4.64 and 0.59-7.62 ppm, respectively. Macronutrients in general were non-significantly and negatively correlated with pH, clay and sand, but positively correlated with organic C and silt. However, micronutrients in general were non-significantly and positively correlated with pH, organic C and clay and negatively with silt and sand. Phosphorus (-0.456*) and Fe (-0.533**) showed significantly negative relationship with pH, while phosphorus and Zn had significantly positive relation with organic C (0.335*, 0.305*) and with clay (-0.378*, 0.372*).The soils of the district were low in nitrogen, sufficient in phosphorus, potassium, and sulphur except for Sitarganj, Jaspur and Bazpur blocks were low in K and Rudrapur block was low in S; however, micronutrients (Fe, Mn, Zn, and Cu) were much higher than the critical level in the district. As mentioned above, a study, while comparing macro- and microelement concentrations in the whole grain of four *Triticum* species, all grown under identical environmental conditions, indicated that the concentrations of the investigated elements were a species-specific character.

3.9. Correlations among Other Soil Characteristics

Correlation constants among 11 soil samples revealed highly significant relationships between $CaCO_3$ % - active lime (1.000**), water saturation-total salt % (0.829**); $CaCO_3$ %- K_2O (0.750**), and active lime- K_2O (0.749**). EC-organic matter % (0.518), EC-K_2O (0.438), K_2O - organic matter % (0.408), K_2O-total salt % (0.400), EC-Ph (0.288), active lime % - EC (0.284), $CaCO_3$ % - EC (0.283), K_2O-saturation with water pH (0.252), $CaCO_3$ % - pH (0.249), $CaCO_3$ % - organic matter % (0.255), active lime %-organic matter % (0.253), active lime %- pH (0.251), pH - water saturation % (0.249), K_2O - water saturation (0.228), pH - total salt % (0.143), EC-total salt % (0.116), and organic matter % - total salt % (0.086)displayed higher but insignificant relationships. Negative insignificant correlation occurred between water saturation-EC (-0.001), $CaCO_3$%-total salt % (-0.062), active lime % - total salt % (-0.062), pH-organic matter % (-0.154), water saturation-$CaCO_3$ % (-0.224), water saturation-active lime % (-0.224), and water saturation- organic matter % (-0.310) (Table 11).

Table 11. Correlations among other soil characteristics.

Characters	CaCO %	Active lime %	E.C ms/cm	K_2O kg/da	Organic matter %	Water saturation %	Saturation with water pH
Total salt %	-0.062	-0.062	0.116	0.400	0.086	0.829**	0.143
Saturation with water pH	0.249	0.251	0.288	0.252	-0.154	0.249	-
Water saturation %	-0.224	-0.224	-0.001	0.228	-0.310	-	
Organic matter %	0.255	0.253	0.518	0.408	-		
K_2O kg/da	0.750**	0.749**	0.438	-			
E.C ms/cm	0.283	0.284	-				
Active lime %	1.000**	-					

* Significant at 0.05, ** significant at 0.01.

3.10. Grain and Soil Characteristics Dendrogram

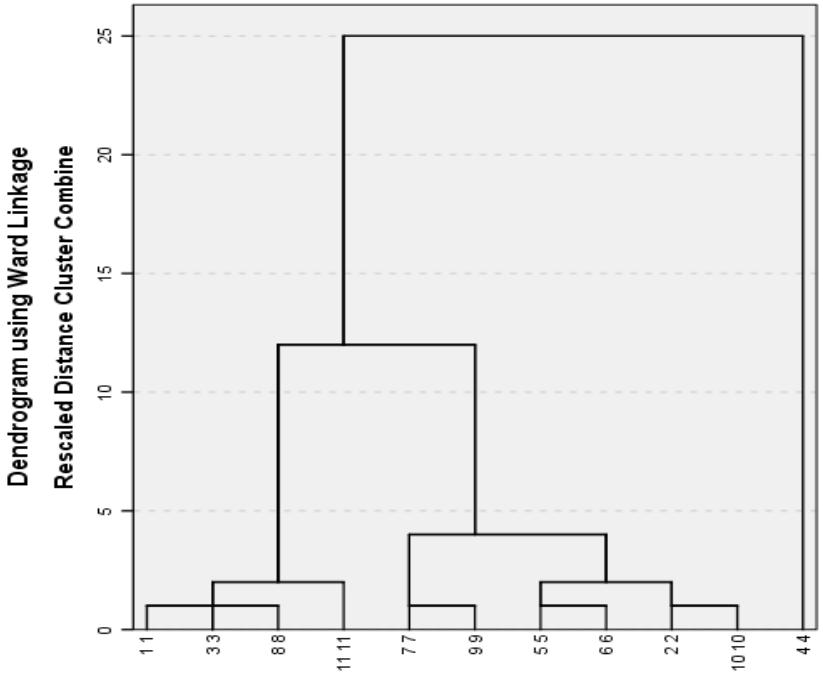
The dendrogram is a kind of clustering technique. A *dendrogram* is a diagram that shows the hierarchical relationship between objects. It is most created as an output from *hierarchical clustering*. The main use of a dendrogram is to work out the best way to allocate objects to clusters (Figure 1). Clustering analysis, on the other hand, is the process of separating information in a data set into groups according to a certain proximity criterion. Each of these groups is called a 'cluster'. The process is called clustering. The simplest definition of clustering is to distinguish data elements with similar characteristics. We, here, preferred dendrogram to establish the proximity and distance relationship of wheat populations and soil samples.

The first main group had two subgroups, while Population-4 stood alone. The first subgroup in the first main group was limited to only Population-1, Population-3, Population-8, and Kunduru-1149. The second main group was also divided into two subgroups. From these groups, the first subgroup had Population-7 and Population-9, and the second subgroup did Population-5, Population-6, Population-2, and Bezostaja-1. Population-4 was very different from the other populations for macro-micro element content and nutritional values. Population-4 was from Enbiya Village, İhsangazi, Kastamonu. It was too different from the others. Factors affecting might have been seed quality and quantity, cultivation, sowing time, harvest season, abandonment, soil, climatic conditions, and irrigation.

3.11. Soil Characteristics by Dendrogram

A dendrogram based on the average of $CaCO_3$, active lime, EC, K_2O, organic matter, P_2O_5, water saturation, saturation with water pH, total salt, total N, Na, K, Fe, Cu, Ca, Zn, Mn, and Mg in 11 soil samples formed two main groups (Figure2). The first main group consisted of five populations and the second main group consisted of two subgroups. The first main group was limited to only Population-2, Bezostaja-1, Population-7, Population-6, and Population-8. The first subgroup in the second main group consisted of Population-3 and Population-5, and the second subgroup obtained Population-9, Kunduru-1149, Population-1, and Population-4.

Figure 1. Dendogram for 11 entries of five einkorn, four emmer, one durum, and one bread wheat based on the averages of 1000 KW, crude protein, energy, raw ash, crude cellulose, raw oil, hectolitre, carbohydrate, starch %, total sugar %, macro elements (N, P, K), and micro elements (Fe, Cu, Zn, Mn).

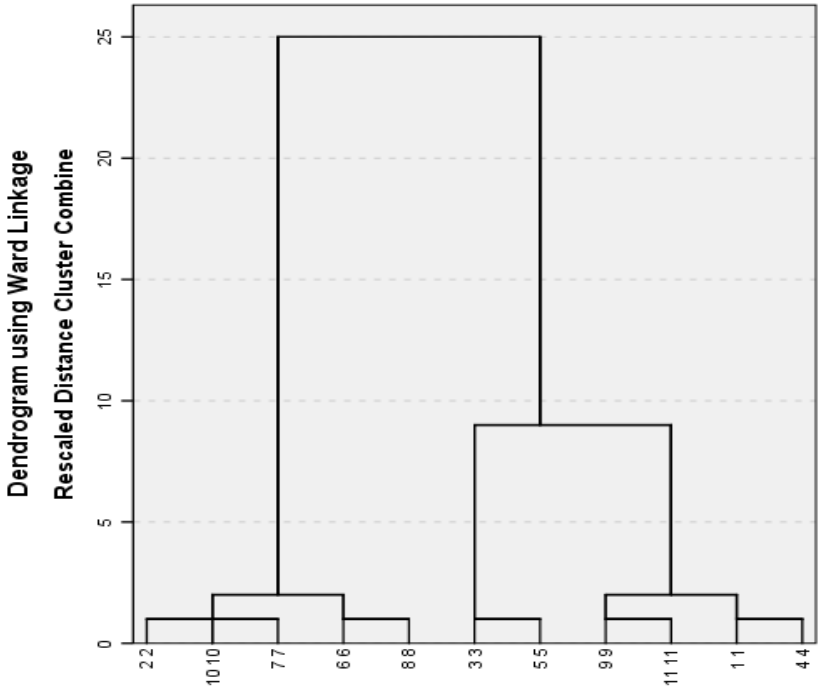


Note: 1; Population-1, 2; Population-2, 3; Population-3, 4; Population-4, 5; Population-5, 6; Population-6, 7; Population-7, 8; Population-8, 9; Population-9, 10; Bezostaja-1, 11; Kunduru-1149.

Nutrition- and health-wise wheat has advantages. Wheat samples here contained 10-16 % protein, which may provide 1/5 of daily protein requirement when 100 gr was consumed per day. If we look at the energy value provided by wheat, it provides 1/3 of our daily energy. Furthermore, fat, ash, cellulose, and nutrients, bioactive molecules, and antioxidants do exist in wheat. Therefore, wheat also is a protective food against colon cancer, constipation, and cholesterol. Crude cellulose use of wheat is beneficial for healthy nutrition of pulp builder, bowel movement regulator and enhancer with a bowel cancer and anti-constipation effect. This is important in weight loss regimes as it is a popular issue these days.

Overall, different results were obtained for macro-micro elements between einkorn, emmer, durum, and bread wheat. Old hulled einkorn and emmer wheats used not to be preferred due to their low yield and difficult harvest. However, nowadays, old hulled wheats are so popular for their health advantages. N, P_2O_5, K_2O, K, Na, Fe, Ca, Zn, Mn, and Mg were highly significantly different (p>0.05) except for Cu (Table 3). Copper richness in the samples may be due to copper mine prevalence in the Western Black Sea Region. More detailed further research, of course, will clarify the issue even better. In particular, the effects of heavy metals on the plants can be taken into consideration because of their toxicity. The tolerances of plants against heavy metals toxicity, the type and the amount of metal, its usefulness, the severity and the type of damage, and the process of damage formation may affect the development and viability of plants. Aluminium, vanadium, arsenic, mercury, lead, cadmium, and selenium are toxic against plants. Excessive accumulation of heavy metals in tissues and organs, whether or not an absolute essential element for plant growth, adversely affects the development of vegetative and generative organs of plants, animals, and humans.

Figure 2. Dendogram of 11 soil samples based on $CaCO_3$, active lime, EC, K_2O, organic matter, P_2O_5, water saturation, saturation with water pH, total salt, total N, Na, K, Fe, Cu, Ca, Zn, Mn, and Mg.



Note: 1; Population-1, 2; Population-2, 3; Population-3, 4; Population-4, 5; Population-5, 6; Population-6, 7; Population-7, 8; Population-8, 9; Population-9, 10; Bezostaja-1, 11; Kunduru-1149.

4. CONCLUSION

Culture landraces and ancient hulled wheat have recently become popular again because of their quality and health concerns raised by humans. Their broad gene pool induced by their heterozygous-heterogenous genetics structures makes their possible successful usage in plant improvement programs. Although their yields are lower and they cannot compete for yield and profitability with modern wheat varieties, their cultivation areas have been continually increasing these days due to the reasons. Many studies like this, therefore, have been carried out; many more still need to be conducted, though.

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The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Nusret Zencirci: Investigation, Resources, Visualization, Writing - original draft. Fatma **Pehlivan Karakaş:** Investigation, Formal Analysis, Writing – Review – Editing. **Bülent Ordu:** Statistical Analysis, Explanation.

Orcid

Nusret Zencirci https://orcid.org/0000-0003-3460-7575
Fatma Pehlivan Karakaş https://orcid.org/0000-0001-5245-6294
Bülent Ordu https://orcid.org/0000-0001-5245-6294

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Research Article

Molecular interactions of some phenolics with 2019-nCoV and related pathway elements

Erman Salih Istifli[1], Arzuhan Sihoglu-Tepe[2], Cengiz Sarikurkcu[3], Bektas Tepe[4,*]

[1]Cukurova University, Faculty of Science and Literature, Department of Biology, TR-01330, Adana-TURKEY
[2]Kilis 7 Aralik University, Vocational High School of Heath Services, Department of Pharmacy Services, Kilis, Turkey
[3]Afyonkarahisar Health Sciences University, Faculty of Pharmacy, Department of Analytical Chemistry, TR-03100, Afyonkarahisar-TURKEY
[4]Kilis 7 Aralik University, Faculty of Science and Literature, Department of Molecular Biology and Genetics, TR-79000, Kilis-TURKEY

Abstract: As of June 2021, the novel coronavirus disease (SARS-CoV-2) resulted in 180 million cases worldwide and resulted in the death of approximately 4 million people. However, an effective pharmaceutical with low side effects that can be used in the treatment of SARS-CoV-2 infection has not been developed yet. The aim of this computational study was to analyze the interactions of twenty-two hydroxycinnamic acid and hydroxybenzoic acid derivatives with the SARS-CoV-2 receptor binding domain (RBD) and host organism's proteases, transmembrane serine protease 2 (TMPRSS2), and cathepsin B and L (CatB/L). According to the RBCI analysis, the ligands with the highest affinity against 4 enzymes in the molecular docking study were determined as 1-caffeoyl-β-D-glucose, rosmarinic acid, 3-*p*-coumaroylquinic acid and chlorogenic acid. It has also been observed that these compounds interacted more strongly with spike RBD, CatB and CatL enzymes. Although the top-ranked ligand, 1-caffeoyl-β-D-glucose, violated the drug-likeness criteria at 1 point (NH or OH>5) and ADMET in terms of AMES toxicity, the second top-ranked ligand rosmarinic acid neither violated drug-likeness nor exhibited incompatibility in terms of ADMET. In conclusion, with its anti-inflammatory properties, rosmarinic acid can be considered and further investigated as a plant-based pharmaceutical that can offer a treatment option in SARS-CoV-2 infection. However, our findings should be supported by additional in vitro and in vivo studies.

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Cathepsins.

1. INTRODUCTION

Corona Virus Disease 2019 (COVID-19), which spread all over the world within months after emerging in China, infected more than 180 million people as of June 2021 and caused the death of approximately four million people (Wu *et al*., 2020; Zhu *et al*., 2020; Worldometers.info, 2021). The World Health Organization (WHO) defined the microorganism causing this disease as the new type of Severe Acute Respiratory Syndrome Corona Virus 2 (2019-nCoV) (Wu *et al*., 2020; Zhou *et al*., 2020; Zhu *et al*., 2020).

*CONTACT: Bektas TEPE ✉ bektastepe@yahoo.com Kilis 7 Aralik University, Faculty of Science and Literature, Department of Molecular Biology and Genetics, TR-79000, Kilis-TURKEY

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It has been determined that the genome of 2019-nCoV consists of a single-stranded RNA molecule with a length of approximately 30.000 nucleotides. As a result of genome analysis, genes encoding the four main components of the virus (nucleocapsid protein, envelope protein, membrane protein and spike glycoprotein) were identified (Wu *et al*., 2020). Among these proteins, especially the spike glycoprotein is the basic structural molecule that enables the virus to bind to receptors on the host cell surface. Therefore, it is one of the most targeted molecules in treatment development strategies against COVID-19 (Luan *et al*., 2020).

Angiotensin converting enzyme 2 (ACE2) is the most critical host component that plays a role in the binding of 2019-nCoV to the host cell. The virus binds to ACE2 via spike glycoprotein, thus ACE2 functions as the key for virus entry into the cell (Li *et al*., 2003; Li *et al*., 2005). The majority of researchers trying to develop an effective drug against COVID-19 have turned to targeting the 2019-nCoV-ACE2 interaction (Letko *et al*., 2020; Zhou *et al*., 2020). Focusing on elucidating the details of the binding interaction between the spike glycoprotein and ACE2, researchers found contact between some amino acids of the viral component in question (Leu455, Phe486, Glu493, Ser494, Asp501 and Tyr505) and mammalian ACE2 (Zakaryan *et al*., 2017; Andersen *et al*., 2020).

Of course, ACE2 is not the only molecule mediating the entry of 2019-nCoV into the host cell. In addition to this molecule, transmembrane protease, serine 2 (TMPRSS2) and cathepsins (CatB and L) were also found to assist in the entry of 2019-nCoV into the cell. TMPRSS2 is a serine protease and cleaves the spike glycoprotein from the S1/S2 site, thus facilitating virus entry and activation (Hoffmann *et al*., 2020). Cathepsins (CatB and CatL) are lysosomal endopeptidases that function in the host cell (Huang *et al*., 2006; Sudhan & Siemann, 2015; Hoffmann *et al*., 2020).

In this study, it was aimed to determine the interaction of some hydroxybenzoic and hydroxycinnamic acids with 2019-nCoV spike glycoprotein and host proteases (TMPRSS2, CatB and CatL).

2. MATERIAL and METHODS

2.1. Structural optimization of ligands

The Protein Data Bank (PDB) files of benzoic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, salicylic acid, protocatechuic acid, ferulic acid, cinnamic acid, vanillic acid, isovanillic acid, chlorogenic acid, gallic acid, syringic acid, rosmarinic acid, 1-*o*-feruloyl-β-D-glucose, 3-feruloylquinic acid, N-caffeoyl-L-aspartic acid, 1-caffeoyl-β-D-glucose, 3-*p*-coumaroylquinic acid, 6-*o*-*p*-coumaroyl-D-glucose, *p*-coumaroyltartaric acid, and chicoric acid (Figure 1) were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov). Detailed information on the structural optimization of ligands was given in the Supplementary file.

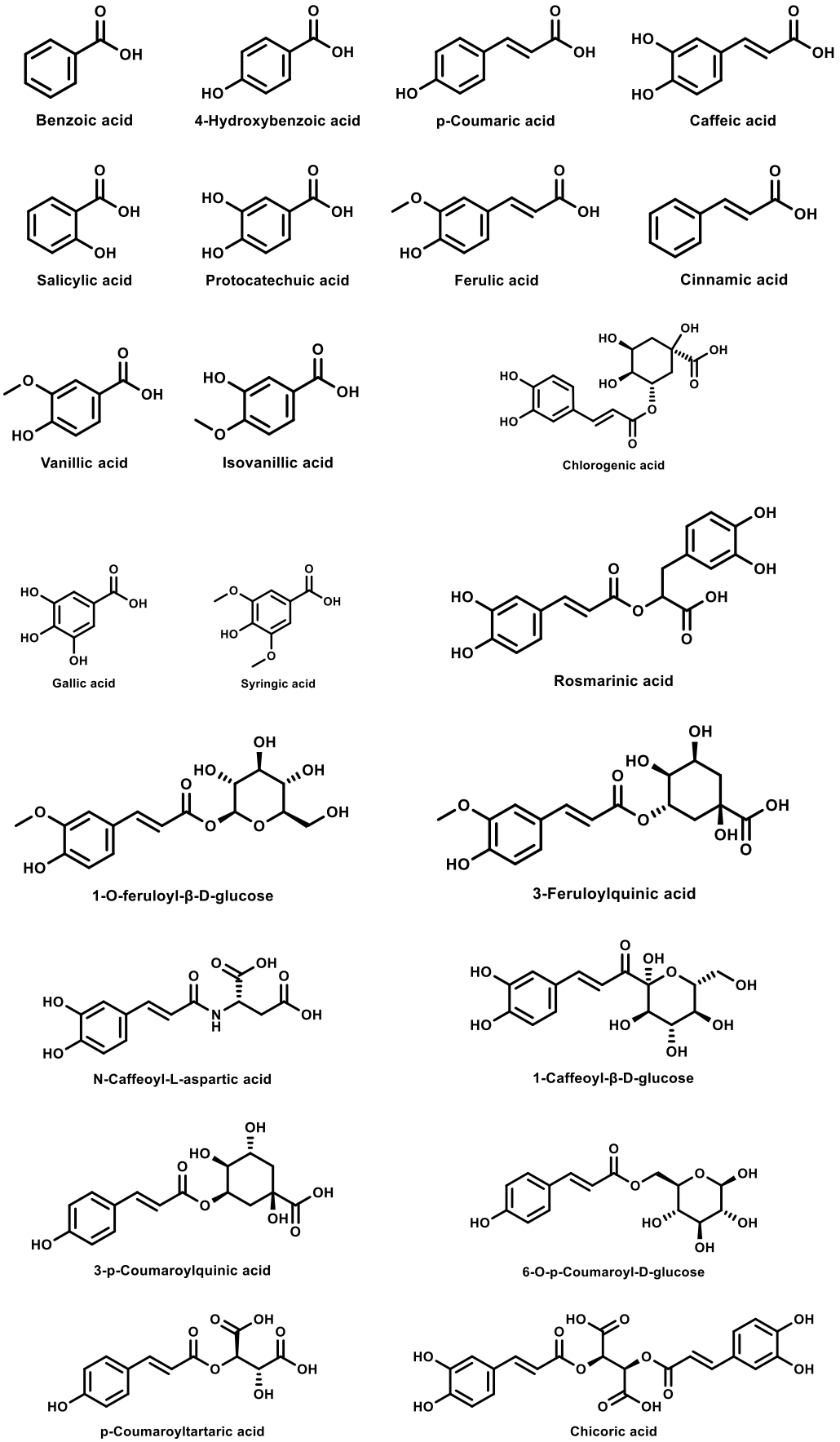
2.2. Protein retrieval and energy minimization of the Spike, TMPRSS2, CatB and CatL proteins using Nanoscale Molecular Dynamics (NAMD)

Detailed information on protein retrieval and energy minimization of the spike and other target proteins are given in the Supplementary file.

