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Research Article

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Research Article

An Investigation of The Biological Activity of Monofloral Honey Produced in South-Western Anatolia

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Abstract: In this study, monofloral honeys (chaste, thyme, citrus, and heather) which were obtained from different sources from members of Beekeeping Associations in South-West Anatolia were studied for their antioxidant capacity, total phenolic amounts, total flavonoid amounts, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and urease inhibition activities.

Antioxidant capacity of honey samples was determined by β-carotene, DPPH, $ABTS^+$ and CUPRAC activity methods. In honey samples, the highest antioxidant activity was found in citrus honey with β-carotene/Linoleic acid color opening with IC_{50}: 7.99 mg/mL, and DPPH free radical removal activity with IC_{50}: 5.28 mg/mL in thyme honey. In CUPRAC activity, it was determined that the highest activity was found in heather honey with IC_{50}: 1.69 mg/mL, in terms of $ABTS^+$ removal activity IC_{50}: 2.80 mg/mL in chaste honey, and metal chelating activity IC_{50}: 1.56 mg/mL in thyme honey.

The total phenolic and flavonoids amounts of honeys ranged from 2.31 and 27.15 (µg PEs/mg) to 4.95 and 25.24 (µg QEs/mg), respectively. In addition, AChE inhibition IC_{50}: 24.25 mg/mL in thyme honey, BChE inhibition IC_{50}: 27.93 mg/mL in thyme honey, and urease inhibition IC_{50}: 34.89 mg/mL with citrus honey were determined concerning the highest activity, consecutively.

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1. INTRODUCTION

Honey is likely known as the oldest natural sweetener food (Kaygusuz *et al*., 2016). Honey is produced by honeybee (Apis mellifera) in almost all countries of the world and is also widely consumed as a food source (Silva *et al*., 2009). It contains sugary ingredients and significant antioxidant substances. It has also many antioxidant properties and these compounds are found naturally in honey. The main natural antioxidants in foods that protect the body against harmful free radicals are vitamins (vitamins A, C and E), flavonoids, carotenoids, and polyphenols. The antioxidant activity of honey generally consists of phenolic compounds, enzymes, ascorbic acid, and peptides (Nicholls & Miraglio, 2003).

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In most studies, it was determined that there was an inverse relationship between the consumption of fruits and vegetables and the formation of certain cancer and heart diseases (Prior & Cao, 2000; Vitaglione *et al*., 2005). Today, in addition to natural antioxidants, many synthetic antioxidants have been produced; however, many studies have reported negative effects of the use of these synthetic antioxidants on the health (Ito *et al*., 1986). Therefore, the use of safer natural antioxidants instead of synthetic antioxidants has become important.

The antioxidant activity and total phenolic content of honey have been found to be parallel (Nicholls & Miraglio, 2003). The most common phenolic compounds in honey are flavonoids and phenolic acids. These phenolic compounds have been shown to play an important role in cardiovascular diseases and cancer treatments as well as antibacterial, anti-inflammatory, anti-allergic, and anti-thrombotic effects (Al-Mamary *et al*., 2002; Pyrzynska & Biesaga, 2009; Zaidi *et al*., 2019).

The enzymes in honey are formed during the processing of nectar by a bee (Badiou *et al*., 2008; Serrano *et al*., 2007). Some of these enzymes originate from plant nectar, bee throat secretion or saliva liquid, diastase (α and β-amylase), invertase (α-glycosidase), glucose oxidase, catalase, and acid phosphatase. In addition, enzymes such as AChE, BChE, urease, and peroxidase are found in a smaller amount (Bertoncelj *et al*., 2007). However, especially among these enzymes, AChE and BChE are important enzymes required for the healthy functioning of the nervous system in our body. AChE is an enzyme that is free in tissues or is compounded with phospholipids and hydrolyzes acetylcholine (Badiou *et al*., 2008).

The cause of Alzheimer's disease, an important disease of our time, is not known exactly. However, the progression of Alzheimer's disease, especially protein accumulation in the brain, disruption of nerve conduction, such as damage to brain cells plays a significant role. In addition to the stimulating effect of acetylcholine in memory, the choline acetyltransferase that allows the synthesis of acetylcholine provides a marked reduction in disease. Inhibitions of acetylcholinesterase (AChE) have been reported to be the most widely used treatment option against Alzheimer's disease (Orhan *et al*., 2006; Vinutha *et al*., 2007; Deveci *et al*., 2018). Therefore, honey containing the enzyme AChE can be considered as a supplementary food.

The aim of this study is to determine the antioxidant capacity, total phenolic amounts, total flavonoid amounts, acetylcholinesterase, butyrylcholinesterase, and urease inhibition activities of monofloral honeys produced in the South-West Anatolia Region, which is a very important region in honey production in Turkey.

2. MATERIAL and METHODS

In this study, monofloral honeys (chaste, thyme, citrus, and heather) which were obtained from South-West Anatolia (Mugla, Antalya, Aydin, and Denizli provinces) were collected from members of Honey Producer Associations. Honey samples were coded and stored in dark and room conditions until the analyses were done. The codes of used honey samples in this study are given in Table 1. In our previous study, the physicochemical components of some monofloral honeys supplied from this region were determined (Karatas *et al*., 2019).

There are several methods for determining antioxidant capacities. However, although there are many methods, a standard method that reflects the useful and antioxidant capacity has not been developed yet. Therefore, researchers state that a single method is not sufficient and several different methods are required for antioxidant capacity determination (Wong *et al*., 2006). In this study, honey's antioxidant capacity was analyzed by β-carotene, DPPH, $ABTS^{+}$ and CUPRAC activity methods. In addition, total phenolic amounts, total flavonoid amounts, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and urease inhibition activities were determined.

Table 1. The codes, origin and region of honey samples.

CODE NO	ORIGIN	PROVINCE (TOWN)
HC1, HC2, HC3, HC4	Chaste	Aydin (Cine)
HK1, HK2, HK3	Chaste	Aydin (Kocarli)
HS1, HS2, HS3	Chaste	Aydin (Soke)
KD1, KD2, KD3, KD4	Thyme	Mugla (Datca)
KK1, KK2, KK3	Thyme	Denizli (Tavas)
KU1, KU2, KU3	Thyme	Mugla (Ula)
ND1, ND2, ND3	Citrus	Mugla (Dalaman)
NF1, NF2, NF3, NF4	Citrus	Antalya (Finike)
NK1, NK2, NK3, NK4, NK5	Citrus	Mugla (Koycegiz)
PC1, PC2	Heather	Aydin (Cine)
PD1, PD2, PD3	Heather	Mugla (Datca)
PK1, PK2, PK3	Heather	Aydin (Kocarli)

2.1. Determination of the Activities of Honeys

2.1.1. *Determination of total carotenoid content*

The antioxidant activities of the honeys studied were examined according to the β-carotene linoleate model system. 40 µL of the honey samples prepared (2.5, 5, 10, 20, 40% by mass) was taken and 80 µl emulsion solution was added. The absorbance of the emulsion solution in 96-well microplates was read at 470 nm. Then, the tubes were allowed to incubate for 120 minutes in total by reading the absorbance values at 45 ^{0}C for half an hour. In addition, deionized water was added instead of honey samples and 80 µL of emulsion solution was added to the control solution. The absorbance of the control solution was read immediately as soon as the emulsion solution was added and allowed to incubate at 45 ^{0}C for half an hour at 120 ^{0}C (Sökmen *et al*., 2004; Habib *et al*., 2014). A standard solution of five different concentrations was prepared from the synthetic antioxidants BHA and the natural antioxidant α-tocopherol at 4000 ppm concentration. The reduction percentage was given according to the below Eq. (1)

Anti-radical activity (%) = [(Abs control – Abs sample) / Abs control] ×100 (1)

2.1.2. *DPPH free-radical scavenging assay*

Free radical removal activities of honey samples were determined using 1,1-diphenyl-2-picrylhydrazase (DPPH). For this purpose, 2.5, 5, 10, 20 and 40% aqueous solutions were prepared by weight (w/v). A standard solution of five different concentrations was prepared from the synthetic antioxidants BHA and the natural antioxidant α-tocopherol at 4000 ppm concentration.

0.4 mM DPPH solution was subjected to dilution with ethanol by controlling the absorbance at 517 nm. Subsequently, 40 µL of different concentrations of honey samples were placed in microplates. Then, 120 µL of ethanol and 40 µl of DPPH solution were added to incubate for 30 minutes in the dark condition. The absorbances were read at 517 nm. The absorbance results of honey samples were examined against control. Free radical removal activity was used as below and % inhibition values were calculated from these absorbance values (Burits *et al*., 2001). Inhibition activity capacities of honey were calculated with the following equation (2).

Anti-radical activity (%) = [(Abs control – Abs sample) / Abs control] ×100 (2)

2.1.3. *$ABTS^+$ cation radical removal activity determination*

Cation removal activities of honey samples were determined using $ABTS^+$ (Re *et al*., 1999). $ABTS^+$ was obtained by reaction between a prepared aqueous ABTS solution with 7 mM and 2.4 mM potassium persulfate ($K_2S_2O_8$). It was kept in the dark for 12-16 hours at room temperature. A solution of 160 µL of $ABTS^+$ was added over 40 µL of the sample at different concentrations. After 10 minutes of incubation at room temperature, absorbance was measured in a 96-well microplate reader at 734 nm. A standard solution of five different concentrations (2.5, 5, 10, 20 and 40% by mass) was prepared from the synthetic antioxidants BHA and the natural antioxidant α-tocopherol at 4000 ppm concentration. The absorbances of the samples were evaluated against control. The capability of scavenging the inhibition activity was calculated using Eq. (2).

2.1.4. *Cupric reducing antioxidant capacity assay*

Copper (II) ion reduction antioxidant capacity was determined by the method used by Apak *et al* (2004). All honey samples were studied at five concentrations (2.5, 5, 10, 20 and 40% by mass). Each of the honey samples prepared as 40 µL in ethyl alcohol was placed in a 96-well microplate. Subsequently, 60 µL of ammonium acetate buffer was mixed with 50 µL of 7,49 mM neocuprin and 50 µL of 10 mM Cu^{+2} solutions. After 1 hour waiting period, the absorbance at 450 nm was measured against the antioxidant-free reference. A standard solution of five different concentrations was prepared from the synthetic antioxidants BHA and the natural antioxidant α-tocopherol at 4000 ppm concentration.

2.1.5. *Determination of total phenolic content*

Total phenolic content was determined by Folin-Ciocalteu reagent (FCR). A standard calibration graph was obtained by measuring the absorbance values of pyrocatechol at various concentrations at 760 nm. In the same way, 250 µL of the samples were taken and 0.1 mL FCR was added and left for 3 minutes. Then, 0.3 mL of Na_2CO_3 solution was added. After 2 hours of incubation, the amounts of phenolic compounds were determined by reading the absorbances. The amounts of phenolic compounds in honey samples were expressed as µg equivalent to pyrocatechase (Öztürk *et al*., 2007; Everette *et al*., 2010; Nayik & Nanda, 2016).

2.1.6. *Determination of total flavonoid content*

Total flavonoid amounts were made according to the method by Moreno *et al*. (2000). Different concentrations of quercetin solutions were prepared and treated with aluminum nitrate and potassium acetate. Then, the standard graph was obtained by measuring the absorbance values at 415 nm.

500 µL of the analysis samples were taken and completed to 4.8 mL with methanol. Then, 100 µL of potassium acetate solution was added and left for 1 min. Finally, 100 mL of $Al(NO_3)_3.9H_2O$ solution was added. After 40 minutes of incubation, the absorbance in 415 nm was read by adding the total amount of flavonoid in honey samples equivalent to µg quercetin (Öztürk *et al*., 2007; Escuredo *et al*., 2013).

2.2. Determination of the Enzyme Activities of Honeys

2.2.1. *Anti-cholinesterase assays*

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition activities were measured by a spectrophotometric method according to Ellman's method (Deveci *et al*., 2018; Ellman *et al*., 1961; Boily *et al*., 2013). AChE and BChE, acetylcholine iodide (AcI) as a substrate, butyrylcholine iodide (BcI) and yellow 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) were used for the measurement of activities. For this, five different concentrations of honey solutions (2.5, 5, 10, 20 and 40% by mass) were prepared and the IC_{50} values were calculated. In this study, galantamine solution was used as the standard inhibitor.

Measurement of AChE and BChE inhibition activities was 10 µL of different concentrations of honey samples added to one well of 96-well microplate. 160 µL of 0.1M phosphate buffer and 10 µL of AChE or BChE enzyme solutions were added. Pure water was used as a control and incubated at 25 ^{0}C for 15 minutes. Then, 10 µL of DTNB solution and 10 µL of AcI were added, and kinetic absorbances were measured for 10 minutes at 412 nm (Ellman *et al*., 1961).

2.2.2. *Urease inhibition activity measurement*

Honey samples of different concentrations (2.5, 5, 10, 20 and 40% by mass) were taken 10 µL of 96-well microplates. After interacting them with 25 µL of urease enzyme, NaH_2PO_4 prepared at pH: 8.2 was added to these concentrations. 50 µL of urea solution was added as a substrate and left for incubation for 15 minutes. Then, 70 µL of sodium hydroxide and phenol reagent containing sodium nitroprusside was added to 45 µL of ammonia solution. Finally, 70 µL of sodium hypochlorous was added and the absorbance at 630 nm was measured after 50 minutes (Khan *et al*., 2004).

3. RESULTS / FINDINGS

3.1. Antioxidant Activity Results of Honey

Antioxidant activity measurements of honey samples were made by using β-carotene, DPPH, $ABTS^+$ and CUPRAC activity methods. Accordingly, the results are determined in IC_{50} values and the results are given collectively in Table 2.

3.1.1. β-*Carotene activity results*

The results are given by determining the IC_{50} values. IC_{50} values of BHA and α-tocopherol were used as a standard for the comparison of the antioxidant activity of honey samples. When these results are examined, it is seen that the antioxidant activities of the honey samples change according to their origin, the region where they grow, and the type of honey (Habib *et al*., 2014).

Considering the IC_{50} values of β-Carotene color exploration activity results, it was determined that all honey showed the highest inhibition at a concentration of 40%. According to the β-carotene discoloration activity method, the highest IC_{50} value was observed in citrus honeys. After the citrus honeys (IC_{50}:7.996), it was determined that they had chaste (IC_{50}: 9.428), thyme (IC_{50}: 14.733), and heather honey (IC_{50}: 15.667), consecutively (Table 2).

3.1.2. *DPPH free radical removal activity results*

When IC_{50} values of DPPH free radical removal activity results were calculated, it was determined that all honeys studied showed the highest inhibition at a concentration of 40%. It was seen that the highest IC_{50} value was in thyme honey. After thyme honey, it was determined to be in heather, citrus and chaste honey, consecutively. The results of the DPPH free radical removal activity of honey samples are consistent with the available literature (Meda *et al*., 2005; Ertürk *et al*., 2014; Philip & Mohd Fadzelly, 2015).

3.1.3. *$ABTS^+$ cation radical removal activity results*

In the $ABTS^+$ cation radical removal activity results, IC_{50} values of BHA and α-tocopherol were used as a standard for comparison in determining the antioxidant activities of honey samples. It was determined that all honey showed the highest inhibition at a concentration of 40%. When the IC_{50} values were calculated, it was seen that the highest inhibition value was found in heather honeys. After the heather honeys, it was determined that they had thyme, chaste and citrus honey, respectively (Table 2).

3.1.4. *Results of CUPRAC*

$A_{0.5}$ values of BHA and α-tocopherol were used as the standard for comparison in determining the antioxidant activity of honey samples. $A_{0.5}$ values were calculated according to the method given in the Cupric Reducing Antioxidant Capacity assay. When the CUPRAC results of all

honey samples were examined, it was seen that the highest absorbance value was in the heather honey, followed by thyme, chaste and citrus honeys, respectively (Table 2) (Ulusoy *et al*., 2010).

Table 2. Antioxidant Activity Results of Monofloral Honeys.

Code	β-Carotene	DPPH	$ABTS^+$	CUPRAC
	IC_{50} (mg/mL)			$A_{0.5}$ (mg/mL)
HC1	29.580±1.34	>40.00±1.44	3.690±1.12	6.002±1.22
HC2	>40.00±1.67	>40.00±2.01	3.780±1.22	4.819±1.55
HC3	26.852±1.78	-	21.063±1.56	10.760±1.28
HC4	17.908±1.34	11.570±1.92	18.952±1.53	9.565±1.56
HK1	12.985±1.24	>40.00±1.56	4.486±1.72	1.172±1.24
HK2	38.305±1.39	11.123±1.45	2.803±1.81	9.847±1.78
HK3	18.715±1.18	>40.00±1.10	16.841±1.92	9.654±1.72
HS1	30.433±1.47	>40.00±1.22	37.469±1.44	9.847±1.68
HS2	34.268±1.83	>40.00±1.24	>40.00±1.51	22.647±1.36
HS3	9.428±1.80	>40.00±1.88	26.023±1.73	9.519±1.89
KD1	>40.00±1.14	34.335±1.12	17.762±1.67	8.875±1.19
KD2	21.062±1.89	6.122±1.78	-	3.682±1.44
KD3	>40.00±1.56	>40.00±1.67	9.576±1.35	9.406±1.77
KD4	27.971±1.65	>40.00±1.62	4.058±1.14	8.700±1.83
KK1	28.763±1.78	>40.00±1.66	16.276±1.83	8.384±2.02
KK2	14.733±1.99	6.603±1.44	5.246±1.82	3.700±1.92
KK3	20.541±2.12	>40.00±1.21	6.647±2.02	8.561±1.82
KU1	>40.00±2.21	6.460±1.50	-	7.142±1.43
KU2	25.189±1.45	5.286±1.53	3.439±2.08	6.752±1.68
KU3	26.520±1.56	10.320±1.78	4.098±1.46	5.733±1.44
ND1	17.641±1.23	>40.00±1.35	5.694±1.13	12.657±1.68
ND2	12.244±1.55	22.210±1.67	12.434±1.18	>40.00±1.15
ND3	18.542±1.78	>40.00±1.14	>40.00±1.68	>40.00±1.56
NF1	7.996±1.22	>40.00±1.43	28.949±1.66	14.993±1.25
NF2	11.658±1.78	>40.00±1.53	38.768±1.12	10.568±1.19
NF3	13.066±1.20	>40.00±1.74	36.024±1.55	20.868±1.57
NF4	22.837±1.20	13.893±1.23	-	8.802±1.77
NK1	19.803±1.23	8.037±1.57	9.419±1.89	20.056±1.42
NK2	>40.00±1.88	18.635±1.34	8.685±1.34	18.372±1.49
NK3	29.045±1.56	15.218±1.87	20.445±1.13	8.633±1.77
NK4	>40.00±1.78	15.501±1.67	20.802±1.75	30.638±1.47
NK5	26.600±1.26	21.684±1.06	25.659±1.13	19.545±1.18
PC1	28.860±1.67	>40.00±1.02	5.291±1.18	4.797±1.55
PC2	18.846±1.45	>40.00±1.10	5.084±1.98	6.948±1.77
PD1	>40.00±1.98	14.631±1.32	5.319±1.44	4.452±1.54
PD2	15.667±1.12	18.208±1.41	3.189±1.77	4.893±1.78
PD3	18.303±1.57	9.846±1.52	2.812±1.17	6.583±1.27
PK1	24.869±1.54	20.548±1.87	7.347±1.82	1.694±1.63
PK2	28.266±1.08	20.436±1.56	2.827±1.41	2.602±1.13
PK3	25.447±1.64	11.094±1.34	3.452±1.86	5.121±1.88
Standards (mg / mL)				
BHA	0.0014 ±0.00001	0.0170 ±0.00018	0.0128 ±0.00050	0.0210 ±0.00001
α-tocopherol	0.0022 ±0.00004	0.0387 ±0.00023	0.0345 ±0.00047	0.0854 ±0.00001

3.1.5. *Total phenolic and total flavonoid measurement results*

Total phenolic and total flavonoid contents of all honey samples were determined according to the method given in the determination of total phenolic and total flavonoid content, respectively. The total phenolic and flavonoid results obtained are given as pyrocatechol (mg PEs/mg honey) and quercetin equivalent (mg QEs/mg honey) in Table 3, respectively.

Table 3. Total Phenolic and Flavonoid Substance Results.

Code	Total Phenolic Substance (µg PEs/mg honey)	Total Flavonoid Substance (µg QEs/mg honey)
HC1	14.06±1.45	13.37±1.23
HC2	17.19±1.23	14.08±1.56
HC3	6.56±1.56	5.90±1.13
HC4	10.31±1.44	20.06±1.24
HK1	4.06±1.12	9.82±1.67
HK2	11.56±1.56	21.22±1.77
HK3	7.65±1.89	10.19±1.55
HS1	5.81±2.04	5.02±1.12
HS2	2.31±1.88	5.33±1.67
HS3	3.44±1.68	7.13±1.83
KD1	24.69±1.20	16.28±1.42
KD2	21.14±1.13	15.21±1.55
KD3	7.81±1.67	10.57±1.72
KD4	3.44±1.93	12.19±1.12
KK1	5.31±1.24	18.33±1.14
KK2	23.44±1.22	18.93±1.77
KK3	19.12±1.16	13.90±1.56
KU1	21.56±1.66	14.70±1.34
KU2	12.81±1.89	23.26±1.22
KU3	27.15±1.34	21.39±1.73
ND1	10.43±1.18	7.88±1.55
ND2	10.41±1.45	7.56±1.78
ND3	10.88±1.83	7.31±1.52
NF1	10.94±1.77	5.68±1.22
NF2	12.19±1.39	4.95±1.56
NF3	12.19±1.99	5.46±1.78
NF4	14.06±1.55	15.95±1.12
NK1	13.21±1.78	6.15±1.68
NK2	10.91±1.35	6.99±1.82
NK3	10.94±1.78	7.55±1.77
NK4	11.56±1.26	6.79±1.45
NK5	13.44±1.34	7.86±1.82
PC1	12.69±1.55	15.77±1.99
PC2	10.31±1.43	12.06±1.91
PD1	12.81±1.42	17.39±1.23
PD2	13.44±1.10	15.70±1.57
PD3	21.56±1.83	20.82±1.88
PK1	23.42±1.45	10.66±1.34
PK2	11.31±1.34	15.64±1.55
PK3	17.81±1.13	16.86±1.78

The phenolic content of the honey samples varied between 2.31 and 27.15 (µg PEs/mg honey), as shown in Table 3. The highest phenolic content value was 27.15 (µg PEs/mg honey) with KU3 coded samples. When the flavonoid content of these honeys was examined, it was found between 5.02-23.26 (µg QEs/mg honey) and the highest value was found in KU2 sample.

The results of the phenolic activity and the total phenolic content of different origin honey samples are consistent with those in the related literature (Al-Mamary *et al*., 2002; Nayik & Nanda, 2016; Uzun, 2011).

3.2. Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) Activity Results

AChE inhibition activity of four different monofloral honeys was compared and the highest activity was determined to be in thyme honeys. Activity fell in citrus honey, heather honey and chaste honey, respectively (Table 4). Also, it was determined that the highest activity in the thyme honey was the KU2 coded sample with a concentration of IC_{50}: 25.27 mg/mL. Accordingly, the studied samples were found to have lower activity in terms of acetylcholinesterase (AChE) compared to Galantamine, the standard inhibitor. However, considering that honey is a functional food, it can be said that it has a moderate activity against acetylcholinesterase (AChE).

Considering the results of BChE inhibition activity, samples of chaste honey showed low activity against BChE. It was determined that the highest activity in the thyme honey was the KD3 coded sample with a concentration of IC_{50}: 27.93 mg/mL (Table 4). These results show that thyme honeys have a high inhibition of BChE. In the same way, citrus honeys were in the second order. After the citrus honey, chaste honeys were in the third place and heather honeys were in the fourth order. Accordingly, thyme and citrus honeys have been shown to have a lower level of inhibition in butyrylcholinesterase (BChE) compared to the standard inhibitor (Galantamine). Such studies have been supported by similar literature (Philip & Mohd Fadzelly, 2015; Uzun, 2011; Wang *et al*., 2016).

When acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity results were compared, it was determined that the highest activity was in thyme honey. Compared to the honey samples studied, citrus honeys showed activity in the second place.

Table 4. Acetylcholinesterase (AChE), Butyrylcholinesterase (BChE) and Urease Activity Results.

Code	Acetylcholinesterase (AChE) IC_{50} (mg/mL)	Butyrylcholinesterase (BChE) IC_{50} (mg/mL)	Urease IC_{50} (mg/mL)
HC1	>80.00±1.66	78.32±1.65	71.24±1.66
HC2	>80.00±1.13	>80.00±1.12	>80.00±1.67
HC3	78.55±1.11	>80.00±1.98	36.58±1.52
HC4	>80.00±1.78	>80.00±1.55	>80.00±1.21
HK1	>80.00±1.44	>80.00±1.66	>80.00±1.98
HK2	72.24±1.67	70.64±1.34	>80.00±1.66
HK3	>80.00±1.34	>80.00±1.78	>80.00±1.35
HS1	>80.00±1.82	>80.00±1.88	35.50±1.36
HS2	>80.00±1.22	>80.00±1.24	>80.00±1.62
HS3	>80.00±1.99	>80.00±1.99	42.81±1.18
KD1	74.16±1.01	>80.00±2.12	>80.00±1.19
KD2	25.24±1.67	75.23±1.45	>80.00±1.84
KD3	61.31±1.55	27.93±1.67	70.26±1.54
KD4	>80.00±1.38	>80.00±1.77	>80.00±1.44
KK1	32.93±1.78	71.65±1.88	>80.00±1.55

Table 4. *Continues.*

KK2	64.55±1.24	69.13±1.23	57.40±1.93
KK3	>80.00±1.22	>80.00±1.33	>80.00±1.21
KU1	>80.00±1.62	>80.00±1.44	35.96±1.46
KU2	25.27±1.77	39.09±1.98	48.25±1.83
KU3	66.26±1.12	>80.00±1.24	34.89±1.34
ND1	>80.00±1.50	>80.00±1.35	>80.00±1.34
ND2	>80.00±1.78	>80.00±1.55	>80.00±1.23
ND3	32.23±1.44	>80.00±1.67	>80.00±1.35
NF1	>80.00±1.35	>80.00±1.77	>80.00±1.92
NF2	49.17±1.46	>80.00±1.22	>80.00±1.64
NF3	>80.00±1.27	66.08±1.12	>80.00±1.73
NF4	>80.00±1.22	37.64±1.18	>80.00±1.77
NK1	>80.00±1.77	>80.00±1.87	>80.00±1.33
NK2	37.79±1.89	>80.00±1.34	>80.00±1.35
NK3	>80.00±1.99	>80.00±1.33	>80.00±1.39
NK4	>80.00±1.23	35.23±1.55	>80.00±1.77
NK5	47.05±1.67	75.62±1.35	>80.00±1.63
PC1	>80.00±1.34	>80.00±1.78	>80.00±1.24
PC2	>80.00±1.25	>80.00±1.24	52.43±1.78
PD1	46.03±1.66	>80.00±1.78	>80.00±1.24
PD2	71.91±1.44	>80.00±1.28	>80.00±1.82
PD3	>80.00±1.56	>80.00±1.99	>80.00±1.76
PK1	69.61±1.23	>80.00±2.10	73.35±1.53
PK2	56.47±1.43	74.92±1.34	>80.00±1.24
PK3	>80.00±1.55	>80.00±1.55	>80.00±1.77
Standard	Galantamin	Galantamin	Thiourea
(mg/mL)	0.00054±0.0001	0.0152±0.00008	0.0166±0.00025

3.3. Urease Activity Results

Urease enzyme inhibition was investigated in five different concentrations (2.5, 5, 10, 20 and 40% by mass). Enzyme activities at some low concentrations could not be calculated. The urease enzyme inhibition results of honey samples were observed in concentrations of 20% and 40% in all honeys.