2.3. Molecular docking analyses of phenolic acids

Detailed information on molecular docking analyses of phenolic acids is given in the Supplementary file.

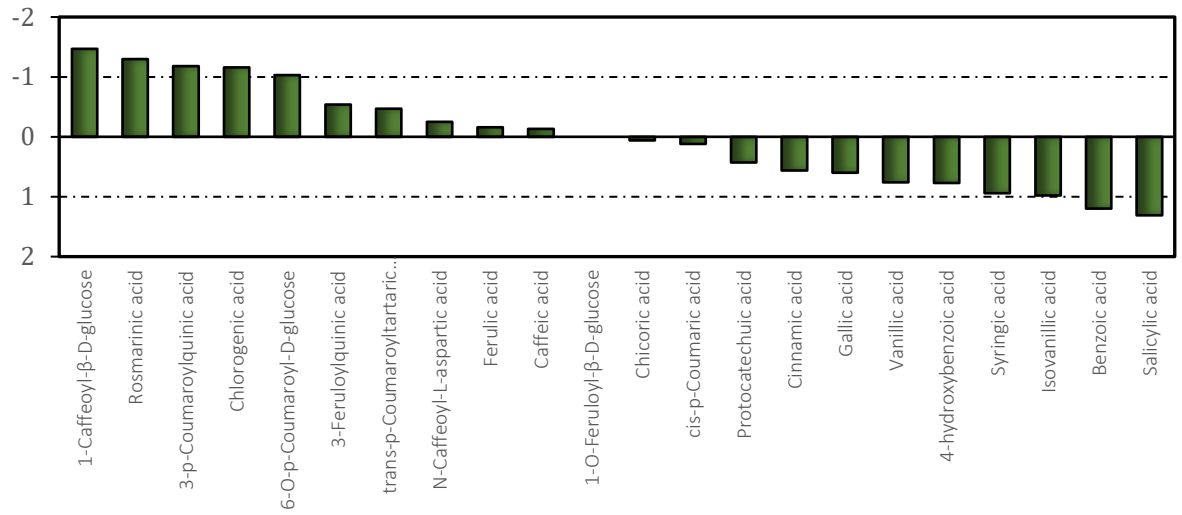
Figure 1. Chemical structures of the phenolic acids.



2.4. Calculation of relative binding capacity index (RBCI)

Detailed information on calculation of RBCI is given in the supplementary file (Figure 2 and Table S1).

Figure 2. RBCI of the phenolic acids.



2.5. Drug-likeness, ADMET profile and target prediction

Detailed information on drug-likeness, ADMET profile and target prediction is given in the Supplementary file.

2.6. Network pharmacology analysis

Detailed information on network pharmacology analysis is given in the Supplementary file.

3. RESULTS / FINDINGS

3.1. Molecular docking results of phenolic acids

In this study, the molecular level interactions of 22 phenolic acids based on molecular docking analyses with target proteins spike glycoproteins, TMPRSS2, CatB and CatL were calculated; however, based on the RBCI analysis, the non-bonded interactions of only 4 compounds (1-caffeoyl-β-D-glucose, rosmarinic acid, 3-*p*-coumaroylquinic acid and chlorogenic acid) determined as 'hit' as a result of RBCI analysis are given (Tables S2, S3, S4 and S5). On the other hand, the binding free energies (ΔG, kcal/mol) and calculated inhibition constants (Ki, μM) of the 22 phytochemicals are given in Table 1.

The interactions of 1-caffeoyl-β-D-glucose with the receptor binding motif (RBM) of spike glycoprotein are shown in (Figure 3A). 1-caffeoyl-β-D-glucose formed a total of nine H-bonds with residues Arg403, Gln409, Lys417, Tyr453, Gly496, Tyr505 and Ser494. 1-caffeoyl-β-D-glucose also formed a pi-alkyl interaction with Lys417 of the RBM (Figure 3A). The interactions of 1-caffeoyl-β-D-glucose with the active site of TMPRSS2 are given in Figure 3B. Analysis of the docking results of this compound revealed that the 1-caffeoyl-β-D-glucose formed mainly 6 H-bonds with residues Val280, His2961, Ser394, Gly439, Ser441 and an electrostatic interaction with Thr393 (Figure 3B). The molecular scale interactions of 1-caffeoyl-β-D-glucose with the active site of CatB mainly consisted of H-bonds with residues Gln231, Gly241, Gly271, Cys291, Gly72 and His197. It was also detected that 1-caffeoyl-β-D-glucose firmly fit the active site of this enzyme (Figure 3C). 1-caffeoyl-β-D-glucose formed a total of 7 H-bonds, a pi-sulfur interaction and two pi-alkyl interactions with residues Cys25, Met70, Asp71, Met161, Asp114, Ala135, Asp162 and Ala215 of CatL (Figure 3D).

Table 1. Free energy of binding and calculated inhibition constant values of the compounds.

No	Compound	Free energy of binding (kcal/mol)				Calculated inhibition constant (μM)			
		Spike RBD	TMPRSS2	CatB	CatL	Spike RBD	TMPRSS 2	CatB	CatL
1	1-Caffeoyl-β-D-glucose	-5.97	-4.71	-6.42	-7.13	42.22	354.40	19.54	5.92
2	Rosmarinic acid	-6.13	-5.10	-5.44	-6.21	31.88	181.71	103.50	28.01
3	3-*p*-Coumaroylquinic acid	-6.38	-4.57	-5.96	-6.31	21.14	449.38	43	23.78
4	Chlorogenic acid	-5.78	-4.92	-6.19	-5.90	57.51	246.66	29.23	47.02
5	6-*o*-*p*-Coumaroyl-D-glucose	-4.87	-4.79	-6.30	-6.75	268.59	310.29	24.16	11.25
6	3-Feruloylquinic acid	-5.61	-4.46	-5.10	-5.90	76.70	534.14	184.15	47.29
7	*p*-Coumaroyltartaric acid	-5.47	-5.16	-4.24	-4.82	97.13	163.79	780.79	290.71
8	N-Caffeoyl-L-aspartic acid	-4.64	-5.16	-4.57	-4.75	395.03	165.48	449.57	328.05
9	Ferulic acid	-4.66	-4.61	-4.67	-5.66	385.89	418.48	378.67	70.64
10	3,4-Dihydroxycinnamic acid	-4.45	-4.69	-4.44	-5.78	544.16	362.04	556.84	57.82
11	1-*o*-Feruloyl-β-D-glucose	-4.83	-4.02	-5.55	-5.52	288.37	1140	85.52	89.98
12	Chicoric acid	-5.63	-4.32	-4.31	-4.66	74.48	675.85	690.09	386.65
13	*p*-Coumaric acid	-4.42	-4.57	-4.02	-5.56	577.62	447.42	1130	83.34
14	Protocatechuic acid	-4.30	-4.75	-3.60	-4.49	706.93	329.83	2310	507.99
15	Cinnamic acid	-4.42	-4.14	-3.80	-5.17	573.77	925.57	1650	162.17
16	Gallic acid	-4.29	-4.61	-3.45	-4.34	722.07	418.15	2970	657.23
17	Vanillic acid	-4.28	-4.33	-3.61	-4.30	727.49	669.40	2270	699.55
18	4-hydroxybenzoic acid	-4.19	-4.42	-3.45	-4.27	846.04	572.24	2970	738.17
19	Syringic acid	-4.21	-4.07	-3.83	-4.14	817.59	1040	1560	918.80
20	Isovanillic acid	-4.20	-4.14	-3.41	-4.20	839.55	915.66	3190	828.60
21	Benzoic acid	-4.15	-3.91	-3.42	-4.00	906.77	1370	3130	1170
22	Salicylic acid	-3.71	-3.96	-3.29	-4.12	1910	1240	3910	961.79

The favorable interactions of rosmarinic acid with the active site (RBM) of spike protein mainly consisted of 6 H-bonds and one pi-alkyl interaction with the residues Arg403, Tyr449, Ser494, Gly496, Gln498 and Tyr505 (Figure 4A). The rosmarinic acid-TMPRSS2 interactions included a total of 4 conventional, 2 carbon-hydrogen and one pi-donor H-bonds with residues His279, Val280, Thr393, Ser460, Gly462 and Ser441. Rosmarinic acid also formed hydrophobic contacts with His296 and Thr293 of TMPRSS2 (Figure 4B). Rosmarinic acid interacted with Gln23, Gly24, Cys29, Asn70, His108, Gly119, Glu120, Gly196 and His197 residues of CatB via a total of 9 H-bonds (Figure 4C). The binding interactions of rosmarinic acid in complex with CatL were mainly consisted of a total of 4 H-bonds with the residues Gly68, Met70, Lys117 and Ser213, an electrostatic interaction with Asp71, two pi-sigma interactions with Ala135 and 214, one pi-sulfur interaction with Met70, and two hydrophobic amide-pi stacked and pi-alkyl interactions with Ala214, 215 and Leu69, respectively (Figure 4D).

Figure 3. Top ranked conformations of 1-caffeoyl-β-D-glucose (A- RBM of the spike glycoprotein of SARS-CoV-2, B- TMPRSS2, C- CatB, D- CatL).

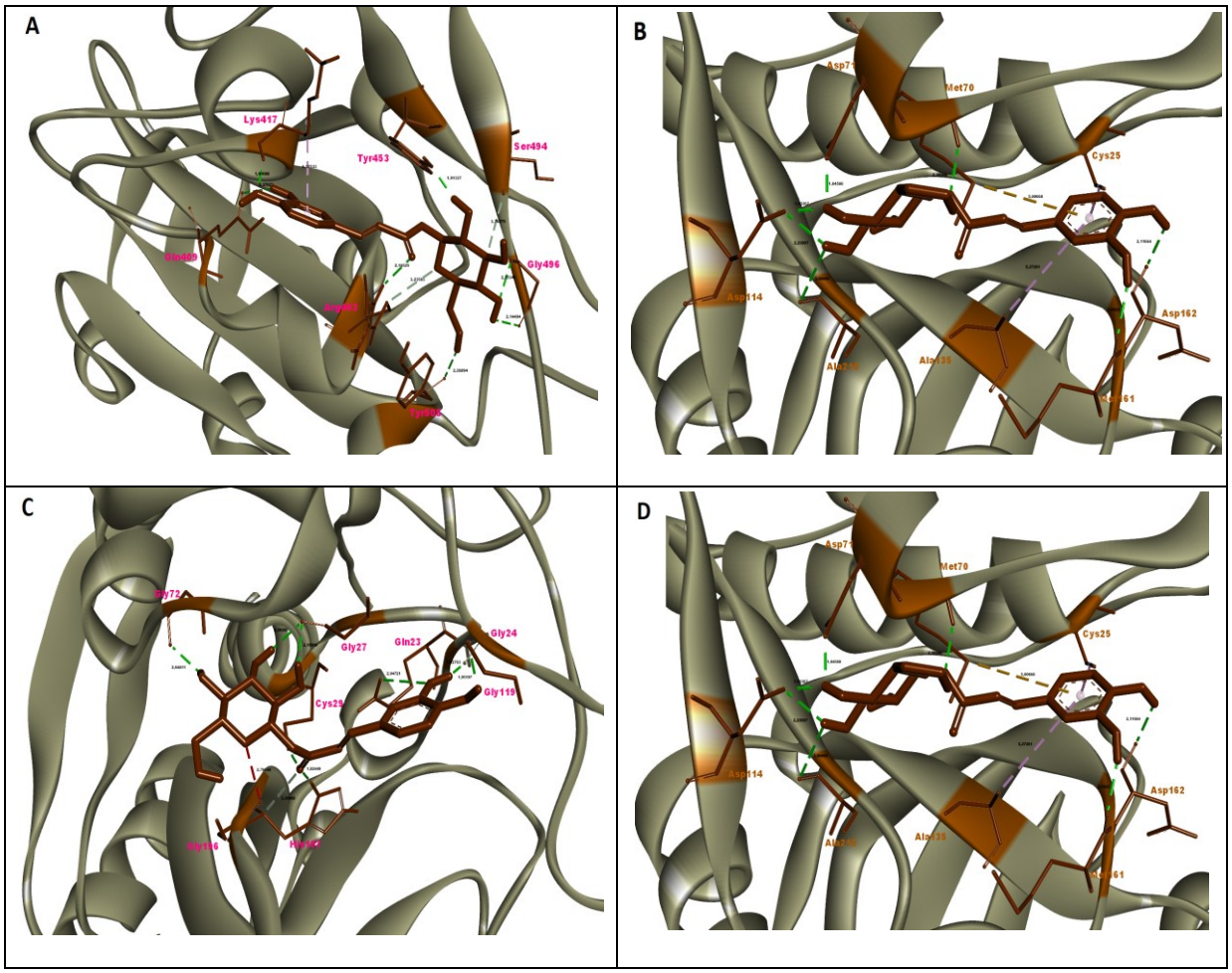
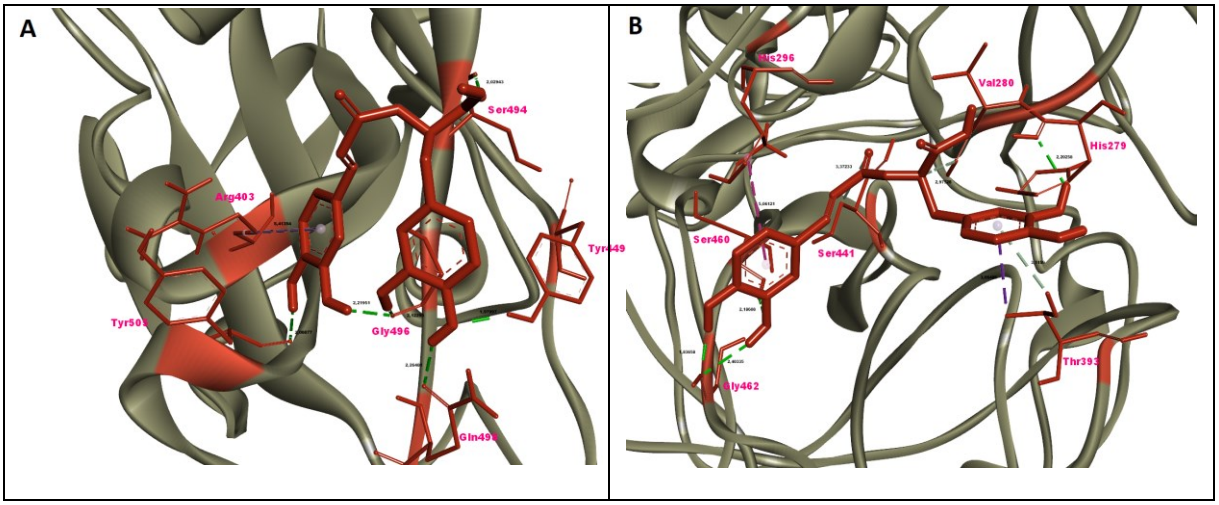
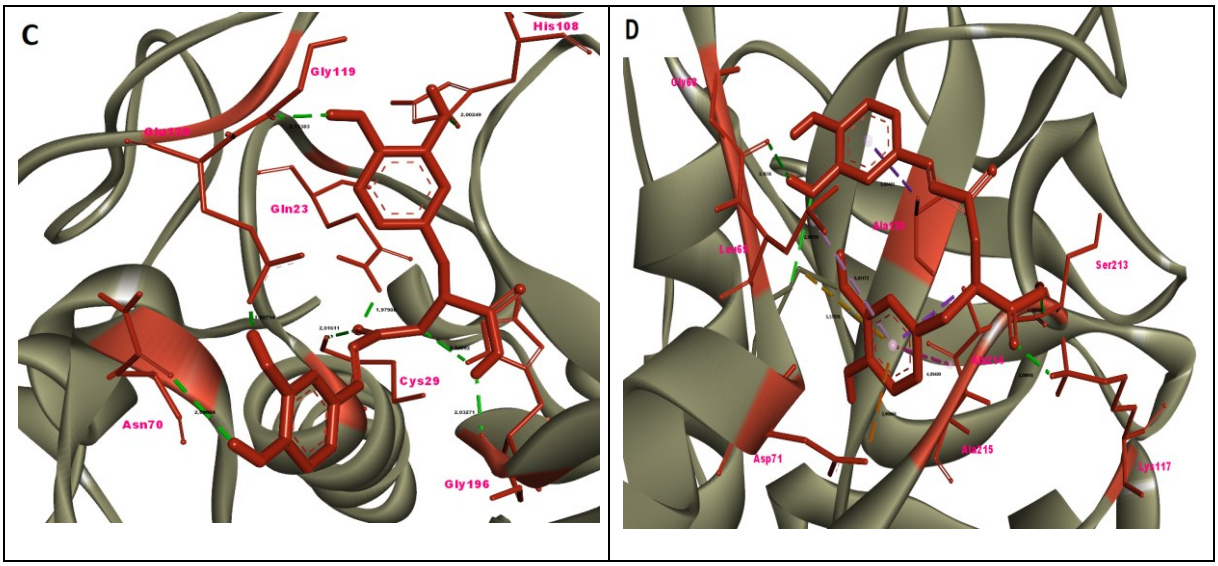


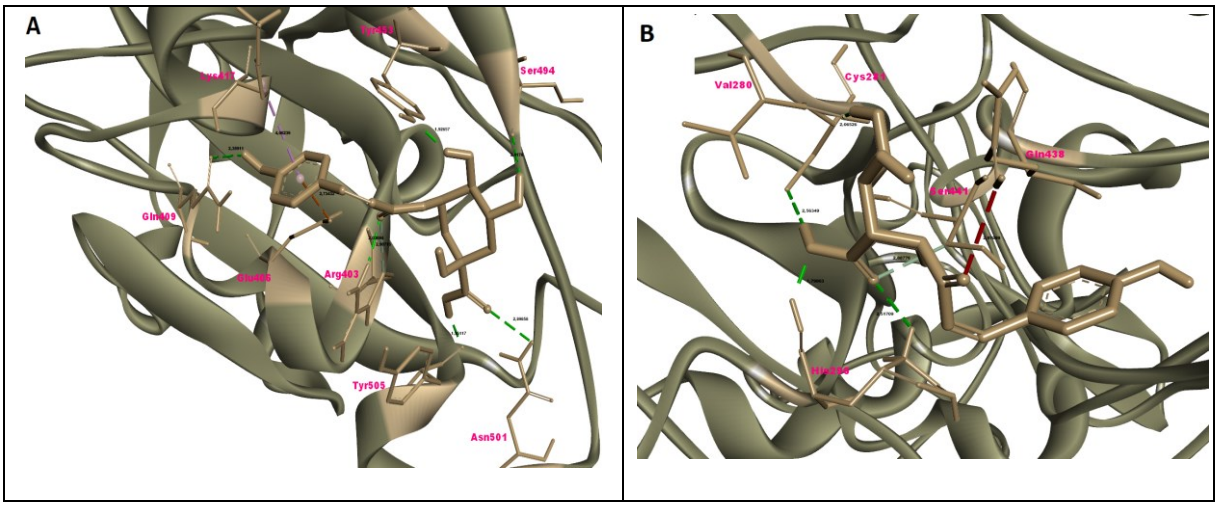
Figure 4. Top ranked conformations of rosmarinic acid (A- RBM of the spike glycoprotein of SARS-CoV-2, B- TMPRSS2, C- CatB, D- CatL).

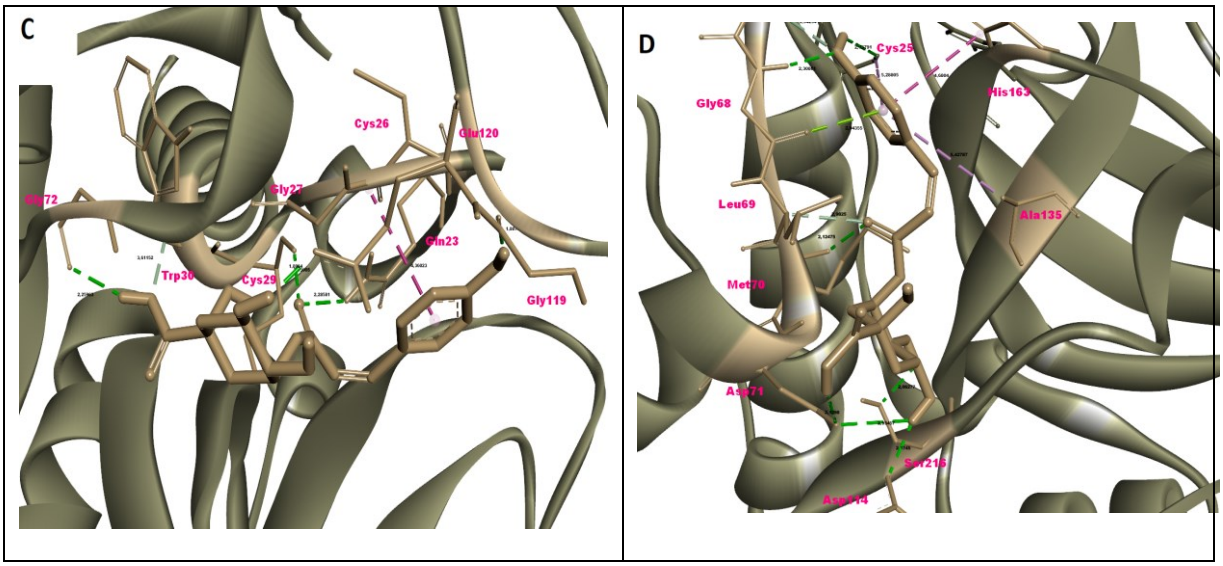




3-*p*-coumaroylquinic acid formed a total of 7 H-bonds with residues Arg403, Gln409, Tyr453, Ser494, Asn501 and Tyr505, one electrostatic interaction with Glu406 and one pi-alkyl interaction with Lys417 of RBM of the spike glycoprotein (Figure 5A). Analysis of the interactions of 3-p-coumaroylquinic acid with TMPRSS2 revealed that the compound in question mainly formed 5 H-bonds with residues Val280, Cys281, His296 and Ser441 (Figure 5B). Figure 5C depicts the binding mode of 3-*p*-coumaroylquinic acid with CatB enzyme. 3-*p*-coumaroylquinic acid interacts with Gln23, Cys29, Trp30, Gly72, Gly119 and Glu120 residues via a total of 7 H-bonds and forms hydrophobic contact with Cys26 and Gly27 (Figure 5C). 3-*p*-coumaroylquinic acid interacts with Cys25, Gly67, Gly68, Leu69, Met70, Asp71, Asp114 and Ser216 residues of CatL via a total of 9 H-bonds. It also shows hydrophobic contacts with Cys25, Ala135, Asp162 and His163 residues of the enzyme (Figure 5D).

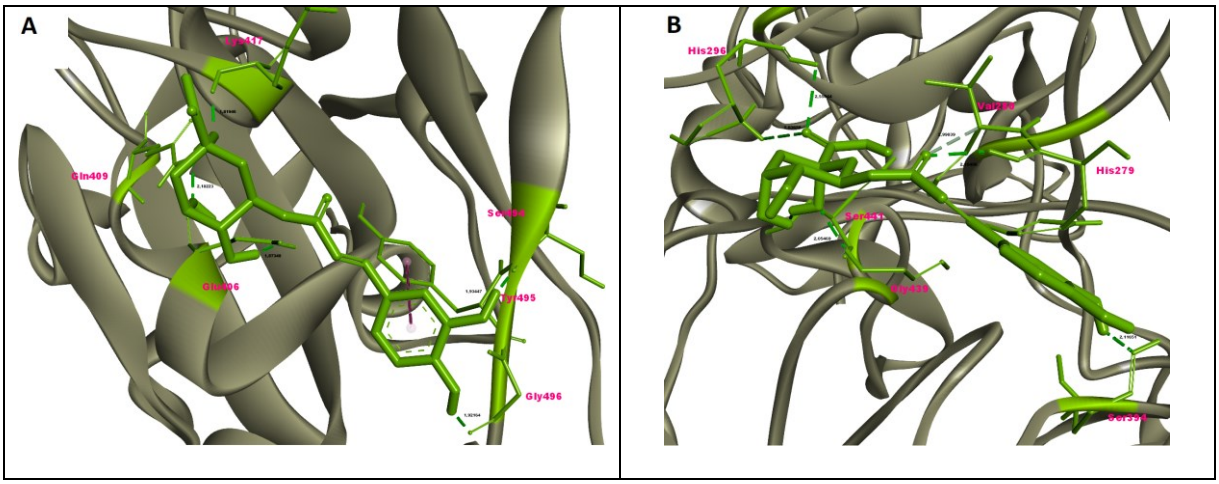
Figure 5. Top ranked conformations of 3-*p*-coumaroylquinic-acid (A- RBM of the spike glycoprotein of SARS-CoV-2, B- TMPRSS2, C- CatB, D- CatL).

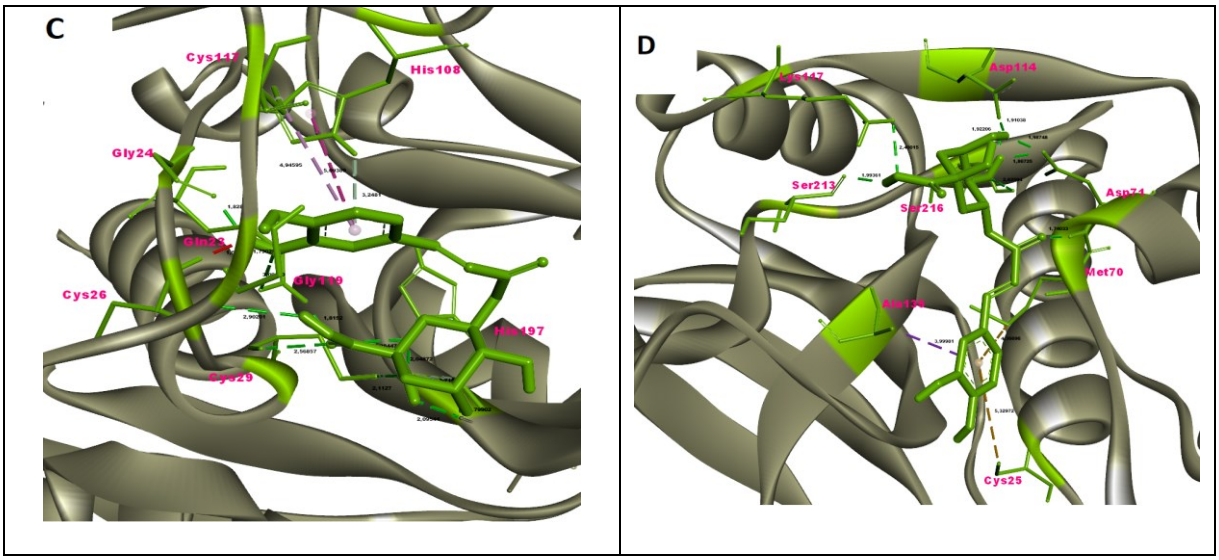




The interactions of chlorogenic acid with the active site of spike glycoprotein mainly consists of 5 H-bonds with residues Glu406, Gln409, Lys417, Ser494 and Gly496 as well as a hydrophobic pi-pi interaction with Tyr495 (Figure 6A). Chlorogenic acid formed a total of 7 H-bonds with the active site residues His279, Val280, His296, Ser394, Gly439 and Ser441 of TMPRSS2 (Figure 6B). Chlorogenic acid-CatB interactions included a total of 13 H-bonds with residues Gln23, Gly24, Cys26, Cys29, His108, Gly119, Gly196 and His197. Chlorogenic acid also formed two hydrophobic interactions with His108 and Cys117 (Figure 6C). The binding interactions of chlorogenic acid in complex with CatL showed a total of 8 H-bonds with residues Asp71, Asp114, Lys117, Ser213 and Ser216 (Figure 6D). Chlorogenic acid formed a hydrophobic pi-sigma interaction with Ala135 and two pi-sulfur interactions with Cys25 and Met70.

Figure 6. Top ranked conformations of chlorogenic acid (A- RBM of the spike glycoprotein of SARS-CoV-2, B- TMPRSS2, C- CatB, D- CatL).





As a result, hydrogen bonds constitute the greatest contribution to the interactions of the top-ranked 4 ligands with their receptors in molecular docking analyzes.

3.2. RBCI values of phenolic acids

In this study, molecular scale interactions between 22 different phenolic acids and 4 different protein targets were investigated using molecular docking analysis. As a result of molecular docking calculations, the binding free energy values (kcal/mol) of each phytochemical against 4 proteins in question were used to calculate the relative binding capacity index (RBCI). The details of this method have been explained in detail in the 'Materials and methods' section. Using the RBCI value, the efficiency of phytochemicals on different proteins can be calculated using different data sets at the same time (Figure 2 and Table S1).

As a result of the RBCI analysis we applied, it was determined that 1-caffeoyl-β-D-glucose, rosmarinic acid, 3-*p*-coumaroylquinic acid and chlorogenic acid were the 'hit' compounds among 22 phytochemicals. Top ranked poses of 1-caffeoyl-β-D-glucose, rosmarinic acid, 3-p-coumaroylquinic acid and chlorogenic acid on the RBD of spike glycoprotein, TMPRSS2, CatB and CatL are presented in Figures 3, 4, 5, and 6, respectively. RBCI analysis also revealed that isovanillic acid, benzoic acid and salicylic acid were the ligands with the weakest affinity for 4 different protein targets.

3.3. Pharmacokinetic properties of phenolic acids

Drug-likeness, ADMET and target profiles of analyzed phenolic acids against spike glycoprotein, TMPRSS2, CatB and CatL are given in Tables 2 and 3, respectively. Except for chicoric acid, all phenolic acids were determined to obey the Lipinski's rule-of-five. Chicoric acid violates this rule because it has N or O > 10, or NH or OH > 5. It was determined that no ligand other than ferulic acid, *p*-coumaric acid, cinnamic acid, 4-hydroxybenzoic acid, benzoic acid and salicylic acid could pass blood-brain barrier (BBB). It has also been determined that no compound other than chicoric acid is a substrate of P-glycoprotein (P-gp). Protocatechuic acid and gallic acid were observed to inhibit CYP3A4, while no other compounds showed an inhibitory effect on cytochrome enzymes. While 1-caffeoyl-β-D-glucose, 6-*o*-*p*-coumaroyl-D-glucose and 1-*o*-feruloyl-β-D-glucose show mutagenicity in the AMES bacterial test system, no other phenolic acid studied showed AMES toxicity. On the other hand, only *p*-coumaroyltartaric acid and N-caffeoyl-L-aspartic acid showed hepatotoxic effect data among 22 ligands studied. The LD_{50} doses of the molecules in the rat ranged from 1.737 mol/kg to 2.811 mol/kg. While the most toxic molecule in the rat was 3-*p*-coumaroylquinic acid (1.737 mol/kg), the phytochemical with the least toxicity was rosmarinic acid (2.811 mol/kg).

Table 2. Drug-likeness properties of docked phenolic acids.

No	Compound	Number of rotatable bonds	TPSA[1]	Consensus Log P	Log S (ESOL[2])	Drug likeness (Lipinski's rule of five)
1	1-Caffeoyl-β-D-glucose	5	156.91	-0.98	-1.41	Yes; 1 violation: NH or OH>5
2	Rosmarinic acid	7	144.52	1.52	-3.44	Yes; 0 violation
3	3-p-Coumaroylquinic acid	5	144.52	-0.03	-1.75	Yes; 0 violation
4	Chlorogenic acid	5	164.75	-0.38	-1.62	Yes; 1 violation: NH or OH>5
5	6-O-p-Coumaroyl-D-glucose	5	136.68	-0.52	-1.20	Yes; 0 violation
6	3-Feruloylquinic acid	6	153.75	0.01	-1.84	Yes; 0 violation
7	p-Coumaroyltartaric acid	7	147.02	-0.42	-1.68	Yes; 0 violation
8	N-Caffeoyl-L-aspartic acid	7	144.16	0.05	-1.45	Yes; 0 violation
9	Ferulic acid	3	66.76	1.36	-2.11	Yes; 0 violation
10	3,4-Dihydroxycinnamic acid	2	77.76	0.93	-1.89	Yes; 0 violation
11	1-O-Feruloyl-β-D-glucose	6	145.91	-0.37	-1.64	Yes; 0 violation
12	Chicoric acid	11	208.12	1.01	-3.58	No; 2 violations: N or O>10, NH or OH>5
13	p-Coumaric acid	2	57.53	1.26	-2.02	Yes; 0 violation
14	Protocatechuic acid	1	77.76	0.65	-1.86	Yes; 0 violation
15	Cinnamic acid	2	37.30	1.79	-2.37	Yes; 0 violation
16	Gallic acid	1	97.99	0.21	-1.64	Yes; 0 violation
17	Vanillic acid	2	66.76	1.08	-2.02	Yes; 0 violation
18	4-hydroxybenzoic acid	1	57.53	1.05	-2.07	Yes; 0 violation
19	Syringic acid	3	75.99	1.02	-1.84	Yes; 0 violation
20	Isovanillic acid	2	66.76	1.07	-2.02	Yes; 0 violation
21	Benzoic acid	1	37.30	1.44	-2.20	Yes; 0 violation
22	Salicylic acid	1	57.53	1.24	-2.50	Yes; 0 violation

[1] TPSA: Topological polar surface area (Å^2)

[2] ESOL: Estimated aqueous solubility [(Insoluble < -10 < Poorly < -6 < Moderately < -4 < Soluble < -2 Very < 0 < Highly), according to Delaney, J.S. (2004)].

Data source: http://www.swissadme.ch/index.php#

3.4. Target predictions and network pharmacology of hit phenolic acids

In this study, the screening of hit phytochemicals against possible intracellular targets was performed using Swiss Target Prediction (http://www.swisstargetprediction.ch/) and STITCH 5.0 (http://stitch.embl.de/) public databases.

In Figure 7A, the intracellular targets of 1-caffeoyl-β-D-glucose are given. According to Figure 7A, various enzymes (28%), lyases (18%) and proteases (16%) appear to make up the majority of the intracellular targets of 1-caffeoyl-β-D-glucose. However, the interaction probability of 1-caffeoyl-β-D-glucose for these enzyme groups was found to be no more than 10% (p = 0.1). Therefore, the interaction of this phytochemical with possible intracellular targets is not statistically significant.

Table 3. ADMET profiles of phenolic acids.

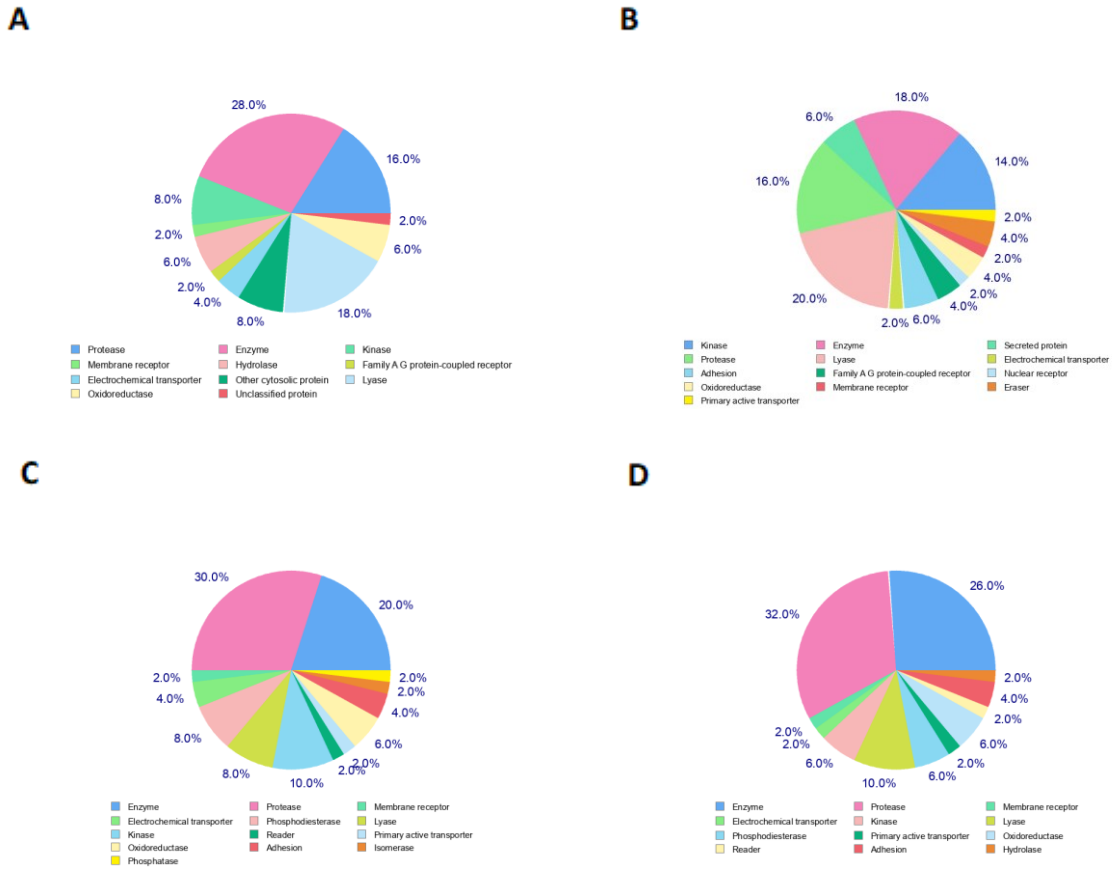
No	Compound	BBB permeation[1,*]	P-gp substrate[2,*]	CYP inhibition[3,*]	AMES Toxicity[4]	Hepatotoxicity[4]	LD_{50} in rat (mol/kg)[4]
1	1-Caffeoyl-β-D-glucose	No	No	No	Yes	No	1.938
2	Rosmarinic acid	No	No	No	No	No	2.811
3	3-p-Coumaroylquinic acid	No	No	No	No	No	1.737
4	Chlorogenic acid	No	No	No	No	No	1.973
5	6-O-p-Coumaroyl-D-glucose	No	No	No	Yes	No	1.933
6	3-Feruloylquinic acid	No	No	No	No	No	2.025
7	p-Coumaroyltartaric acid	No	No	No	No	Yes	2.295
8	N-Caffeoyl-L-aspartic acid	No	No	No	No	Yes	2.084
9	Ferulic acid	Yes	No	No	No	No	2.282
10	3,4-Dihydroxycinnamic acid	No	No	No	No	No	2.383
11	1-O-Feruloyl-β-D-glucose	No	No	No	Yes	No	2.157
12	Chicoric acid	No	Yes	No	No	No	2.445
13	p-Coumaric acid	Yes	No	No	No	No	2.155
14	Protocatechuic acid	No	No	CYP3A4	No	No	2.423
15	Cinnamic acid	Yes	No	No	No	No	2.094
16	Gallic acid	No	No	CYP3A4	No	No	2.218
17	Vanillic acid	No	No	No	No	No	2.454
18	4-hydroxybenzoic acid	Yes	No	No	No	No	2.255
19	Syringic acid	No	No	No	No	No	2.157
20	Isovanillic acid	No	No	No	No	No	2.487
21	Benzoic acid	Yes	No	No	No	No	2.170
22	Salicylic acid	Yes	No	No	No	No	2.282

[1] BBB: Blood Brain Barrier
[2] P-gp: P-glycoprotein substrate
[3] CYP: Cytochrome P
[4] http://biosig.unimelb.edu.au/pkcsm/prediction
[*] https://www.swissadme.ch

Figure 7B shows that possible intracellular targets of rosmarinic acid comprise lyases (20%), various enzymes (18%), proteases (16%), and kinases (14%). Quite impressively, rosmarinic acid is likely to interact with each of these targets at 96% (p = 0.96), a statistically very significant value. This finding suggests that rosmarinic acid exerts possible inhibitory/activator effect on these enzyme groups. Most of the intracellular targets of 3-*p*-coumaroylquinic acid consist of proteases (30%), various enzymes (20%) and kinases (10%) (Figure 7C). 3-*p*-coumaroylquinic interacts with these targets at a statistically insignificant probability level (p = 0.10 - 0.41).

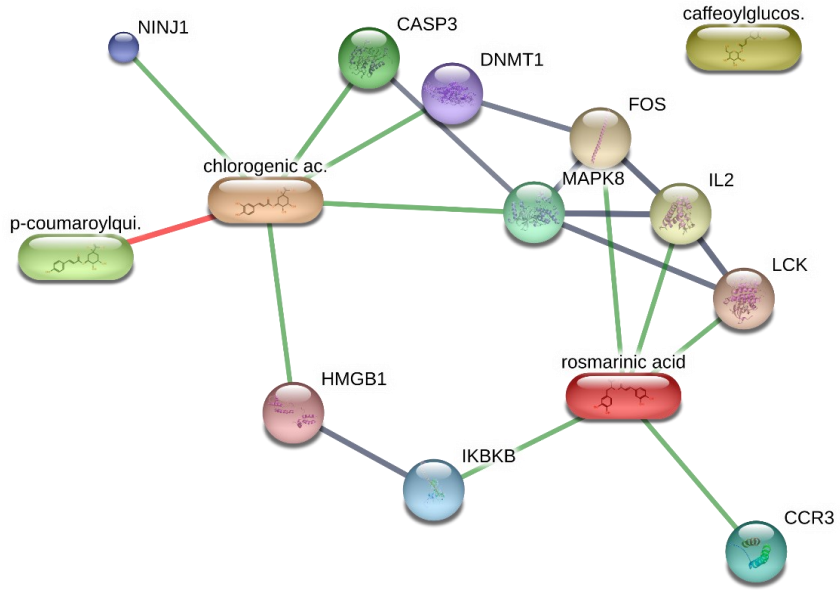
Intracellular targets of chlorogenic acid generally consist of proteases (32%), various enzymes (26%) and lyases (10%) (Figure 7D). Although chlorogenic acid shows a statistically insignificant level of probability (p = 0.09 - 0.33) in terms of interaction with the proteases and lyases, its interaction with various intracellular enzymes appears slightly significant (p = 0.77 - 0.80).

Figure 7. Target prediction of A - 1-caffeoyl-β-D-glucose B - rosmarinic acid, C - 3-*p*-coumaroylquinic acid, D - chlorogenic acid.