The highest urease inhibition IC_{50} value of the honeys was determined in KU3 coded thyme honey with 34.89 mg/mL. When the IC_{50} values calculated for honey samples are taken into consideration, it is seen that thyme honey is highly inhibited urease than the other honeys. Chaste honeys are in the second place. Compared to other honeys subject to the study, citrus honeys showed the lowest level of urease inhibition.

4. DISCUSSION and CONCLUSION

Four different monofloral honeys (Chaste, Thyme, Citrus, Heather) with high production potential in the South-West Anatolian region have been the subject of this specific research. Monofloral honey's antioxidant capacities were revealed by β-carotene, DPPH, $ABTS^+$ and CUPRAC activity methods. Also, total phenolic amounts, total flavonoid amounts, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and urease inhibition activities were determined. In this study, the values obtained were compatible with those of similar studies.

Previous studies have found a positive relationship between antioxidants and anti-cholinesterase activity in plants and vegetables as these studies indicate a strong positive correlation between antioxidant and anticholinesterase activities (Philip & Mohd Fadzelly,

2015). In the analysis, it was determined that all honeys showed antioxidant activity and especially thyme honeys had higher activity than that of other monofloral honeys. In terms of enzyme inhibition activities, it was revealed that thyme honey showed higher activity than the other honeys did in the study. When the results of the phenolic activity and the total phenolic content of different origin honey samples are taken into consideration, it can be seen that thyme honeys have the highest average value with 16.58 (µg QEs/mg honey). As a result, it can be said that all the honeys used in the study, especially thyme and heather honey, have higher antioxidant properties than those of the others (Table 3).

It was determined that all monofloral honeys analyzed had a significant antioxidant capacity. It has also been found that honey samples have an effective activity against AChE and BChE enzymes. However, it was determined that it was less effective against urease inhibition than the other enzymes studied. Furthermore, especially thyme and heather honeys have been found to have higher antioxidant and enzyme capacity than the others have.

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Declaration of Conflicting Interests and Ethics

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

Authorship contribution statement

Sukru Karatas: Investigation, Resources, Software, Formal Analysis and Writing. **Abdurrahman Aktumsek**: Methodology, Supervision and Validation. **Mehmet Emin Duru:** Original draft, Visualization.

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Research Article

Investigation of *in vitro* Enzyme Inhibitory Properties and Antioxidant Activity of *Moltkia coerulea* (Willd.) Lehm. (Boraginaceae) Growing in Raman Mountain - Batman

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Abstract: The province of Batman is located in the Southeastern Anatolia Region of Turkey, and it is significant in terms of its ecosystem and plant biological diversity. Recently, researching economically important plant species has become a necessity in the province. In this context, while the members of the Boraginaceae genus have found a wide application area in traditional medicine in many countries from ancient times until today, they have been used for many purposes in Turkey. Most of the members of this family are medically important plants containing secondary metabolites such as flavonoids, terpenoids, alkaloids, fatty acids, glycosides, phytosterols, and various proteins. *Moltkia coerulea* (Willd.) Lehm. is found Anatolia, Lebanon and Crimea. This study aimed to determine the enzyme inhibition and antioxidant activity of *M. coerulea* (Willd.) Lehm, which has not been studied before, and grows in the untouched Raman Mountain in Batman. α-amylase and α-glucosidase inhibition results of methanolic (MeOH) and aqueous (Aq) extracts of *M. coerulea* were calculated as acarbose equivalents (ACAEs/g extract). Tyrosinase inhibition results of MeOH and Aq extracts of *M. coerulea* were calculated as kojic acid equivalent (mmol KAEs/g extract). Additionally, the extracts were tested against the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free-radical to analyze their antioxidant activity. The highest antioxidant activity was found in the leaf extract (MeOH) as 61.2 % with for the DPPH• method. These results showed that *M. coerulea* could be used as a potential source of natural antioxidant.

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1. INTRODUCTION

The Boraginaceae family is represented in the world with 130 genera and 2300 species, mainly annual, biennial or perennial herbs and shrubs, some trees and a few lianas, and it is distributed in temperate and subtropical areas of the Northern and Southern Hemispheres (Akçin & Binzet 2009; Tufa *et al*., 2019). In the last arrangement made by APG IV (2016), in light of molecular phylogeny, the Boraginaceae family was placed in the order Boraginales, which was formed within the clades of Eudicots – Superasterids - Asterids and Lamiids (Chacon *et al*., 2016).

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In Turkey, it includes 357 taxa, including 34 genera, 325 species, 16 subspecies, and 16 varieties. Turkey ranks in top 10 with the broadest distribution of taxa in the Boraginaceae family. Of these, 31 genera and 320 species naturally grow; 3 genera and 5 species are cultivated, 1 species is naturalized, and there are 2 types of hybrids. Among the natural species, the endemism rate was reported as 42.2 % (Davis 1978; Yıldırım 2000). Most plants belonging to the family are used as ornamental plants and obtain spices and dyestuffs (Pehlivan *et al*., 2001). The family includes important plants that have a wide range of use in medicine, pharmacology and cosmetics. The therapeutic effect of these herbs is related to their contents of many biologically active compounds, including naphthoquinones, flavonoids, terpenoids, and phenols. Components isolated from these plants exhibit antimicrobial, antitumor, antiviral, anti-inflammatory, cardiotonic, contraceptive and antiplatelet activities (Sharma *et al*., 2009; Papp *et al*., 2011; Taravati *et al*., 2014; Dresler *et al*., 2017). *M. coerulea*, which constitutes the biological material of this study, belongs to the Boraginaceae family. The vernacular name of this species is "Mavi kesen". In the Anti-Taurus Mountains in the province of Niğde in Turkey, the flowers of the plant are consumed by children because of their taste, while its flowers and roots are also used to treat diarrhea and abdominal pain (Özdemir & Alpınar 2010-2011; Özdemir & Alpınar 2015). In Sivas, the leaves of *M. coerulea* are consumed as food (Orhan *et al*., 2021).

The α-amylase and α-glucosidase enzymes are key enzymes involved in carbohydrate digestion. Oligosaccharides such as starch, α-dextrin, maltose are hydrolyzed by amylase. α-glucosidase hydrolyzes disaccharides and oligosaccharides into glucose units in the small intestines (Sing *et al*.,2010). Therefore, the inhibition of α-amylase and α-glucosidase enzymes is an important strategy in reducing increased blood glucose levels (Laube 2002). Drugs that lower blood glucose levels have side effects such as severe hypoglycemia, lactic acidosis, neurological disorders, upset stomach, headache, and even death. Considering the side effects of the long-term use of insulin and other hypoglycemic drugs, it is needed to develop safe and effective drugs, especially plant-based ones (Grover *et al*., 2002; Watkins 2003). Different traditional medical systems use raw plant extracts or active compounds derived from the plant to treat diabetes. Additionally, after the recommendation made by the World Health Organization (WHO), research on the hypoglycemic activities of medicinal plants has become more important (WHO 1980). The literature review in this study revealed that there are few studies (such as Zengin *et al*., 2017; Orhan *et al*., 2021) on *Moltkia* species that grow or are grown in Turkey. The fact that the area where the study material was collected had not been investigated reveals the necessity of shedding light on the properties of this plant in the area. Moreover, this study was needed for reasons such as differences like the altitudes of the habitats of plants and the soil structure affecting the secondary metabolites produced by plants. Thus, in this study, I aimed to investigate the *in vitro* enzyme inhibitory properties and antioxidant activity of the MeOH and aq extracts of the dried and ground parts (above-ground parts: leaves and flowers) of *Moltkia coerulea* (Willd.).

2. MATERIAL and METHODS

2.1. Plant Material

Moltkia coerulea which constitutes the study material, was collected from the campus of Batman University during the vegetation period in May 2020 (Figure 1). The botanical identity of the plant was confirmed by Dr. Alevcan Kaplan. Voucher specimens have been deposited at Batman University (voucher no.2020/12). The plants collected have been identified using the 6th volume of the Flora of Turkey (Davis 1978). The collected plant was washed to remove impurities and dried in the shade at room temperature. The samples obtained were ground in the blender.

2.2. Plant Extraction

The Aq and MeOH extracts of dried and ground plant parts (above-ground parts: leaves and flowers) were prepared. First of all, the aq extract was prepared. The dried and ground plant parts were extracted with 100 mL hot water (2 % w/v) for 6 hours on a heated magnetic stirrer and filtered. The residues were then recovered with 100 mL of water, again using the same procedure. The filtered aq extracts were combined, lyophilized and stored at +4 ºC pending experiments. After this, the MeOH extract was prepared. The dried and ground plant parts were treated with 200 mL of MeOH on a shaker for one day (24 hours) at room temperature and then filtered. This process was repeated with the same procedure. Finally, the extracts were pooled and concentrated using a rotary evaporator.

2.3. Enzyme Inhibitory Activity

2.3.1. α-amylase inhibitory activity

For determining α-amylase inhibition, a 25 µL sample of the extract (Aq and MeOH), and 50 µL of an α-amylase solution prepared in a sodium phosphate buffer (pH 6.9) were added to test tubes, and the samples were incubated at 37 ºC for 10 minutes. The reaction was initiated by adding 50 µL of a starch solution to the wells. A blank was prepared containing denatured enzyme samples. The samples and the blank were incubated at 37 ºC for 10 minutes. The reaction was stopped by adding 25 µL of 1M HCl, and 100 µL of a potassium iodide solution was added. Samples and blank were read at 630 nm. The α-amylase inhibition effect of the extracts were expressed in units of mmol Acarbose equivalent per g dry weight (mmol ACAEs/g extract) (Sarıkurkcu *et al*., 2018).

2.3.2. α-glucosidase inhibitory activity

For determining α-glucosidase inhibition activity, 50 µL of the extract (Aq and MeOH), 50 µL glutathione, 50 µL α-glucosidase solution prepared in a phosphate buffer (pH 6.8) and 50 µL 4-N-trophenyl-α-D-glucopyranoside. (PNPG) were added into test tubes, and the resulting samples were incubated at 37 ºC for 15 minutes. A blank containing denatured enzyme samples was also prepared. The reaction was stopped by adding 50 µL of 0.2 M sodium carbonate. The samples and the blank were read at 400 nm. Acarbose, an α-glucosidase inhibitor, was chosen as a reference. The α-glucosidase inhibition effect of the extracts were expressed in units of mmol Acarbose equivalent per g dry weight (mmol ACAEs/g extract) (Sarıkurkcu *et al*., 2018).

2.3.3. Tyrosinase inhibitory activity

To determine tyrosinase inhibition activity, 25 µL of the aq and MeOH extracts, 100 µL of a phosphate buffer (pH 6.8) and 40 µL of a tyrosinase solution were added, and the samples were incubated at 25 ºC for 15 minutes. The reaction was initiated by adding 40 µL of an L-DOPA solution to the tubes. A blank containing denatured enzyme samples was also prepared. The samples and the blank were read at 492 nm after waiting for 10 minutes and at 25 ºC. The tyrosinase inhibition activity of the extracts were expressed in mmol kojic acid equivalent per g dry weight (mmol KAEs/g extract) (Sarıkurkcu *et al*., 2018).

2.4. Antioxidant Activity

2.4.1. DPPH scavenging activity

Free radical activities of the extracts were determined using the DPPH free radical (Gezer *et al*., 2006). For the experiment, the concentrate was prepared by dissolving 4 mg DPPH in 100 mL MeOH. 3.2 mL of the DPPH radical and 200 µL (500 µg/mL) of the extract solutions were

added for each sample. After 30 minutes of incubation at room temperature in the dark, absorbance was measured at 517 nm. For control, 200 µL of the extract solution was added to the test tube. Each trial was made with triplicate. The following formula was used to determine the % DPPH radical scavenging activities of the samples.

$$\% \text{ DPPH scavenging activity} = [(A_{control} - E_{extract}) / A_{control}] \times 100$$

2.5. Statistical Analysis

All experiments in the study were carried out in triplicates. For the DPPH assay, the % scavenging activities of the samples were calculated using the Microsoft Excel program, and standard error bars were added to the plots. For the other analysis, the descriptive statistical data (Mean ± Standard Deviation) of the variables are shown in tables. The statistical analysis were performed using SPSS for Windows (version 21.0).

3. RESULTS and DISCUSSION

M. coerulea which constitutes the study material, was collected from the campus of Batman University during the vegetation period in May 2020 (Figure 1).

Figure 1. Natural photos of *Moltkia coerulea*: A- general view of plant, B- flowering part.

The yield amounts of the extracts are given in Table 1. Accordingly, when the effects of the solvents on the yield were examined based on due to the extraction process, the highest performance in terms of the two different solvents was obtained in the experiments using the aq as a solvent. A lower yield was obtained when MeOH was used as the solvent. The inhibitory effects of the Aq and MeOH extracts of *M. coerulea* against three different enzymes were determined (Table 2).

Table 1. Extract yields of *Moltkia coerulea* in the Aq and MeOH solvents.

Samples	Solvent system	%	mg /g dry matter
MCL	Aq	21.37	213.7
MCL	MeOH	11.12	111.2
MCF	Aq	21.65	216.5
MCF	MeOH	12.15	121.5

MCL: *Moltkia coerulea* leaf; *Moltkia coerulea* flower

± standard deviation was used. n: 3

Table 2. Enzyme inhibitory activities of the Aq and MeOH extract of *M. Coerulea*.

Sample	Part	Extract type	α-amylase inhibition activity (mmol ACAEs/g extract)	α-glucosidase inhibition activity (mmol ACAEs/g extract)	Tyrosinase inhibition activity (mmol KAEs/g extract)
M.coerulea	Leaf	Aq	0.296±0.12	0.481±0.06	0.860±0.00
		MeOH	0.325±0.01	0.610±0.01	0.761±0.00
	Flower	Aq	0.450±0.05	0.516±0.22	0.480±0.01
		MeOH	0.358±0.07	0.524±0.71	0.610±0.04

KAE: Kojic acid equivalent; ACAE: Acarbose equivalent
Values expressed are means ± S.D. of three parallel measurements

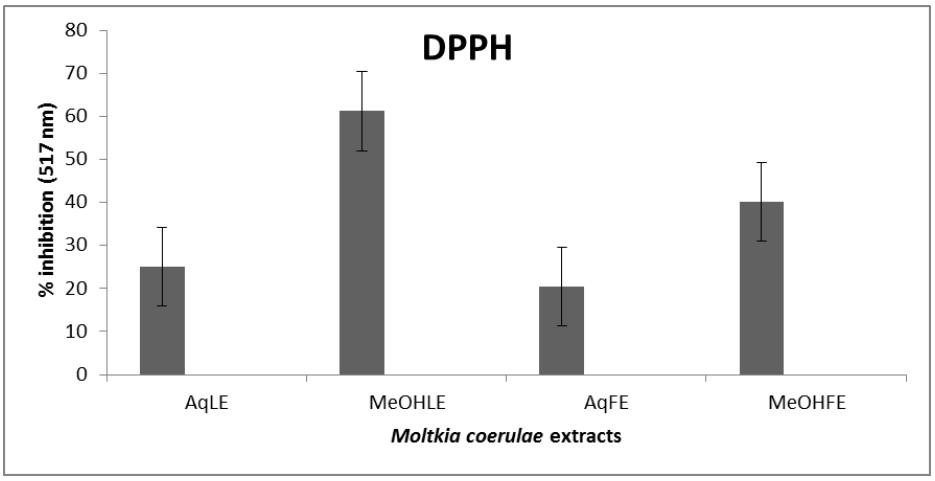
Diabetes is characterized by chronic hyperglycemia and has become a significant health problem all over the world. A consistently high glucose level in the blood leads to cardiovascular disease, neuropathy, retinopathy, nephropathy and other disorders. Hypoglycemic drugs used today manage to normalize serum glucose levels, but they cause gastrointestinal complaints. Thus, it is important to find effective therapeutic agents that inhibit α-amylase and α-glucosidase and have no side effects (Liu *et al*., 2017). The α-glucosidase and α-amylase inhibition effects of the Aq and MeOH extracts of the flowers and leaves of the *M. coerulea* plant are shown in Table 2. The α-amylase inhibition effect of the Aq and MeOH (0.450 and 0.358 mmol ACAEs/g extract, respectively) extracts of flowers was stronger than the Aq and MeOH (0.296 and 0.325 ACAEs/g extract, respectively) extracts of leaves. The α-glucosidase inhibition effect of the Aq and MeOH (0.481 and 0.610 mmol ACAEs/g extract, respectively) extracts of leaves was closest with the Aq and MeOH (0.516 and 0.524 mmol ACAEs/g extract, respectively) extracts of flowers. In general, the results obtained in this study on the α-amylase inhibition effects of the extracts were similar to those reported in the literature. The α-glucosidase inhibition effects of the extracts in this study were lower than those in some studies and higher than those in some others (Zengin *et al*., 2017;Orhan *et al*., 2020). The different substrates can explain these differences in the inhibition of two enzymes that affect sugar metabolism.

Likewise, in various studies investigating the enzyme inhibition effects of phenolic compounds, some phenolic compounds have been reported to have effective glycosidase inhibition activities (Kubola *et al*., 2008; Vadivel & Biesalksi, 2011; Wang *et al*., 2012). This also explains the inverse relationship between the consumption of foods rich in phenolic compounds and diabetes rates. Moreover, tyrosinase is a key enzyme that catalyzes the production of the pigment melanin that helps prevent damage induced by UV light exposure. Melanin synthesis occurs in melanocytes by the transformation of tyrosine into dihydroxy phenylalanine (DOPA), then DOPA-quinone, and then into black-brown eumelanin and yellow-red pheomelanin via the tyrosinase enzyme. With this function, tyrosinase is takes part in the formation of skin and hair color (Canovas *et al*., 1982; Rodriguez-Lopez *et al*., 1991; Cooksey *et al*., 1997). Its excess can lead to hyperpigmentation and neurodegenerative diseases such as Parkinson's disease. Although many synthetic inhibitors, especially kojic acid, have been developed to inhibit tyrosinase activity, the long-term toxic effects of these inhibitors have made their use questionable, and studies to determine natural inhibitors as alternatives to these have become the focus of attention (Tocco *et al*., 2009). Accordingly, in line with the data obtained it this study, the inhibitory effect of the Aq and MeOH (0.860 and 0.761 mmol KAEs/g extract, respectively) leaf extracts on the tyrosinase enzyme was stronger than that of the Aq and MeOH (0.480 and 0.610 mmol KAEs/g extract, respectively) flower extracts. Zengin *et al*. (2017), also determined that the aeiral parts of the plant samples' extract

of the same plant was more effective in inhibiting the tyrosinase enzyme (34.97 ± 0.50 mg KAEs/g extract). In addition, they stated that rutin, which is the major compound with phenolic groups, has been previously reported to inhibit tyrosinase through an inhibitory mechanism similar to that of copper chelators. That is, rutin competes competitively with the substrate L-DOPA in the active site pocket, inducing hydrophobic surface exposure (Si *et al*., 2012; Zengin *et al*., 2017).

Oxidative stress is recognized as the main pathological trigger for many diseases, including type II diabetes and Alzheimer's. Antioxidants are thought to be therapeutic tools to inhibit the activity levels and formation of oxidative stress (Liu *et al*., 2017). In this study, the antioxidant capacity of the MeOH and aq extracts of *M.coerulea* was determined using the DPPH test (Figure 2). The DPPH radical is widely used to evaluate free radical scavenging activity due to its ease of reaction. When the DPPH radical is scavenged with an antioxidant compound through hydrogen donation to form a stable DPPH-H molecule, the color of the solution turns from purple to yellow (Gangwar *et al*., 2014). If the results of this study are evaluated in general, it may be stated that the MeOH extracts of leaves and flowers (MeOHLE 61.2 % and MeOHFE 40.17 %, respectively) showed a better DPPH scavenging activity than the aq extracts (AqLE 25.1 %; AqFE 20.4 %). Koncˇic´ *et al*. (2010) collected the leaves of the *Moltkia petraea* plants from two regions named Sveti Jure and Snijezˇnica in Croatia, and they found that the leaves of the plants collected from the Snijezˇnica locality generally showed better antioxidant activity than the ones collected from the Sveti Jure locality. Orhan *et al*. (2021) investigated in the *in vitro* enzyme inhibition properties, antioxidant and phytochemical profiles antimicrobial and anti-tyrosinase activity of *M. aurea* and *M. coerulea*. As a result of their analysis of the total antioxidant capacity, they revealed that the ethyl acetate extracts exhibited a remarkable antioxidant potential compared to the extracts prepared using other solvents. While the superoxide scavenging activity of the water, MeOH and EA (ethyl acetate) extracts of the roots of both species was found promising, the MeOH extracts of all samples had significant DPPH free radical scavenging activities. The researchers stated that the antioxidant and antidiabetic effects of the extracts may have occurred due to their rutin and rosmarinic acid contents. It has been thought that the reason for the variations between *M. coerulea* species collected from different localities and between different species (such as *M. petraea*) in other studies was differences affecting these plants' biochemical and physiological structures such as species, organ, physiological age, harvest time, and locality. Consequently, in this study, it was determined that the plant extracts had a high rate of radical scavenging activity. Based on the results, it is thought that the extracts of *M. coerulea* can be used as natural source of antioxidants in areas such as food, cosmetics and pharmacology, in treatment, as a preservatives and as additives.

Figure 2. DPPH free radical scavenging activity of *Moltkia coerulea* extracts. AqLE; aqueous leaf extract, MeOHLE; methanol leaf extract, AqFE; aqueous flower extract, MeOHFE; methanol flower extract.



4. CONCLUSION

Due to the recently increasing concerns of the public regarding the effects of synthetic compounds on human health, natural compounds have gained a significant position. This field of research has revealed new, safe and natural sources for natural compounds, which are among the most popular topics in the scientific world. The results obtained in this study demonstrated that the extracts of *M. coerulea* can be considered as a source of natural biological agents. Therefore, these results show that the tested *M. coerulea* plant has a wide range of pharmaceutical uses.

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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

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Research Article

Antibacterial and Antioxidant Activity of Isoflavans from the Roots of *Rhynchosia ferruginea* and *In Silico* Study on DNA Gyrase and Human Peroxiredoxin

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Abstract: *Rhynchosia ferruginea* (Udusalim, Afan Oromo) is a medicinal plant traditionally used to treat skin infection, intestinal problems and amoebiasis. Silica gel chromatographic separation of dichloromethane/methanol (1:1) roots extract yielded isoflavan (**1**), isoflavene (**2**) and 1, 3-dilinoleoyl-2-stearoylglycerol (**3**), reported herein for the first time from the genus. Antibacterial activity was examined using disc diffusion method against *E. coli, S. aureus, P. aeruginosa* and *S. pyogenes.* AutoDoc vina 4.2 software was used for molecular docking analysis of compounds against human peroxiredoxin 5 and DNA gyrase B enzymes. Isoflavan (**1**) displayed zone of inhibition of 9.67 ± 0.58 mm and 10.67 ± 0.58 mm whereas isoflavene (**2**) showed 10.33 ± 1.15 mm and 10 ± 1.00 mm against *E. coli* and *S. aureus*, respectively, compared to ciprofloxacin (15.67±0.58 mm for both strains). DPPH radical scavenging assay of the dichloromethane/methanol (1:1) roots extract and isoflavan (**1**) exhibited better radical scavenging activity with IC_{50} value of 17.7 and 32, respectively. Molecular docking analysis revealed that **1** and **2** exhibited similar binding affinity of -7.4 kcal/mol compared to ciprofloxacin (-7.3 kcal/mol). *In silico* analysis against human peroxiredoxin 5 (PDB ID: 1HD2) revealed minimum binding affinity of - 3.7 and - 2.0 kcal/mol for compounds **1** and **2**, respectively, compared to ascorbic acid (-4.9 kcal/mol). The *in vitro* antibacterial and antioxidant activity of compounds **1** and **2** suggest the potential use of these compounds as drug lead candidates which corroborate with the traditional uses of the roots of *R. ferruginea*.

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1. INTRODUCTION

From the very beginning of human existence man has familiarized himself with plants and used them in a variety of ways throughout the ages. The relationship between plants and man led to the discovery of many plants used as medicines (Petrovska, 2012). Out of 255 drugs which are considered as basic and essential by the World Health Organization (WHO), 11% are obtained

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from plants and a number of synthetic drugs are also obtained from natural precursors (Joseph*et al*., 2013).A huge number of natural product derived compounds in various stages of clinical development highlighted the existing viability and significance of the use of natural products as sources of new drug candidates, chemical models or templates for the design, synthesis, and semi synthesis of novel substances for treating humankind's diseases.

The genus*Rhynchosia* consists of approximately 300 species circulated throughout the tropical and subtropical areas around the world (Rungsung*et al*., 2015). The most evident substances in *Rhynchosia* species are C-glycosides, O-glycosides, prenylated flavonoids and aglycones. Some of the isolated compounds of *Rhynchosia* genus and their plant extracts exhibit interesting biological activities, including antioxidant, antiinflammatory, antifungal, antibacterial and antiproliferative (Muhammad*et al*., 2019). *Rhynchosia ferruginea* A. Rich (Figure 1) is known with common name Udusalim (Afan Oromo) by traditional healers in Barbare district Bale Zone Oromia region of Ethiopia used for treatment of different ailments including skin infection, wound, stomachache and amoebiasis (Jima andMegersa, 2018).

Figure 1. Picture of *R. ferrugenia* (Picture taken by Kalid Hussien, 17/8/2020).

2. MATERIALS and METHODS

2.1 Plant Material Collection and Identification

The plant materials were collected from Bale Zone, Berbere district, which is located at about 526 km to southeast of Addis Ababa. Berbere district is situated between 06°33'N and 06°75'N and 039°40'E and 040°29'E. The plant material was identified with the help of a botanist (MelakuWondaferash) and voucher specimen (number KH-001) was deposited at the National Herbarium of Ethiopia, Addis Ababa University, Ethiopia.

2.2 Instruments Used

Column chromatography was carried out using silica gel 60-120 mesh ASTM. TLC was performed using precoated aluminum-backed supported silica gel 60 F254 (0.3 mm thickness). Melting point was measured in glass capillaries using Thiele tube. TLC visualization was done using UV lamp at 254 and 365 nm. The ultraviolet and visible (UV-Vis) spectrum was run using Spectroscopic Genesys™ 2PC UV-Vis scanning spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker avance 400 MHz spectrometer using $CDCl_3$ solvent and Tetramethylsilane (TMS) was used as an internal standard.