The STITCH platform was used to predict putative targets of hit phenolic acids in the human proteome. Thus, the direct interactions of 1-caffeoyl-β-D-glucose, rosmarinic acid, 3-p-coumaroylquinic acid and chlorogenic acid with different proteins were mapped (Figure 8). Prior to mapping target-component interactions, the minimum required interaction score was set to a high confidence score which was ≥ 0.7. A high confidence score indicates a strong interaction between hit phytochemicals and protein(s). According to the targets-components interaction network in Figure 8, it can be observed that 2 ligands other than rosmarinic acid and chlorogenic acid do not directly interact with the human proteome. Rosmarinic acid interacts with FOS, IL2, LCK, CCR3 and IKBKB proteins, while chlorogenic acid interacts with NINJ1, CASP3, DNMT1, MAPK8 and HMGB1 proteins. For rosmarinic acid, it is noteworthy that kinases (LCK, IKBKB) play a central role among protein targets predicted by both SwissTargetPrediction and STITCH platforms (Figures 7A, 7B, 7C, 7D and Figure 8).

Figure 8. Targets-components analysis (chemical-protein interactions) of top 4 hit phytochemicals performed via the STITCH platform (http://stitch.embl.de). Note the explicit role of rosmarinic acid in the targets-components network.



4. DISCUSSION and CONCLUSION

Although the novel coronavirus disease (SARS-CoV-2), which emerged in Wuhan in 2019, affected the whole world as a pandemic in a short time, an effective drug molecule has not yet been found in the treatment of this virus. Therefore, a safe orally administered pharmaceutical would be a breakthrough in the treatment of SARS-CoV-2. In this context, organic molecules of plant origin always maintain their potential among possible treatment options against viral diseases (Chavez *et al*., 2006; Ruibo *et al*., 2017; Taguchi *et al*., 2017; Wu *et al*., 2017; Jahan & Onay, 2020; Piccolella *et al*., 2020; Cano-Avendaño *et al*., 2021). On the other hand, although herbal compounds are biologically diverse and accessible, drug discovery is an arduous process and molecular docking-based bioinformatics approach has become an increasingly important tool in this field in recent years (Meng *et al*., 2011).

In the present study, the molecular interactions of twenty-two polyphenolic compounds, derivatives of hydroxycinnamic acid and hydroxybenzoic acid (Table 1, Figure 1), with the receptor binding domain (RBD) of SARS-CoV-2 spike glycoprotein, TMPRSS2, CatB and CatL were analyzed. According to the RBCI analysis we applied, it was determined that 1-Caffeoyl-β-D-glucose was the phytochemical with the highest affinity (binding free energy) against all target proteins (Table 1). There is no docking or molecular dynamic study of 1-Caffeoyl-β-D-glucose with the four target proteins (spike RBD, TMPRSS2, CatB and CatL) in the literature. Therefore, our results regarding this ligand constitute the first record in terms of literature. In our study, the second top-ranked ligand, rosmarinic acid, showed a high binding affinity for spike, TMPRSS2, CatB and CatL proteins (-6,13, -5,10, -5,44 and -6,21 kcal/mol, respectively) (Table 1). Although there is no other study showing the binding affinity of rosmarinic acid against spike protein, this ligand was found to show a strong binding (MolDock score of -89.17) against the host cell furin (a protein convertase) enzyme that the spike protein of SARS-CoV-2 uses to enter the cell using the Molegro Virtual Docker program (Kumar Verma *et al*., 2021). On the other hand, in support of our study, it was reported in a different study using the Glide XP docking program that rosmarinic acid showed a reasonably high binding affinity (-5.6 kcal/mol) for the TMPRSS2 enzyme (Coban *et al*., 2021). However, the

molecular docking study of rosmarinic acid regarding CatB and CatL enzymes has not been found in the literature. Regarding 3-*p*-coumaroylquinic acid (Table 1), the third promising ligand in this study, no molecular docking studies on spike, TMPRSS2, CatB or CatL enzymes were found in the literature. However, it has been reported that 3-*p*-coumaroylquinic acid showed a very favorable binding affinity (-7.2 kcal/mol) against non-structural protein 10 (nsp10) of SARS-CoV-2 in molecular docking simulation (Mohammad *et al*., 2021). This result supports our finding we obtained with SARS-CoV-2 spike glycoprotein. In a molecular docking study conducted with the post fusion core of the SARS-CoV-2 spike S2 subunit, spike glycoprotein open state and spike glycoprotein closed state structures, chlorogenic acid, our 4th top-ranked ligand (Table 1), has been reported to bind to these different spike protein states with high affinities (-101.663, - 108,993 and -92,121 MolDock scores) (Adem *et al*., 2021). These results are also in agreement with our study.

In addition, there are molecular docking studies reporting that gallic acid, *p*-coumaric acid, ferulic acid, chicoric acid, cinnamic acid and 4-hydroxybenzoic acid, the other polyphenolic phytochemicals in our study, show high binding affinity for spike, TMPRSS2, CatB and CatL enzymes (Georgousaki *et al*., 2020; Adem *et al*., 2021; Guler *et al*., 2021; Srivastava *et al*., 2021; Surucic *et al*., 2021).

In this present study, 1-caffeoyl-β-D-glucose, rosmarinic acid, 3-*p*-coumaroylquinic acid, and chlorogenic acid, which we determined as the first four top-ranked ligands, have drug-likeness properties according to Lipinski's Rule of Five (1-caffeoyl-β -D-glucose 1 violation [NH or OH>5]; chlorogenic acid 1 violation [NH or OH>5]). 6-tri-*o*-caffeoyl-β-D-glucopyranose (TCGP), an isomer of 1-caffeoyl-β-D-glucose isomer, has been reported to effectively inhibit the entry of HIV-1 Env pseudovirus into host cells (IC_{50} = 5.5 µg/mL) (Dong *et al*., 2013). Therefore, although the spatial arrangement of the atoms of these two isomers are different, it can be expected that these two close isomers will show similar antiviral activity. It has been also shown that rosmarinic acid and chlorogenic acid have wide-ranging pharmacological effects on the inflammatory response, tumor occurrence and development, antioxidant, antimicrobial, anti-inflammatory and hepatoprotective effects in the host organism (Maalik *et al*., 2016; Guan *et al*., 2021). In general, it is obvious that the ligands in our study have various pharmacological effects (Table 2). However, considering the ADMET profiles, 1-caffeoyl-β-D-glucose was positive in the bacterial gene mutation test (AMES test), indicating that 1-caffeoyl-β-D-glucose may be a potential mutagen (Table 3).

Although our top-four ligands generally interact with cellular enzymes, lyases, proteases and kinases according to SwissTarget analysis (Figures 7A, 7B, 7C and 7D), based on probability analysis, only rosmarinic acid showed potential for interaction (p = 0.96) with its putative targets (lyases, various enzymes, proteases and kinases). Interestingly, it should be noted that rosmarinic acid showed an anti-inflammatory effect by inhibiting the PI3K-Akt pathway stimulated by IL2 in mice gastric cells (Nam *et al*., 2020) and by inhibiting the kinase IKK-β (inhibitor of nuclear factor kappa-B kinase subunit beta) activity in human dermal fibroblasts (Lee *et al*., 2006). This reported that inhibitory potential of rosmarinic acid on interleukins and kinases in the inflammatory pathway is in high agreement with the 'targets-components analysis' we performed on the STITCH public server. Based on this analysis, putative targets of rosmarinic acid include IKK-β (IKBKB), CCR3 and IL2, which play central roles in the inflammatory pathway (Figure 8).

In this study, it was investigated whether 22 polyphenolic phytochemicals (hydroxycinnamic acid and hydroxybenzoic acid derivatives) have effective pharmaceutical properties in the fight against SARS-CoV-2 by determining their binding affinities against the spike glycoprotein, TMPRSS2, CatB and CatL via molecular docking method. Considering the molecular docking scores in combination with the drug-likeness and intracellular target predictions, it can be

suggested that rosmarinic acid may be the most promising ligand among these 22 phytochemicals. As known, SARS-CoV-2 viral replication causes an aggressive inflammation (cytokine storm) in patients infected with this virus, and increased plasma levels of IL-2, IL-7, IL-10, GCSF, IP-10, MCP-1, MIP- 1A and TNF-α are frequently observed in affected individuals (Fu *et al*., 2020). Therefore, based on its additional anti-inflammatory effects, we believe that further molecular optimization and investigation of the efficacy of rosmarinic acid in pre-clinical in vitro and in vivo studies may be promising in drug development efforts against SARS-CoV-2.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Erman Salih Istifli: Methodology, Resources, Visualization, Software, Formal Analysis. **Arzuhan Sihoglu Tepe**: Investigation, Resources, Validation, and Writing -original draft. **Cengiz Sarikurkcu:** Methodology, Formal Analysis, Software. **Bektas Tepe:** Investigation, Resources, Validation, and Writing -original draft.

Orcid

Erman Salih ISTIFLI https://orcid.org/0000-0003-2189-0703
Arzuhan SIHOGLU TEPE https://orcid.org/0000-0001-8290-9880
Cengiz SARIKURKCU https://orcid.org/0000-0001-5094-2520
Bektas TEPE https://orcid.org/0000-0001-8982-5188

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6. APPENDIX: SUPPLEMENTARY

6.1. Structural optimization of ligands

The Automatic Topology Builder (ATB) online server was utilized for geometry optimization of all the ligands using density functional theory DFT/B3LYP/6-31G* basis set. Following geometry optimization, the energetically minimized 3D ligand structures generated by the ATB server was exported in pdb file format for further use in molecular docking analyzes (Malde *et al.*, 2011).

6.2. Homology modeling of TMPRSS2

The crystallographic structure of human TMPRSS2 enzyme has not been resolved until today, therefore, a homology model was generated for this enzyme to utilize in further molecular docking and molecular dynamics analyses. The amino acid sequence of TMPRSS2 was downloaded from UniProtKB (https://www.uniprot.org/uniprot/O15393). Template search for TMPRSS2 catalytic domain was performed against the SWISS-MODEL template library with BLAST and HHBlits. BLAST was used to search the TMPRSS2 catalytic domain target sequence against the primary amino acid sequence in the SMTL (Remmert *et al*., 2012).

ProMod3 was used to carry out model building for TMPRSS2 catalytic domain based on the target-template alignment. The rest of the procedure was carried out as previously described (Guex et al., 2009).

The model quality (global and per-residue) of TMPRSS2 obtained was evaluated with the QMEAN scoring function (Studer et al., 2020). A near-zero QMEAN score was a good value in terms of the quality of the fit between model structure and the experimental structure. According to the QMEAN score, however, scores of 4.0 and below indicated that the model was of poor quality. Therefore, among the top 5 TMPRSS2 models we obtained as a result of homology modeling, we determined the 5ce1.1.A (model 06) model as the target structure in the molecular docking analysis.

In addition, whether our model has an energetically favorable conformation, we generated a Ramachandran plot (Figure S1) using the PROCHECK web server (Laskowski et al., 1993). Also, ERRAT web-based tool (Figure S2) was also deployed to calculate the overall quality factor (OQF) for non-bonded atomic interactions (Colovos and Yeates, 1993).

6.3. Protein retrieval and energy minimization of the Spike, TMPRSS2, CatB and CatL proteins using Nanoscale Molecular Dynamics (NAMD)

The purpose of performing energy minimization (optimization) of proteins is the physical significance of the obtained 3D structures: the optimized structures often resemble to their native conformation as they found in nature. Thus, this process searches to find the lowest energy conformation of the proteins in question. The spike glycoprotein was retrieved by removing the ACE2 subunit from the angiotensin-converting enzyme 2 - 2019nCoV RBD complex in Discovery Studio Visualizer v16. This model was downloaded from "https://swissmodel.expasy.org/interactive/HLkhkP/models/03" (PDB ID: model_03.pdb) (Camacho *et al.*, 2009; Remmert *et al.*, 2012). Since, the crystallographic structure of human TMPRSS2 enzyme has not still been defined, using SWISS-MODEL, we generated a homology model of this enzyme to utilize in further molecular docking analyses. The details of the homology modeling procedure can be found in the study of Istifli *et al.* (2020). The PROCHECK web server was utilized whether our generated model has an energetically favorable conformation (Figure S1) and the ERRAT web-based tool was also employed to calculate the protein overall quality factor (OQF) (Figure S2) (Colovos & Yeates, 1993; Laskowski *et al.*, 1996). The crystal structures of CatB (PDB ID: 1GMY) and CatL (PDB ID: 2YJ9) enzymes were retrieved from Protein Data Bank.

Water molecules, co-crystallized ligands and non-interacting ions were removed from proteins before energy minimization. During the protein energy minimization step, the atom types and electrical charges of the spike glycoprotein, TMPRSS2, CatB and CatL were fixed using CHARMM22_PROT force field and Gasteiger-Marsili charges in the Vega ZZ software (Pedretti *et al.*, 2004). Next, for the energy minimization of all the proteins using NAMD, the parameters were loaded from a template file. The number of time steps (number of minimization steps) were set to 10.000 and CHARMM22_PROT was set as the force field. When the energy minimization was completed, the 3D structures corresponding to the last minimization step of all proteins were saved as the lowest energy conformation.

6.4. Molecular docking analyses of phenolic acids

Molecular docking analyses between spike glycoprotein, TMPRSS2, CatB and CatL and the selected phenolic acids were performed using AutoDock 4.2.6. Correspondingly, the docking scores (binding free energy) of the ligands with the spike receptor binding motif, TMPRSS2, CatB (PDB ID: 1GMY) and CatL (PDB ID: 2YJ9) were determined. AutoDockTools-1.5.6 was used to prepare the targets and ligands as well as the parameters prior to initiating molecular docking using AutoDock 4.2.6 (Sanner, 1999). The grid box coordinates determined in molecular docking studies were adjusted to allow the analyzed phytochemicals to interact with the catalytic amino acid residues (active sites) of the target proteins. Accordingly, the grid box sizes were adjusted as: a) 80 × 90 × 40 Å points (x: -34.42, y: 27.69, z: 5.37) for the spike glycoprotein; b) 60 × 110 × 86 Å points (x: 14.67, y: -3.01, z: 6.88) for TMPRSS2; c) 86 × 84 × 44 Å points (x: 22.86, y: 5.73, z: 27.96) for CatB; and d) 54 × 52 × 60 Å points (x: 5.31, y: 6.05, z: 0.12) for CatL.

Docking calculations were performed using 100 genetic algorithm (GA) runs, an initial population of 150 individuals, max. number of 3.000.000 energy evaluations, and a max. number of 27.000 generations. The mutation and crossover rates were set as default values, 0.02 and 0.8, respectively. After 100 independent docking runs, all the ligand binding modes (conformations) were clustered and ranked on the basis of the most negative free energy of binding (kcal/mol). The best poses of receptor-ligand pre-reactive complexes obtained by AutoDock 4.2.6 were visualized and examined with BIOVIA Discovery Studio Visualizer v16.

6.5. Calculation of relative binding capacity index (RBCI)

In this study, the relative binding capacity index (RBCI) was applied to statistically rank the activity potentials of phytochemicals using the binding free energy values obtained from the binding analysis (Figure 2 and Table S1) (Istifli *et al.*, 2020). Using RBCI, it is possible to compare statistically relevant data with different scientific meanings. Since the binding energies of ligands are different for each protein, phytochemicals can only be ranked in terms of their potential at this parameter if they are performed in the light of their binding energies only to one protein. However, sequencing based on only one of these proteins cannot represent the full activity potential of these molecules. The most common method used to calculate the interaction between each receptor and ligand is the "central bias" in which components are ranked according to the mean value of each component.

If the values (binding free energy) in each data set are converted into standard scores, it is possible to compare them with each other. Arithmetic mean and standard deviation values were calculated for each protein by using the binding free energies of the ligands. Raw standard scores were obtained by subtracting the binding free energies of each protein for each ligand from this arithmetic mean and then dividing by the standard deviation value (see equation given below) (Sharma, 1995). The RBCI values of each phytochemical were then calculated by taking the average of these standard scores obtained separately for each protein target.

$$Standard\ score = \frac{(x - \mu)}{\sigma}$$

where 'x' is the raw data, 'μ' is the mean, and 'σ' is the standard deviation.

Although RBCI is a relative measure and does not represent the specific binding capacities of the components, it makes it possible to rank components reasonably based on their binding free energy values. Therefore, it can be used as an integrated approach to evaluate the molecular interaction of the components, considering all parameters.

6.6. Drug-likeness, ADMET profile and target prediction

The determination of drug-likeness, ADMET and target profiles of promising hit compounds in structure-based drug design studies is important in terms of reducing their side effects on the target organism. In this study, web-based SwissTargetPrediction and pkCSM tools were used to investigate such effects of analyzed phenolic acids (Pires *et al.*, 2015; Daina *et al.*, 2019).

6.7. Network pharmacology analysis

The one-drug/one-target approach in drug discovery has some deficiencies in terms of safety and efficacy (Chandran *et al.*, 2015). Therefore, analyzing the mutual interactions of small molecules with the protein network in discovering the possible side effects of hit or lead compounds or the elucidation of novel therapeutic effects requires the application of a network pharmacology approach. In our study, the targets-components analysis of identified hit phytochemicals was performed by selecting the target organism as '*Homo sapiens*' through the STITCH (http://stitch.embl.de/) public database.

Table S1. RBCI values of phenolic acids.

Compound	Total RBCI
1-Caffeoyl-β-D-glucose	-1.47
Rosmarinic acid	-1.30
3-p-Coumaroylquinic acid	-1.18
Chlorogenic acid	-1.16
6-O-p-Coumaroyl-D-glucose	-1.03
3-Feruloylquinic acid	-0.54
p-Coumaroyltartaric acid	-0.47
N-Caffeoyl-L-aspartic acid	-0.25
Ferulic acid	-0.16
Caffeic acid	-0.13
1-O-Feruloyl-β-D-glucose	0.00
Chicoric acid	0.06
p-Coumaric acid	0.12
Protocatechuic acid	0.43
Cinnamic acid	0.56
Gallic acid	0.60
Vanillic acid	0.76
4-hydroxybenzoic acid	0.77
Syringic acid	0.94
Isovanillic acid	0.98
Benzoic acid	1.20
Salicylic acid	1.31

Table S2. Molecular interactions between the phenolic acids and RBD of the spike glycoprotein of 2019-nCoV.

No	Compound	Classical H-bond	Van der Waals	Non-Classical H-bond (C-H, Pi-Donor)	Hydrophobic interaction		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
					π-π interaction	Mixed π/Alkyl		
1	1-caffeoyl-β-D-glucose	Arg403, Gln409, Lys417, Tyr453, Gly496, Tyr505[1]	Glu406, Gly416, Ile418, Gln493[1], Tyr495, Ala419, Gln493[1], Phe497, Asn501[1]	Arg403, Ser494[1]	-	Lys417	-	-
2	Rosmarinic acid	Tyr449, Ser494[1], Gly496, Gln498, Tyr505[1]	Tyr453, Gln493[1], Tyr495, Phe497	-	-	Arg403	-	-
3	3-p-Coumaroylquinic acid	Arg403, Gln409, Tyr453, Ser494[1], Asn501[1], Tyr505[1]	Gly416, Ile418, Ala419, Gln493[1], Tyr495, Gly496, Phe497, Gln506	Arg403	-	Lys417	Glu406	-
4	Chlorogenic acid	Glu406, Gln409, Lys417, Ser494[1], Gly496	Arg403, Asp405, Gly416, Ile418, Tyr453, Gln493[1], Phe497, Asn501[1], Tyr505[1]	-	Tyr495	-	-	-

[1]Amino acid residues involved in binding to ACE2 in the RBM of 2019-nCoV (Leu455, Phe486, Gln493, Ser494, Asn501, and Tyr505)

Table S3. Molecular interactions between the phenolic acids and TMPRSS2.

Compound	Classical H-bond	Van der Waals	Non-Classical H-bond (C-H, Pi-Donor)	Hydrophobic interaction		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
				π-π interaction	Mixed π/Alkyl		
1-caffeoyl-β-D-glucose	Val280, His296[1], Ser394, Gly439, Ser441[1]	His279, Cys281, Cys297, Gln317, Trp384, Glu395, Gln438, Ser460	-	-	-	Thr393	-
Rosmarinic acid	His279, Ser460, Gly462	Val278, Cys281, Gln438, Gly439, Trp461, Ser463, Gly464	Val280, Thr393, Ser441[1]	His296[1]	Thr393	-	-
3-p-Coumaroylquinic acid	Val280, Cys281, His296[1]	His279, Cys297, Trp308, Thr393, Ser436, Cys437, Gly439, Ser460, Trp461, Gly462, Ser463, Gly464, Cys465	Ser441[1]	-	-	-	-
Chlorogenic acid	His279, His296[1], Ser394, Gly439, Ser441[1]	Cys281, Cys297, Gln317, Trp384, Cys437	Val280	-	-	-	-

[1]The active amino acid residues of TMPRSS2 (His296, Asp345, Ser441)

Table S4. Molecular interactions between the phenolic acids and CatB.