2.3. Extraction and Isolation

The collected roots of *R. ferruginia* were air dried at room temperature and grounded into powder using grinding mills. The grounded roots (500 g) of *R. ferruginia* were extracted with 2.5 L dichloromethane/methanol (1:1) for 72 h with occasional shaking at room temperature. The solution was filtrated and concentrated by a vacuum rotary evaporator at 50 ºC to afford 24.48 g (4.89%) dark red crude extract. The marc left was further extracted with methanol (2 L) for 72 h with occasional shaking at room temperature. The solution was filtered and concentrated by a vacuum rotary evaporator at a temperature of about 50 ºC to afford 17.55 g (3.51%) dark red crude extract. The dichloromethane/methanol (1:1) crude extract (14g) was adsorbed on 14 g of silica gel (mesh size 60-120) and subjected to silica gel column chromatography (180 g of silica gel, using *n*-hexane for packing) and eluted with increasing gradient of ethyl acetate in *n*-hexane followed by methanol in ethyl acetate. A total of 115 fractions each 100 mL were collected. The purity of each fraction was checked by using TLC. Fractions that showed similar R_f values and the same characteristic color on TLC (visualized in UV lamp at 254 and 356 nm) were combined. Fraction 28 showed single spot on TLC (EtOAc/*n*-hexane as eluent, 3:7, R_f= 0.44, 40 mg) to afford compound **3**. Fractions 35-38 showed single spot with similar R_f values to afford isoflavan (**1**) (EtOAc/*n*-hexane as eluent, 1:1, R_f= 0.52,120 mg). Fractions 43-45 showed single spot with similar $R_f$values to afford isoflavene (**2**) (EtOAc/*n*-hexane1:1 as eluent, R_f=0.43, 50 mg).

2.4. Antibacterial Activity

The antibacterial activities of crude extracts and isolated compounds were done in collaboration with the Department of Applied Biology, Adama Science and Technology University, Ethiopia. *In vitro* antibacterial activity of crude extracts and isolated compounds was investigated by using Mueller-Hinton Agar (MHA) disc diffusion method against four bacterial strains *E. coli, S. aureus, P. aeruginosa*, and *S. pyogenes.* The medium was prepared by dissolving 38 g of Mueller Hinton agar medium in 1000 mL of distilled water and the autoclaved at 121 °C for 15 min. The autoclaved medium was poured into sterile plates (20-25 mL/plate) and the plates were allowed to solidify under sterile condition at room temperature. After solidification, the plates were seeded with colonies of pure bacterial culture approximately 1.5 x 10^8 CFU/mL with a sterile cotton swab on to the surface of the medium until an even distribution of the inoculum was achieved.

Using sterile forceps, antibiotic disk of standard drugs (ciprofloxacin), and disk soaked with crude extract and pure compounds of *R. ferrugenia*were placed on the surface of the inoculated and dried plate. After 24 h of incubation at 37 °C, thediameter of the zones of growth inhibition around the disks was measured with a ruler and compared with ciprofloxacin. The experiments were done in triplicates and results were presented as mean value ± standard deviation.

2.5. Antioxidant Activity

DPPH is widely used to test the ability of compounds to act as free radical scavengers and to evaluate antioxidant activity of compounds. It is a stable free radical, which is due to the delocalized electron. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 5l7 nm (Hangun-Balkir and McKenney, 2012). The decrease in absorbance of DPPH radical is caused by antioxidants because of the reaction between antioxidant molecules and radicalswhich results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity (Ansari*et al*., 2013). Four different concentrations of the samples and positive control (1 mL each), ascorbic acid (100, 50, 25 and 12 µg/mL), were taken and mixed with 4 mL of DPPH solution that was prepared by dissolving

4 mg of DPPH in 100 mL of MeOH. The resulting solution was placed in an oven at 37°C for 30 min and subjected to UV-Vis spectrophotometer to record absorbance at 517 nm.

The percentage DPPH inhibition was calculated according to the following formula (Proestos*et al*., 2013).

$$\% \text{ of } I = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where I = DPPH inhibition (%), A_O = absorbance of control sample (t = 0 h) and A = absorbance of a tested sample at the end of the reaction (t = 30 min).

2.6. Molecular Docking Study of Compounds

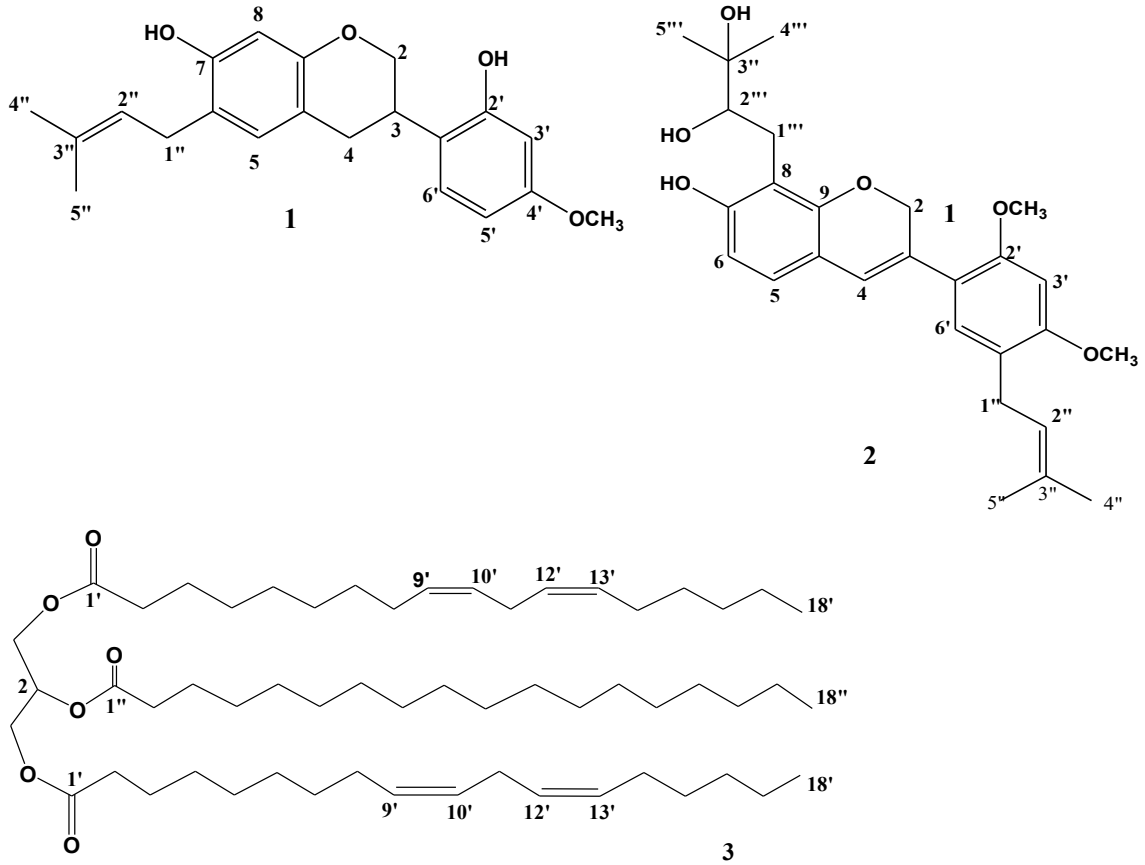
To have a better understanding about the inhibitory mechanism as well as the mode of interactions of the phytochemical compounds of the crude extract, molecular docking analysis was accomplished using the ADT version 1.5.2 and AutoDock vina version 4.2 docking program. The crystal structure of receptor molecule *E. coli* gyrase B (PDB ID: 6F86) and human peroxiredoxin (PDB ID 1HD2) were downloaded from protein data bank. The protein preparation was done using the reported standard protocolby removing the co-crystallized ligand, selected water molecules and cofactors, the target protein file was prepared by leaving the associated residue with protein by using auto preparation of target protein file AutoDock 4.2 (MGL tools1.5.7) (Eswaramoorthy *et al*., 2021). The graphical user interface program was used to set the grid box for docking simulations. The grid was set so that it surrounded the region of interest in the macromolecule. The docking algorithm provided with AutoDock Vina 4.2 was used to search for the best docked conformation between ligand and protein. During the docking process, a maximum of nine conformers were considered for each ligand. The conformations with the most favorable (least) free binding energy were selected for analyzing the interactions between the target receptor and ligands by discovery studio visualizer and PyMOL. The ligands are represented in different color and H-bonds and the interacting residues are represented in ball and stick model representation (Mansourian*et al*., 2015; Galma *et al*., 2021; Anza *et al*., 2021).

3. RESULTS and DISCUSSION

The root extracts of *R. ferruginea* after silica gel column chromatography furnished isoflavan (**1**), isoflavene (**2**) and 1, 3-dilinoleoyl-2-stearoylglycerol (**3**, Figure 2), are reported herein for the first time from the genus. The detailed characterization of these compounds ispresented in Figure 2.

Compound **1**was isolated as yellow solid (R_f=0.52 using 50 % EtOAc in *n*-hexane as eluent, melting point of 136-137 ^{0}C). Its ^{1}H NMR spectrum (Table 1) displayed a set of aliphatic proton signals at δ4.31 (dd, *J* = 15.7, 5.3 Hz, H-2), 3.99 (m, H-3), 2.94 (dd, *J* = 15.9, 5.2 Hz, H-4) attributed to isoflavan nucleus (ring-C) in agreement with previous literature (Bedane *et al*., 2016). In agreement with the skeketon, methylene protons at C-4 appeared at δ2.94 (dd, *J* = 15.9, 5.2 Hz) and 2.86 (dd, *J* = 15.7, 5.2 Hz) as diastereotopic protons in agreement with the presence of chiral center C-3 position. This observation was also supported by the carbon resonances at δ70.0, 31.7 and 30.0 attributed to C-2, C-3 and C-4, respectively, of which the former is pointing down in DEPT-135 spectrum in agreement with methylene moiety. A set of ortho-coupled aromatic protons with AB spin pattern at δ6.90 (H-6', d, *J*= 8 Hz) and δ6.36 (H-5', d, *J*= 8 Hz) and three aromatic protons having ABX spin pattern were observed atδ6.36 (H-5', d, *J*=8Hz), 6.90 (H-6', d, *J*=8Hz), 6.38 (H-3', d, *J* = 2.2 Hz). Signals at δ1.78 (H-5", s), 1.73 (H-4", s), 3.26 (H-1", d, J= 7.5) and5.31 (H-2", t) suggest the presence of a prenyl group.

Figure 2. Compounds isolated from the roots of *R. ferruginea*.



The ^{13}C NMR and DEPT-135 spectrum (Table 1) revealed the presence of eight quaternary carbons, four of which are sp^2 oxygenated quaternary aromatic carbons at δ 154.8 (C-7), 152.8 (C-9), 155.2 (C-2') and 156.4 (C-4'). Aromatic methine peaks observed at δ99.2(C-8), 130.0 (C-5), 103.2 (C-3'), 107.7(C-5') and 128.3 (C-6') of which the upfeild chemical shift value of C-8 and C-3 are in good agreement with 1,3-dioxygenated substitution pattern that contributed to shielding effect possibly due to delocalization of lone pair electrons of oxygen atoms attached to adjacent carbons (oxygenation at C-7 & C-9 affecting chemical shift of C-8 and oxygenation at C-2' & C-4' affecting chemical shift of C-3'). The peak at δ55.5 suggest the presence of methoxy moiety. Thus, based on the above spectral data and comparison with literature, compound **1** was found to be identical with that of 7, 2'-dihydroxy-4'-methoxy-6-(3", 3"-dimethylallyl) isoflavan (**1**) (Figure 2, Table 1) previously reported from *Glycyrrhizauralensis* (Fan *et al*., 2020). This is the first report of the compound from the genus *Rhynchosia*.

Compound **2** was isolated as yellow solid (R_f=0.43using 50 % EtOAc in *n*-hexane as eluent, melting point of 124-125 ^{0}C). Its ^{1}H NMR spectrum (Table 2) revealed signals atδ 4.44 (s, H-2) and 6.78 (s, H-4) which are characteristic signals of flavan-3-ene ring C skeleton. On the basis of literature precedence, the signals at δ2.73 (H-1'"), δ 3.28 (H-2'"), 1.16 (H-4'") and 1.12 (H-5'") with corresponding carbon signals at δ70.5 (C-3'") and δ77.2 (C-2'") suggest the presence of 2,3-dihydroxy-3-methylbutyl moiety (Kamdem *et al.*, 2005; Nyandoro *et al.*, 2017). The presence of a prenyl group was evident from the signals at δ1.62 (H-5", s), 1.60 (H-4", s), 3.12 (H-1", d, *J*= 8.1), 5.17 (H-2", t) with corresponding carbon peaks at δ 24.1 (C-1"), 122.7 (C-2"), 131.4 (C-3"), 17.0 (C-4") and 25.2 (C-5"). Two methoxy groups are observed at δ 3.67 (s, 3H) and 3.68 (s, 3H).

Table 1. ^{1}H, ^{13}C and DEPT-135 NMR spectral data of 7, 2'-dihydroxy-4'-methoxy-6-(3", 3"-dimethylallyl) isoflavan (**1**) in $CDCl_3$.

Position	**1**			Fan *et al*., 2020	
	^{1}H NMR	^{13}C NMR	DEPT-135	^{1}H NMR	^{13}C NMR
1	-	-	-	-	-
2 ax 2 eq	4.31 (dd, *J* = 15.7, 5.3 Hz), 3.99 (t)	70.0	70.0	4.11 (dd, *J*= 4.8,10.4 Hz); 3.86 (t, *J*=10.0 Hz)	69.1
3	3.50 (m)	31.7	-	3.30 (m)	31.2
4 ax 4 eq	2.94 (dd, *J* =15.9, 5.2 Hz) 2.86 (dd, *J* = 15.7, 5.9 Hz)	30.0	30.0	2.85 (m) 2.68 (dd, *J* = 4.0, 10.4 Hz)	29.8
5	6.84 (s, 1H)	130.0	130.0	6.69 (s, 1H)	129.7
6	-	122.5	-	-	119.8
7	-	154.8	-	-	153.6
8	6.40 (s, 1H)	99.2	99.2	6.23 (s, 1H)	102.3
9	-	152.8	-	-	152.4
10	-	113.6	-	-	112.4
1'	-	120.0	-	-	119.8
2'	-	155.2	-	-	155.8
3'	6.38 (d, *J* = 2.2 Hz)	103.2	103.2	6.42 (d, *J* = 2.4 Hz)	101.3
4'	-	156.4	-	-	158.7
5'	6.36 (d, *J*= 8 Hz)	107.7	107.7	6.34 (dd, *J*= 8.8, 2.4 Hz)	104.3
6'	6.90 (d, *J*= 8 Hz)	128.3	128.3	6.97 (d, *J*= 8.8 Hz)	127.6
1"	3.26 (d, *J*= 7.5 Hz)	27.8	27.8	3.11 (d, *J*=7.2 Hz)	27.4
2"	5.31 (t, 1H)	123.0	123.0	5.23 (t, 1H)	123.1
3"	-	132.0	-	-	130.4
4"	1.73 (s, 3H)	17.8		1.67 (s, 3H)	17.6
5"	1.78 (s, 3H)	25.9		1.73 (s, 3H)	25.5
OCH_3	3.77 (s, 3H)	55.5		3.66 (s, 3H)	54.8

The ^{13}C NMR and DEPT-135 spectra (Table 2) revealed the presence of eleven quaternary carbons, four of which are sp^2 oxygenated aromatic quaternary carbons at δ 155.4 (C-7), 152.7 (C-9), 156.0 (C-2'),155.9(C-4') and sp^3oxygenated quaternary carbon at δ 70.5 (C-3'"). The peak at δ77.23 suggests sp^3 oxygenated methine (C-2'"). Aromatic non-oxygenated quaternary carbon appeared atδ113.5 (C-8), 118.7 (C-10), 121.7 (C-1') and 122.6 (C-5'). Non aromatic sp^2quaternary carbon appeared at δ127.4 (C-3) and 131.4 (C-3"). Aromatic methines were observed at δ129.5 (C-5), 102.2 (C-6), 98.7 (C-3') and 129.8 (C-6'). Non aromatic sp^2methines were observed at δ127.2 (C-4) and 122.7 (C-2"). The upfeild chemical shift values for C-3' are in good agreement with 1, 3-dioxygenated substitution pattern at C-2', 4' positions as in the proposed structure. The peak at δ69.8 suggest oxygenated methylene, pointing down in DEPT-135 spectrum, and peaks atδ54.9 and 54.8 suggest methoxygroups supported by DEPT-135 spectrum pointing up. From the above spectral data and comparison with literature (Tanaka *et al*., 2002), compound **2** was identified and labeled as 7-hydroxy-2', 4'-di-methoxy-8-(2'",3'"-dihydroxy-3'"-methylbutyl)-5'-(3",3"-dimethylallyl) isoflav-3-ene (**2**) (Figure 2, Table 2).

Table 2. ^{1}H, ^{13}C and DEPT-135 NMR spectral data of 7-hydroxy-2', 4' di-methoxy-8-(2''', 3'''-dihydroxy-3'''-methylbutyl)-5'- (3'', 3''-dimethylallyl) isoflav-3-ene (**2**) in $CDCl_3$.

Position	**2**			Tanaka *et al*., 2002	
	^{1}H NMR	^{13}C NMR	DEPT-135	^{1}H NMR	^{13}C NMR
1	-	-	-	-	-
2	4.44 (s, 2H)	69.8	69.8	4.97 (s, 2H)	68.4
3	-	127.4	-	-	128.9
4	6.78 (s, 1H)	127.2	127.2	6.5 (s, 1H)	121.9
5	6.71 (d, *J* = 9.0 Hz)	129.5	129.5	6.82 (d, *J*= 8.1 Hz)	125.0
6	6.25 (d, *J* =8.9 Hz)	102.2	102.2	6.40 (d, *J*= 8.1 Hz)	108.7
7	-	155.4	-	-	155.2
8	-	113.5	-	-	114.4
9	-	152.7	-	-	151.9
10	-	118.7	-	-	117.1
1'	-	121.7	-	-	120.8
2'	-	156.0	-	-	158.4
3'	6.21 (s, 1H)	98.7	98.7	6.42 (s, 1H)	99.2
4'	-	155.9	-	-	156.6
5'	-	122.6	-	6.41 (dd, *J*=8.1,2.2 Hz)	107.3
6'	6.59 (s, 1H)	129.8	129.8	7.1 (d, *J*=8.1 Hz)	129.4
2 x OCH_3	3.67 (s, 3H) 3.68 (s, 3H)	54.8 54.9	54.8 54.9	3.75 (s, 3H) -	55.4 -
1''	3.12 (d, *J*= 8.1 Hz),	24.1	24.1	3.42(d, *J*= 7.3)	22.4
2''	5.17 (t, 1H)	122.7	122.7	5.27 (t, *J*=7.3)	121.8
3''	-	131.4	-	-	134.5
4''	1.60	17.0	17.0	1.74	17.9
5''	1.62	25.2	25.2	1.82	25.8
				(Nyandoro *et al*. 2017)	
1'''	2.73 (d, *J* =15 Hz)	29.2	29.2	2.73 m	26.0
2'''	3.28 (d, *J* =15 Hz)	77.2	77.2	3.61m	77.7
3'''	-	70.5	-		72.0
4'''	1.16 (s, 3H)	31.3	31.3	1.19 (s, 3H)	24.3
5'''	1.12 (s, 3H)	28.0	28.0	1.23 (s, 3H)	23.6

Compound **3** was obtained as white semi solid with R_f value of 0.44 (EtOAc/*n*-hexane, 3:7 as eluent) and melting point of 43-45 ^{0}C. Its ^{1}H NMR spectrum (Table 3) displayed peaks at δ0.90 and 0.89 suggesting the presence of two terminal methyl protons. The peaks at δ1.27 and δ 1.62 indicate protons of aliphatic methylene ($-CH_2$) group, a peak at δ2.06 indicates the presence of protons of a methylene group that is bonded to C=C bond and the triplet peak

atδ2.36 indicates the presence of methylene protons α to a carbonyl group. The glycerol moiety exhibited doublet of doublets at δ4.17 for oxygenated methylene protons H-1, 3 and a multiplet at δ5.10 attributed to oxygenated methine proton H-2 whereas olefinic protons were observed at δ5.37 as multiplet.

The ^{13}C NMR and DEPT-135 spectra (Table 3) revealed the acyl function attributed to esters carbonyls at δ174.0 and 173.9, oxygenated methylene at δ65 pointing down in DEPT-135 spectrum, and oxygenated methine at δ68, pointing up in DEPT-135 spectrum. Four sp^2 methines are clearly evident at δ130.2, 130.0, 128.1 and 127.9. Overlapped methylene peaks are observed at δ28-29 and two terminal methyl carbons appeared at δ14.1 and 14.2. From the above spectral data the compound was found to be triglyceride compound named as 1, 3-dilinoleoyl-2-stearoylglycerol (**3,** Figure 2) by relating with a compound previously reported from plant *Moringastenopetala* (Bekele *et al*., 2013).

Table 3. ^{1}H, ^{13}C and DEPT-135 NMR spectral data of 1, 3-dilinoleoyl-2-Stearoyl glycerol (**3**) in $CDCl_3$.

Position	**3**			Bekele *et al*., 2013	
	^{1}H NMR	^{13}C NMR	DEPT-135	^{1}H NMR	^{13}C NMR
1	4.17(dd, *J*=10.6, 5.0 Hz)	65	65	4.20	62.1
2	5.10 m	68	68	5.25	68.9
3	4.17(dd, *J*=10.6, 5.0 Hz)	65	65	4.20	62.1
1', 1"	___	174.0, 173.9	___	___	173.3, 172.8
2', 2"	2.36 (t)	34.1	34.1	2.33	34.1, 34.2
3', 3"	1.64	31.9, 31.5	31.9, 31.5	1.60	31.9, 31.5
4', 4"	1.30	22.7	22.7	1.25	22.7
5', 5"	1.30	22.6	22.6	1.25	22.7
6', 6"	1.30	24.9	24.9	1.25	24.9
7', 7"	1.30	24.9	24.9	1.25	24.9
8', 8"	2.05	27.2	27.2	2.03	27.2
9', 9"	5.37 (m)	130.9, 29.7	130.9, 29.7	5.40	129.7, 130.2
10', 10"	5.37 (m)	128.1, 29.7	128.1, 29.7	5.40	127.9, 129.7
11', 11"	2.05	25.6, 29.7	25.6, 29.4	2.80, 2.03	25.6, 27.2
12',12"	5.37 (m)	130.0, 29.4	130.0, 29.4	5.40, 1.25	127.9, 128.1
13',13"	5.37 (m)	127.9, 29.4	127.9, 29.3	5.40, 1.25	131.9, 130.2
14',14"	1.30	29.3	29.2	2.03, 1.25	27.2
15',15"	1.30	29.2	29.2	1.25	29.2–29.7
16',16"	1.30	29.1	29.1	1.25	29.2–29.7
17',17"	1.30	29.1	29.1	1.25	29.2–29.7
18',18"	0.90	14.2, 14.1	14.2, 14.1	0.90	14.2, 14.1

3.1. Antibacterial Activity

In vitro antibacterial activity of dichloromethane/methanol (1:1) crude extract and isolated compounds were tested for their antibacterial activity against the bacterial species *E. coli, S. aureus*, *P. aeruginosa* and *S. pyogenes*at two different concentration (1 mg/mL and 1.5 mg/mL) (Table 4). Compound **1** (1.5 mg/mL) displayed 9.67 ± 0.58 mm and 10.67 ± 0.58 mm zone of inhibition whereas compound **2** (1.5 mg/mL) showed 10.33 ± 1.15 mm and 10 ± 1.00 mm zone

of inhibition against *E. coli* and *S. aureus,* respectively, compared to ciprofloxacin (15.67 ± 0.58 mm zone of inhibition for both strains). The values displayed by compounds **1** and **2** against these two strains can be considered as promising antibacterial results. All compounds and crude extract showed weak zone of inhibition against *P. aeruginosa* and *S. pyogenes*. The antibacterial activity of dichloromethane/methanol (1:1) extract and compound **3** were found to be less than that of compound **1** and **2** with weak antibacterial activity against *E. coli* and *S. aureus*. The result obtained is comparable with previous report on essential oil of *Rhynchosia minima* displaying inhibition zone diameter of 18.2 and 12.5mm against *E. coli* and *S. aureus*, respectively, at concentration of 100 µg/mL (Gundidza*et al*., 2009).

Table 4. Zone of bacterial growth inhibition diameter (mm).

Sample	Concentration in mg/mL	Inhibition diameter (mm) ± SD			
		E. coli	*S. aureus*	*P. aeruginosa*	*S. pyogenes*
methanol/dichloromethane (1:1) extract	1	6.67±0.58	6.67±0.58	6.00±0.00	6.00±0.00
	1.5	7.33±0.58	7.33±0.58	6.33±0.58	6.00±0.00
1	1	8.67±0.58	10.33±0.58	8.33±0.58	6.33±0.58
	1.5	9.67±0.58	10.67±0.58	8.67±0.58	7.33±0.58
2	1	9.67±1.15	9.67±0.58	6.67±0.58	6.00±0.00
	1.5	10.33±1.15	10±1.00	7.33±0.58	6.33±0.58
3	1	6.33.58	6.67±0.58	6.33±0.58	6.00±0.00
	1.5	6.67±0.58	7.67±0.58	6.67±0.58	6.00±0.00
Ciprofloxacin	1	15.33±0.58	15.33±0.58	15.67±0.58	16.33±0.58
	1.5	15.67±0.58	15.67±0.58	17.33±1.53	17.33±1.00

SD = standard deviation

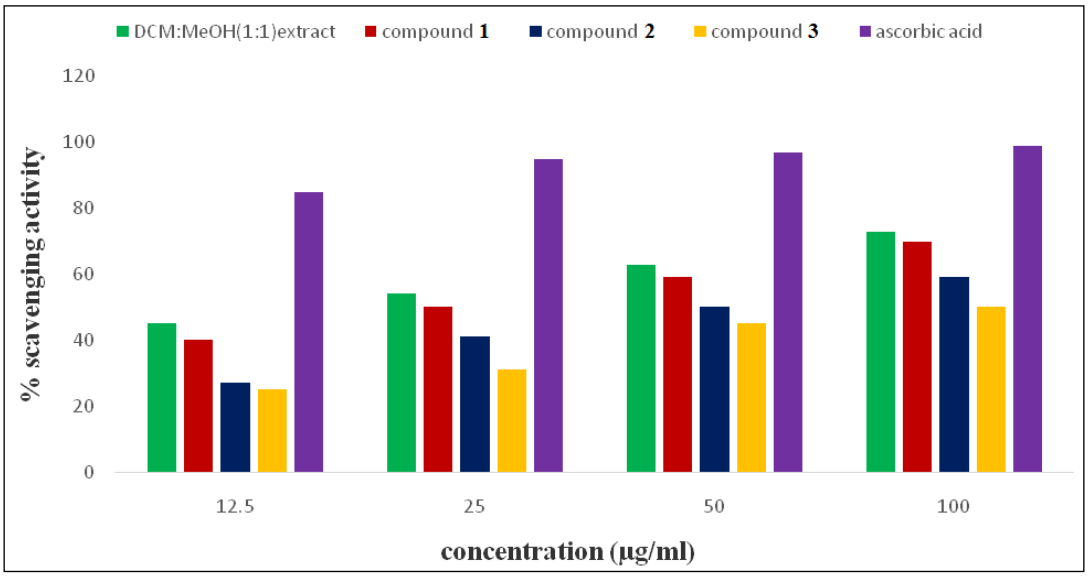
3.2. Antioxidant Activity

DPPH is widely used to test the ability of compounds to act as free radical scavengers and to evaluate antioxidant activity of compounds. The radical scavenging activity of the extracts and isolated compounds were assessed by measuring the reduction of DPPH absorbance at 517 nm. Crude extracts and pure compounds of *R. ferrugenia* reduced the DPPH in dose-dependent manner (Table 5, Figure 3). Compound **1**, DCM/methanol (1:1) extract, compound **2** and compound **3** displayed 70%, 73%, 59% and 50 % inhibition, respectively, at concentration of 100 µg/mL. The positive control, ascorbic acid (Figure 3) showed maximum scavenging effect from very low to high concentration (85, 93, 97 and 99 % at 12, 25, 50 and 100 µg/mL, respectively). Overall, the dichloromethane/methanol (1:1) extract and compound **1** displayed promising radical scavenging activity with IC_{50} of 17.7 and 32, respectively.

Table 5. Radical scavenging activity of DCM/MeOH (1:1) extract and compounds (**1**-**3**).

Concentration	DCM/MeOH(1:1) extract		**1**		**2**		**3**	
	% scavenging activity	IC_{50}	% scavenging activity	IC_{50}	% scavenging activity	IC_{50}	% scavenging activity	IC_{50}
12.5	45	17.7	40	32	27	64.5	25	90.6
25	54		50		41		31	
50	63		59		50		45	
100	73		70		59		50	

Figure 3. DPPH scavenging activity (%) of DCM/MeOH (1:1) extract and compounds (**1-3**).



3.3. Molecular Docking Study Against DNA Gyrase B (PDB ID 6F86)

Molecular docking analysis of isolated compounds was done in comparison with ciprofloxacin to predict the orientation and binding affinity of ligand molecules at the active site of the receptor. Compounds**1**and **2** (Table 6) displayed similar binding affinity of -7.4 kcal/mol and exhibit hydrogen bond with three active site amino acid residue compared to ciprofloxacin -7.3 kcal/mol (Table 6, Figure 7). Compound **1**binding conformation within the active pocket of PDB ID 6F86 exhibits four hydrogen bonding interaction. The green dash lines indicates a possible hydrogen bond formed between the connections residues and pink lines indicated hydrophobic bond interaction (Figure 4).

Table 6. Molecular docking results of compounds (**1-3**) against *E.coli* DNA gyrase B (PDB ID 6F86).

Ligands	Affinity (kcal/mol)	Amino acid residue form H-bond with ligands	Residual Pi-Alkyl Hydrophobic/Pi-Cation/Pi-Anion/ interactions
1	-7.4	Asp-73, Asn-46, Val-71	Ile-78, Glu-50, Thr-165, Pro-79, Ala-47, Val-43, Val-167, Gly-77, Arg-136
2	-7.4	Asp-73, Asn-46, Thr-165	Ile-78, Val-43, Ala-47, Val-167, Val-120, Asp-49, Gly-77, Gly-119, Ser-121
3	-5.2	Asn-46, Val-120	Arg-76, Ile-78, Pro-79, Ile-94, Val-93, Val-90, Val-93, Val-43, Val-47, Val-71, Val-167
Ciprofloxacin	-7.3	Asp-73, Asn-46, Arg-76	Ala-47, Glu-50, Gly-77, Ile-78, Pro-79, Ile-94

Compound **2** showed hydrogen bond interaction with three active site amino acid residue Asp-73, Asn-46, and Thr-165 (Table 7). The hydrogen bond formed (Figure 5) between O atom of C ring with Asn-46, O atom of methoxy on C-4' with Thr-165 and H atom of methoxy on C-4' with Asp-73. The two methyls of prenyl moiety attached to C-5' displayed hydrophobic interaction with Val-43, Ala-47, Val-167 and Val-120. Compound **3**showed binding affinity of -5.2 kcal/mol (Table 7). The oxygen atoms on esters functional group of compound **3** form hydrogen bonding with two amino acids Asn-46 and Val-120 (Figure 6). In this study, compounds **1** and **2** exhibited better docking efficiency with DNA gyrase and may act as potential inhibitors of DNA gyrase enzyme.

Figure 4. The 2D and 3D binding interactions of compound **1** against DNA gyrase B (PDB ID: 6F86).

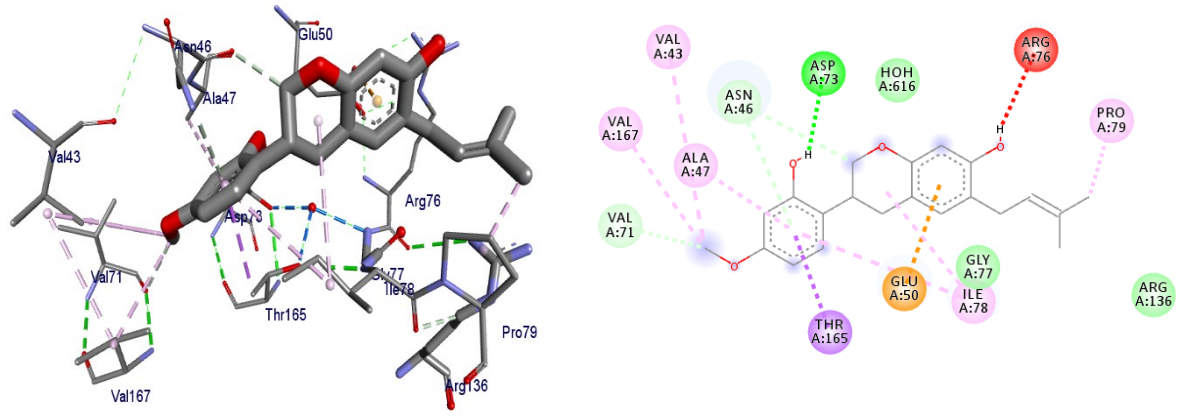


Figure 5. The 2D and 3D binding interactions of compound **2** against DNA gyrase B (PDB ID: 6F86).

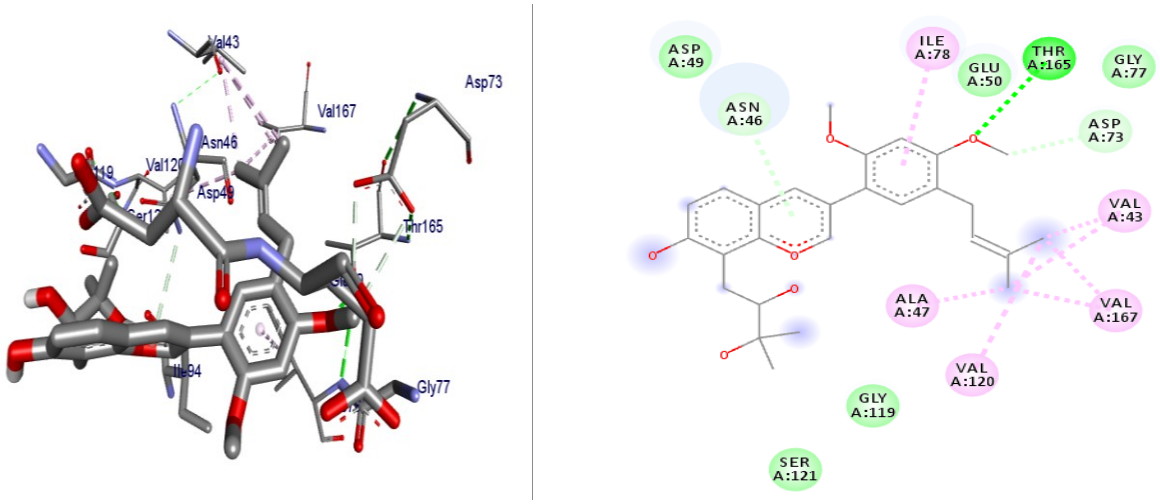
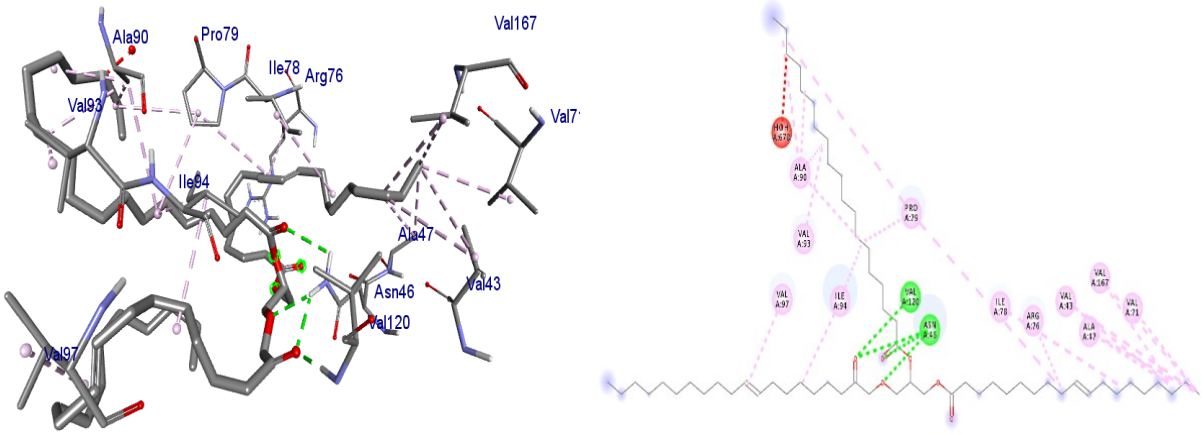


Figure 6. The 2D and 3D binding interactions of compound **3** against DNA gyrase B (PDB ID: 6F86).



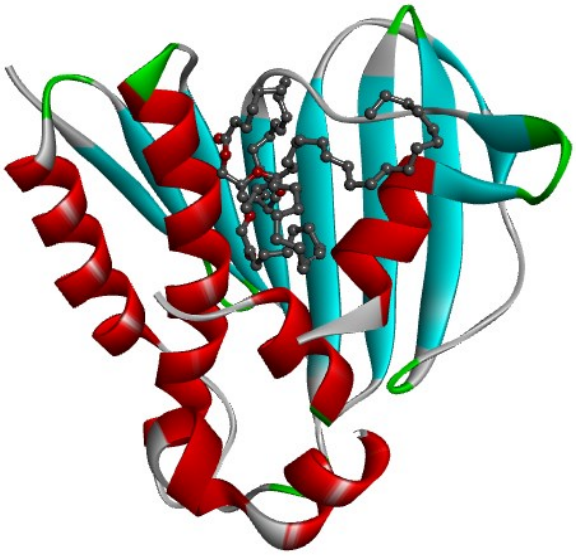


Figure 7. The 2D and 3D binding interactions of ciprofloxacin against DNA gyrase B (PDB ID:6F86).

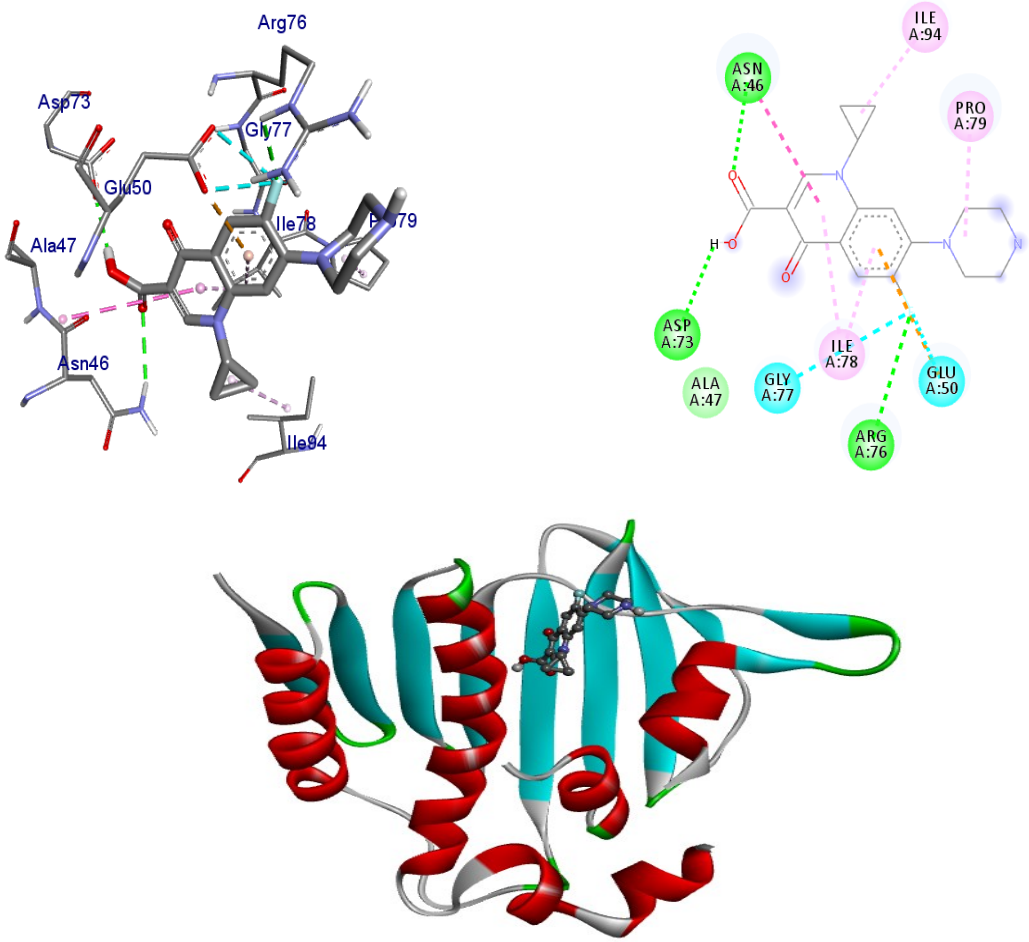
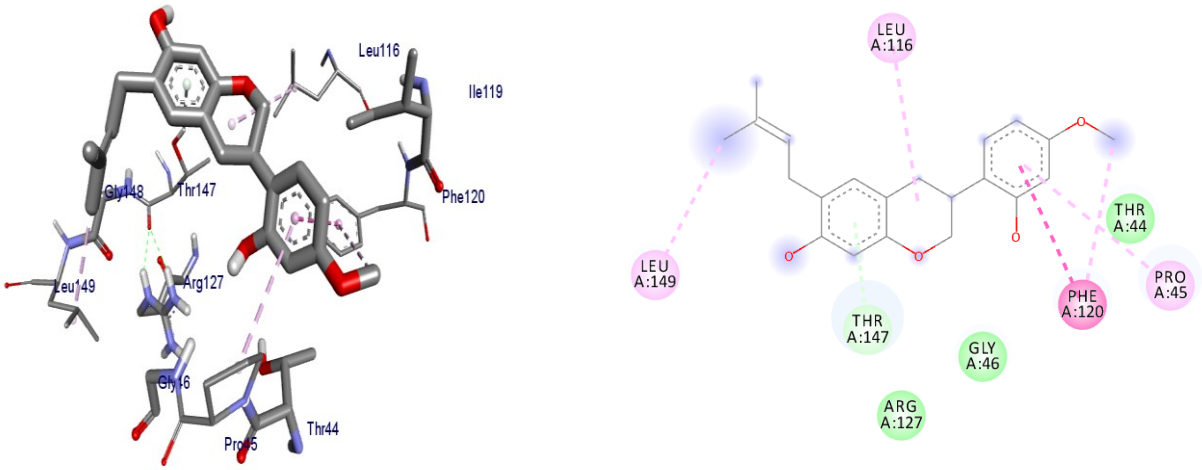


Figure 8. The 2D and 3D binding interactions of compound **1** against human peroxiredoxin 5 (PDB ID: 1HD2). Hydrogen bond between compounds and amino acids are shown as green dash lines, hydrophobic interaction are shown as pink lines.



3.4. Molecular Docking Binding Analysis Against Human Peroxiredoxin 5 (PDB ID:1HD2)

The molecular docking analysis of the isolated compounds was carried out to investigate their binding pattern with human peroxiredoxin 5 (PDB ID: 1HD2) and compared with the natural antioxidant ascorbic acid. Isoflavan (**1**) and Isoflavene (**2**) were found to have minimum binding energy of -3.7 and - 2.0 kcal/mol, respectively (Table 7). The molecular docking analysis also support the better docking affinity (-3.7 kcal/mol) for compound **1** within the binding pocket of human peroxiredoxin 5. The key amino acid residues within the active sites of peroxiredoxin 5 are Cys 47, Thr-44, Gly-46 and Thr-147. Compound **1** formed hydrogen bond interaction with Thr-147 (Figure 8) whereas compound **2** formed hydrogen bond interaction with Asp-145 and residual van darwaals interaction with residual amino acids Thr-147, Thr-44, Pro-45, Arg-127, Ile-119, Phe-120 (Figure 9, 10, Table 7).

Table 7. Molecular docking value of isolated compounds (**1** and **2**) and ascorbic acid against human peroxiredoxin (PDB ID: 1HD2).

Compounds	Affinity (kcal/mol)	H-bond	Residual Amino acid Interactions	
			Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	Van-der Walls interactions
1	-3.7	Thr-147	Pro-45, Phe-120, Leu-116, Leu-149	Thr-44, Pro-40, Gly-46, Arg-127
2	-2.0	Asp-145	Cys-47, Pro-40, Leu-149	Thr-147, Thr-44, Pro-45, Arg-127, Ile-119, Phe-120
Ascorbic Acid	-4.9	Cys-47, Thr-44, Gly-46, Thr-147	Pro-40, Pro-45, Phe-120, Arg-127, Leu-149	--

Figure 9. The 2D and 3D binding interactions of compound **2** against Human peroxiredoxin 5 (PDB ID: 1HD2). Hydrogen bond between compounds and amino acids are shown as green dash lines, hydrophobic interaction are shown as pink lines.

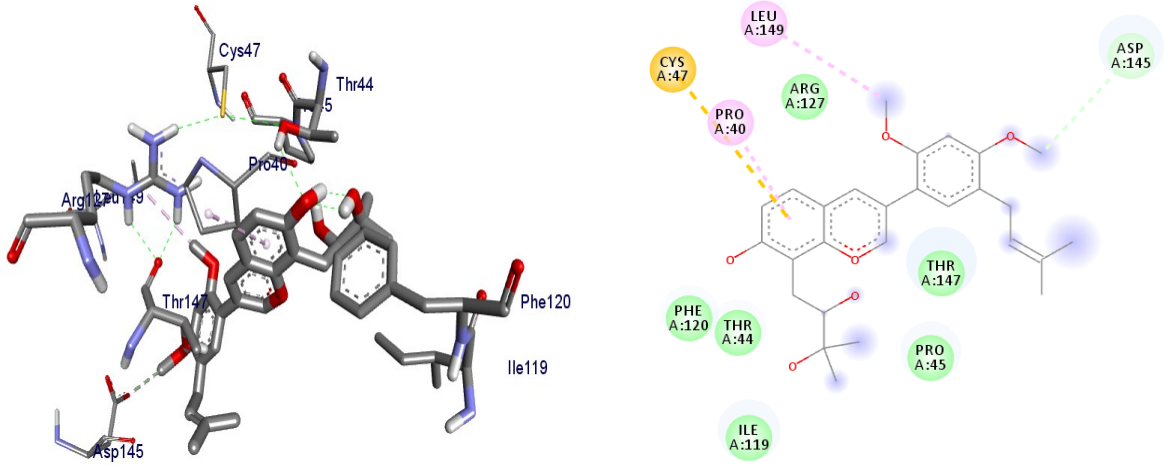
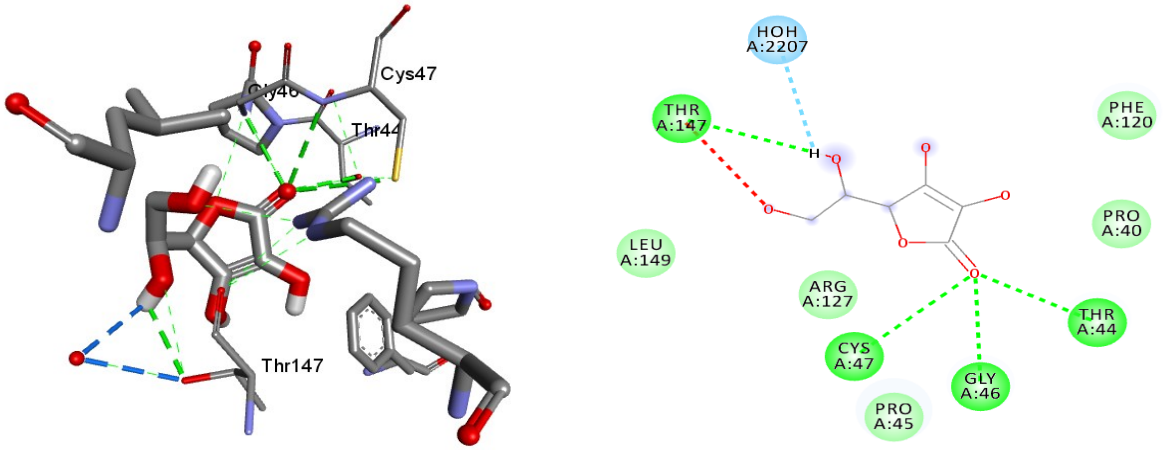


Figure 10. The 2D and 3D binding interactions of compound Ascorbic Acid against Human peroxiredoxin 5 (PDB ID: 1HD2). Hydrogen bond between compounds and amino acids are shown as green dash lines; hydrophobic interaction are shown as pink lines.



4. CONCLUSION

*R. ferrugenia*is one of traditional medicinal plants used by local traditional healers for treatment of skin infection, wound, stomachic and amoebiasis. Silica gel column chromatographic analysis of dichloromethan/methanol (1:1) extract furnished 7,2'-dihydroxy-4'-methoxy-6-(3",3"-dimethylallyl) isoflavan (**1**), 7-hydroxy-2',4'-dimethoxy-8-(2"',3"'-dihydroxy-3"'-methylbutyl)-5'-(3",3"-dimethylallyl) isoflav-3-ene (**2**) and 1,3-dilinoleoyl-2-stearoyl glycerol (**3**), reported herein for the first time from the genus. *In vitro* antibacterial activity of dichloromethane: methanol (1:1) and isolated compounds were tested for their antibacterial activity against the bacterial species *E. coli, S. aureus*, *P. aeruginosa* and *S. pyogenes.* Compound **1** displayed 9.67 ± 0.58 mm and 10.67 ± 0.58 mm zone of inhibition whereas compound **2** showed 10.33 ± 1.15 mm and 10 ± 1.00 mm zone of inhibition against *E. coli* and *S. aureus,* respectively, compared to ciprofloxacin (15.67±0.58 mm zone of inhibition for both strains). Dichloromethane/methanol (1:1) extract and isoflavan (**1**) showed promising DPPH

radical scavenging activity with IC_{50} of 17.7 and 32, respectively, compared to ascorbic acid. Molecular docking analysis against DNA gyrase B (PDB ID: 6F86) revealed minimum binding affinity value of -7.4 kcal/mol for both compounds**1** and **2**compared to ciprofloxacin -7.3 kcal/molsuggesting the compounds may act as potential inhibitors of DNA gyrase enzyme. *In silico* analysis against human peroxiredoxin 5 (PDB ID: 1HD2) revealed minimum binding energy of - 3.7 and - 2.0 kcal/mol for compounds **1** and **2**, respectively, compared to ascorbic acid (-4.9 kcal/mol). The *in vitro* antibacterial activity and antioxidant activity of compounds **1** and **2**suggest the potential use of these compounds as potential drug lead candidates which corroborate with the traditional uses of the roots of *R. ferrugenia*.

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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

Kalid Hussein: conducted experimental work and drafted the manuscript. **Rajalakshmanan Eswaramoorthy:** conducted the computational study. **Yadessa Melaku** and **Milkyas Endale:** supervised the experimental work and edited the manuscript.

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Research Article

Phenolic profile, antioxidant and enzyme inhibitory activity of the ethyl acetate, methanol and water extracts of *Capparis spinosa* L.

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Abstract: In this study, it was aimed to determine the phytochemical compositions and biological activities of ethyl acetate (EtOAc), methanol (MeOH) and water extracts obtained from the aerial parts of *Capparis spinosa* L. As a result of spectrophotometric analyzes, MeOH extract was found to be richer in terms of both phenolics and flavonoids compared to other extracts [81.45 mg GAEs (gallic acid equivalent)/g and 36.57 mg RE (rutin equivalent)s/g, respectively], while chromatographic analyzes showed that the extract in question contains a significant amount of hepseridin (72927.48 µg/g), quercetin (1335.88 µg/g), hyperoside (1227.73 µg/g), and 4-hydroxybenzoic acid (924.08 µg/g). Phosphomolybdenum, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging, Cupric Reducing Antioxidant Power (CUPRAC) and Ferric Reducing Antioxidant Power (FRAP) reducing and ferrous ion chelating activity tests resulted in superiority of MeOH extract [371.0, 44.93, 56.46, 91.77, 52.61 mg TEs (trolox equivalent)/g and 14.85 mg EDTAEs/g, respectively]. On the other hand, EtOAc extract exhibited higher activity than other extracts in acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α-amylase, and α-glucosidase inhibitory activity tests [3.29, 2.12 mg GALAEs (galanthamine equivalent)/g, 541.01 and 1584.20 mg ACEs (acarbose equivalent)/g, respectively]. The tyrosinase inhibitory activity test resulted in the superiority of MeOH extract [41.90 mg KAEs (kojic acid equivalent)/g]. A strong correlation was determined between the phenolic and flavonoid contents of the extracts and their antioxidant activities.

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1. INTRODUCTION

Plants can be used in industries such as medicine, pharmacy, food, cosmetics, etc., due to their pharmacological/biologically active phytochemicals, and therefore, new plants are attracting the attention of researchers every day (Orphanides *et al.,* 2016). Researchers have revealed that many phytochemicals such as polyphenols, flavonoids, flavonoids, etc. can be used as critical functional compounds in the treatment of many metabolic diseases (Ng *et al.,* 2012). Since

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plants have low cost and are sustainable sources of phytochemicals, it is of great importance to use those with proven biological/pharmacological activities for industrial use as an alternative to synthetic compounds (Samy *et al.,* 2005; Ng *et al.,* 2020).

Foods deteriorate over time due to lipid oxidation and lose their quality (Yanishlieva & Marinova, 2001). The deterioration of foods with high lipid content can be delayed by the addition of compounds that prevent oxidation during processing. The most effective way to control lipid oxidation is to benefit from antioxidant compounds (Shahidi & Zhong, 2015). These compounds can be found widely in plants and animals, as well as chemically synthesized. Polyphenols and tocopherols are among the most potent antioxidant compounds and are abundant in many vegetables, fruits and grains. In addition, there are various reports of compounds with antioxidant effects in fish, algae and shellfish (Shahidi & Amarowicz, 1996; Amarowicz *et al.,* 1999; Athukorala *et al.,* 2003). In the last decades, researchers have focused on many plant species for the discovery of new and more effective natural compounds that can be used instead of synthetic antioxidants to extend the shelf life of foods. As a result of these studies, many antioxidant phytochemicals have been identified (Liyana-Pathirana *et al.,* 2006; Shahidi & Zhong, 2007; Cumby *et al.,* 2008).