No	Compound	Classical H-bond	Van der Waals	Non-Classical H-bond (C-H, Pi-Donor)	Hydrophobic interaction		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
					π-π interaction	Mixed π/Alkyl		
1	1-caffeoyl-β-D-glucose	Gln23[1], Gly24[1], Gly27[1], Cys29[1], Gly72, His197	Ser25, Cys26[1], Ser28[1], Trp30[1], Gly71, Pro74[1], His108, Cys117, Thr118, Glu120, Ala198	His197	-	-	-	-
2	Rosmarinic acid	Gln23[1], Cys29[1], Asn70, His108, Gly119, Glu120, Gly196, His197	Gly24[1], Ser25, Gly27[1], Cys26[1], Ser28[1], Gly71, His109, Cys117, Val174, Trp219	Gly24[1]	-	-	-	-
3	3-p-Coumaroylquinic acid	Gln23[1], Cys29[1], Gly72, Gly119, Glu120	Gly24[1], Ser25, Gly27[1], Ser28[1], Asn70, Gly71, His108, Cys117, Gly196, His197, Trp219	Trp30[1]	-	Cys26[1], Gly27[1]	-	-
4	Chlorogenic acid	Gln23[1], Gly24[1], Cys26[1], Cys29[1], Gly119, Gly196, His197	Ser25, Gly27[1], Ser28[1], His109, Thr118, Glu120, Val174, Leu179, Met194, Gly195, Trp219 Gln23[1], Ser25, Gly27[1], Cys69, Gly71, His108, Cys117, Gly119,	His108, His197	His108	Cys117	-	-

[1]The active amino acid residues of CatB (Gln23, Gly24, Cys26, Gly27, Ser28, Cys29, Trp30, Gly73, Pro74, His110, His111, His199, Trp221).

Table S5. Molecular interactions between the phenolic acids and CatL.

No	Compound	Classical H-bond	Van der Waals	Non-Classical H-bond (C-H, Pi-Donor)	Hydrophobic interaction		Electrostatic	Miscellaneous (Lone pair/Pi-sulphur)
					π-π interaction	Mixed π/Alkyl		
1	1-caffeoyl-β-D-glucose	Met70[1], Asp71, Met161[1], Asp114, Asp162[1], Ala215	Trp26[1], Gly68[1], Leu69[1], Phe112, Ser133, His163, Gly164, Ser216, Ala214	-	-	Cys25[1], Ala135[1]	-	Met70[1]
2	Rosmarinic acid	Gly68[1], Met70[1], Lys117, Ser213	Cys25[1], Trp26[1], Phe74, Asp114, Ser133, Met161[1], Asp162[1], His163, Gly164, Ser216	-	-	Leu69[1], Ala135[1], Ala214, Ala215	Asp71	Met70[1]
3	3-p-Coumaroylquinic acid	Cys25[1], Gly68[1], Met70[1], Asp71, Asp114, Ser216	Gly23[1], Trp26[1], Ser133, Gly164, Ala214, Ala215	Gly67[1], Leu69[1]	Asp162[1], His163	Cys25[1], Ala135[1]	-	Gly68[1]
4	Chlorogenic acid	Asp71, Asp114, Lys117, Ser213, Ser216	Trp26[1], Gly68[1], Leu69[1], Tyr72, Phe112, Met161[1], Asp162[1], His163, Gly164, Ala214	-	-	Ala135[1]	-	Cys25[1], Met70[1]

[1]The active amino acid residues of CatL (Gln19, Gly20, Gln21, Cys22, Gly23, Ser24, Cys25, Trp26, Gly61, Asn66, Gly67, Gly68, Leu69, Met70, Ala135, Met161, Asp162, Trp189)

Figure S1. Ramachandran plot of TMPRSS2 model.

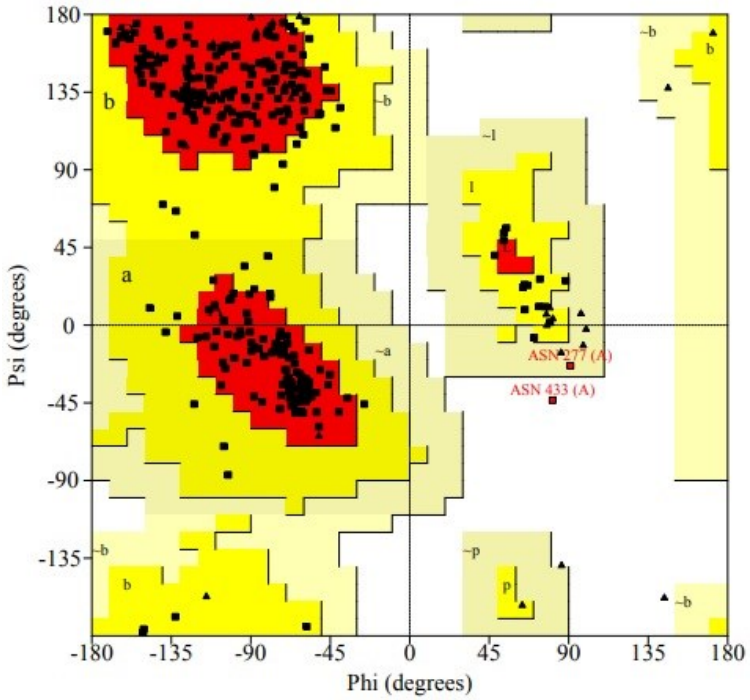
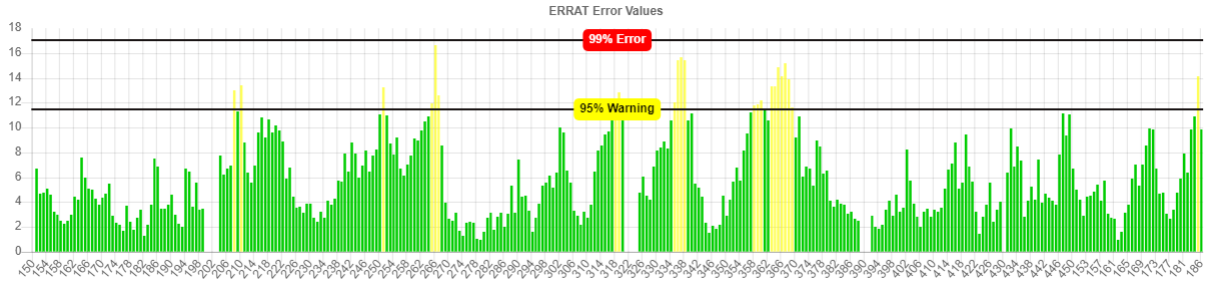


Figure S2. ERRAT error values for TMPRSS2 model.



(Protein regions show misfolding at 95% confidence level were indicated with yellow bars. Green bars, on the other hand, point to regions that show correct folding)

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Research Article

Chemical composition and antimicrobial and antioxidant activities of essential oils of *Polytrichum commune* (Hedw.) and *Antitrichia curtipendula* (Hedw.) Brid. grown in Turkey

Tayyibe Beyza Yucel [1,*]

[1]Department of Medical Services and Techniques, Vocational School of Health Services, Giresun University, 28200, Giresun, Turkey

Abstract: The aim of this study is to analyze the volatile composition and antimicrobial and antioxidant activities of the essential oils of *Polytrichum commune* and *Antitrichia curtipendula*. The essential oils obtained by hydro-distillation (HD) from each species were identified by GC-MS/FID. The main components were biformene (13.06%), *α*-pinene (6.53%), and bornyl acetate (8.10%) in *P. commune.* Nonanal and tetradecanal as major compounds were 19.96% and 20.23% in *A. curtipendula* essential oils, respectively. Antioxidant activity of obtained essential oils was evaluated using in-vitro antioxidant models. There was no significant difference within the groups according to DPPH activity. Also, the essential oil from *P. commune* showed higher metal-ion chelating activities than that of the essential oil of *A. curtipendula.* Metal-ion chelating activities varied between 4.1% and 67.4% at the 800 µg/mL concentration, respectively. The antimicrobial activity was tested by a minimal inhibition concentration test. Each moss species showed good antimicrobial activity against microorganisms according to the results of minimal inhibition concentration experiments.

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1. INTRODUCTION

Bryophytes, divided into three classes; namely, Bryophyta (mosses, 14000 species), Marchantiophyta (liverworts, 6000 species), and Anthocerotophyta (hornworts, 300 species), have about 23000 species in the worldwide and comprise the second largest group of plants after Magnoliophyta – the flowering plants (350000 species) (Asakawa *et al.*, 2013; Asakawa & Ludwiczuk, 2018; Tonguç-Yayıntaş & İrkin, 2017). Bryophytes spread all over the world from the deserts to the glaciers, except for the seas (Chandra *et al.*, 2017). Bryophytes are used in several sectors varying from aquatic bioindicators to treatment of waste or from radioactivity indicator to the treatment of packing (Asakawa, 2007).

The mosses are represented by approximately 25000 taxa around the world (Smith, 2004). In the previous studies, despite their broad coverage, the mosses have been unable to preserve their actual place as a prior research. Mosses are used for biomonitoring/bioindicator of waters

*CONTACT: Tayyibe Beyza YÜCEL beyza.yucel@giresun.edu.tr Department of Medical Services and Techniques,Vocational School of Health Services, Giresun University, 28200, Giresun, Turkey

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and air pollution in addition to its use for the determination of heavy metal accumulation (Harmens *et al.*, 2007). Lately due to the fact that algae have new and/or bioactive compounds, there has been an increase in the number of pharmacological studies recently (Yayıntaş *et al*., 2019; Wang *et al.*, 2017). Also, mosses possess various natural products that exhibit biological activity and are used as food supplements or spices and medicals. That moss species have a wide variety of substances of active metabolite makes it possible to use it as an antioxidant as well as antimicrobial (Dey & De, 2012; Russel, 2010). The mosses *Atrichum undulatum* and *Mnium hornum* produce vitamin E, vitamin K, and plastoquinon, while *Dicranum scoparium*, *Leucobryum* species contain prostaglandin-like highly unsaturated fatty acids playing an important role as an antioxidant in the human body (Ichikawa *et al.*, 2008; Tedone *et al.*, 2011). Several mosses species have been used as medicinal plants such as *Bryum, Mnium, Philonotis* species, and *Polytrichum juniperinum* by North American Indians to treat burns, bruises, and wounds. Terpenoids are other valuable compounds with antibacterial, antifungal, anti-inflammatory, and cytotoxic activities (Chen *et al.,* 2018). β-cyclocitral, β-ionone, and geosmin are the most common monoterpenoids detected in mosses while *Mnium, Taxiphyllum, Plagiothecium, Homalia, and Plagiomnium* genus of mosses contain the volatile terpenoids (Asakawa, 1995).

Polytrichum commune as moss class consumed as boiled tea was chosen as the study material for this specific study because it is used in the treatment of many diseases such as wound healing, antipyretic, antidotal activity, dissolving kidney and gallbladder stones, antipyretic and antipyretic, and colds (Chandra *et al.*, 2017; Hallingback *et al.*, 2000; Greeshma & Murugan 2018). There are a few reports on the antibacterial, cytotoxicity, and antimicrobial activities of solvent extract of *Polytrichum commune* grown in different parts of the world including Turkey (Klavina *et al.*, 2015; Nikolajeva *et al.*, 2012; Sevim *et al.*, 2017).

Antitrichia curtipendula is used to prepare moss costumes during the annual festival to celebrate important historical wars in Spain (Mártinez-Abaigar & Núñez-Olivera, 2001) and this species is reported to be used today for packaging mushrooms in the Pacific Northwest (Glime, 2007). *Antitrichia curtipendula* was chosen as another moss species in this study because there are not many studies about the antioxidant and antimicrobial activity of this moss in the literature (T. Yayıntaş *et al.*, 2019).

In different studies to date, approximately 3000 essential oils have been described from plants that include mosses by using various methods. The number of studies investigating the content, quality, quantity, and biological activities of essential oils has been increasing, especially in recent years due to the fact that they are both cheaply available sources rich in polyunsaturated fatty acids (Bayaz, 2017; Morteza-Semnani *et al.*, 2012) and also they are effective antimicrobial used in the livestock industry and pharmacology as well as in the fields of cosmetics, perfumery, aromatherapy, and soft drinks due to some components it contains (Şahin *et al.*, 2004; Öztürk & Özbek, 2005).

We used mosses species of *Polytrichum commune* and *Antitrichia curtipendula* in this study. In Turkey, there are three species of *Polytrichum* genus that belong to Polytrichaceae family and two species of *Antitrichia* genus that belong to Leucodontaceae family. The essential oils of these species were extracted with hydro-distillation using a Clevenger apparatus. Antimicrobial activity of essential oils was examined by microdilution methods against *Escherichia coli* ATCC25922, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, and *Streptococcus mutans*. The antioxidant capacity of essential oils was determined by DPPH scavenging assay and metal-ion chelating assay. Chemical compositions of essential oils were analyzed using GC-MS/FID. Each biological activity test was done twice in duplicate and the results are expressed as mean

± standart deviation (SD). The statistical analysis was performed using a one-way ANOVA (p <0.05).

2. MATERIAL and METHODS

2.1. Plant Material

In this study, the fresh moss materials were separated and divided into small pieces. The leaves of *P. commune* were collected on 23rd March 2018 from Taflancik Village, Hayrat, Trabzon, Turkey at an altitude of 478 m. *A. curtipendula* (Hedw.) was collected on 20th March 2018 from National Park of Altindere Valley, Macka, Trabzon, Turkey at an altitude of 848 m. Voucher specimens were deposited in the Herbarium of the Department of Biology, Karadeniz Technical University, Turkey, respectively (KTUB 1614, KTUB 1608).

2.2. Isolation of Essential Oils

The essential oils from each moss species (approximately 50 g each) were subjected to hydro-distillation for 3h using a Clevenger type apparatus with the cooling bath (12 °C) system (4 h) (yields: 0.13% and 0.11% (v/w), respectively). The obtained oils were dissolved in HPLC grade *n*-hexane (0.5 mL) and dried over anhydrous sodium sulphate and stored at 4-6 ºC in a sealed brown vial. 1 µL of the essential oils was directly injected separately into the GC-MS instrument.

2.3. GC-MS Analysis of Essential Oils

The GC-MS analysis was performed using Shimadzu 2010 Plus gas chromatograph coupled to a Shimadzu QP2010 Ultra mass selective detector. The gas chromatography-flame ionization detector (GC-FID) was used. The fibre containing the extracted aroma compounds was injected into GC/MS injector. The split mode was used. The separation was performed by means of a Restek Rxi-5MS capillary column, 60 m length, 0.25 mm i.d., and a 0.25 µm phase thickness. The oven program was as follows: Initial temperature was 60 °C for 2 min, which was increased to 240 °C at 3 °C min^{-1}. 250 °C was maintained for 4 min. Helium (99.999%) was used as carrier gas with a constant flow-rate of 1 µL min^{-1}. Detection was carried out in electronic impact mode (EI) and ionization voltage was fixed to 70 eV. Scan mode (40-450 m/z) was used for mass acquisition. The volatile compounds were identified by comparison with the mass spectra of the two libraries (FFNSC1.2 and W9N11) and using the standard compounds (β-pinene, camphene, limonene oxide, isopinocarveol, 1-terpinen-4-ol, β-ionone, caryophyllene oxide, biformene, and nonanal) (Renda *et al*., 2019).

2.4. Antioxidant Activity

2.4.1. Measurement of metal-ion chelating capacity

The experiments as to the chelation of ferrous ions by the essential oils were performed as described by Decker and Welch. Different concentrations of essential oil (25, 50, 100, 200, 400, and 800 µg/mL) were added to the reaction mixture (Decker & Welch, 1990). The absorbance of the reaction mixture was measured at 562 nm. EDTA and Trolox were used as standards in the same concentration of essential oils. The percentage of chelating capacity of the test sample was calculated as follows:

$$\text{Chelating capacity\%} = [(A_1 - A_2)/A_1 \times 100]$$

where A_1 is the absorbance of control and A_2 is the absorbance in the presence of essential oil or EDTA.

2.4.2. DPPH scavenging activity assay

The antioxidant activity of each species' essential oil was first determined by measuring the DPPH scavenging ability. The essential oil at various concentrations (25, 50, 100, 200, 400,

and 800 µg/mL) was added to the reaction mixture including DPPH. When DPPH reacts with an antioxidant in the essential oil that can donate hydrogen, it gets its reduced form as the resulting decrease in absorbance at 517 nm was recorded using a UV-Vis spectrophotometer (Jasco, V-630, Tokyo, Japan) (B.Williams *et al.*, 1995). In this study, Trolox and BHT were used as antioxidant standards. DPPH scavenging activities of the test samples were calculated as follows:

$$DPPH\ (\%) = 100- (A_0-A_1/A_0\ x100)$$

2.5. Antimicrobial Activity

The essential oils showed moderate antibacterial activity against three gram-positive (*Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212 and *S. mutans*) and two gram-negative bacteria (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853). Due to the presence of activity in antimicrobial activity studies against gram positive and gram negative microorganisms of these mosses in previous studies, an antimicrobial activity against *E. coli, S. mutans, E. faecalis*, *P. aeruginosa,* and *S. aureus* as microorganisms with different ATCC numbers was preferred in this study. Antibacterial susceptibility testing was performed according to the CLSI laboratory standards for broth microdilution assays (CLSI, 2012). For this purpose, antibiotic stock solutions were prepared for the antibiotic test and serial dilutions were made in separate tubes from here. An overnight culture on a nonselective medium such as bloody agar was used in the experiment. A standard inoculum of 0.5 McFarland units (108 colony forming units/µL) was prepared in MRS broth. It was diluted 1:30 (5x 106 CFU/mL) and 50µL (2.5x105 CFU/mL) was inoculated into each well excluding the control well. Therefore, bacteria inoculum of approximately 2.5x105 CFU/mL was adjusted in a final volume of 100 µL in each well. MHB was used in bacterial suspension and antibiotic dilutions were prepared by DMSO. A well of each plate was used as a reproductive control and no antibiotics were added. A well of each plate was used as a sterilization control and included only the broth. The same antibiotics stock solutions and dilutions of the same antibiotics were analysed on appropriate ATCC strains in another plate for the quality control of the experiment. The plates were incubated at 35-37 °C for 24 hours in a normal atmosphere. Ampicillin (10.000 µg/mL), fluconazole (5.000 µg/mL), and streptomycin (10.000 µg/mL) were used as standard antibacterial and antifungal. When MIC values of quality control strains were appropriate and the results of the reproductive control, the sterilization control, the inoculum purify control, and the inoculum density control were valid, the plates were read by a microplate reader in 600 nm. The results obtained were calculated as % inhibition.

2.6. Statistical Analysis

The obtained essential oils were tested for antioxidant and antimicrobial activities. A statistical package (SPSS version 20.0) was used for data analysis**.**

3. RESULTS / FINDINGS

The chemical composition of essential oils of *P. commune* and *A. curtipendula* were identified with GC-MS. Altogether, 35 essential compounds were identified with Restek Rxi-5MS column. Chemical compounds were classified into ten classes, viz., monoterpenes, oxygenated monoterpenes, oxygenated monoterpenes related, sesquiterpenes, oxygenated sesquiterpenoids, oxygenated sesquiterpenoids related, diterpene, aldehydes, carboxyllic acids, and others.

In this study, metal ion chelating activity of essential oil of *P. commune* was found to be more effective than that of the essential oil of *A. curtipendula.* Both of the essential oils represented similar DPHH and antimicrobial activity with no significant difference within the group.

3.1. Chemical Composition of Essential Oils

Both the *P. commune* and *A. curtipendula* extracted the identified compounds in respective orders starting with a total of eight monoterpenes (30.31%, 1.11%), three oxygenated monoterpenes (3.42%, -), eight oxygenated monoterpenes related (19.25%, 18.22%), three sesquiterpenes (2.86%, 1.44%), three oxygenated sesquiterpenoids (5.83%, -), oxygenated sesquiterpenoids related (9.99%, 14.26%), one diterpene (16.06%, -), three aldehydes (-, 44.21%), two carboxyllic acid (10.76%,-), and four others compounds (-, 8.37%), respectively (Table 1).

In *P. commune* essential oil, respectively twenty-five components were identified (Table 1), representing almost 95.48% of total oils. The main components were biformene (13.06%), hexahydro farnesyl acetone (9.99%), (9*Z*,12*Z*)-octadecadienoic acid (9.51%), bornyl acetate (8.10%), and *α*-pinene (6.53%), respectively. Biformene, a labdane-type diterpene was reported in *Bazzania francana,* which is a moss-like plant (Metoyer *et al.*, 2018). It is known that the labdane diterpenes have been shown to possess cardiovascular effects, anti-fungal activity, and anti-inflammatory and cytotoxic effects (Demetzos *et al.*, 2001; Lahlou *et al.*, 2007). In essential oil components of *A. curtipendula*, fifteen components were characterized, representing almost 85.61% of the essential oil (Table 1). The major components were tetradecanal (20.23%), nonanal (19.96%), hexahydrofarnesyl acetone (14.26%), and *β*-ionone (10.43%). Generally, the number of essential components in the oil of *P. commune* is more than that in *A. curtipendula*.

Table 1. Chemical composition of essential oils of mosses.