Plants are of particular interest to researchers because they contain phytochemicals with enzyme inhibitory activity as well as antioxidant activities. In the enzyme inhibitory activity studies intensified in recent years, it has been reported that some plant species or some phytochemicals found in these species exhibit inhibitory activity such as cholinesterase (Hung *et al.,* 2008; Loizzo *et al.,* 2010; Pinho *et al.,* 2013), α-amylase/α-glucosidase (Liu *et al.,* 2017; Rasouli *et al.,* 2017; Tan *et al.,* 2017), tyrosinase (Kubo & Kinst-Hori, 1999; Likhitwitayawuid, 2008; Maisuthisakul & Gordon, 2009), etc.

Capparis spinosa L. is an industrial plant species distributed in western and central Asia and along the Mediterranean coastline (Trombetta *et al.,* 2005; Rahimi *et al.,* 2020). This herb has been traditionally used by people for many years in the treatment of various diseases (gout, rheumatism, etc.) (Romeo *et al.,* 2007; Aliyazicioglu *et al.,* 2013; Zhang *et al.,* 2018). Local people living in countries bordering the Mediterranean coastline also frequently benefit from *C. spinosa*'s analgesic properties (Fu *et al.,* 2008). In studies conducted by researchers, it has been reported that the aerial parts, roots or seeds of the plant exhibit many biological/pharmacological activities (anti-allergic, immunomodulatory, anti-inflammatory, antimicrobial, anti-histaminic, antiviral, etc.) (Trombetta *et al.,* 2005; Tlili *et al.,* 2011; Kulisic-Bilusic *et al.,* 2012).

The aim of this study was to determine the chemical compositions of ethyl acetate (EtOAc), methanol (MeOH) and water extracts obtained from *C. spinosa* by qualitative and quantitative chromatographic methods, *in vitro* antioxidant and to document their inhibitory activities on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α-amylase, α-glucosidase, and tyrosinase.

2. MATERIAL and METHODS

2.1. Plant Material and Extract Preparation

Aerial parts of *C. spinosa* was collected from Camlibel village, Kavaklidere, Mugla-Turkey (780 m., 37° 24 849'N 28° 27 688'E) (Herbarium number: O.1196). Dr. Olcay Ceylan (Mugla Sitki Kocman University) performed the identification of the plant material. Aerial parts of the plants were used as the study material to obtain EtOAc, MeOH and water extracts [extract yields: 10.20, 11.31 and 21.58% (w/w), respectively]. Details of the extraction procedure can be found in supplementary file.

2.2. Determination of the Phenolic Compositions of the Extracts

Details of the spectrophotometric and chromatographic analysis were given in supplementary file (Zengin *et al.,* 2017; Cittan & Çelik, 2018).

2.3. Biological Activity

Details of the antioxidant (Apak *et al.,* 2006; Tepe *et al.,* 2011; Kocak *et al.,* 2016; Zengin *et al.,* 2017; Sarikurkcu *et al.,* 2020) and enzyme inhibitory activity (Ozer *et al.,* 2018) tests were given in supplementary file.

2.3. Statistical Analysis

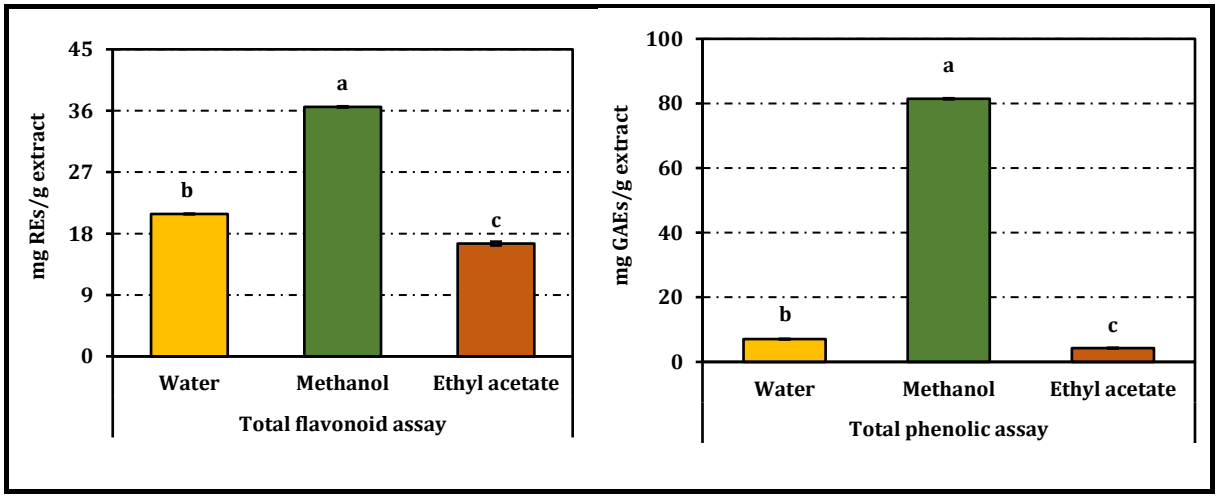
Details of the relative antioxidant capacity index (RACI) (Sun & Tanumihardjo, 2007) and statistical analysis can be found in the supplementary file.

3. RESULTS / FINDINGS

3.1. Chemical Composition

The total amounts of phenolic and flavonoid compounds of the extracts are given in Figure 1. According to the data obtained by spectrophotometric method, MeOH extract was found to be rich in both phenolics and flavonoids. The total amount of phenolic and flavonoid compounds of this extract was 81.45 mg GALAEs/g and 36.57 mg REs/g, respectively. Although the amounts of phenolic and flavonoid compounds of EtOAc and water extracts were close to each other, the chemical compositions of these extracts were statistically different from each other (p <0.05).

Figure 1. Amounts of total flavonoids and phenolics in the extracts of *C. spinosa*. Different letters (a, b, c) on the bars show that the relevant data are statistically different from each other (p <0.05).



The chemical composition data of the extracts obtained by chromatographic methods are given in Table 1. According to the data in the table, it was understood that none of the extracts contained *(+)*-catechin, luteolin 7-glucoside, apigenin 7-glucoside, pinoresinol, kaempferol and luteolin. Chromatographic analyses showed that hepseridine was present in high amounts in the MeOH extract (72927.48 µg/g). Quercetin (1335.88 µg/g), hyperoside (1227.73 µg/g), 4-hydroxybenzoic acid (924.08 µg/g) were also found in the MeOH extract. In addition to these phytochemicals, *p*-coumaric acid was also found in high amounts in EtOAc and water extracts.

3.2. Antioxidant Activity

The total antioxidant activities, reducing powers, radical scavenging and chelating capacities of the extracts are given in Figure 2. On the figures, the statistical relationship between the antioxidant activities of the extracts and each other is also indicated with small letters.

In all antioxidant test systems presented in Figure 2, the MeOH extract exhibited significantly higher activity than the others. The activity value of this extract in phosphomolybdenum, DPPH and ABTS radical scavenging, CUPRAC and FRAP reducing and ferrous ion chelating assays were 371.0, 44.93, 56.46, 91.77, 52.61 mg TEs/g and 14.85 mg EDTAEs/g, respectively. In the radical scavenging and ferrous ion chelating activity tests, the MeOH extract was followed by the water extract (18.80, 35.36 mg TEs/g and 9.97 mg EDTAEs/g, respectively), while the EtOAc extract ranked second in the phosphomolybdenum and CUPRAC reducing assays (329.40 and 48.34 mg TEs/g, respectively). In the FRAP reducing assay, however, no statistically significant difference was found between the antioxidant activities of water and EtOAc extracts.

Table 1. Concentrations of selected phenolic compounds in the extracts of *C. spinosa* (µg/g extract).

Compound	EtOAc	MeOH	Water
Gallic acid	3.84±0.02^{b}	8.46±0.40^{a}	3.80±0.10^{b}
Protocatechuic acid	646.52±0.37^{a}	179.25±2.86^{b}	13.36±0.29^{c}
3,4-Dihydroxyphenylacetic acid	13.36±0.43^{a}	14.28±0.37^{a}	14.76±0.19^{a}
Pyrocatechol	26.39±0.14^{b}	42.93±2.92^{a}	33.11±1.04^{b}
(+)-Catechin	nd	nd	nd
Chlorogenic acid	4.33±0.01^{a}	4.06±0.10^{b}	4.19±0.01^{ab}
(-)-Epicatechin	2.61±0.09^{b}	3.08±0.13^{a}	2.41±0.02^{b}
2,5-Dihydroxybenzoic acid	16.93±0.62^{a}	11.75±0.90^{b}	14.51±1.32^{ab}
4-Hydroxybenzoic acid	648.78±1.04^{b}	924.08±2.26^{a}	106.19±3.28^{c}
Vanillic acid	215.30±17.44^{b}	305.77±14.43^{a}	138.24±5.15^{c}
Caffeic acid	12.91±0.85^{b}	24.96±1.84^{a}	14.13±0.43^{b}
Syringic acid	9.74±0.06^{b}	53.98±6.66^{aa}	5.65±0.10^{b}
3-Hydroxybenzoic acid	10.64±0.60^{a}	11.74±0.77^{a}	11.39±0.23^{a}
Vanillin	7.85±0.22^{c}	19.42±2.16^{b}	31.79±1.21^{a}
Verbascoside	5.78±0.08^{a}	6.04±0.13^{a}	5.90±0.03^{a}
Taxifolin	7.11±0.16^{c}	15.60±0.11^{a}	8.77±0.26^{b}
p-Coumaric acid	144.54±0.56^{b}	693.59±10.15^{a}	34.32±2.24^{c}
Sinapic acid	4.97±0.13^{c}	33.80±0.14^{a}	6.06±0.04^{b}
Ferulic acid	71.80±1.21^{b}	176.20±4.65^{a}	13.39±2.38^{c}
Luteolin 7-glucoside	nd	nd	nd
Hyperoside	4.53±0.19^{b}	1227.73±16.22^{a}	9.32±0.29^{b}
Hesperidin	260.27±3.47^{a}	72927.48±659.21^{b}	155.34±1.26^{a}
Rosmarinic acid	16.83±1.01^{b}	28.82±0.63^{a}	25.49±1.89^{a}
Apigenin 7-glucoside	nd	nd	nd
2-Hydroxycinnamic acid	3.02±0.09^{b}	2.06±0.07^{c}	3.54±0.05^{a}
Eriodictyol	9.56±0.12^{b}	46.83±2.88^{a}	13.64±0.11^{b}
Pinoresinol	nd	nd	nd
Quercetin	5.42±0.15^{c}	1335.88±6.51^{a}	100.11±1.39^{b}
Kaempferol	nd	nd	nd
Luteolin	nd	nd	nd

Different letters (a, b, c) within the same row show that the relevant data are statistically different from each other (p <0.05). nd: Not detected.

The relative antioxidant capacity index (RACI) data (Figure 3), in which extracts were compared with each other according to their activity potentials, taking into account all the activities obtained from the antioxidant activity tests, confirmed the data obtained from the antioxidant activity tests. According to the data presented in the figure, the MeOH extract ranked first with a RACI value of 1.20. It was followed by water and EtOAc extracts with RACI values of -0.55 and -0.62, respectively.

Figure 2. Antioxidative capacity of the extracts of *C. spinosa*. Different letters (a, b, c) on the bars show that the relevant data are statistically different from each other (p <0.05).

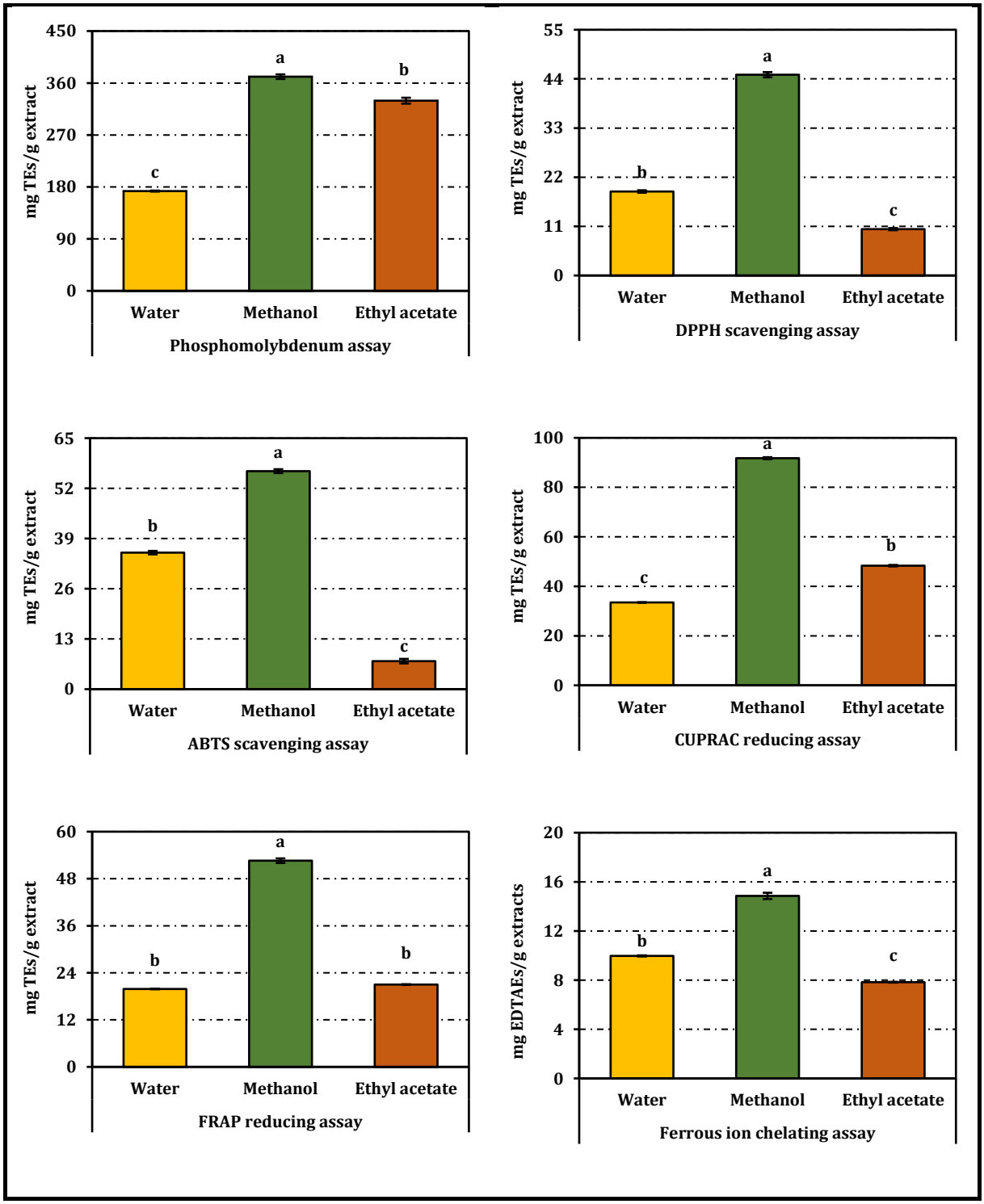
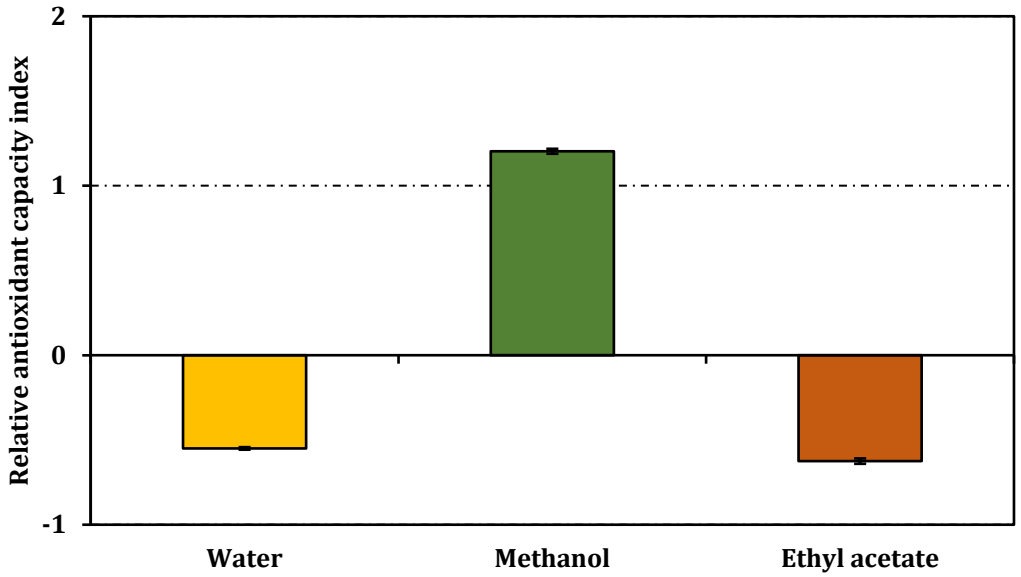


Figure 4 shows the correlation between the antioxidant activities of the extracts and their RACI values. A high correlation was found between the antioxidant activities of the extracts and the RACI values in all tests except the phosphomolybdenum assay. However, in the phosphomolybdenum test, the correlation between the total antioxidant activity of the EtOAc extract and the RACI value was found to be lower than in the other test systems.

Figure 3. RACI of the extracts of *C. spinosa*.



3.3. Enzyme Inhibitory Activity

Figure 5 shows the inhibitory activity potentials of *C. spinosa* extracts on AChE, BChE, α-amylase, α-glucosidase and tyrosinase.

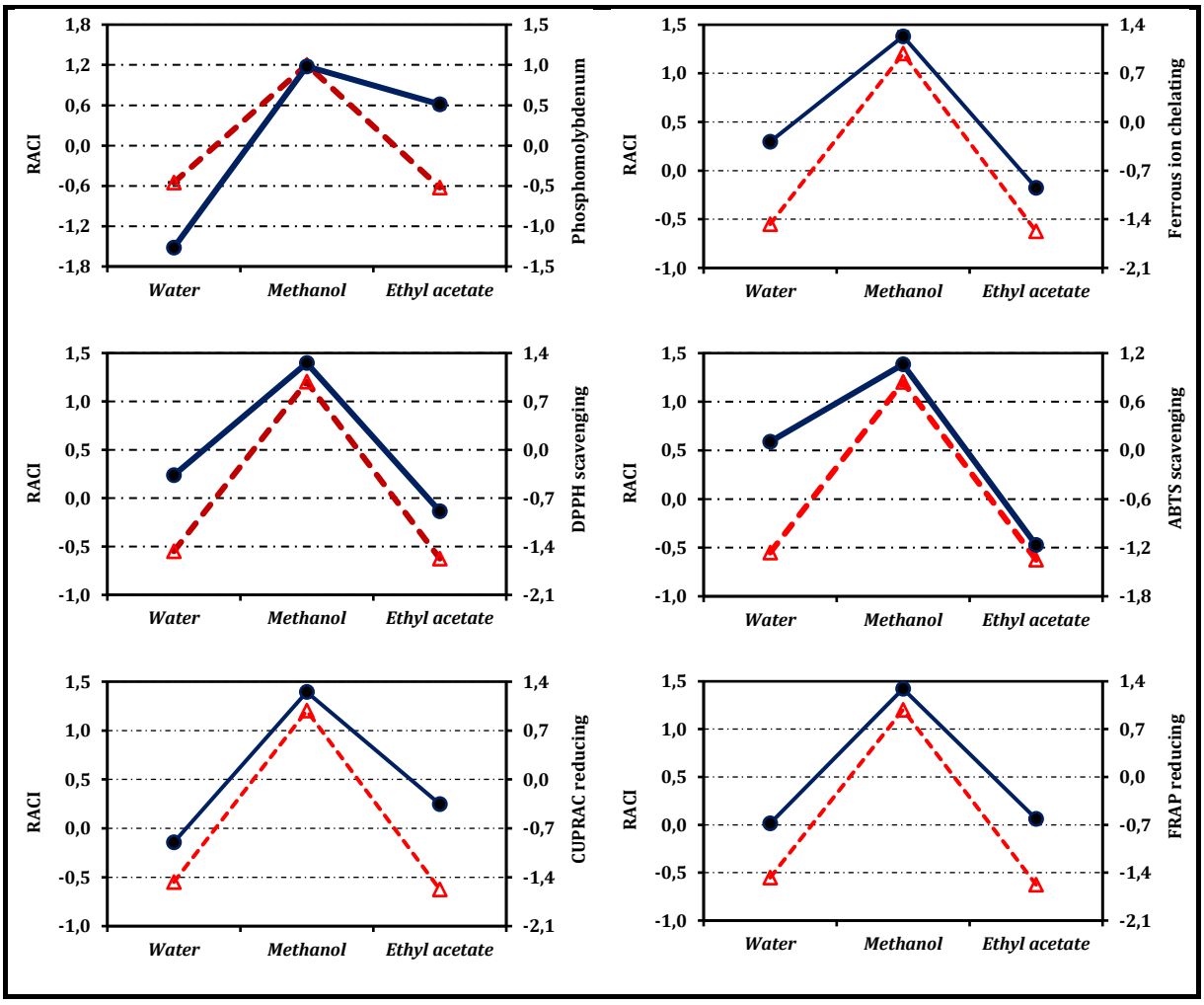
As can be seen from Figure 5, EtOAc extract exhibited higher inhibitory activity than the others in all test systems except the tyrosinase inhibitor activity test. The inhibitory activities of the extract in question in AChE, BChE, α-amylase and α-glucosidase tests were 3.29, 2.12 mg GALAEs/g, 541.01, and 1584.20 mg ACEs/g, respectively. The tyrosinase inhibitory activity test resulted in the superiority of MeOH extract (41.90 mg KAEs/g). In this assay, no statistical difference was found between the activity potentials of the water and EtOAc extracts. While water and MeOH extracts were not active in the BChE inhibitory activity tests, the water extract remained inactive in the α-glucosidase inhibitor activity assay.

3.4. Correlation Coefficients

Table 2 shows the correlation between the biological activity data of the extracts in the tests given above and their chemical compositions.

According to the correlation coefficients given in Table 2, there was a strong correlation between the phenolic and flavonoid contents of the extracts and their antioxidant activities (correlation coefficients were above 0.9). The relationship between these compounds and tyrosinase inhibitory activity was also found to be high. In addition, the relationship between protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, ferulic acid, hesperidin, hyperoside, and quercetin contents of the extracts and their antioxidant and tyrosinase inhibitor activities were also found statistically significant.

Figure 4. Antioxidant activity (solid dark blue line with circle) and RACI (dashed red line with triangle) of the extracts of *C. spinosa*.



4. DISCUSSION and CONCLUSION

There are some data on the chemical composition of *C. spinosa* in the literature. According to these data, the presence of some tannins, saponins, alkaloids and flavonoids has been detected in this plant so far (Anwar *et al.,* 2016; Snoussi *et al.,* 2017). It is of course possible to elaborate on these studies. However, it is seen that some phytochemicals specific to this species come to the fore in some studies. In a study by Fu *et al.* (2007), cappariloside A and stachydrin were found to be the main components, while in some other studies, it was reported that rutin, which is a flavonoid, is found in high amounts in the plant (Stefanucci *et al.,* 2018; Mollica *et al.,* 2019). A review by Anwar *et al.* (2016) documented the flavonoids, alkaloids and essential oil components identified so far in *C. spinosa*. However, none of these studies included the presence of hepseridine and hyperoside, which were identified as the main compounds in the current study. Therefore, the presence of these compounds in *C. spinosa* was brought to the literature for the first time with this study.

Figure 5. The capacity of the extracts of *C. spinosa*. to inhibit some enzymes. Different letters (a, b, c) on the bars show that the relevant data are statistically different from each other (p <0.05).

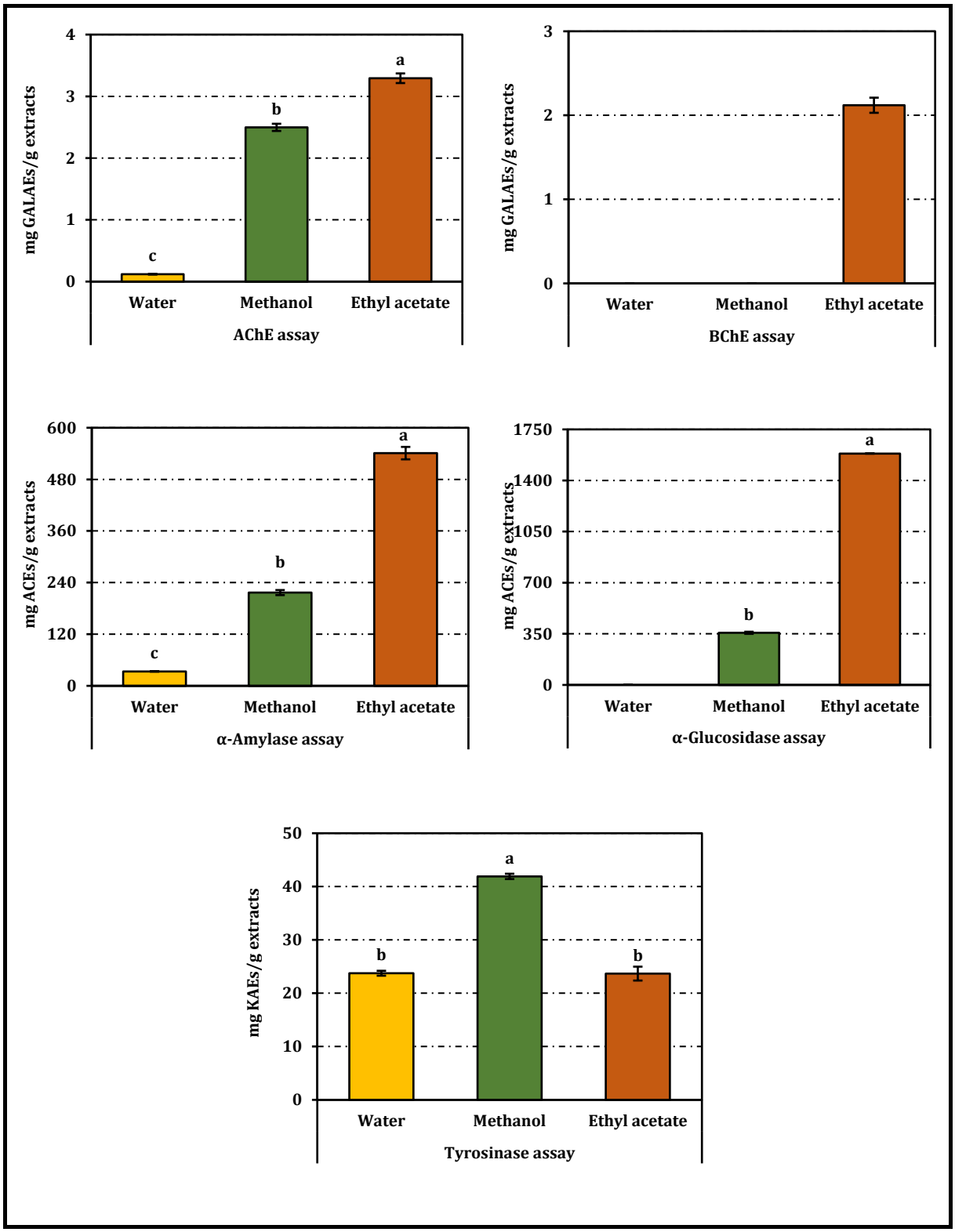


Table 2. Correlations among chemical composition and assays.

	22	1	2	3	4	5	7	8	9	10	11
1	0.986										
2	0.989	0.999									
3	0.990	0.999	0.999								
4	0.987	0.999	0.999	0.999							
5	0.985	0.999	0.999	0.999	0.999						
6	0.988	0.999	0.999	0.999	0.999	0.999					
7	-0.976	-0.992	-0.991	-0.993	-0.991	-0.989					
8	-0.993	-0.999	-0.999	-0.999	-0.999	-0.999	0.987				
9	0.991	0.997	0.998	0.997	0.998	0.998	-0.981	-0.999			
10	-0.974	-0.998	-0.996	-0.996	-0.997	-0.997	0.996	0.993	-0.989		
11	-0.989	-0.999	-0.999	-0.999	-0.999	-0.999	0.993	0.999	-0.997	0.997	
12	0.989	0.999	0.999	0.999	0.999	0.999	-0.991	-0.999	0.998	-0.996	-0.999
13	0.989	0.999	0.999	0.999	0.999	0.999	-0.993	-0.999	0.997	-0.997	-0.999
14	0.986	0.999	0.999	0.999	0.999	0.999	-0.991	-0.999	0.997	-0.997	-0.999
15	0.987	0.999	0.999	0.999	0.999	0.999	-0.993	-0.999	0.997	-0.997	-0.999
16	0.980	0.997	0.997	0.995	0.997	0.998	-0.979	-0.996	0.997	-0.992	-0.995
17	0.986	0.999	0.999	0.999	0.999	0.999	-0.992	-0.999	0.997	-0.997	-0.999
18	0.989	0.999	0.999	0.999	0.999	0.998	-0.996	-0.998	0.995	-0.997	-0.999
19	0.989	0.999	0.999	0.999	0.999	0.999	-0.993	-0.999	0.997	-0.997	-0.999
20	0.989	0.999	0.999	0.999	0.999	0.999	-0.993	-0.999	0.997	-0.997	-0.999
21	0.989	0.999	0.999	0.999	0.999	0.999	-0.993	-0.999	0.997	-0.997	-0.999

1: DPPH, 2: ABTS, 3: CUPRAC, 4: FRAP, 5: FICA, 6: RACI: 7: AChEIA, 8: BChEIA, 9: TIA, 10: AAIA, 11: AGIA, 12: Total flavonoid, 13: Total phenolic, 14: Protocatechuic acid, 15: 4-Hydroxybenzoic acid, 16: Vanillic acid, 17: p-Coumaric acid, 18: Ferulic acid, 19: Hesperidin, 20: Hyperoside, 21: Quercetin, 22: TAP

As detailed in Part 3, the MeOH extract of *C. spinosa* showed remarkable antioxidant activity. There are many studies on the antioxidant activity of *C. spinosa* in the literature (Nadaroglu *et al.,* 2009; Tlili *et al.,* 2017; Yu *et al.,* 2017; Al-Azawi *et al.,* 2018). In many of these studies, the plant species in question exhibited remarkable antioxidant and radical scavenging activity. Therefore, the data obtained from the present study confirm the literature data. Also, according to the literature data, quercetin (Selway, 1986; Rauha *et al.,* 2000; Guardia *et al.,* 2001; Williams *et al.,* 2004), 4-hydroxybenzoic acid (Duke *et al.,* 2003; Manuja *et al.,* 2013) and *p*-coumaric acid (Bonina *et al.,* 2002; Ahmad *et al.,* 2006) obtained from this plant may be phytochemicals responsible for the antioxidant activity. However, as mentioned above, the presence of hesperidin and hyperoside in this plant was brought to the literature for the first time with this study. There are some literature data on the contribution of these compounds to antioxidant activity with some other plants or with these compounds themselves (Ku *et al.,* 2014; Hao *et al.,* 2016; Yatao *et al.,* 2018; Gao *et al.,* 2019; He *et al.,* 2019; Kim *et al.,* 2019; Musa *et al.,* 2019; Aggarwal *et al.,* 2020; Huang *et al.,* 2020). These findings support the correlation coefficient data obtained from the present study.

There are some reports in the literature regarding the cholinesterase inhibitory activity of *C. spinosa*. In a study carried out by Mollica *et al.* (2019), cholinesterase inhibitory activities of extracts obtained from *C. spinosa* by different methods were investigated and it was reported that the highest activity was exhibited by the extract obtained by microwave extraction. In another study by Wojdylo *et al.* (2019), it was reported that extracts obtained from different developmental stages of *C. spinosa*, especially those rich in flavonols (quercetin, kaempferol, myricetin, and isorhamnetin derivatives), showed significant cholinesterase inhibitory activity. Similar findings were also reported by Mekinic *et al.* (2018).

In the current study, the EtOAc extract exhibited the highest cholinesterase inhibitory activity, and according to the data in Table 1, this extract contains high amounts of protocatechuic and 4-hydroxybenzoic acids. There are some reports in the literature that these compounds themselves or some extracts containing high amounts of these compounds exhibit significant cholinesterase inhibitory activity (Szwajgier & Borowiec, 2012; Ertas *et al.,* 2014; Zengin *et al.,* 2017). These reports corroborate the data from the present study.

According to literature data, *C. spinosa* is considered to be a remarkable anti-hyperglycemic agent, in addition to its biological activities given above. In a study by Mollica *et al.* (2017), it was reported that *C. spinosa* leaves or buds normalized biochemical parameters and reversed liver/lung damage in streptozocin-induced diabetic rats. The inhibitory activity of *C. spinosa* phytochemicals on α-amylase and α-glucosidase was also analyzed by *in silico* methods (Ogunwa *et al.,* 2017). In the aforementioned study, it was reported that naringin and rutin show high affinity for α-amylase and α-glucosidase. In the present study, EtOAc extract from *C. spinosa* exhibited the highest inhibitory activity on both enzymes. As can be seen from the data in Table 1, protocatechuic and 4-hydroxybenzoic acids are present in high amounts in this extract. Literature data indicate that both compounds may be responsible for the anti-diabetic activity of the extract (Saltan *et al.,* 2017; Alegbe *et al.,* 2019).

There are also some reports in the literature regarding the tyrosinase inhibitory activity of *C. spinosa*. It was determined that quercetin increased tyrosinase expression in B16 murine melanoma cells treated with *C. spinosa* extract at a concentration of 0.03% (w/v) (Matsuyama *et al.,* 2009). Similar findings have been reported in a different report of the same research group (Matsuyama *et al.,* 2009). In the current study, MeOH extract exhibited the highest tyrosinase inhibitory activity. According to the data in Table 1, it is thought that the main compounds of the MeOH extract contribute significantly to this activity. However, the presence of 1335.88 µg/g quercetin in the MeOH extract creates a contradiction between the data obtained from the current study and the literature data. Therefore, biological activity-guided fractionation is needed to elucidate the compounds that contribute to the activity.

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

Bulent Kirkan: Investigation and Resources. **Olcay Ceylan:** Resources. **Cengiz Sarikurkcu:** Methodology, Visualization, Software, Formal Analysis and Validation. **Bektas Tepe:** Investigation, Supervision and Writing -original draft.

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Research Article

Chemical composition and antioxidant activities of essential oils and extracts from cones of *Tetraclinis articulata* (Vahl) Masters

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Abstract: This study was carried out to evaluate the in vitro antioxidant activity and the chemical composition of essential oils and organic extracts of Moroccan *Tetraclinis articulata* cones (Khemisset region). The GC–MS analysis of essential oils identified a total 23 volatile components. The major constituents of the oil were α-pinene (18.33%), cis-verbenone (10.02%), and L-pinocarveol (8. 32%). For phytosterols analysis of hexane extract, β-sitosterol constitutes the majority with a percentage of 77.74%. The amount of total phenolic and flavonoid contents was high in the methanol extract (78.54±2.8 mg GAE / g and 41.11± 4.5 mg QE/g, respectively) and the Antioxidant capacity determined by DPPH method showed a strongest radical scavenging activity exhibition by the methanol extract (IC50=0.038±0.006 mg/mL). The results indicated that *T. articulata* contains bioactive compounds which are responsible for its antioxidant activity. Therefore, this plant could be potential candidates for the preparation of a natural antioxidant drug or an additive preparation.

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1. INTRODUCTION

Reactive oxygen species (ROS) include species, such as the hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and superoxide (O_2^-) readily react with most biological macromolecules and leads to their oxidative modification and consequently resulting in the loss of their activities (Kapoor *et al.*, 2019). They have the ability, to damage all biomolecules, causing peroxidation of lipids, oxidation of proteins, and damage to nucleic acids, enzyme inhibition (Madkour, 2020) which causes chemical alterations of these molecules (Mitra, 2020) leading to many chronic diseases such as pulmonary diseases (Park *et al.*, 2006), cancer (Sosa *et al.*, 2013), renal

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diseases (Massy & Nguyen-Khoa, 2002), neurodegenerative disorders (Uttara *et al.*, 2009), metabolic and cardiovascular diseases (Incalza *et al.*, 2018).

Synthetic phenolic antioxidants (SPAs) such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and propyl gallate (PG) are closely correlated with human life due to their extensive usages, and increasing concerns have been raised on their biosafety (Liu & Mabury, 2020). Previous studies have found that some synthetic antioxidants and related transformation products showed toxicity effects including hepatic toxicity, endocrine disrupting effects, and even carcinogenicity (Yang *et al.*, 2018; Dassarma *et al.*, 2018). Recently there has been a considerable interest in finding natural antioxidants from natural resources such as medicinal plants to replace synthetic ones and to find more information concerning the antioxidant potential of plant (Jamshidi-kia *et al.*, 2020).

In searching for novel natural antioxidants, The Thuya of Berber, *Tetraclinis articulata* (Vahl) Masters, known byits name "*El ârâr*" in Arabic, belongs to Cupressaceae family, is an endemic species to North Africa and in particular to the Maghreb countries (Morocco, Algeria and Tunisia). It is also found in some very specific areas, in the south-east of Spain (region of Almeria) and on the island of Malta (Kouider & Assia, 2017). In addition, this forest species has an important sociological and economical role (Tsouli Faroukh *et al.*, 2017).It is also widely used in traditional medicine because of its multiple therapeutic effects. Indeed, different parts of Thuya are recommended in the treatment of intestinal infections, gastric pain, respiratory diseases, diabetes, severe diarrhea, hypertension, and fever (Teixidor-Toneu *et al.*, 2017; Hind *et al.*, 2017). These uses reveal that extracts and essential oils of *T. articulata* contain compounds which exert numerous biological activities such as antibacterial, antioxidant, immunostimulatory, antifungic, anti-inflammatory, cytotoxic properties, anticholinesterase, anti-tyrosinase, antidiarrheal, and anti-ulcerative activities (Sadiki *et al.*, 2019; Rabib *et al.*, 2020; El-Shemy, 2020; Rached *et al.*, 2018; Fidah *et al.*, 2016; Jlizi *et al.*, 2018; Calderón-Montaño *et al.*, 2018; Daoudi *et al.*, 2013; Ababsa *et al.*, 2019; Saber *et al.*, 2021). The objective of this work is the evaluation of antioxidant activity and chemical characteristics of the essential oil and the extracts of the cones of *Tetraclinis articulata* from Khemisset.

2. MATERIAL and METHODS

2.1. Plant collection and preparation

The cones were harvested in March 2017 from Khemisset region and dried in the open air, at room temperature and protected from humidity. All measurements are performed in triplicate.

2.1.1. *Preparation of essential oil (EO)*

The extraction of essential oils was carried out by hydrodistillation in a Clevenger type apparatus (Clevenger, 1928). Briefly, 100g of the dry plant is placed in a balloon filled to 2/3 with water; the whole is brought to the boil for 6 hours. The oil is recovered and then stored at a temperature of 4 °C for the tests.

2.1.2. *Preparation of extracts*

Firstly, 40 g of crushed Thuya cones are taken then put it in a cartridge before extracting it using a solvent (hexane, methanol) using the Soxhlet as an extraction material (Jensen, 2007). The system is heated at reflux for 6 hours, until discoloration. Samples are prepared by two different solvents used a successive extraction: hexane (for delipidation) then with methanol. The extracts obtained are evaporated to dryness using a rotary evaporator equipped with a vacuum pump and then stored at 4 ° C for the tests.

2.2. Phytochemical screening

Chemical assays for screening and identification of bioactive constituents in *T. articulata* cones were performed with extracts prepared by qualitative characterization reactions.

2.2.1. *Flavonoid detection*

Briefly, 1mL of extract with a few drops of concentrated HCl, and then adds a few milligrams of magnesium turnings. The presence of flavonoids is confirmed by the appearance of a pink to red orange color (N'Guessan *et al.*, 2009).

2.2.2. *Alkaloid detection*

100 mg of extract with 3 ml H_2SO_4 (1%), the whole is brought to a boil in a water bath at 100 ° C for 5 min. After cooling and filtration, 5 drops of Mayer's reagent are added. The formation of a white precipitate indicates the presence of alkaloids (Mojab *et al.*, 2010).

2.2.3. *Tannin detection*

The extract dissolved in distilled water and added drops of a solution of $FeCl_3$ (1%). The appearance of a blue-black color indicates the presence of gallic tannins and the appearance of a green-blackish color indicates the presence of catechetical tannins (Y *et al.*, 2004).

2.2.4. *Saponosides detection*

Mix 1ml of the extract with 2 mL of hot distilled water and stir for 15 seconds then let stand for 10 min. A height of persistent foam, greater than 1 cm indicates the presence of Saponosides (Bekro *et al.*, 2016).

2.2.5. *Sterols and polyterpenes detection*

The extract is diluted in 2 mL of acetic anhydride. The addition of a few drops of concentrated H_2SO_4 allows the appearance of a violet color which indicates the presence of sterols and polyterpenes (N'Guessan *et al.*, 2009).

2.3. Determination of the total phenolic content

The determination of the total polyphenols of the methanol extract is carried out by the Folin-Ciocalteu method (Singleton *et al.*, 1999). Briefly, 200 µL of extract or reference (Gallic acid) with 800 µl of the Na_2CO_3 solution (7.5%), After stirring (5 min), 1 mL of the Folin-Ciocalteu solution (diluted with distilled water 1:10) is added to the whole, after 2 hours of incubation at room temperature, the absorbance is read at 765 nm against a blank without extract. The results are expressed in micrograms of Gallic acid equivalent per milligram of extract (µg EAG / mg of extract).

2.4. Determination of flavonoids content

The determination of flavonoids in our experiments is carried out by the method (Quettier-Deleu *et al.*, 2000). In test tubes 1 mL of methanol extract or standard (quercitin) and 1 mL of methanol solution of aluminum chloride (2%). After 15 min of incubation at room temperature, the absorbances are read using a UV spectrophotometer visible at 430 nm against a blank (methanol added to $AlCl_3$). The results are expressed in µg Quercetin equivalent per milligram of extract (µg EQ / mg of extract).

2.5. Analysis of Essential oil

The gas chromatography/mass spectrometry (GC/MS) device is made by Perkin Elmer ClarusTM GC-680 with Q-8 MS. It is equipped with an auto-sampler, which gives access to the automatic injection of samples into the injector and a capillary column type RxiR-5Sil MS traversed by Helium gas. The mass spectrometer is powered by a SMART electronic ionization source. This source can ionize and vaporize the different molecules as well as a quadrupole filter to separate the different ions in their m/z ratio. The GC/MS system is computer-controlled with Turbomass (TM) software, which allows programming of analytical methods as well as qualitative and quantitative identification of detected species. The analysis parameters are as follows: analysis time: 2 hours, vector gas flow rate: 1 mL/min, ionization energy: 70 eV,

injector temperature: 260 °C, oven temperature: 40 °C for 2 minutes, then rise from 10 °C / min to 290 °C, and the injected volume is 0.5 µL.

2.5. Determination of the composition of sterols

Sterol composition was quantified according to the (ISO 6799, 1991), standard method using capillary gas chromatography (CGC) on an apolar column (Chroma pack) (30m× 0.32 mm, DI: 0.25 µm, phase: CPSIL8CB). The Varian CP-3800 chromatograph is equipped with a divider injector type 1079 (T: 300°C) and an FID (T: 300°C) and using helium as carrier gas (flow: 1.5 mL/min).

2.6. Evaluation of antioxidant activity (DPPH test)

The scanning activity of the DPPH radical is measured according to the protocol described by (Lopes-Lutz *et al.*, 2008). A methanol solution of 0.3 mM of DPPH is mixed with different concentrations of the samples from the cones of *T. articulata* (0.0025; 0.005; 0.01; 0.025; 0.05; 0.1; 0.25; 0.5; 1; 2 mg / mL). In a test tube, 2.5 mL of samples and controls solution (Ascorbic acid, Gallic acid and Quercetin) are added to 1 mL of the methanol solution of DPPH, after incubation for 30 min in the dark and at room temperature, the absorbances are measured at 517 nm against a blank which contains pure methanol. The negative control is composed of 1 mL of the methanol solution with DPPH and 2.5 mL of methanol.

3. RESULTS / FINDINGS

3.1. Yield

Table 1 summarizes the results of the study of the yield of the essential oil and extracts of *T. articulata* cones collected in the Khemisset region.

Table 1. Yield obtained from the different samples.

Samples	Yields (%)
Essential Oil	0.33 ± 0.05
Hexane extract	2.46 ± 0.20
Methanol extract	13.22 ± 1.01

The yield of the methanol extract is higher than that of the hexane extract by the order of 13.22 ± 1.01% and 2.46± 0.20%, respectively (Table 1). The yield of essential oils calculated after 6 hours of extraction from the dry plant material is 0.33±0.05%. These results are close to 0.31% found by (Buhagiar *et al.*, 2000), have studied the essential oils cones of a cultivated stand of *T. articulata* growing in Malta (spain). Different yields are present in the literature, especially in Algeria. It was reported that the EO yield extracted from fresh cones of *T. articulata* from the Hammam Melouane region was 0.80% and that of Tipazaregion was 1.60% (Chikhoune *et al.*, 2013). Also, the EO yield collected in Ain-Defla region was of the order of 0.52% (Djouahri *et al.*, 2014). This yield being lower than that obtained by the sawdust of our other study (2.19%) (Saber *et al.*, 2021).

3.2. Phytochemical screening

The flavonoids and saponosides test indicate the presence with a strongly positive amount, then the alkaloid test reveals the presence in methanol extract. For the sterols and polyterpenes test shows the absence of these in the methanol extract. Also, the tannins test shows the absence of catechetical tannins and gallic tannins as can be seen in Table 2.

Table 2. Results of the phytochemical screening of the methanol extract of the cones studied.

Secondary metabolites	Flavonoids	Saponosides	Alkaloids	Sterols and polyterpenes	Catechetical tannins	Gallic tannins
Methanol extract	+++	+++	++	-	-	-

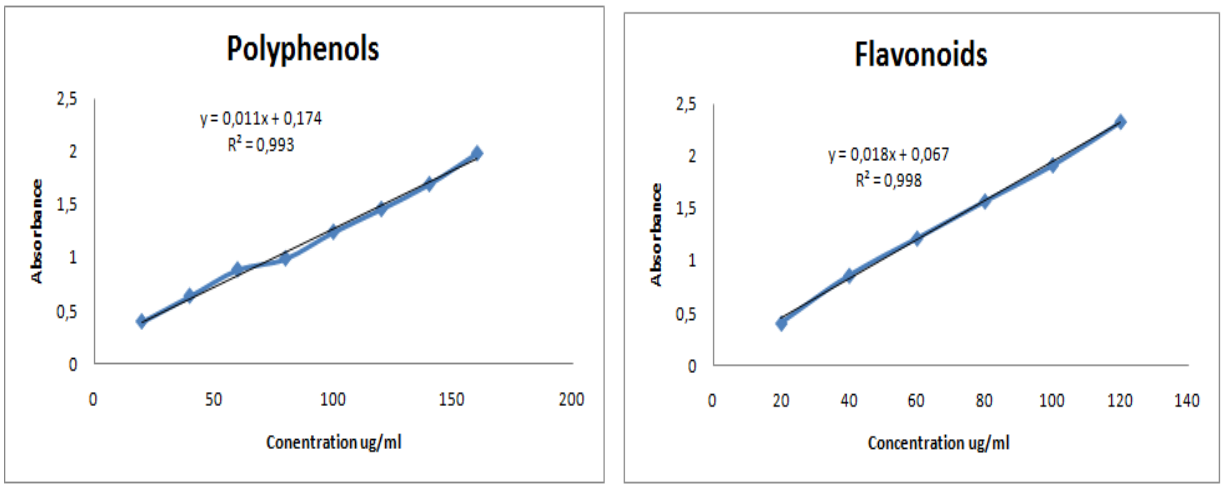
(+++): Strongly positive test
(++): Positive average test
(+): Low positive test
(-): Negative test

The presence of flavonoids and saponosides in our extract is probably responsible for the free radical scavenging effects observed. Indeed, flavonoids are phenolic compounds inplant medicinal which recognized by their antioxidant potentials (Ait Lahcen *et al.*, 2020). Also, saponosides have a wide range of pharmacological activities, including expectorant, anti-inflammatory, vasoprotective, gastroprotective and antimicrobial properties (Koczurkiewicz *et al.*, 2015). For Alkaloids they have shown to exert a broad spectrum of antimicrobial, anticancerogenic, and antimutagenic activity (Račková *et al.*, 2004).

3.3. Total phenolic and flavonoid content

The content of phenolic compounds in two extracts was determined from the calibration curve for gallic acid and the results are expressed in µg gallic acid equivalent per mg of extract (µg EAG/mg). On the other hand, the flavonoid content is expressed in µg quercetin equivalent per milligram of extract (µg EQ / mg of extract). The calibration curves are shown in Figure 1. Based on these results, the methanol extract showed high phenolic and flavonoid compounds (78.54 ± 2.8 µg GAE/mg and 41.11± 4.5 µg QE/mg,respectively).

Figure 1. Gallic acid (phenolic) and quercetin (flavonoid) calibration curves.



2.4. Chemical compounds of Essential oils

The GC-MS analysis of cones TAEO revealed the presence of 23 volatile compounds, the compounds identified are reported in Table 3. These compounds are belonging to different classes, including oxygenated monoterpenes (52.44%) and hydrocarbons monoterpene (27.78%). From these results, we note that this EO is mainly made up of α-pinene (18.33%), cis-Verbenone (10.02 %), L-pinocarveol (8.32%), bicyclo[4.1.0]hept-2-ene (6.76%),α-campholenal (6.10%), and D-limonene (5.75%). To our knowledge, this study is the first carried

out in Morocco, no study has been mentioned in the literature on the chemical composition of the Moroccan *T. articulata* cones.

Table 3. Chemical composition of the essential oil of *T. articulata* cones.

Compounds	RT	%
α-pinene	4.033	18.33
2,4-thujadiene	4.325	1.99
p-Xylene	5.045	0.66
o-cymene	5.347	1.71
D-limonene	5.410	5.75
Styrene, 2,5-dimethyl-	3.353	2.11
Linalool	6.474	0.85
α-campholenal	6.940	6.10
L-Pinocarveol	7.168	8.32
Cis-verbenol	7.232	2.96
3-Methylenecyclohexene	7.301	3.18
D-Pinocamphone	7.502	2.15
Bicyclo[4.1.0]hept-2-ene	7.571	6.76
Terpinen-4-ol	7.745	1.26
Thymol	7.846	2.53
α-terpineol	7.941	1.60
Myrtenal	8.052	5.22
cis-Verbenone	8.259	10.02
trans-2-Caren-4-ol	8.360	5.01
cis-Carveol	8.540	0.94
D-Carvone	8.757	2.62
Bornylacetate	9.387	2.18
Terpinylacetate	10.292	0.68
Monoterpenehydrocarbons		27.78
Oxygenatedmonoterpenes		52.44
Sesquiterpenehydrocarbons		-
Oxygenatedsesquiterpenes		-
Others		12.71
Total		92.93

The comparative study carried out in the literature on *T. articulata* cones EOs reveal a homogeneity of major compounds. Indeed, Buhagiarand collaborators identified α-pinene (68.2%), limonene (16.6%) (Buhagiar *et al.*, 2000). Djouahri, who worked on *T. articulata* as an antimicrobial and anti-inflammatory plant, cited α-campholenal (16.34%); trans-pinocarveol (15.45%); verbenone (13.36%); cis-verbenol (12.36%) (Djouahri *et al.*, 2014). In another study (Chikhoune *et al.*, 2013), Chikhoune *et al.* reported a chemical composition dominated by α-pinene; β-myrcene; limonene for two EOs of the cones studied. These results are close to the results found by Boussaïd et al (Boussaïd *et al.*, 2016) who examined the chemical composition of six EO of *T. articulate* cones collected in six different regions of Algeria with a quantitativedifference.

3.5. Sterols compounds

The sterols analyzed by CPG detected eight phytosterols for the hexane extract as summarized in Table 4. According to the results of Table 4, β-sitosterol constitutes the majority sterol with a percentage of 77.74%, followed by 10.55% of compesterol; brassicasterol (4.06%); stigmasterol (2.56%); cholesterol (0.48%); Δ-5-Avenosterol (0.37%); Δ-7-Avenosterol (0.27%) and Δ-7-stigmasterol (0.09%).

Table 4. Chemical composition in sterols of hexane extract of Thuya cones.

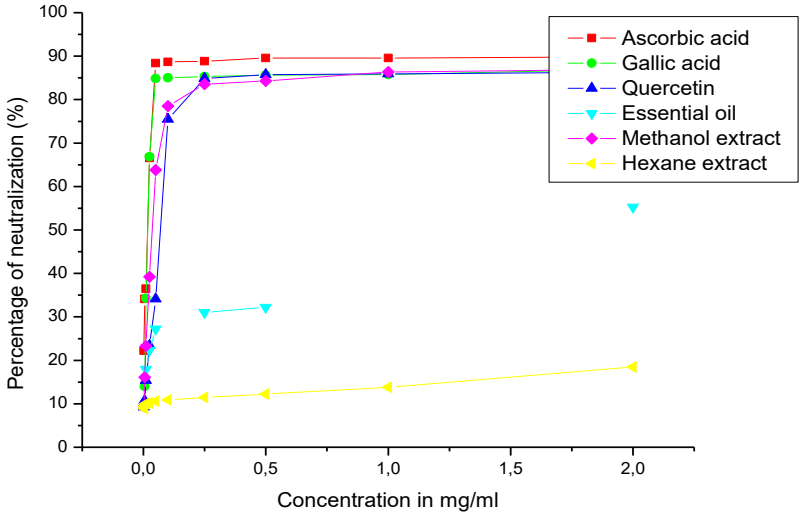
Phytosterols	Pourcentage (%)
Cholesterol	0.48
Brassicasterol	4.06
Compesterol	10.62
Stigmasterol	2.56
β-Sitosterol	77.21
Δ-5-Avenosterol	0.37
Δ-7-Stigmasterol	0.09
Δ-7-Avenosterol	0.27

Among these, the most abundant phytosterols is β-sitosterol, which has a broad range of *in vivo* biological functions including antitumor, anti-inflammatory, antidiabetic, and gallstone-reducing activities (Soleimanian *et al.*, 2020).To our knowledge, this study is the first carried out on the composition of phytosterols, no study has been mentioned in the literature on the composition of phytosterols in *T. articulata* cones.