Number	Retention time[a]	Compounds	A % Area	B % Area	RI LIT[a]	RI[b]
	Monoterpenes					
1	6.90	*α*-pinene	6.53	-	939	935
2	7.36	Camphene	6.31	-	956	961
3	8.30	*β*-pinene	3.78	-	979	982
4	9.74	Limonene	1.45	-	1029	1033
5	10.03	*Z*-*β*-Ocimene	1.75	-	1037	1042
6	10.25	*E*-*β*-Ocimene	6.48	-	1050	1050
7	11.40	*m*-Cymenene	2.46	-	1085	1083
8	11.94	Terpinolene	1.55	1.11	1089	1090
	Oxyganeted monoterpenes					
9	14.71	*trans*-limonene oxide	1.09	-	1142	1141
10	14.90	Camphor	1.57	-	1146	1148
11	18.25	*β*-Cyclocitral	0.76	1.48	1221	1222
	Oxygenated monoterpenes related					
12	16.37	Terpinen-4-ol	4.98	-	1177	1178
13	17.17	Myrtenal	0.73	-	1196	1195
14	17.75	Verbenone	1.57	3.09	1207	1205
15	21.08	Bornyl acetate	8.10	-	1289	1291
16	24.93	Geranyl acetate	0.89	-	1381	1377
17	25.70	*E*-*α*-Damascenone	-	1.16	1385	1387
18	26.63	*α*-Ionone	-	2.06	1430	1432
19	28.47	*β*-Ionone	2.98	10.43	1489	1492
	Sesquiterpenes					
20	24.67	*α*-cubebene	-	1.44	1351	1354
21	26.35	*trans*-Caryophyllene	1.13	-	1418	1420
22	27.48	*α*-Humulene	1.73	-	1452	1456

Table 1. *Continues*

	Oxyganeted sesquiterpenes					
23	31.22	Caryophyllene oxide	4.76	-	1582	1585
24	32.83	*α*-Cadinol	1.07	-	1654	1650
	Oxygenated sesquiterpenoids related					
25	36.06	Hexahydro farnesyl acetone	9.99	14.26	1847	1848
	Diterpenes					
26	41.53	Biformene	13.06	-	2026	2030
	Aldehydes					
27	10.21	Benzene acetaldehyde	-	2.02	1042	1046
28	13.28	Nonanal	-	19.96	1100	1101
29	31.30	Tetradecanal	-	20.23	1613	1617
	Carboxyllic acids					
30	39.10	*cis*-13-eicosenoic acid	1.25	-	1915	1918
31	42.13	9,12(*Z,Z*)-octadecadienoic acid	9.51	-	2149	2150
	Others					
32	7.28	Cyclohexanone	-	2.14	952	955
33	8.42	3-Octanone	-	2.66	984	986
34	8.89	1-Decene	-	1.50	990	991
35	10.39	Octanol	-	2.07	1068	1065
		Monoterpenes	30.31	1.11		
		Oxygenated Monoterpenes	3.42	-		
		Oxygenated Monoterpenes related	19.25	18.22		
		Sesquiterpenes	2.86	1.44		
		Oxygenated Sesquiterpenes	5.83	-		
		Oxygenated Sesquiterpenes related	9.99	14.26		
		Diterpene	13.06	-		
		Aldehydes	-	44.21		
		Carboxyllic acid	10.76	-		
		Others		8.37		
		Total	95.48	85.61		

[a]Retention times relative to that of n-alkanes C_7-C_{30}.
[b]RI calculated from retention times relative to that of n-alkanes (C_7-C_{30}) on the non-polar Rxi-5MS column.
A: *P. commune*, B: *A. curtipendula*

3.2. Antioxidant and Antimicrobial Activity

Metals such as iron and copper can form reactive radicals such as superoxide in biological systems due to the formation of redox reactions. Excessive accumulation of these metals causes the accumulation of reactive oxygen and consequently oxidative stress. Oxidative stress causes DNA damage, lipid peroxidation, and protein modification underlying many diseases from many cancers to neurodegenerative diseases (Jomova & Valko, 2011). Chelation of redox-active metals prevents oxidative damage avoiding them from forming a redox reaction. The DPPH method is based on the reduction of free radical DPPH in the presence of a hydrogen donating antioxidant. The reaction results in the formation of non-radical DPPH-H and can be measured at 517 nm. Chelation of redox-active metals prevents oxidative damage avoiding them from forming a redox reaction. In this study, maximum metal ion chelating activity was observed in *P. commune* as the value of 67.42±0.39. Essential oils of moss materials represented similar activity (18.11±7.14 and 19.32±7.04) to reduce DPHH (Table 2) with no significant

difference within the group. The standards of metal chelate and DPPH activities are shown in Table 2.
± values indicate the standard deviation. As a result of Anova test, a statistical significance was observed in metal chelate data between samples (p<0.05), while no statistical significance was observed between samples in DPPH data (p<0.953).

Table 2. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities and Metal ion-chelating of essential oils obtained from *P. commune* and *A. curtipendula*. (Inhibition% ± SD)

	P. commune	*A. curtipendula*
% DPPH radical scavenging[a]	18.11±7.14	19.32±7.04
% Metal ion-chelating[b]	67.42±0.39	4.17±040
BHT	94.69±0.04	
Trolox	90.57± 0.06	
EDTA	97.06± 0.01	

[a]Values are given as mean±S.D. (n= 3), and there is no significant difference at p<0.05.
[b] Values are given as mean±S.D. (n= 3), and considered to be significantly different at p<0.05.

The essential oils showed moderate antibacterial activity against three gram-positive (*S. aureus, E. faecalis* and *S. mutans*) and two gram-negative bacteria (*E. coli* and *P. aeruginosa*). The % inhibition values of essential oils from five species determined in a broth microdilution assay are shown in Table 3. According to the results, essential oils from each species showed a potent inhibitory effect on the growth of microorganisms without significant differences between groups. Antimicrobial effect values were found ranging from 91.27 to 95.12%. The essential oils showed higher activities than other microorganisms antibacterial activities against *S. mutans, E. faecalis,* and *P. aeruginosa.*

Table 3. Minimal inhibitory concentrations (µg/mL) of essential oils against.

Microorganism	Sample	% Inhibition[a]
E.coli ATCC 25922	*P. commune*	94.63±0.07
	A.curtipendula	95.09±0.06
E.faecalis ATCC29212	*P. commune*	94.64±0.09
	A.curtipendula	94.35±0.05
S. mutans	*P. commune*	94.91±0.06
	A.curtipendula	95.12±0.09
P.aeruginosa ATCC27853	*P. commune*	94.85±0.05
	A.curtipendula	94.80±0.05
S. aureus ATCC25923	*P. commune*	91.27±0.04
	A.curtipendula	91.37±0.03

[a]Values are given as mean±S.D. (n= 3) and considered to be significantly different at p<0.05.

4. DISCUSSION and CONCLUSION

The essential oil of *P. commune* contained biformene-diterpene as a major component (Table 1). Diterpenes are the basis of many biologically active substances such as retinol, retinal, taxol, as well as diterpenoids exert anticancer, antioxidant, and anti-inflammatory effects (Costa *et al.*, 2012; Costa *et al.*, 2014). In this study, the essential oil of *P. commune* represented highly inhibitory effects on microorganism. According to our results it's DPPH activity and metal chelating capacity are the highest (22.84±7.14 and 67.42±0.39, respectively). Therefore, we can say that biformene exhibits antioxidant properties as in the literature (Öztürk, 2008; Öztürk *et*

al., 2009). At the same time, according to the findings we obtained, the inhibition effect against microorganisms was quite high as shown in Table 3.

Fu *et al.* (2009) determined the components by separating the extracts of *P. commune* in methanol and different organic solvents by column chromatography. They were identified as the structures of Ohioensin, Communin B, and Communin A and the new compounds were evaluated for cytotoxicity against a small panel of cancer cell lines. In this study, the essential oil of *P. commune* was identified biformene as new compounds.

In another study by Nikolajeva *et al.,* the antimicrobial activity of aqueous and ethanolic extracts of 11 *Bryophyta* species including *P. commune* Hedw. and 9 *Marchantiophyta* species collected in Latvia was tested against *Staphylococcus aureus* MSCL 334*, Escherichia coli* MSCL 332 and *Bacillus cereus* MSCL 330. Extract of *P. commune* did not have a significant influence ($p > 0.05$) on the growth of *E. coli*. The growth of *Bacillus cereus* was inhibited by the aqueous extracts of *P. commune* (MIC80 was not achieved). Minimal inhibitory concentration of *P. commune* aqueous and ethanolic extracts (in %) against *Staphylococcus aureus* was found >33 and 30. In this study, the essential oil of *P. commune* was determined as a good antimicrobial activity against *P. aeruginosa, E. faecalis, E. coli,* and *S. mutans.*

Monoterpenes compounds are known to be found in many plants and exhibit antioxidant, anticancer, antiviral, cardioprotective, and cytotoxic effects (Pirbalouti *et al.,* 2014). According to the previous studies, monoterpenes are the major ingredients in essential oil and moderate antimicrobial activity has been observed against the bacteria *Y. pseudotuberculosis, P. aeruginosa, S. aureus, E. faecalis, B. cereus,* and *M. smegmatis* (Dragomanova *et al.,* 2018; Kozioł *et al*., 2014; Zielinska-Błajet & Feder-Kubis, 2020). In our study, the essential oil of *P. commune* contained more monoterpene (30.31%) and less oxygenated monoterpenes and oxygenated monoterpenes related (22.67%) and represented highly antioxidant and antimicrobial activity against *P. aeruginosa, S. mutans*, *E. faecalis*, and *E. coli*. In this respect, it can be said that there are different microorganisms used in this study.

P. commune grown Chinese is used for the treatment of fever, hemostatic, traumatic injury, pneumonia, uterine prolapse, and especially lymphocytic leukemia (Zhonghua, 1999; Mishra *et al.*, 2014). In 2013, Cheng showed that ethyl acetate extract of this species stimulates apoptosis and increases oxidative stress in L1210 cells (Cheng *et al.,* 2013). In another study with methanol extract of *P. commune*, the species have been shown to have an effective antimicrobial effect on *P. larve* isolates (Sevim *et al.,* 2017). These studies support the high antimicrobial activity that this species exhibited in our study.

In the study of Tonguç-Yayıntaş, it was determined that ethanol and methanol extracts of *A. curtipendula* by Soxhlet extraction did not exhibit an antioxidant activity in the analysis by radical scavenging capacity method (DPPH) (Tonguç-Yayıntaş *et al.*, 2019). These results differ from the results of radical scavenging capacity (DPPH) of the essential oil of *A. curtipendula* in our study.

In a study of chemical profile of the methanol and chloroform extracts of *P. commune* by Klavina *et al. (2015),* sterols were found in comparatively higher concentrations in extract and also high ratio carboxyllic acids as tetradecanoic acid, pentadecanoic acid, and octadecanoic acid were found as determined (9*Z,*12*Z*)-octadecadienoic acid and cis-13-eicosenoic acid. In this respect, it can be said that there is a similarity between the two studies. In the literature about *P. commune* and *A. curtipendula*, chemical profile of the essential oils of the mosses showed big differences as in our case, which can be explained by the environmentally, locality, and the subspecies of the mosses used.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Authors are expected to present author contributions statement to their manuscript such as; **Tayyibe Beyza YUCEL**: Methodology, Investigation, Resources, Visualization, Formal Analysis, and Writing -original draft.

Orcid

Tayyibe Beyza YUCEL https://orcid.org/ 0000-0002-2632-8325

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Research Article

Polyphenolic composition and Antioxidant Effect of Aerial Parts and Roots Extracts from *Scorzonera veratrifolia*

Duygu Taskin [1,*], **Mert Gecim** [2], **Ahmet Dogan** [3], **Ayfer Beceren** [4]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, University of Health Sciences, Istanbul, Turkey
[2]Department of Biochemistry, Faculty of Pharmacy, Marmara University, Istanbul, Turkey
[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, Marmara University, Istanbul, Turkey
[4]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

Abstract: Antioxidant activities of the different extracts (*n*-heptane, chloroform, methanol) from the roots and aerial parts of *Scorzonera veratrifolia* by maceration method, as well as total phenolic and flavonoid content were examined first time in this study. The findings revealed that the methanol extract from *S. veratrifolia* aerial parts exhibited greater DPPH radical scavenging (IC_{50}: 0.62±0.60 mg/mL) and iron (III) reduction capacity (1.56±0.03 mM Fe^{2+}/mg extract). Furthermore, aerial parts methanol extract has the highest concentration of total phenolic (46.3±1.1 mgGAE/g extract) and flavonoid (0.013±0.002 mg QE/mg extract) compounds. Based on these findings, the main phenolic content of aerial parts methanol extract was analyzed by LC-ESI-QTOF/MS, as this extract was found to contain the strongest antioxidant as well as the highest amount of phenolics and flavonoids as compared to the others. Quinic acid, chlorogenic acid, rutin, liquiritin, quercetin hexoside, luteolin-7-*O*-rutinoside, and di-*O*-caffeoylquinic acid compounds were identified as major compounds in methanol extract. The findings showed that aerial parts of *S. veratrifolia*, rather than its roots, could be used as a source of antioxidants.

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1. INTRODUCTION

The accumulation of reactive species formed under oxidative stress conditions defined as the disruption of antioxidant and pro-oxidant balance in the organism causes irreparable damage to biological macromolecules in living cells. As a result, oxidative stress causes various diseases such as cancer, coronary heart disease, diabetes, hypertension, cellular deterioration, mutations, and immune system disorders (Chedea *et al*., 2010). Internal enzymatic defenses against oxidative damage are not entirely effective, and a series of internal and external free radical-scavenging antioxidants act as the second defense system. Antioxidants are compounds that prevent or delay the oxidation of that compound at a lower concentration than that of the oxidizable compound (Akyüz *et al*., 2013). Antioxidants are either a group of enzymes that

*CONTACT: Duygu Taskin ✉ duygu.taskin@sbu.edu.tr University of Health Sciences, Istanbul, Turkey, Department of Analytical Chemistry, Faculty of Pharmacy, Selimiye Mah. Tıbbiye Cad. No:38 34668 Üsküdar, İstanbul

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strengthen the antioxidant capacity of the cell, or molecules that inactivate free radicals or prevent chemical reactions initiated by free radicals by participating in physiological, biochemical, or cellular processes (Halliwell, 2008; Papetti *et al*., 2006). Phenolic compounds are secondary metabolites found in large amounts in plants. The antioxidant effects of herbal products are due to the phenolic compounds in their ingredients (Turumtay *et al*., 2014). Phenolic compounds exert antioxidant effects by destroying free radicals and chelating with metal ions that can form lipid peroxidation (Huyut *et al*., 2017).

Scorzonera species (Asteraceae) are found in traditional medicine as analgesic, antirheumatic, anthelmintic, automatic, diuretic and wound-healing, hypertension, pulmonary edema, kidney disorders, diabetes, and diarrhea (Acıkara *et al*., 2013; Sarı *et al*., 2009). *Scorzonera* species have been found to contain dihydroisocoumarin, bibenzyl derivatives, flavonoids, lignans, stilbene derivatives, quinic and caffeic acid derivatives, sesquiterpene, sesquiterpene lactones and triterpene compounds (Sarı, 2010). It has been determined by literature search that *Scorzonera* species show antioxidant, analgesic, anti-inflammatory, and wound-healing activities (Tsevegsuren *et al*., 2007; Wang *et al*., 2009). *Scorzonera veratrifolia* Fenzl plant is a perennial herbaceous plant. This plant spreads in Eastern Anatolia and grows on dry rocky slopes. The roots and latex of this plant are used as wound healing. Benzylfithalide, scorzoveratrin, scovoveratroside, chlorogenic acid, chlorogenic acid methyl ester, cryptochlorogenic acid, 4,5-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid were isolated from only the root of *Scorzonera veratrifolia*, and their antimicrobial activity was examined (Sarı *et al*., 2009; Sarı, 2010).

No studies on the antioxidant activity of *Scorzonera veratrifolia* (*S. veratrifolia*) and the analysis of phenolic compounds have been found in the literature searches. The first aim of this study was to examine the antioxidant activities of *n*-heptane, chloroform and methanol extracts obtained from aerial parts and roots of this plant. The last aim of our study is to analyze the phenolic contents of the methanol extract from the above-ground parts of the plant, which shows the strongest activity with LC-MS/MS system.

2. MATERIAL and METHODS

2.1. Identification and Collection of Plant Samples

The aerial parts and roots of *S. veratrifolia* were collected from Tunceli in Turkey by Dr. Ahmet Dogan. The taxonomic description of the plant samples was made by Dr. Ahmet Dogan from Marmara University, Pharmacy Faculty (MARE:13917).

2.2. Preparation of Different Extracts and Extract Yield

The plant samples were dried at room temperature. Using the maceration procedure with *n*-heptane (4x1000 mL), chloroform (3x700 mL) and methanol (4x1000 mL) solvents, extracts were produced from the aerial parts of the plant (200 g). Also, extracts were obtained from the roots of the plant (50 g) using *n*-heptane (4x300 mL), chloroform (3x400 mL) and methanol (4x500 mL) solvents, respectively. The solvents were then filtered via filter paper and evaporated under low pressure in a rotary evaporator, with the raw extracts preserved in the refrigerator. Table 1 shows the yield percentages and extract quantities of the various extracts from the plant. The aerial components of the methanol extract (10.12 g) were found to be more abundant than the other extracts. Furthermore, when the yield percentages of the extracts were compared, it was discovered that the methanol extract (11.96%) made from the plant roots had the highest yield percentage.

Table 1. Amount and percentage yield (%) of *S. veratrifolia* in different extracts.

Extracts	Amount (g)	Yield (%)
Aerial parts *n*-heptane	7.55	3.78
Aerial parts chloroform	3.64	1.82
Aerial parts methanol	10.12	5.06
Root methanol	5.98	11.96
Root chloroform	2.45	2.05
Root heptane	1.12	1.02

2.3. Determination of Total Amount of Phenolic Substances

Distilled water was added to the extracts prepared at concentrations of 1-5 mg/mL and taken into tubes of 0.1 mL each, and their volume was 4.6 mL. 0.1 ml Folin-Ciocalteu reagent and 0.3 mL of 2% sodium carbonate solution added to the absorbance of the color that occurs after being kept in a shaking water bath for 2 hours under room conditions. It was measured at 760 nm in comparison to a standard (Samatha *et al*., 2012).

Preparation of the calibration curve of gallic acid: total phenolic substance was determined by using Folin-Ciocalteu reagent to the gallic acid solutions prepared at concentrations of 0.05-0.40 mg/mL. The calibration curve was prepared by plotting the concentrations against the measured absorbances and the correct equation was obtained [(Abs=75.63 [GA]x-0.044 (R^2 = 0.9963)]. From the obtained equation, the total phenolic amounts of the samples were calculated as the equivalent of mg gallic acid (mg GAE/g extract).

2.4. Determination of the Total Amount of Flavonoid Substances

Total flavonoid amounts of plant extracts were determined by aluminum chloride color measurement method equivalent to quercetin (Samatha *et al*., 2012). Briefly, 0.5 mL of each of the different extracts was taken and mixed with distilled water of 2 mL. Then, 0.15 mL of 5.0% (w/v) $NaNO_2$ solution was added, and this mixture was left for 6 minutes. Then, 0.15 mL of 10% $AlCl_3$ solution was added to the mixture, and after 6 minutes of incubation, 2 mL of 4.0% (w/v) NaOH solution was added to the mixture. Finally, the total volume of the mixture was completed with 5 mL of distilled water, and the absorbance values against the reference solution were measured at 510 nm, in which the pink colored flavonoid-aluminum complex in the alkaline medium gave maximum absorbance after 15 minutes.

2.5. DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to test the extracts' free radical scavenging activity (Wei *et al*., 2010). 3.9 mL of 0.1 mM DPPH solution was added to 0.1 mL of extracts prepared at known concentration and standard solution (ascorbic acid). After mixing the mixture with vortex, it was left at room temperature and in the dark for 30 minutes. UV-vis spectroscopy was used to determine the activity of the extracts, with at least three duplicate measurements taken at 517 nm. The control was prepared under the same conditions using 0.1 mL methanol instead of sample and standard substance. The absorbance of the control was measured daily. Before the IC_{50} value was calculated, the % DPPH radical scavenging activity was calculated with the formula given below:

$$\text{DPPH radical removal capacity} = [(A_0 - A_1)/A_0] \times 100]$$

A_0: Absorbance of the control solution,

A_1: Plant extracts and absorbance of standard solutions

IC_{50} is the concentration of extract or standard material that causes a 50% reduction in initial DPPH concentration. This value was calculated using the correct equation obtained by placing

the % free radical removal activity values against the studied concentrations and the results were given as IC_{50} = mg/mL.

2.6. Iron (III) Reduction/Antioxidant Power (FRAP) Method

Acetate buffer (pH 3.6) 25 mL of 300 mM, 2.5 mL of TPTZ solution (solution of 10 mM TPTZ in 40 mM HCl), and 2.5 mL of 20 mM $FeCI_3.6H_2O$ were mixed and kept at 37 ºC for 30 minutes. In the fourth minute, an increase in absorbance was detected at 593 nm. against the reference prepared by mixing 3.8 mL of the FRAP reagent with 0.2 mL of extract and by adding distilled water instead of the extract. The absorbance values of the extracts at 593 nm were compared with the values of the calibration plot [Abs=0.8226 [$FeSO_4$]x-0.0338 (R^2=0.9940)] prepared with $FeSO_4.7H_2O$, and the FRAP value was expressed as mM $FeSO_4$/mg extract (Benzie & Strain, 1996).

2.7. LC-ESI-QTOF/MS Analysis

The phenolic compounds of methanol extract from aerial parts of plant were determined by LC-ESI-QTOF/MS technique. An Agilent 6530 was used for the separation and analysis of phytochemicals. The chromatographic separation was performed on reverse phase Agilent Poroshell C18 (3.0 x 150 mm, 2.7 µm) analytical column. The column temperature was set to 30 °C. The separation was carried with a gradient binary mixture of solvent A (0.1% aqueous formic acid) and solvent B (acetonitrile) at a flow rate of 0.4 mL/min:0-2 min 10% B; 2-6 min 10-50%B; 6-8 min 50%B; 8-12 min 10-90% B; 12-14 min 10-90% B; 14-14.01 min 90-10% B and stop time is 20.00 min. The electrospray ionization mass spectrometer (ESI/MSn) in negative ion mode was used to create the MS spectra of the most abundant ion. Helium was employed as the collision gas. Nitrogen was employed as a nebulizing gas, a 10-arbitrary-unit auxiliary gas flow, and a 35-arbitrary-unit sheath gas flow. The spray voltage was set to 5.00 kV, while the capillary temperature and voltage were set to 300 degrees Celsius and 35.00 volts, respectively. 10 mg of the extract was dissolved in 3 mL of methanol-water solution (2:1 v/v). The filtrate was then filtered into the vial to 1.5 mL using a filter and syringe and 10 µL sample was injected to LC.