3.5. Antioxidant activity of samples

From Figure 2, it is quite clear that the percentages of neutralization of DPPH radical increase with increasing concentration of samples and standards (ascorbic acid, gallic acid and quercetin). The antioxidant activity is expressed in IC_{50}, it defines the concentration of the extract or reference tested necessary to reduce 50% of the DPPH radical. In addition, the lower the IC_{50} values, the stronger the antioxidant power.

Figure 2. Evolution of the neutralization percentage (%) for samples and standards.



The concentrations which provide 50% inhibition (IC_{50}) are grouped together in Table 5, where it is found that the IC50 values calculated for the methanol extract and references confirmed the reactivity of these samples with respect to DPPH. The results obtained demonstrated that the methanol extract (IC_{50} = 0.038±0.006 mg/mL) has an antioxidant activity close to that of the standards and greater than the essential oils and hexane extract (absence) over the entire range of concentrations studied.

Table 5. Antioxidant activity expressed in IC_{50}.

	IC_{50} (mg/mL)
Ascorbic acid	0.018±0.001
Gallic acid	0.020±0.003
Quercetin	0.070±0.004
Essential oil	1.677±0.026
Methanol extract	0.038±0.006
Hexane extract	Absence

In comparison with the literature, the value of methanol extract obtained is higher than those obtained in the work carried out by Bensebia and collaborators (IC_{50} = 0.14 mg/ml) were obtained from the *T. articulata* leaves extracted with 80 % ethanol (Bensebia, s. d.). In addition, our essential oil shows better activity than two samples of essential oils from cones in two different sites in Algeria which showed low activity (Chikhoune *et al.*, 2013).

4. DISCUSSION and CONCLUSION

The aromatic plants currently present a reliable source of active ingredients known for their therapeutic properties, in particular anti-oxidant activity. This work is interested in the study of the antioxidant activity of essential oil and organic extracts from the cones of *T. articulata.* The phytochemical tests carried out by the characterization reactions made it possible to highlight alkaloids, flavonoids and saponosides in the Thuya cones of the Khemisset region. The determination of phenolic compounds of methanol extract revealed considerable contents of polyphenols and flavonoids. Regarding antioxidant activity, we studied the antioxidant power through the capacity of DPPH radical scavenging, the methanol extract studied revealed a significant antioxidant potential. This study may find important application in the pharmaceutical and food industries.

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

Mohammed Saber: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing-original draft. **Hicham Harhar:** Methodology, Supervision, and Validation. **Mohamed Tabyaoui:** Methodology, Supervision, and Validation. **Latifa El Hattabi:** Visualization, Software and Formal Analysis. **Gokhan Zengin:** checked and corrected the final version. **Abdelhakim Bouyahya:** validation, checking and correction of the manuscript.

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Research Article

Biological evaluation of *Stachys iberica* subsp. *stenostachya* (Boiss.) Rech.f. and *Scutellaria orientalis* subsp. *sosnowskyi* (Takht.) Fed. growing in eastern Anatolia

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Abstract: Lamiaceae is one of the largest families in the plant kingdom, including the genus *Stachys* and *Scutellaria,* which are used in many folk medicines throughout the world for the prevention and also the treatment of several disorders. *In vitro* biological potential of *Stachys iberica subsp. stenostachya* and *Scutellaria orientalis* subsp. *sosnowskyi* were investigated in the current study. The aerial parts of the plants were extracted using different solvents such as *n*-hexane, chloroform, and methanol. In addition, infusions of each plant were prepared. The antioxidant potential of the samples was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), ABTS assays, ferrous ion-chelating, and ferric reducing antioxidant power (FRAP) assays. Anticholinesterase activity of the extracts was also determined. Spectrophotometric analysis was used to assess the total phenolic content of the samples. The antimicrobial activities of samples were determined by minimal inhibitory concentration (MIC) against seven bacteria and three *Candida spp*. yeast. According to the findings, the infusion demonstrated significant antioxidant properties, whilst the extracts demonstrated high-to-moderate antioxidant effects. The *n*-hexane extracts showed higher antifungal activity against *C.parapsilosis* and *C.tropicalis*. These outcomes suggest that these two species from Turkey could be employed in the manufacture of phytopharmaceuticals.

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1. INTRODUCTION

For centuries, herbal medicine has been practiced all around the world and remains an important part of treatment not only as a support in therapy but also as the prevention of several disorders (Saad *et al.,* 2005). Nowadays, the value of herbal medicines is increasing day by day in the global pharmaceutical market, which encourages studies on both their chemical composition and pharmacological activities on plants (Pieters *et al.,* 2005). In particular, the members of

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Lamiaceae family have gained attention because these plants have been shown to exhibit a wide variety of biological activities by providing a diverse chemical composition (Frezza *et al.,* 2019).

Stachys L. is considered to be one of the most extensive genera in the Lamiaceae family with 370 species (435 taxa) found around the world (Akçiçek *et al.,* 2016; Açar & Satıl*,* 2019). In Turkey, the genus is represented by 92 species (118 taxa), 53.3% of which are endemic (Güner *et al.,* 2021). These species commonly known as 'mountain tea' are used in many traditional medicines as anti-spasmodic, antimicrobial agent, and herbal tea in order to alleviate asthma problems, to decrease the symptoms of earaches, and to stop the growth of genital tumors and a malignant ulcer (Baytop, 1999; Ebrahimabadi *et al.,* 2010; Sarikurkçu *et al.,* 2016; Satıl & Açar*,* 2020; Tomou *et al.,* 2020). Many studies conducted on these species have confirmed remarkable anti-inflammatory, antioxidant, antimicrobial, anxiolytic, hypotensive, hyaluronidase, anti-Helicobacter pylori, and anti-nephritic activities (Shang *et al.,* 2010; Tundis *et al.,* 2014; Kocak *et al;* 2017; Satıl & Açar, 2020; Tomou *et al.,* 2020). From the chemical point of view, *Stachys* species were discovered rich in different types of secondary metabolites such as iridoids, di- and triterpenes, alkaloids, phenylethanoid glycosides, flavonoids, phenolic acids, and essential oil (Ahmad *et al.,* 2008; Bahadori *et al.,* 2020; Demirci *et al.,* 2016; Kartsev *et al.,* 1994; Kaya *et al.,* 2001).

One of the largest genera of the family Lamiaceae is *Scutellaria* L., which consists of approximately 350 species across the world (Georgieva *et al.,* 2021). In Turkey, it is represented by 39 taxa, almost half of which are endemic (Çiçek, 2012). In several folk medicines, the aerial parts of the species have been used for the treatment of several medical problems, including constipation, genital herpes, kidney stones, stomach ulcers and also used for their neuroprotective, antihelminthic, and diuretic functions (Tao *et al.,* 2016). In Turkish traditional medicine, the infusion and decoctions of the *Scutellaria* species are utilized as a tonic, hemostatic, and wound healing agent (Baytop, 1999; İçen *et al.,* 2016). In regard to the phytochemical analysis of the species, over 300 compounds were identified, which showed the presence of different kinds of substances such as alkaloids, flavonoids, polysaccharides, phytosterols, iridoid glycosides, phenylethanoid glycosides, terpenes, and essential oil (Formisano *et al.,* 2011; Mamadalieva *et al.,* 2017; Doğan *et al.,* 2019; Yılmaz *et al.,* 2019; Zhao *et al.,* 2019). A wide range of biological activities were observed in *Scutellaria* extracts, which was attributed to their diverse chemical composition (Zengin *et al.,* 2019). The pharmacological activities of several *Scutellaria* species have been investigated and some traditional usage has been approved such as antioxidant, antimicrobial, anticancer, hepatoprotective, anti-angiogenesis, anticonvulsant, and neuroprotective activities (Nie *et al.,* 2010; Shang *et al.,* 2010; Vergun *et al.,* 2019; Zhao *et al.,* 2019).

Intensive oxidative stress in the body may result in a number of degenerative illnesses including dermatological problems, cancer, and coronary disease and neurological disorders such as Alzheimer's disease (AD) and Parkinson's disease (Bibi Sadeer *et al.,* 2020). Antioxidants are considered to retard or prevent oxidation or the development of oxidizing chain reactions during the oxidation phase (Shahidi, 2000). Synthetic antioxidants have gained prominence in recent years, however, several studies have revealed that they have severe side effects, limiting their use in the body (Koşar *et al.,* 2008). As a result, researchers have focused their efforts on discovering natural antioxidants. Several studies conducted on natural medicinal plants in order to determine their antioxidant activity demonstrated that the raw extracts or extracted pure secondary metabolites from them were found strong to moderate activity compared to that of synthetic antioxidants (Li *et al.,* 2007; Zhang *et al.,* 2015; Toplan *et al.,* 2017). Numerous investigations have established that plant phenolic compounds have significant free radical scavenging capabilities due to their reactivity as hydrogen-or electron-

donating agents and their metal ion-chelating properties. Hence, determining the total phenol content of food plant extracts would be beneficial (Zengin *et al.,* 2019).

Alzheimer's disease (AD), a progressive neurological condition marked by changes in thinking and behavior and, especially in the developed world, has become one of the major public health issues (Orhan *et al.,* 2011). The primary therapeutic method for AD is to restore diminished acetylcholine levels in the brain by inhibiting acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes (Akkol *et al.,* 2012). For the treatment of AD, researchers have concentrated on determining the phytochemical composition and inhibitory effects of chemical compositions of various plant fractions on enzyme activities (Şenol *et al.,* 2010).

Infectious illnesses are a significant public health problem in hospitals and communities (Gibbons, 2005). Indeed, there is a growing need for plant-derived bioactive as an alternative to antimicrobial synthetic medicines since their extensive and continuous usage has resulted in the mutation of resistant bacteria, decreasing the therapeutic efficacy of these treatments (Orhan, et al., 2010). Plants, especially belonging to the Lamiaceae family, are a fascinating study subject due to their reported inhibitory impact on cholinesterases, showing excellent antioxidant, antibacterial, and antifungal activities (Frezza *et al.,* 2019).

In Turkish traditional medicines, *Stachys* and *Scutellaria* species have been widely used for cough, digestive disorders, and wound healing, remain important, and are suggested by local healers (Minareci *et al.,* 2017; Bardakci *et al.,* 2019; Satıl & Açar, 2020). The goal of this study is therefore to investigate the antibacterial, antifungal, antioxidant, and anticholinesterase properties of various extracts obtained from *S. orientalis* subsp. *sosnowskyi* and *S. iberica* subsp. *stenostachya* growing naturally in eastern Anatolia.

2. MATERIAL and METHODS

2.1. Plant Material

The aerial parts of *S. orientalis* subsp. *sosnowskyi* and *S. iberica* subsp. *stenostachya* were collected in Elle Hamlet, Gölyüzü Village, Doğubeyazıt, Ağrı, Turkey during the flowering stage (2017). After their collection, both of the species were air-dried in a dark place at room temperature, separately, and after that, the laboratory mill was used to powder the materials. The plant was identified by one of us (Gülay Ecevit-Genç). Voucher specimens of *S. orientalis* subsp. *sosnowskyi* and *S. iberica* subsp. *stenostachya* were stored at the Herbarium of the Pharmacy Faculty of Istanbul University (ISTE Number: 116566 and 116567, respectively).

2.2. Preparation of Extracts

Air-dried and powdered aerial parts of *S. orientalis* subsp. *sosnowskyi* and *S. iberica* subsp. *stenostachya* were extracted respectively with *n*-hexane, chloroform, and methanol using the Soxhlet apparatus. An infusion was also prepared via the maceration procedure. The infusion was first filtered and then the filtrates were frozen at −80 °C in an ultra-low temperature freezer, lyophilized, and stored at -20 °C until analysis. Solvents were evaporated to dryness under reduced pressure by a rotary evaporator at a maximum temperature of 50 °C. After the evaporation of the solvents, the crude extracts obtained were stored at +4 ºC until the analysis and used for all experiments.

2.3. Determination of Total Phenolic Contents in Extracts

To each 0.1 mL tube was poured 4.5 mL of water, and varied amounts of extracts (1-5 mg/mL) were mixed. Then, 0.3 mL of 2% sodium carbonate solution and 0.1 mL of the Folin-Ciocalteu reagent (diluted 1/3 with distilled water) were added to the mixture.

After allowing the combination to remain at room temperature for two hours, the absorbance was measured at 760 mm. (4). The total phenolic compounds contained in the extracts were given as mg gallic acid equivalents/mg extract (Ozsoy *et al.,* 2008).

2.4. Antioxidant Activity

2.4.1. *Determination of DPPH• Radical Scavenging Activity*

The free radical scavenging activity of the extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (1). Briefly, DPPH solution (0.1 mM, 240 µL) was added to the extracts (10 µL) prepared at different concentrations (1-5 mg/mL). Then, the mixture was let to rest for 30 minutes at room temperature. A microplate reader (AMR-100, Allsheng) was used to compare the mixture's absorbance to the reference at 517 nm.

The experiment was done three times and using the Graphpad Prism 5 Demo application, the averages of the values and standard deviation were calculated. The concentration of extracts and standard substance, which causes a 50% reduction in initial DPPH concentration, was defined as IC_{50}. The results obtained in the experiment were given as IC_{50} = mg / mL (Wei *et al.,* 2010).

2.4.2. *Determination of the reducing power of (CUPRAC) assay*

The reducing power capacity of the samples was measured using the CUPRAC method. A plate was combined with 1 mL of Cu (II) (1.10-2 M), neocuproine ethanolic mixture (7.3.10-3 M), and 1 M NH_4Ac buffer solution. Extracts 1 mL and 0.1 mL pure EtOH were added to the initial mixture to make the final volume: 4.1 mL. Then, after ten seconds of vortexing, the absorbance of the solution was measured at 450 nm against a reagent blank. Samples of CUPRAC measurements have been demonstrated as equivalents for Trolox (mM Trolox/mg extract). (Apak *et al.,* 2004)

2.4.3. *Ferric reducing antioxidant power (FRAP) assay*

The FRAP reagent was prepared by dissolving 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of the TPTZ solution (10 mM TPTZ in 40 mM HCI) and 2.5 mL of 20 mM $FeCl_3.6H_2O$. Then the FRAP reagent was kept at 37 °C for 30 minutes in an incubator device (Nuve). After 4 minutes, the absorbance of the mixture was measured against a reference at 593 nm using 3.8 mL of the FRAP reagent with 0.2 mL of extract. (3). FRAP values of the samples were reported as mM Fe^{+2}/mg extract (Benzie *et al.,* 1996).

2.5. Determination of Antimicrobial Activity of the Samples

In this study for *in vitro* antimicrobial activities of various extracts obtained from the aerial parts of *S. orientalis* subsp. *sosnowskyi* and *S. iberica* subsp. *stenostachya* were evaluated against *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750. The antimicrobial effectiveness of the samples was determined using the broth microdilution technique as described by the Clinical and Laboratory Standards (CLSI, 1997; CLSI, 2020). The MIC values of the samples were defined as the lowest antibiotic concentration that completely inhibited an observable growth. For the positive control, antifungal and antibacterial agents were used such as cefuroxime, cefuroxime-sodium, ceftazidime, amikacin, amphotericin B, and clotrimazole.

2.6. Anticholinesterase Activity of the Samples

The inhibition of cholinesterases was determined using a 96-well microplate reader based on the method developed by Ellman et al. (1961), with some changes. Tris-HCl buffer (50 mM, pH 8.0) was used to prepare all reagent solutions (daily). Shortly, AChE solution (20 μl) was

mixed with 20 μl of the sample and 40 μl of Tris-HCl buffer, and the mixture was kept at room temperature (25 °C) for 10 minutes. Then, 20 μl of ATChI (50 mM) was mixed into the combination and the total mixture was incubated for 5 min. at 25 °C. Then, 100 μl of 20 mM DTNB (containing 1M NaCl and 0.2 M $MgCl_2.6H_2O$) was added to the mixture and its absorbance was read at 412 nm against the reference. Each experiment was conducted in triplicate. Galanthamine was used as a positive control (Ellman *et al.,* 1961).

2.7. Statistical Analysis

Results were expressed as the means ± standard deviation (SD) of three parallel and independent measurements. One-way analysis of variance (ANOVA) was performed, and important differences between means were determined using Tukey's multiple comparisons test. Statistical significance was set at $p < 0.05$.

3. FINDINGS

The antioxidant, anticholinesterase, and antimicrobial activities, as well as the phenolic content, of different solvent extracts obtained from two commonly used Lamiaceae plants are investigated in the present study.

The extract yield and total phenolic content of the samples are presented in Table 1. Of all the extracts, the methanol extracts of both plants had the highest extract yield percentage. When the phenolic components in the extracts were revealed, it was observed that the methanol extract of *S. iberica* subsp. *stenostachya* contained the highest phenolic amounts compared with the other extracts, while the infusion and methanol extracts of *S. orientalis* subsp. *sosnowskyi* contained the high phenolic amounts nearly with similar percentages. Interestingly, the amount of phenolic compound in chloroform extract was found higher than infusion in *S. iberica* subsp. *stenostachya* studied. Additionally, the lowest total phenolic percentages were determined in the *n*-hexane extract of *S. iberica* subsp. *stenostachya.* The *n*-hexane and chloroform extracts of *S. orientalis* subsp. *sosnowskyi* contained a moderate amount of phenolic compounds.

Table 1. The yield and total phenolic content of the samples from *S. iberica* subsp. *stenostachya* and *S. orientalis* subsp. *sosnowskyi.*

Samples	Extracts	Yield[1]	Total phenolics[2]
Stachys iberica subsp. *stenostachya* (1)	*n*-hexane	279.6	6±1.4
	chloroform	497.9	46±2.7
	methanol	5977.2	**74±1.6**
	infusion	504.4	21±1.9
Scutellaria orientalis subsp. *sosnowskyi*. (2)	*n*-hexane	272.6	20±0.5
	chloroform	447	28±0.8
	methanol	3175.3	**52±1.9**
	infusion	510.1	**59±1.2**

[1]Extract yields expressed as milligrams of extract per gram (dry weight) of aerial parts of the plant.

[2]The total phenolic compounds contained in the extracts were given as mg gallic acid equivalents/mg extract.

The evaluation of the antioxidant capacity of the plants is recommended with more than one test in the literature (Zengin *et al.,* 2019). For this purpose, three complementary assays have been conducted to determine the antioxidant potential of different extracts of both plants including DPPH• free radical scavenging, FRAP (ferric reducing antioxidant power), and CUPRAC activity methods. The results are given in Table 2.

Table 2. The antioxidant capacity of the samples from *S. iberica* subsp. *stenostachya* and *S. orientalis* subsp. *sosnowskyi.*

Samples	DPPH (mg AaE/g extract)	CUPRAC (mMtrolox/mg extract)	FRAP assay (mM Fe^{2+}/mg extract)
1-H	-	0.057±0.006	0.085±0.008
1-C	12.7±0.7	**0.103±0.002**	**0.325±0.020**
1-M	**62.3±0.3**	**0.100±0.001**	0.062±0.014
1-I	**53.8±2.3**	0.081±0.005	0.020±0.013
2-H	-	0.059±0.005	0.094±0.018
2-K	-	0.069±0.004	**0.308±0.038**
2-M	**62±0.3**	0.099±0.002	0.082±0.004
2-I	**57.2±0.6**	0.098±0.004	0.105±0.007
BHT		1.1±0.12	
BHA			1.622±0.12

Values are mean of triplicate determination (n =3) ±standard deviation; $^{*}p$ <0.05 compared with the positive control

1: *Stachys iberica* subsp. *stenostachya*, 2- *S. orientalis* subsp. *sosnowskyi*, H: *n*-hexane extracts, C: Chloroform extracts; M: Methanol extracts; I: Infusion; BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene; AaE: ascorbic acid equivalent

DPPH• is frequently employed to assess the antioxidant capacity of samples, which is the most straightforward, least expensive, and easiest method. The DPPH test results indicated that infusion and methanol extracts of both species exhibited the strongest radical scavenging activity. On the other hand, no activity was observed among other extracts except chloroform extract of *S. iberica* subsp. *stenostachya.* In ferric reducing power assay, FRAP values of the chloroform extracts from both plants were found to be approximately the same and also the greatest compared with those of the other extracts. The cupric reducing antioxidant capacity of chloroform and methanol extracts of *S. iberica* subsp. *stenostachya* was demonstrated to be quite similar and to possess strong effects, while the infusion and *n*-hexane showed moderate effects. Besides, the infusion and methanol extracts of *S. orientalis* subsp. *sosnowskyi* exhibited higher cupric reducing power than that of *n*-hexane and chloroform extracts.

There is vast research on the antioxidant properties of *Stachys* and *Scutellaria* species and remarkable effects have been observed in most of these studies (Koçak *et al.,* 2017; Mamadalieva *et al.,* 2017; Elfalleh *et al.,* 2019; Georgieva *et al.,* 2021). It is generally known that these species are rich in essential oil and phenolic compounds, which may be linked to potent antioxidant action (Sarikurkcu *et al.,* 2016; Zengin *et al.,* 2019).

Generally many previous studies established that there is a positive correlation between the phenolic content and antioxidant capacity (Kartsev *et al.,* 2019; Zengin *et al.,* 2019; Zhao *et al.,* 1994). Following the literature, our study also shows that methanol extracts possessed strong antioxidant activity with high total phenolic content. However, other investigated extracts showed strong to moderate antioxidant potential independent of their total phenolic content. Consequently, the antioxidant capability was present in different degrees in the samples studied. The synthetic antioxidants, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) were used as a positive control and the antioxidant capacity of extracts and their infusions were compared with the capacity of samples. Nevertheless, all samples showed lower activity when compared with the standards.

The Ellman technique was subjected to the comparison of the inhibitory potential of the acetylcholinesterase enzyme in various extracts with galantamine which was used as a reference compound. The results are given in Table 3. As a result, only the infusion and methanol extracts of both species demonstrated inhibition against the acetylcholinesterase enzyme. The other investigated samples were found to be inactive. These findings indicated that the enzyme inhibition properties of the infusion of *S. iberica* subsp. *stenostachya* (92.63%) were almost equaled by galantamine (94.52 %). An early study examined the antioxidant and enzyme inhibitory activity of 33 *Scutellaria* species from Turkey, including *Scutellaria orientalis* subsp. *sosnowskyi*. Its methanol extract showed poor anticholinesterase activity, while strong DDPH radical scavenging activity was determined in both ethyl acetate and methanol extracts (Şenol *et al.,* 2010).

Table 3. The anticholinesterase activity of different extracts from *S. iberica* subsp. *stenostachya* and *S. orientalis* subsp. *sosnowskyi.*

Samples	Enzyme inhibition (%) (500 µg/mL)
1-H	-
1-C	-
1-M	82.032±1.116
1-I	**92.635±1.597**
2-H	-
2-C	-
2-M	75.492±0.721
2-I	**85.206±0.220**
Galanthamine	94.52±0.14

1: *Stachys iberica* subsp. *stenostachya*, 2: *S. orientalis* subsp. *sosnowskyi*, H: *n*-hexane extracts, C: Chloroform extracts; M: Methanol extracts; I: Infusion.

The antimicrobial activities of extracts obtained from the aerial parts of *S. iberica* subsp. *stenostachya* and *S. orientalis* subsp. *sosnowskyi* were studied against 7 bacteria and 3 *Candida spp.* yeast using the broth micro dilutions method. Since the infusions were not found active, only the results of extracts are reported in Table 4. According to our results, the *n*-hexane extract of *S. iberica* subsp. *stenostachya* showed *a* moderate inhibitory effect with MIC values of 625 ug/mL and 156.2 ug/mL against *E. faecalis* and *C. parapsilosis*, respectively. Furthermore, the strong antimicrobial activity was identified in methanol extract of *S.iberica* subsp. *stenostachya* against *S. epidermis* with a MIC value of 625 ug/mL. As to the antimicrobial results of *S. orientalis* subsp. *sosnowskyi,* the *n*-hexane extract showed notably antifungal effects against *C. tropicalis* with a MIC value of 312.5 ug/mL, while methanol extract showed strong to moderate inhibitory effects against *E. faecalis* and *C. tropicalis* with a MIC value of 312.5 ug/mL and 625 ug/mL, respectively. None of the extracts and infusion of both species were active against *E.coli*, *K. pneumoniae*, *P. mirabilis, S. aureus,* and *P. aeruginosa*. The existence of antimicrobial activity in *Scutellaria* and *Stachys* species has been approved by previous investigations despite the fact that their effectiveness rates show changes depending on using different solvents and also the composition of the extracts (Sato *et al.,* 2000).

Table 4. The antimicrobial activity of several extracts from *S. iberica* subsp. *stenostachya* and *S. orientalis* subsp. *sosnowskyi.*

Strains	Extracts					
	1-H	1-C	1-M	2-H	2-C	2-M
P. aeruginosa	>2500	>2500	>2500	>2500	>2500	>2500
E. coli	>2500	>2500	>2500	>2500	>2500	>2500
K. pneumoniae	>2500	>2500	>2500	>2500	>2500	>2500
P. mirabilis	>2500	>2500	>2500	>2500	>2500	>2500
E. faecalis	**625**	>2500	>2500	>2500	>2500	**625**
S. epidermidis	>2500	>2500	**625**	>2500	>2500	>2500
S. aureus	>2500	>2500	>2500	>2500	>2500	>2500
C. albicans	>2500	>2500	>2500	>2500	>2500	>2500
C. parapsilosis	**156.2**	>2500	>2500	>2500	>2500	>2500
C. tropicalis	>2500	>2500	>2500	**312.5**	>2500	**312.5**

1: *Stachys iberica* subsp. *stenostachya*, 2- *S. orientalis* subsp. *sosnowskyi*, H: *n*-hexane extracts, C: Chloform extracts; M: Methanol extracts; I: Infusion; Reference compounds: Ceftazidime: 2.4 mg/L For *P. aeruginosa,* Cefuroxime-Na: 4.9 mg/L for *E. coli* and *K. pneumoniae,* Cefuroxime-Na: 2.4 mg/L for *P. Mirabilis,* Cefuroxime-Na: 1.2 mg/L for *S. aureus*, Cefuroxime: 9.8 mg/L for *S. epidermidis*, Amikacin: 128 mg/L for *E. faecalis*, Clotrimazole: 4.9 mg/L *C. albicans*, Amphotericin B: 0.5 mg/L for *C. parapsilosis*, Amphotericin B: 1 mg/L for *C. tropicalis.*

4. DISCUSSION and CONCLUSION

Not only have plants often offered significant opportunities for pharmaceutical development but also they have produced a wide range of secondary metabolites for their defense systems, allowing us to discover new bioactive chemicals. Lamiaceae family has also been extensively investigated due to the presence of considerable therapeutic potential in its members. *Stachys* and *Scutellaria* are one of the most investigated genus whose extracts possess considerable potential in many biological activities. Hence, investigations into them appear to be extremely beneficial in terms of identifying the potential sources of herbal medicines.