2.8. Statistical Analysis

All the results were performed in triplicate and illustrated in terms of mean±SD. One-way analysis of variance was performed following ANOVA procedures, and significant differences between means were determined by Tukey Multiple Comparison test. $p< 0.05$ was considered statistically significant.

3. RESULTS / FINDINGS

3.1. Determination of Total Flavonoid and Phenolic Content

Total phenolic and flavonoid amounts of different extracts of plant were determined in this study. According to the results, 46.3±1.1 gallic acid equivalents per gram extract was detected as the number of total phenolics of the methanol extract from plant's aerial parts. Besides, it was determined that the methanol extract (0.013±0.020 quercetin equivalents per milligram extract) from plants aerial parts had the highest amount of flavonoid compounds. In this study, the phenolic compounds content of the aerial parts and root extracts of the plant was compared, and it was found that all extracts from the aerial parts contained a higher amount of phenolic and flavonoid compounds than the root extracts (Table 2).

The total flavonoid and phenolic contents of the different extracts (water, ethyl acetate, chloroform, ether, *n*-butanol) of the aerial parts and roots of the *S. paradoxa* was determined. According to the results, it was determined that the *n*-butanol extracts from the aerial parts contain higher amounts of phenolic (11.86±0.10 mg tannic acid equivalents/g extract) and

flavonoid (6.42±0.04 mg rutin equivalents/g extract) compounds than the other extracts (Nasseri et al., 2015). In our study, it was observed that methanol extract of *S. veratrifolia* aerial parts contains higher amounts of phenolic (46.3±1.1gallic acid equivalents per gram extract) and flavonoid (0.013±0.020 quercetin equivalents per milligram extract) compounds than other extracts.

Table 2. Total phenolic and flavonoid contents of different extracts from various parts of *S. veratrifolia*.

Extracts	Total phenolic (mgGAE/g extract)	Total flavonoid (mg QE/mg extract)
Aerial parts methanol	46.3 ± 1.1[a]	0.013±0.002[a]
Aerial parts chloroform	16.5 ±1.5[b]	0.0055±0.011[b]
Aerial parts *n*-heptane	8.1±2.3[c]	0.002±0.003[c]
Root methanol	10.0 ±1.6[d]	0.004±0.011[d]
Root chloroform	4.1±2.5[e]	0.001±0.025[e]
Root *n*-heptane	1.2 ±1.9[f]	0.001±0.032[f]

The mean of three independent determinations (n=3) is used to calculate the standard deviation. GAE–Gallic acid equivalents; QE-Quercetin equivalents. a-f Means in a row without a common superscript letter differ ($p < 0.05$), as analyzed by one-way ANOVA.

3.2. Scavenging Activity of DPPH Radical Assay

The DPPH test is a widely used spectrophotometric method to determine the antioxidant capacity of plants or foods (Bursal *et al*., 2020). The IC_{50} values of the extracts and standards for DPPH radical scavenging were found as; ascorbic acid (0.004±0.003)> aerial parts methanol (0.62±0.60)>roots chloroform (1.14±0.01)>roots heptane (1.37±0.21)>roots methanol (2.76±0.28)>aerial parts chloroform (2.82±0.04)>aerial parts *n*-heptane (4.15±0.02). According to the findings, the methanol extract of aerial parts had much higher DPPH radical scavenging activity than the other extracts. (Table 3).

3.3. Ferric Ions (Fe^{3+}) Reduction Abilities of Different Extracts

The reduction potentials of different extracts obtained from the plants aerial parts and roots were determined by reduction systems including FRAP capability. Fe^{3+} reducing powers of the extracts and standards were decreased as; BHT (1.2±0.21)>aerial parts methanol (1.56±0.03)>roots methanol (1.02±0.05)>aerial parts chloroform (0.980±0.002)>roots chloroform (0.97±0.47)> roots *n*-heptane (0.82±0.51)> aerial parts *n*-heptane (0.22±0.06). In this study, it was found that methanol extracts from the aerial parts and roots of the plant had potent iron reduction capacity from other extracts, while they had a lower capacity than the standard compound (Table 3).

Table 3. Free radical scavenging activity and FRAP of different extracts from various parts of *S. veratrifolia.*

Extracts/standards	DPPH· (IC_{50}: mg/mL)	FRAP assay (mM Fe^{2+}/mg extract)
Aerial parts methanol	0.62±0.60[a]	1.56± 0.03[a]
Aerial parts chloroform	2.82±0.04[b]	0.98± 0.002[b]
Aerial parts *n*-heptane	4.15±0.02[c]	0.22± 0.06[c]
Root methanol	2.76±0.28[d]	1.02± 0.05[d]
Root chloroform	1.14±0.01[e]	0.97± 0.47[e]
Root *n*-heptane	1.37±0.21[f]	0.82± 0.51[f]
Ascorbic acid	0.004±0.003[g]	
BHT		1.2± 0.21[g]

Values are mean of triplicate determination (n=3) ± standard deviation; a-g Means in a row without a common superscript letter differ ($p< 0.05$), as analyzed by one-way ANOVA.

3.4. Identification of phytochemical compounds by LC-ESI-QTOF/MS

In the present study, the antioxidant activities of the extracts obtained from different parts of the plant using different solvents, as well as the total phenolic and flavonoid substance amounts were compared among themselves. The methanol extract from the plants aerial parts was shown to have the highest antioxidant activity as well as total phenolic and flavonoid contents. In line with this result, the LC-QTOF/MS device was used to qualitatively analyze the main phenolic molecules in methanol extract that might be responsible for the activity (Figure 1). The presence of quinic acid, chlorogenic acid, rutin, liquiritin, quercetin hexoside, luteolin-7-*O*-rutinoside and di-*O*-caffeoylquinic acid (Table 4) in the methanol extract from aerial parts was determined in this investigation.

Figure 1. MS base peak chromatogram (BPC) of methanol extract from *S. veratrifolia*.

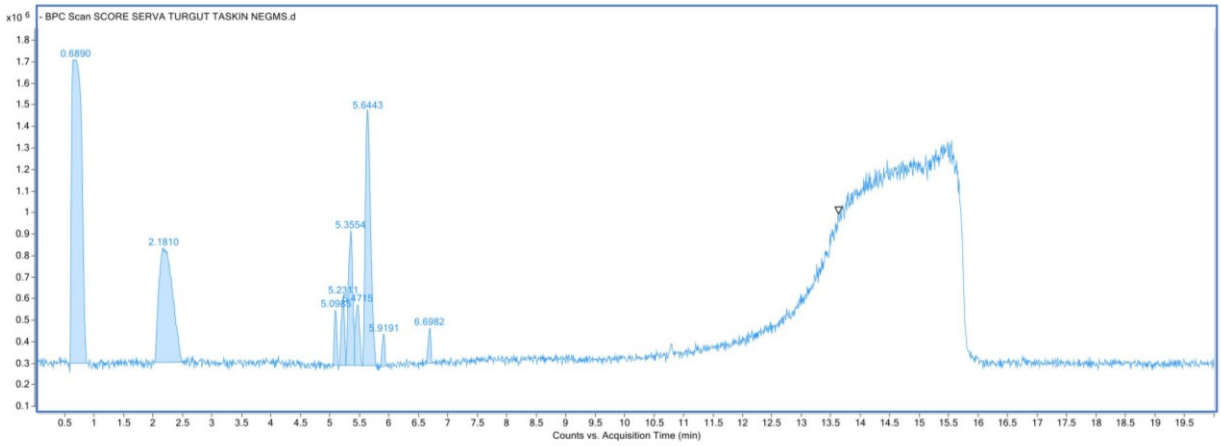

Table 4. Identification of polyphenols in *S. veratrifolia* by LC-ESI-TANDEM MS data

Rt (Min)	$[M-H]^-$	Other MS-MS ions (M/Z)	Identification	Reference
0.7173	191.0569	171,155,127,93,85	quinic acid	*
2.0564	353.0907	191	chlorogenic acid	*
2.1073	707.1843	353,191	chlorogenic acid dimer	*
5.0809	609.1528	300,271,151	rutin	*
5.2578	417.1193	255,169	liquiritin	(Simirgiotis *et al.,* 2015).
5.2829	463.0923	300,271,151,112,69	quercetin hexoside	Hofmann *et al.,* 2016
5.3589	593.1568	285,255,151	luteolin-7-*O*-rutinoside	Simirgiotis *et al.,* 2015
5.4431	515.1266	353, 179,135	di-*O*-caffeoylquinic acid	Schütz *et al.,* 2005

*Compounds identified by comparing retention times and MS data with those of reference

As shown in Supporting Information, quinic acid gave a molecular ion at m/z 191. This compounds MS/MS fragmentation pattern revealed a distinct m/z at 171, 155 with water loss and then 93 fragments of mass with the loss of carbon dioxide. Chlorogenic acid produced deprotonated molecules at m/z 353. The MS/MS fragmentation pattern obtained from chlorogenic acid revealed a characteristic m/z at 191 and 161 by separating the caffeoyl portion and separating the quinic and one molecule of water, respectively. The precursor ion at m/z 707 was identified as chlorogenic acid dimer, and the fragment ion at m/z 353 was a fragment of chlorogenic acid (m/z 354) that lost a hydrogen ion fragment when mass spectrometry was used in negative mode. The fragment of quinine (m/z 192), which lost a hydrogen ion fragment, was another ion at m/z 191. Rutin produced deprotonated molecules at m/z 609 and the sugar portion was separated and gave a fragment of quercetin aglycone at a molecular weight of 300 g/mol. The peak with retention time at 5.26 was liquiritin and gave m/z 255, the molar mass of the

4',7-dihydroxyflavanon (Simirgiotis *et al*., 2015). At m/z 463, quercetin hexoside formed a deprotonated molecule, and a fragment of the aglycon ion peak provided a quercetin monosaccharide at m/z 300.0303 ([M-H-162]$^-$ loss of hexose fragments) (Hofmann *et al*., 2016). There is a parent ion at m/z 593 and fragment ions corresponding to the luteolin aglycon at m/z 285, 255 and 151. This compound has been proven in the relevant literature to be luteolin-7-*O*-rutinoside. (Simirgiotis *et al*., 2015). The peak with retention time at 5.44 was tentatively deduced as di-*O*-caffeoylquinic acid (Schütz *et al*., 2005), which gave fragment ion at m/z 353 M-H-caffeoyl as follows; m/z 173 fragments was formed by the separation of M-H-caffeoyl-quinic molecule ions.

4. DISCUSSION and CONCLUSION

According to the literature research, there are just a few research studies on this species. It was also found that the water extract (3.36±0.28 mg tannic acid equivalents/g extract) from the roots contains high amounts of phenolics, while the *n*-butanol extract (0.15±0.01 mg rutin equivalents/g extract) contains high amounts of flavonoids (Nasseri *et al*., 2015). Unlike this study, the methanol extract of *S. veratrifolia* roots was found to possess greater levels of phenolic (10.00±0.01 gallic acid equivalents per gram extract) and flavonoid (0.004±0.011 quercetin equivalents per milligram extract) compounds than other extracts. In this study, the antioxidant potential of root and aerial parts extracts of the *S. veratrifolia* for the first time was examined in comparison with the standard.

The antioxidant capacities of *Scorzonera* species have been investigated in the studies. It was stated that water extract from the aerial parts of *S. suberosa* (IC_{50}: 42.33±1.60 mg/mL) S. laciniata (IC_{50}: 77.07±1.88) *S. latifolia* (IC_{50}: 29.36±1.46) showed median DPPH radical scavenging activity (Erden *et al*., 2013). In the present study, it was observed that the methanol extract (IC_{50}: 0.62±0.60 mg/mL) from *S. veratrifolia* aerial parts has higher DPPH radical scavenging activity than the water extract from aerial parts of *S. suberosa, S. laciniata* and *S. latifolia*. The DPPH radical scavenging potential of the methanol:water (80:20 v/v) extract from the aerial parts and roots of the *S. latifolia* was determined. It has been determined that the aerial parts (IC_{50}:1.036 mg/mL) have an effective radical scavenging effect compared to the root extract (IC_{50}:4.102 mg/mL) (Açıkara *et al*., 2017). In the present study, the DPPH radical scavenging activity of the aerial parts and root parts of *S. veratrifolia* was examined in parallel with the literature. When the results were examined, it was discovered that methanol extracts from *S. veratrifolia* aerial parts (IC_{50}: 0.62±0.60 mg/mL) and roots (IC_{50}: 2.76±0.28 mg/mL) had higher radical scavenging activity than methanol: water (80:20 v/v) extracts from *S. latifolia* aerial parts and roots.

A previous study found that the methanol extract (10.8%, 50 µg/mL) from the aerial parts of *S. tomentosa* L showed lower radical scavenging activity compared to ascorbic acid (96.78 %, 100 µg/mL) (Karagöz *et al*., 2015). In our present study, it was determined that all extracts of *S. veratrifolia* showed lower activity than ascorbic acid in parallel with the literature.

It was stated that the methanol extract (IC_{50}: 18.81 mg/mL) from the *S. paradoxa* aerial parts showed more effective DPPH radical scavenging activity than the root extract (IC_{50}: 88.9 mg/mL) (Nasseri et al., 2015). According to our findings, it was determined that the methanol extract of *S. veratrifolia* aerial parts (IC_{50}: 0.62±0.60 mg/mL) has more effective DPPH radical scavenging activity than the root (IC_{50}: 2.76±0.28 mg/mL) extract and has a stronger radical scavenging effect than the methanol extracts from *S. paradoxa* aerial parts and roots.

In the literature search, benzylfithalide, scorzoveratrin, scovoveratroside, chlorogenic acid, chlorogenic acid methyl ester, cryptochlorogenic acid, 4,5-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid compounds were isolated from the root of the *S. veratrifolia*, but no study was found about the analysis of phenolic compounds both in the aerial parts by LC-ESI-

QTOF/MS system (Sarı *et al*., 2009; Sarı, 2010). The phenolic compounds analysed in methanol extract of aerial parts, which possesses strong antioxidant activity, were investigated for the first time in this work. It was analyzed by HPLC system that the methanol: water (80:20 v/v) extract from aerial parts of the *S. latifolia* contained high amounts of chlorogenic acid and hyperoside compounds (Açıkara *et al*., 2017). In our current study, it was determined by HPLC system that the methanol extract from *S. veratrifolia* aerial parts contains chlorogenic acid like *S. latifolia* in parallel with the literature.

This study provided information about antioxidant activity and phytochemical composition of *S. veratrifolia*. The antioxidant activity of different extracts from the root and aerial parts of the plant was examined and the aerial parts methanol extract was determined to have the highest antioxidant activity. Furthermore, when compared to other extracts, the methanol extract contained the highest amount of phenolics and flavonoids. It was analyzed by LC-ESI-QTOF/MS that the methanol extract from aerial parts substantially contained quinic acid, chlorogenic acid, rutin, liquiritin, quercetin hexoside, di-*O*-caffeoylquinic acid, and luteolin-7-*O*-rutinoside compounds. These analysed compounds show antioxidant properties because they contain many hydroxyl groups attached to their aromatic rings. Hence, this extract can be used as a natural medicinal and nutritional source in the future after detailed analysis tests.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Authors are expected to present author contributions statement to their manuscript such as; **Duygu Taskin**: Investigation, Writing-original draft, Supervision. **Mert Gecim**: Investigation. **Ahmet Dogan**: Plant collection **Ayfer Beceren**: Supervision.

Orcid

Duygu Taskin https://orcid.org/0000-0002-5279-0900
Mert Geçim https://orcid.org/0000-0002-9575-7801
Ahmet Dogan https://orcid.org/0000-0003-0603-5100
Ayfer Beceren https://orcid.org/0000-0002-6770-6089

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6. APPENDIX

Supporting Information

Figure S1. MS/MS spectra and fragmentation patterns of quinic acid in *S. veratrifolia.*

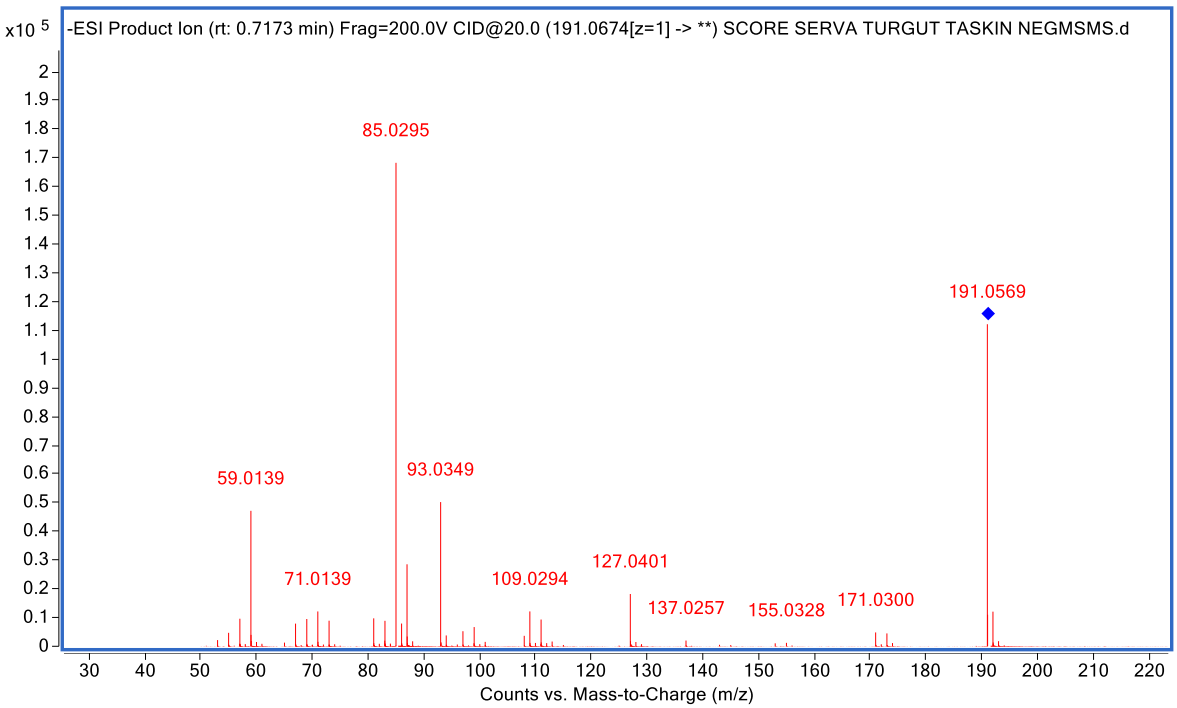

Figure S2. MS/MS spectra and fragmentation patterns of chlorogenic acid in *S. veratrifolia.*

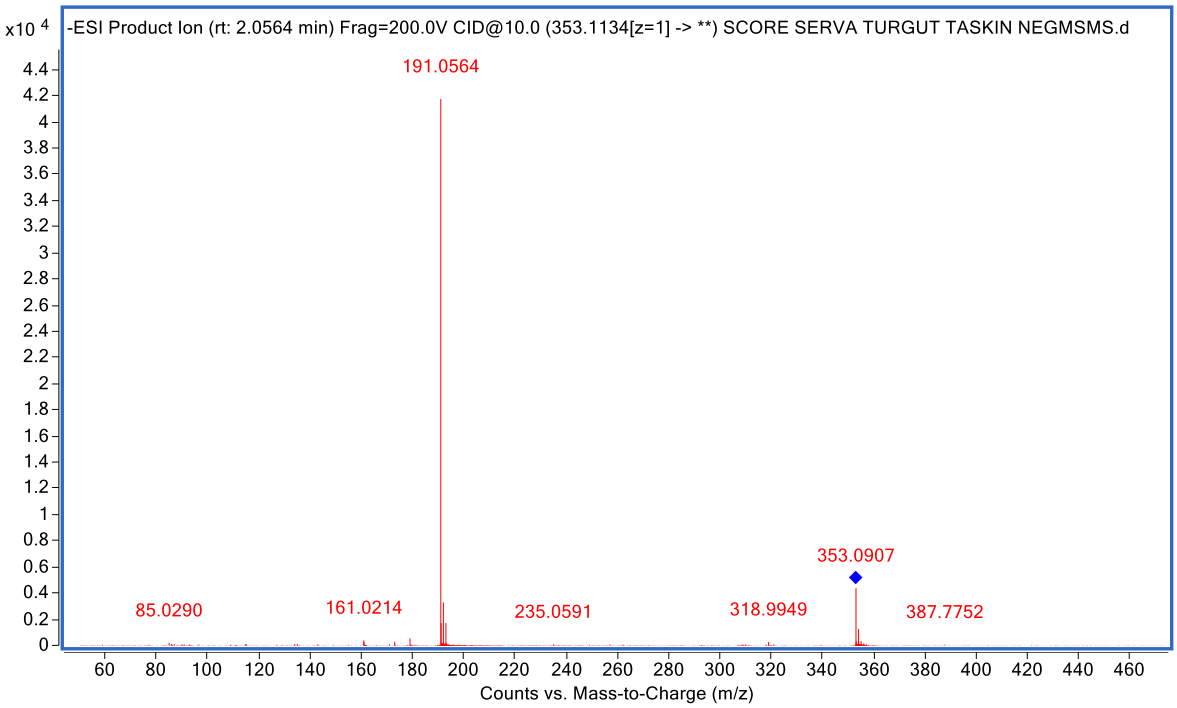

Figure S3. MS/MS spectra and fragmentation patterns of chlorogenic acid dimer in *S. veratrifolia.*

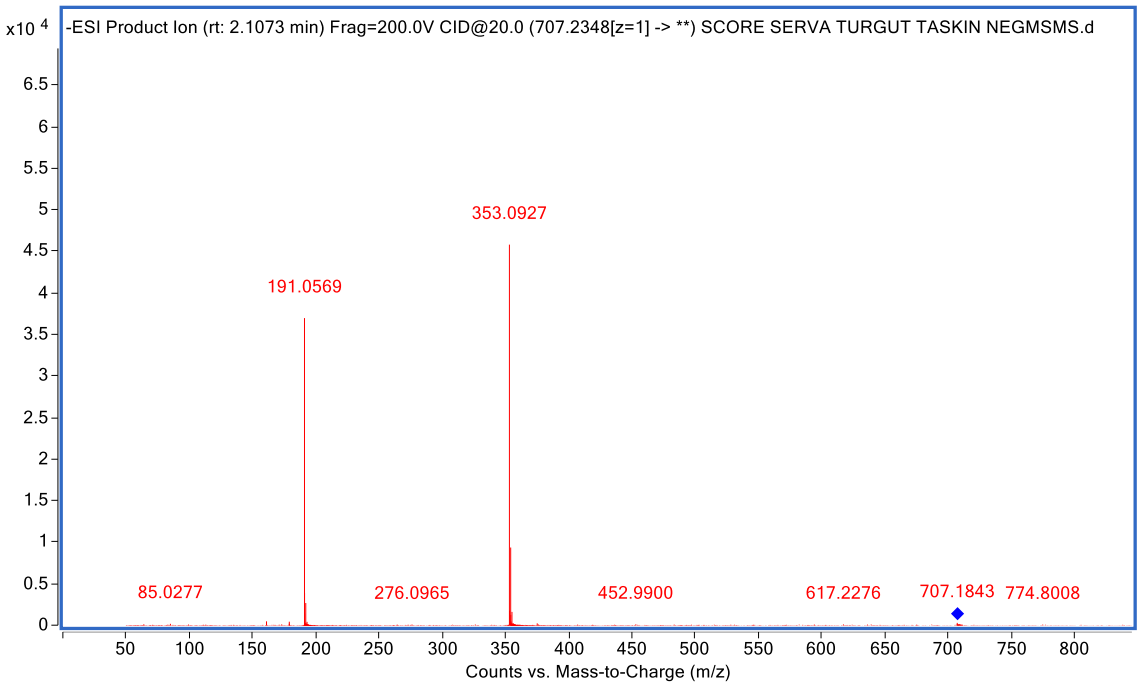

Figure S4. MS/MS spectra and fragmentation patterns of rutin in *S. veratrifolia.*

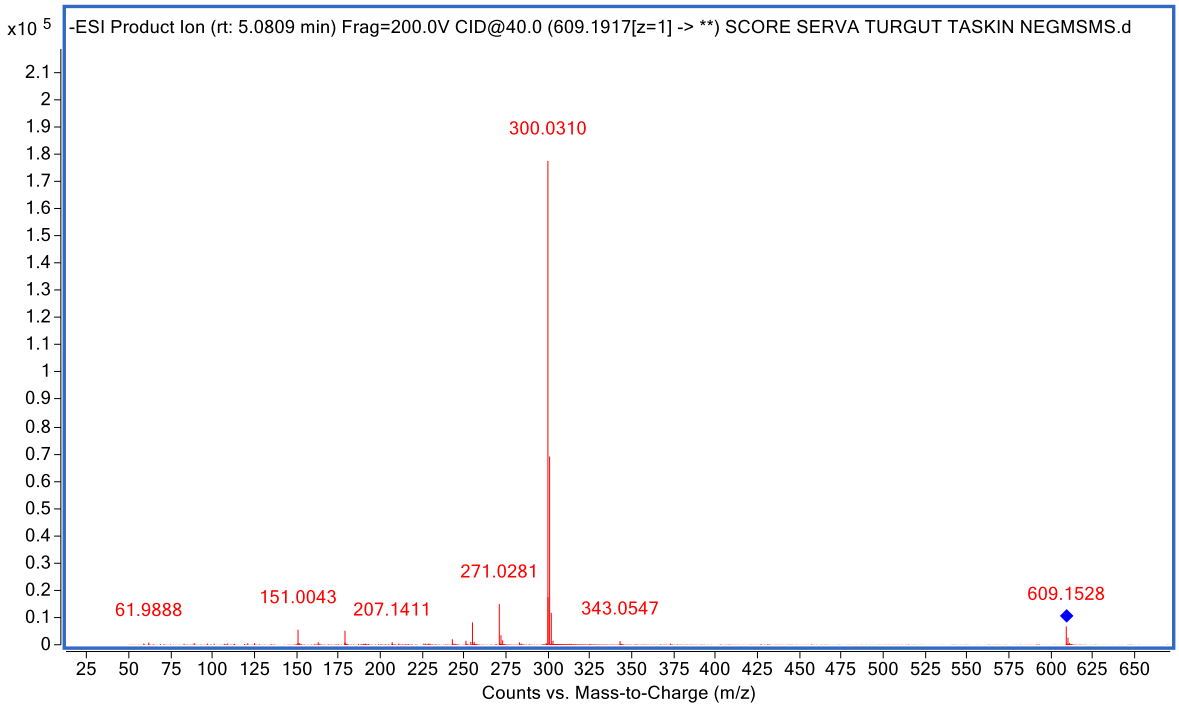

Figure S5. MS/MS spectra and fragmentation patterns of liquiritin in *S. veratrifolia.*

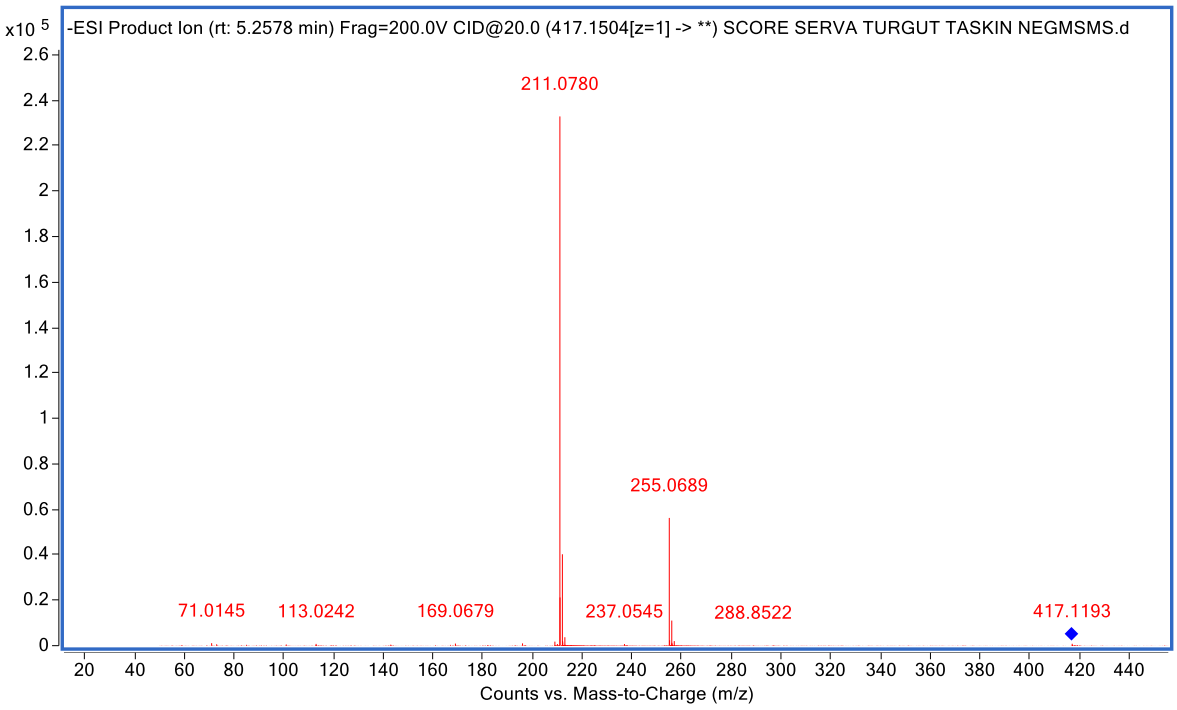

Figure S6. MS/MS spectra and fragmentation patterns of quercetin hexoside in *S. veratrifolia.*

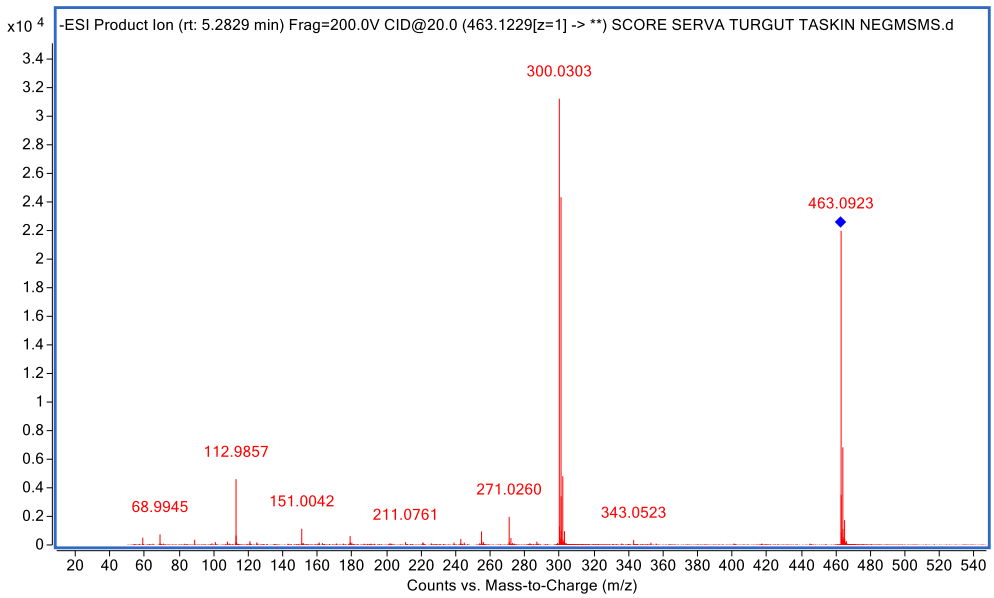

Figure S7. MS/MS spectra and fragmentation patterns of luteolin-7-O-rutinoside in *S. veratrifolia.*

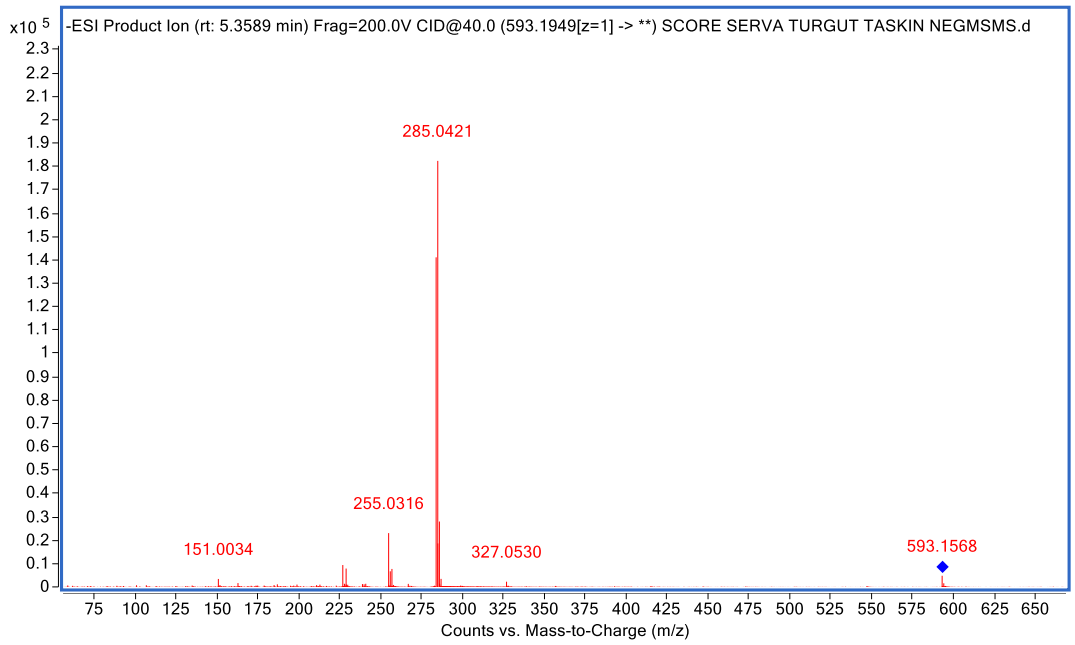

Figure S8. MS/MS spectra and fragmentation patterns of di-O-caffeoylquinic acid in *S. veratrifolia.*

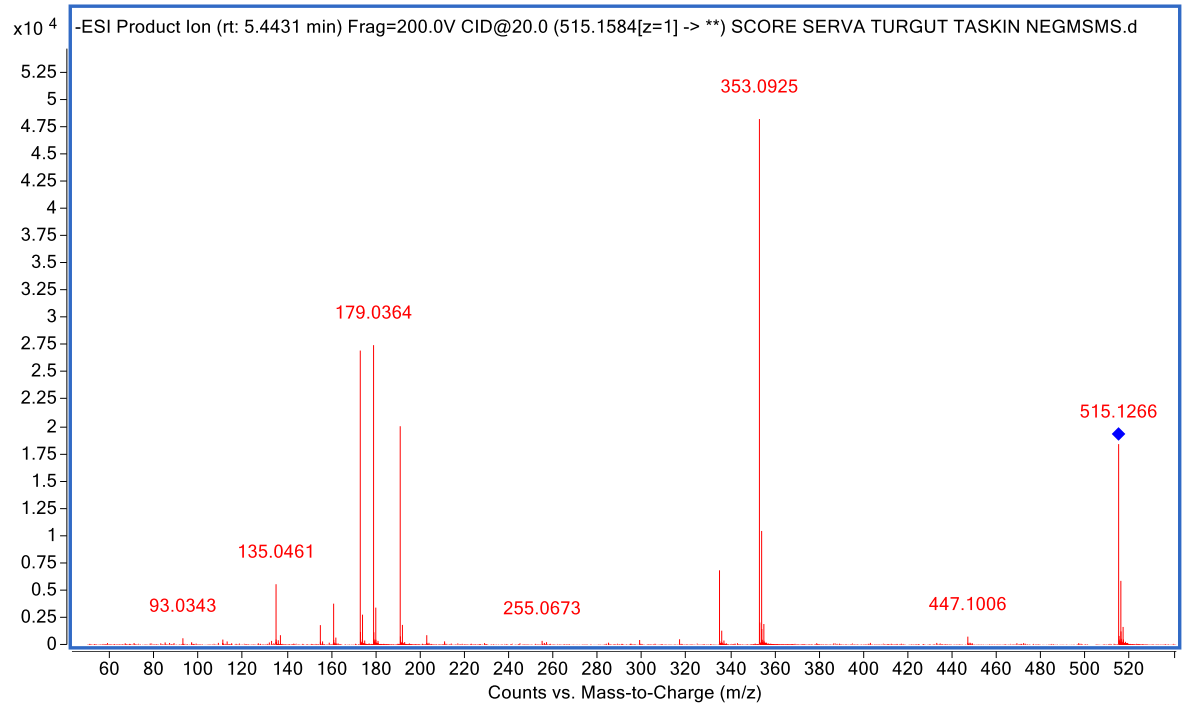

Figure S9. An unknown compounds MS/MS spectra and fragmentation patterns at 5.63 retention time.

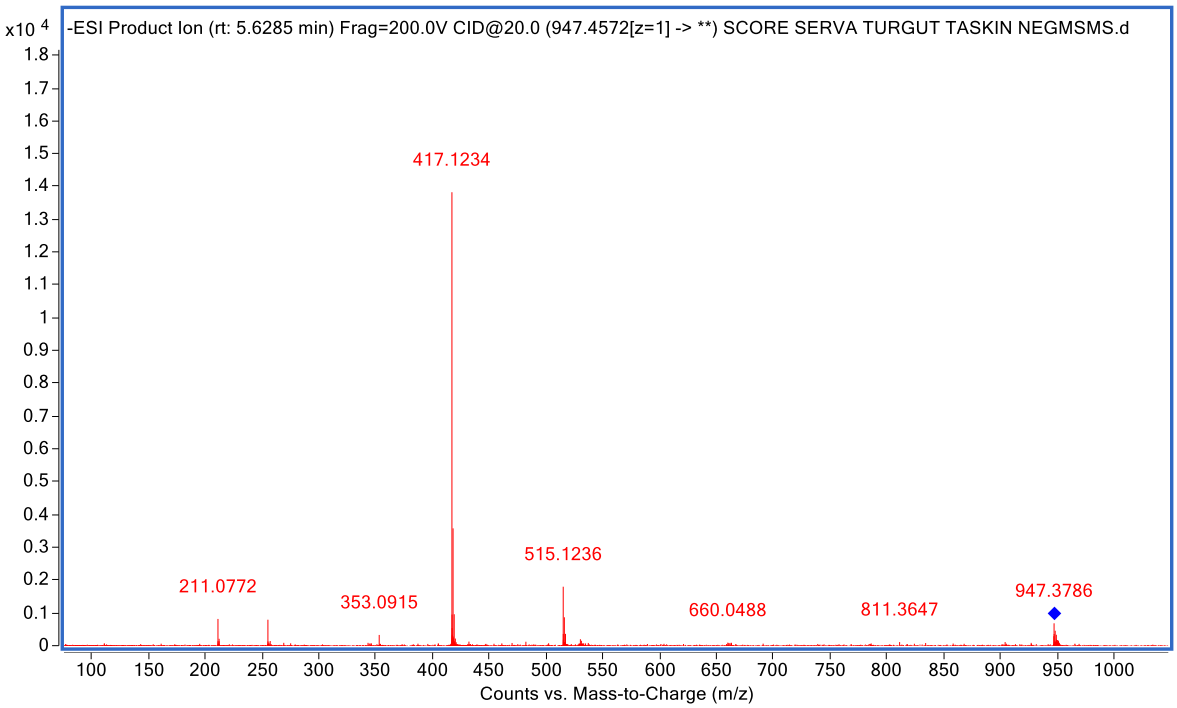

Figure S10. An unknown compounds MS/MS spectra and fragmentation patterns at 5.91 retention time.

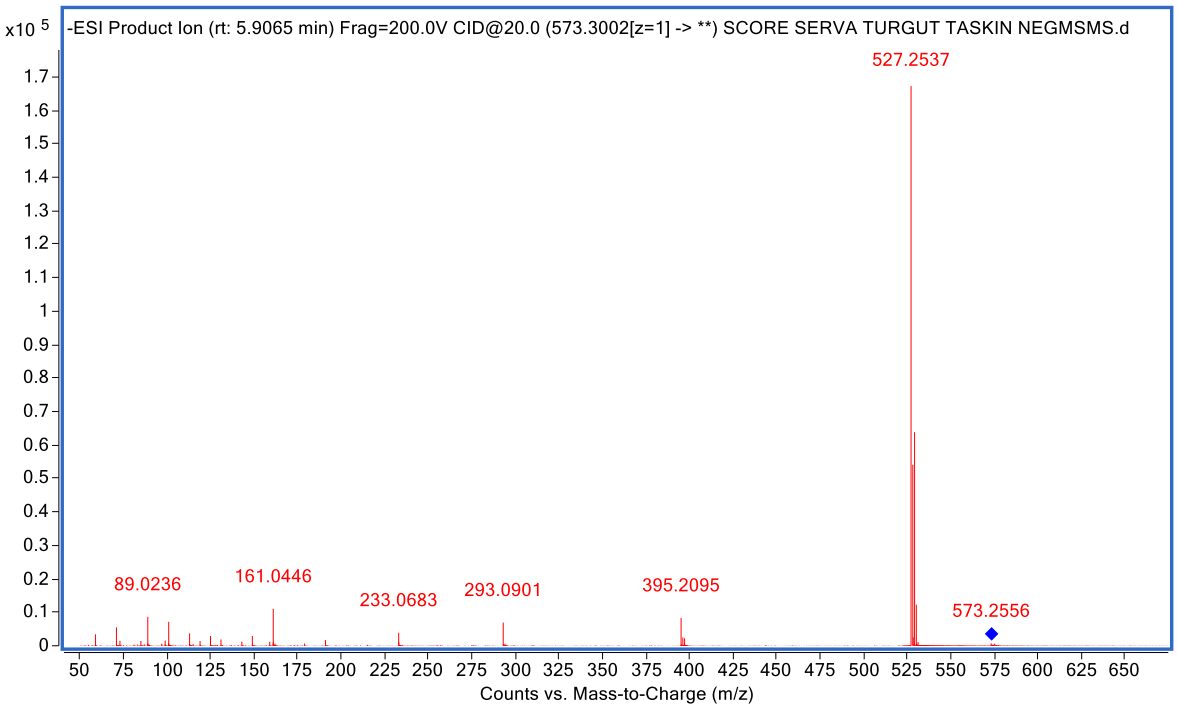

Figure S11. An unknown compounds MS/MS spectra and fragmentation patterns at 6.67 retention time.