Previous studies on the essential oil of the aerial parts of *Stachys iberica* subsp. *stenostachya* showed linalyl acetate (42.2%), linalool (18.9%), geranyl acetate (8.2%), and α-terpineol (5.3 %) (Kaya *et al.,* 2001). In another study, antimicrobial potential of the methanol extract of *S. orientalis* subsp. s*osnowskyi* was investigated and moderate antibacterial and anticandidal effects were observed (Yılmaz *et al.,* 2020). In this study, the biological potential of different solvent extracts of these two species has been evaluated since they are still used for treatment purposes by the local residents. The activities of several extracts prepared from aerial parts were studied using a variety of methodologies including antioxidant, antibacterial, antifungal, and anticholinesterase activity tests. The antioxidant potential of the extracts ranged from moderate to high while considerable activity was observed in infusion and methanol extracts of both species. The *n*-hexane extract of *S. iberica* subsp. *stenostachya* exhibited notable antimicrobial activity. Further studies are needed to investigate the phytochemical composition of the active extracts in order to find out responsible substances.

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

Authors are expected to present author contributions statement to their manuscript such as; **Gizem Gulsoy Toplan**: Investigation, Resources, Analysis, and Writing-original draft, Supervision. **Ayse Civas**: Analysis **Emel Mataraci Kara:** Analysis **Turgut Taskin**: Analysis **Gulay Ecevit Genc:** Identification of plants.

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Research Article

Biological activities and chemical composition of *Xanthoria* lichens from Turkey

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Abstract: This study presents the biopharmaceutical potential and bioactive composition of *Xanthoria* lichens (*X. candelaria*, *X. elegans*, *X. parietina*) that wildly grown and traditionally utilized as medicine in North Eastern Anatolia, Turkey, which has specific microclimatic and ecological zones.

Chromatographic findings revealed significant levels of parietin compound (35 to 49 mg/g extract), low levels of various fatty acids and a volatile compound; α-terpinene in the extracts. The extracts exhibited pronounced antioxidant potential through reducing and scavenging mechanisms; FCR: 33-38 mg gallic acid equivalent, FRAP: 511-815 µ mol Fe^{2+}, ORAC: 1032-1355 µ mol Trolox equivalent per gram extract, respectively and DPPH: IC_{50}: 1.1-2.7, ABTS: IC_{50}: 2-2.3, CUPRAC: IC_{50}: 0.7-1.2, phosphomolybdenum: IC_{50}: 2-2.9, metal chelation: IC_{50}: 1.3-2.3 mg extract/ml, respectively. Concerning enzyme inhibitory activities, the extracts effectively suppressed the activity of acetylcholinesterase (IC_{50}: 0.5-0.75 mg/ml), butyrylcholinesterase (IC_{50}: 0.7-1.1 mg/ml), tyrosinase (IC_{50}: 0.6-0.7 mg/ml), amylase (IC_{50}: 1.7-2 mg/ml), glucosidase (IC_{50}: 0.6-3 mg/ml) and lipase (IC_{50}: 55-79 µg/ml) enzymes.

These findings showed that *Xanthoria* lichens are dominated by parietin as the major key compound and high-tolerated lichen taxa towards to different ecological and climatic conditions. These lichens might be promising sources of novel antioxidant and enzyme inhibitory activities such as *Xanthoria candelaria* as antioxidant and antilipase, *Xanthoria elegans* as anticholinesterase, and *Xanthoria parietina* as antiamylase and antiglucosidase.

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1. INTRODUCTION

Lichens are stable, self-supporting, harmoniously, and continuous autotrophic complex symbiotic plantlike associations of photosynthetic green algae and/or cyanobacteria (photobiont) with at least one fungus (mycobiont). Such an association provides a unique

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structure, which is utterly different from algae and fungi in terms of morphology, anatomy, physiology, and bioactivity.

These natural sources are able to grown in extreme habitats and ecological conditions such as arctic, alpine, and tropical rainforest zones and are significant sources of bioactive metabolites, which mainly composed of lichen acids and phenolic compounds (up to 10% dry weight of the thallus). These compounds absorb harmful UV-B radiation and minimize the harmful effects of environmental stresses such as high concentrations of salinity and protection against of the herbivores (Nybakken *et al*., 2004; Boustie & Grube, 2005; Lina *et al*., 2015; Karthikadevi *et al*., 2011; Calcott *et al*., 2018).

Such accumulation of biologically active compounds in high quantities provides multiple biological and pharmacological effects to the lichens (Boustie & Grube, 2005). Until now, a couple of drugs were developed from lichen sources such as Isla-Moos® and Broncholind® from Icelandic lichens (especially *Cetraria islandica*) and an antimycobacterial drug called clofazimine (Lamprene®) from Irish lichen (*Buellia canescens*) (Reddy *et al*., 1999; Ingolfsdottir, 2002).

Lichens have been used by locals as food, fodder, perfume, spice, dyes and traditional medicines for a long time across the world. For instance, *Cetraria islandica* is used to treat lung, kidney, wound, bladder diseases and cancer; some species of *Xanthoria* are used in the treatment of jaundice; species of *Lobaria* and *Parmelia* are used to treat of lung and neural diseases (Chevallier 1996; Bown 2001; Hawksworth, 2003). In Anatolia, *Xanthoria* species are traditionally used for various ethno-lichenological purposes including medicinal utilization (antipyretics and jaundice). Also, they have been used as dyeing agents (personal communication, 2018).

Anthraquinones including parietin are among common bioactive compounds of lichens. Multiple scientific studies reported the chemical composition and some biological activities (with limitation on enzyme inhibitory potential) of *Xanthoria* lichens grown across the world and biopharmacological properties of their major compound "parietin" (Nybakken *et al.,* 2004; Kumar *et al*., Basile *et al*., 2015; Cornejo *et al*., 2016; Ali & Hameed, 2019). However, a very limited information is available in scientific literature on biologically active compounds and potential biological activities of *Xanthoria* lichens grown in Turkey. Environmental factors such as radiation, UV, and climatic variables are among the main modulators of chemical compounds accumulation in natural sources including plants, fungi, and lichens (Pirie *et al*., 2013). The North Eastern Anatolia Region of Turkey includes many microclimatic and ecological zones which enriched the development of diverse flora and fauna. Therefore, the chemical nature of *Xanthoria* lichens grown in this region may differ from those of originating from other parts of the world. Considering abovementioned aspects, this study aimed to research the biological activities (antioxidant and enzyme inhibition) of methanol-based extracts obtained from thalluses of *Xanthoria* lichens (*Xanthoria Candelaria, Xanthoria elegans*, and *Xanthoria parietina*) grown and traditionally utilized as food and medicine in Turkey and identify their chemical composition using proper chromatographic methodology.

2. MATERIAL and METHODS

2.1. Lichen Materials

Thallus samples of *Xanthoria* species (Figure 1) were harvested properly from wild as described below and transferred to the laboratory within 4 hours. Lichenological identities of lichen materials were done at Van Pharmaceutical Herbarium (VPH), Pharmacy Faculty, Van Yuzuncu Yil University, Van / Turkey and voucher specimens were deposited at VPH. The herbarium informations of the samples are detailed as follows:

i. *Xanthoria elegans* (Link) Th.Fr.; Rize, Camlihemsin; Hayiki plateau, on *Fagus orientalis,* GPS coordinates 40°57'15''N 41°04'30''E, 1400 m, Turkey, August 2018, Herbarium code: VPH-353; Collector code: AA825.

ii. *Xanthoria parietina* (Link) Th.Fr.; Rize, Camlihemsin; Hayiki plateau, on *Fagus orientalis,* GPS coordinates 40°57'15''N 41°04'30''E, 1400 m, Turkey, August 2018, Herbarium code: VPH-354; Collector code: AA826.

iii. *Xanthoria candelaria* (L.) Th. Fr.; Artvin, Savsat; Merkezadam hill, siliceous rock, GPS coordinates 41°15′ N 42°22′E, 1350 m., Turkey, August 2018, Herbarium code: VPH-352; Collector code: AA827.

Figure 1. *Xanthoria* taxa from North Eastern Anatolia.

Xanthoria elegans *Xanthoria parietina* *Xanthoria candelaria*

2.2. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA) and were of analytical or HPLC grade.

2.3. Preparation of Extracts

Methanol has been reported as the most efficient and proper solvent for obtaining active ingredients from lichen matrix (Zambare & Christopher, 2012) and hence, the extractions were prepared based on methanol solvent. The powder of lichen samples (1 gr) was treated with a 10-fold volume (10 ml) of methanol (80% methanol) and incubated for 2 h at room temperature (22°C) with shaking. Then, homogenized mixture was filtered using vacuum filtration (45 µm) with the supernatants collected and subsequently were evaporated using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland). The derived concentrated fraction was freeze-dried using a lyophilizator (Alpha1-2 LDplus, Christ, Germany) under a vacuum at -51°C to obtain fine lyophilized methanol powder.

2.4. Folin-Ciocalteu Reducing Assay

Folin-Ciocalteu reducing capacities of the extracts were measured as described previously by Ainsworth & Gillespie (2007) and were expressed as mg gallic acid equivalents per gram of dry weight of the lyophilized extracts, based on gallic acid standard curve and against a blank control. The analyses were conducted in triplicate.

2.5. Antioxidant Capacity

Ferric reducing antioxidant power (namely total reducing; FRAP) assay was conducted according to (Benzie & Strain, 1996) and total reducing capacities of the extracts were expressed as µmol of iron (Fe^{2+}) per gram of dry weight of lyophilized extracts based on an iron sulphate standard (Fe_2SO_4) curve against a blank control. Oxygen radical scavenging

(ORAC) capacities of the extracts were measured as described previously by Dalar & Konczak (2013) and the results were expressed as µmol Trolox equivalent per gram of dry weight of lyophilized extract based on a Trolox standard curve.

The total antioxidant (TAC; phosphomolybdenum method), DPPH radical scavenging, ABTS radical cation scavenging, the cupric ion reducing (CUPRAC), and metal chelating activities of the extracts were determined as described previously described by Uysal *et al*. (2017) and the results were expressed as IC_{50}-half maximal inhibitory concentration (mg extract/ml).

2.6. Enzyme Inhibitory Activities

Cholinesterase (ChE), α-amylase, α-glucosidase, and tyrosinase inhibitory activities of the extracts were determined according to Zengin (2016) and expressed as IC_{50}-half maximal inhibitory concentration (mg extract/ml). The pancreatic lipase activity was assayed as described previously (Dalar and Konczak, 2013) using 4-methylumbelliferyl oleate (0.1 mmol) as a substrate and the results were expressed as IC_{50}-half maximal inhibitory concentration (mg extract/ml). All enzyme inhibitory analyses were conducted in triplicate.

2.7. HPLC-MS/MS Analysis

Identification and quantification of phenolic compounds by high performance liquid chromatography-diode array detector (HPLC-DAD) and liquid chromatography-photo-diode array–mass spectrometry (LC-PDA–MS/MS) (Thermo Fisher Scientific, Waltham, MA, USA) analysis were conducted as described previously (Dalar & Konczak, 2013). The composition of phenolic compounds was characterized based on their UV spectrum, retention time, co-chromatography with commercial standards, when available, and MS/MS fragmentation patterns. MS experiments in the full scan (parent and product-specific) and the selected reaction monitoring (SRM) mode were conducted. All analyses were conducted in triplicate.

2.8. GC-MS Analysis

Volatile compounds and fatty acids were analyzed by gas chromatography mass spectrometry (GC/MS) (3800 Varian GC, Agilent Technologies, Istanbul, Turkey) using a head space solid phase micro extraction and identified by the fragment ions and relative retention indices of their peaks with those of the MS library standards as described previously (Uzun *et al*., 2017). All analyses were conducted in triplicate.

2.9. Data Analysis

The mean values were calculated based on at least three determinations (n = 3). One-way ANOVA followed by the Bonferroni *post-hoc* test was performed to assess differences between the samples at the level of $p<0.05$ through Graphpad Prism 5 (Graphpad Software, CA, USA).

3. RESULTS and DISCUSSION

3.1. Total Phenolic Contents and Antioxidant Capacities

The pathological and detrimental environmental conditions disrupt the antioxidant balance in the cells and tissues and thus elevated the amount of the reactive oxygen species or other free radicals and finally resulted in oxidative stress which was associated with many metabolic and neurological diseases (Moldovan, 2004; Prior, 2015).

Table 1 presents antioxidant activities of the samples through various assays which have different and complementary mechanisms. A pattern of *X. candelaria* > *X. parietina* > *X. elegans* was observed in total phenolic content and all assays of antioxidant measurements. Solely, *X. elegans* exhibited superior metal chelation activities than those of *X. candelaria* and *X. parietina* (Table 1). Although the lichen extracts were found as effective antioxidant sources

that capable both of single electron and hydrogen atom transfer, and as well as metal binding mechanisms, they showed weaker antioxidant activities compared to synthetic antioxidant agents such as ascorbic acid, butylated hydroxyanisole, Trolox, and ethylenediamine tetraacetic acid which were used as positive controls in the present study (Table 1).

Kumar *et al*. (2014) investigated antioxidant activities of several lichen samples grown high altitudes of cold deserts and reported *Xanthoria elegans* as active antioxidant source among 14 lichens evaluated. The authors also reported total phenolics content of *Xanthoria elegans* (19.7 mg gallic acid equivalent/g extract) which is approximately 0.5-fold that of our finding. This difference can be explained by the varieties in climatic and environmental factors of Trans-Himalayan Cold Desert (harsh climatic conditions) and Northern Eastern Anatolia (warm and rainy climatic conditions). The positive correlations between total phenolic contents and antioxidant determination (a range of $r^2 = 0.8$-0.98) suggest phenolic compounds as the potential major contributor of the antioxidant activities (except metal chelation activities) of the extracts, which was in agreement with previous antioxidant reports of lichen samples (Manojlovic *et al*., 2012; Atalay *et al*., 2011; Fernandez-Moriano *et al*., 2016). Based on these findings, it can be suggested that *Xanthoria* lichens might be promising sources of novel antioxidant agents particularly as oxygen radical scavengers.

Table 1. Total phenolic contents and **a**ntioxidant activities of *Xanthoria* extracts.

	Xanthoria Lichens			Positive control (Synthetic antioxidants)			
Antioxidant activity	*Xanthoria elegans*	*Xanthoria parietina*	*Xanthoria candelaria*	Ascorbic acid	Butylated hydroxyanisole	Trolox	Ethylenediamine tetraacetic acid
Folin-Ciocalteu reducing (Total phenolics) [1]	34±2d	39±1c	47±2b	-	-	-	-
Ferric reducing antioxidant power [2]	511±14d	591±35c	815±17b	5138±19a	-	-	-
Oxygen radical absorbance capacity [3]	1033±67d	1263±38c	1355±26b	-	6092±82a	-	-
DPPH radical scavenging activity [4]	> 3d	2.7±0.6c	1.1±0.1b	-	-	0.06±0.01a	-
ABTS radical scavenging activity [4]	> 3d	2.3±0.1c	2±0.1b	-	-	0.18±0.02a	-
Cupric ion reducing antioxidant capacity [4]	1.2±0.0c	1.1±0.0c	0.7±0.0b	-	-	0.11±0.01a	-
Phosphomolybdenum total antioxidant capacity [4]	> 3d	2.9±0.4c	2±0.2b	-	-	0.55±0.1a	-
Metal chelation activity [4]	1.3±0.0b	2.3±0.1d	1.8±0.2c	-	-	-	0.03±0.0a

Means with different letters in the same row were significantly different at the level ($p< 0.05$), n=3.

[1] mg Gallic acid Equivalent/g extract,

[2] µ mol Fe^{2+} /g extract,

[3] µ mol Trolox Equivalent/g extract,

[4] IC_{50}-half maximal inhibitory concentration (mg extract /ml).

3.2. Enzyme Inhibitory Activities

Enzyme inhibitory strategy such as utilization of acarbose, orlistat, voglibose, captopril etc. is commonly applied towards several metabolic diseases globally. However, their unwanted effects (diarrhea, abdominal pain, gas, etc.) (Copeland, 2000; Gonçalves & Romano, 2017), induces exploring novel safe and effective enzyme inhibitors from natural sources to minimize abovementioned side effects (Dalar & Konczak, 2013; Zengin, 2016; Uysal *et al*., 2017; Uzun *et al*., 2017).

Lichens are potent novel candidates of enzyme inhibitors due to high levels of their bioactive components. For instance, effective antidiabetic potential of lichens has been revealed through the inhibitory activity mechanism of carbohydrate hydrolyzing enzymes (α- amylase and α-glucosidase) and their hypoglycemic action has been linked to the presence of high amount of phenolic compounds (Raj *et al*., 2014; Shivanna *et al*., 2015; Valadbeigi & Shaddel, 2016; Zambare & Christopher, 2012; Kekuda *et al*., 2019). Moreover, novel effective cholinesterase inhibitor agents such as biruloquinone, lobaric acid, and perlatolic acid were recently isolated from lichens (Kekuda *et al*., 2019), which indicate their effective enzyme suppressive abilities.

Table 2. Enzyme inhibitory activities of *Xanthoria* extracts.

			Xanthoria elegans	*Xanthoria parietina*	*Xanthoria candelaria*
Enzyme inhibitory activity	Acetylcholinesterase	IC_{50} (mg/ml)	0.5±0.0a	0.8±0.0c	0.6±0.0b
		Galanthamine Equivalent (mg/g extract) *	5.5±0.1a	3.9±0.1c	4.5±0.1b
	Butyrylcholinesterase	IC_{50} (mg/ml)	0.7±0.0a	1.1±0.0c	0.8±0.0b
		Galanthamine Equivalent (mg/g extract) *	4.7±0.1a	3.1±0.0c	4±0.2b
	Tyrosinase	IC_{50} (mg/ml)	0.7±0.0b	0.6±0.0a	0.6±0.1a
		Kojic acid Equivalent (mg/g extract) *	127±1b	134±0.6a	132±0.2a
	Alpha-Amylase	IC_{50} (mg/ml)	2.1±0.1b	1.7±0.1a	2.0±0.1b
		Acarbose Equivalent (mmol/g extract) *	0.5±0.0b	0.6±0.0a	0.5±0.0b
	Alpha-glucosidase	IC_{50} (mg/ml)	>3b	0.6±0.0a	NA
		Acarbose Equivalent (mmol/g extract) *	0.7±0.1b	11±0.1a	NA
	Pancreatic Lipase	IC_{50} (µg/ml)	79±5c	68±5b	55±3a
		Orlistat Equivalent (µmol/g extract) *	38±1c	41±1b	69±2a

Means with different letters in the same row were significantly different at the level ($p< 0.05$), n=3. NA: not active (no any inhibition was observed).
*The equivalent of commercial standards calculated based on a standard curve and against control.

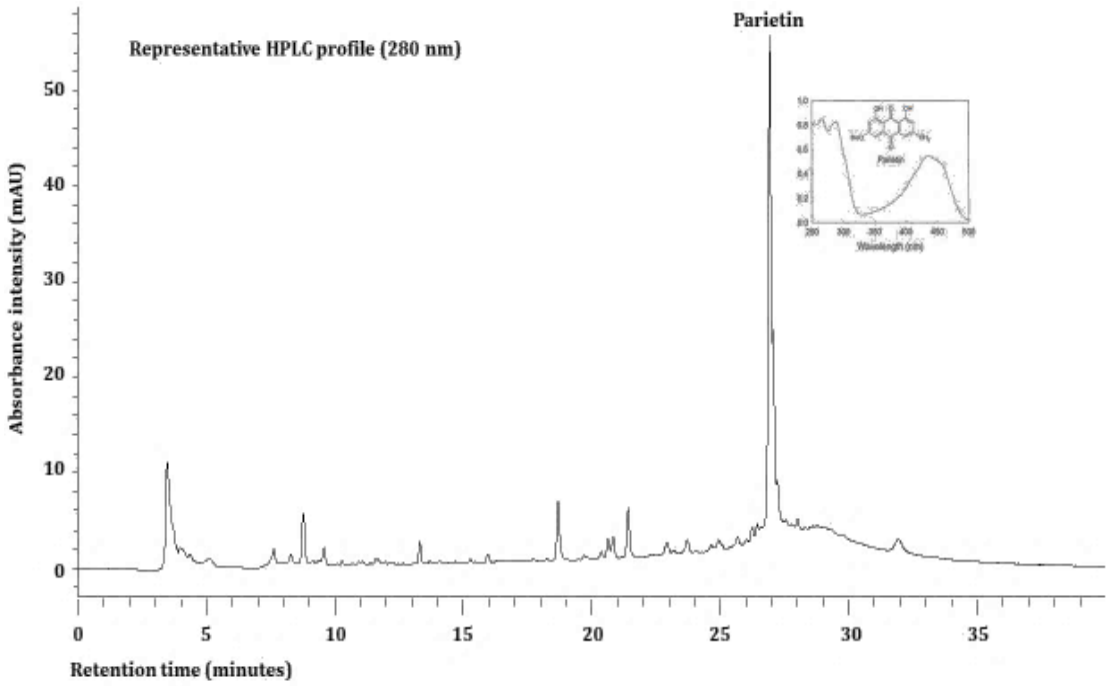
X.elegans was detected as the most effective inhibitor of AChE, BChE, and tyrosinase followed by *X. candelaria* and *X. parietina*. Concerning diabetes related enzymes; all extracts showed low inhibitory activity ($IC_{50} \geq 1.7$ mg extract/ml) in α-amylase and α-glucosidase ($IC_{50} \geq 3$ mg extract/ml; with the exception of *X. parietina* ($IC_{50} = 0.5$ mg extract/ml)). With regards to pancreatic lipase inhibition, all extracts exhibited pronounced levels (IC50 ≥ 55 µg extract/ml) (Table 2). These findings revealed that *Xanthoria* lichens might be among new

promotive sources of enzyme inhibitors; specifically, *Xanthoria candelaria* as antilipase, *Xanthoria elegans* as anticholinesterase, and *Xanthoria parietina* as antiamylase and antiglucosidase.

3.3. Chemical Profiles

Chemical profiling of lichen samples was shown in Table 3 and Figures 2-3. HPLC-MS/MS data showed that the dominant compound of the extracts gave a negatively charged molecular ion $(M1]^-$) at m/z 283 and MS/MS fragment was 283 m/z. Based on neutral loss, fragmentation pattern, molecular weight, absorbance spectrum and co-chromatogram analyses, it was tentatively identified as parietin. This compound was found in all Xanthoria species and contributed 90% ≥ of the phenolic compositions of the extracts. Other phenolic compounds were detected in low amounts correspond to their trace levels individually (contribution of ≤ 2% of total phenolics) (Figure 1). These findings were in coherence with chemical composition of *Xanthoria* species reported previously (Ali & Hameed, 2019), which showed that *Xanthoria* lichens were high-tolerated lichen taxa towards to different ecological and climatic conditions.

Figure 2. Representative HPLC profile of *Xanthoria* taxa (*Xanthoria candelaria*).

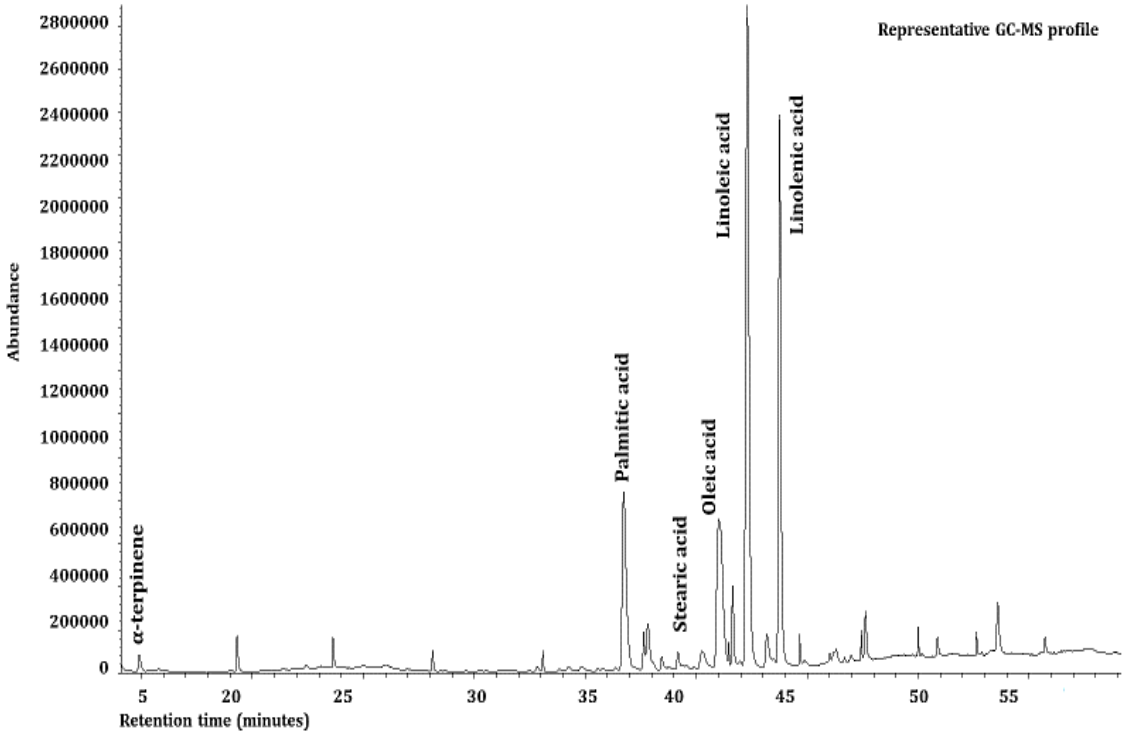


Parietin is an anthraquinone that responsible from the orange yellowish color of *Xanthoria* species, which is able to absorb blue light (Basile *et al*., 2015). It was reported that parietin was a biologically active compound that exhibit effective antibacterial, anticancer, antiproliferative, and antifungal activities (Lopez-Tobar *et al*., 2016), and pronounced antioxidant activity (Gundogdu *et al*., 2019).

With regards to GC-MS analyses, five fatty acids (palmitic, stearic, oleic, linoleic and α-linolenic acid) and a volatile compound (alpha-terpinene) were identified in the extracts (Table 3 and Figure 3). The presence of alpha-terpinene in *Xanthoria* lichens has not been reported previously in our knowledge. Torres *et al*. (2003) investigated fatty acid composition of *Xanthoria parietina* and reported the presence of palmitic, stearic and very long chain fatty acids, which was slightly similar to our findings. Piervittori *et al*. (1994) researched the fatty acid variations of *Xanthoria parietina* and reported a similar composition to our findings. The

authors also emphasized a general trend of decreasing fatty acid unsaturation with elevation. At an elevation of 1300 m, the authors found a minor increase in palmitic acid, but a drastical decrease in linoleic acid. This finding showed that there is a negative correlation between the elevation and accumulation of linoleic acid in lichen tissues. Although a scalar quantity in fatty acid composition of the extracts, their levels were at low quantities and it can be speculated that they have a secondary contribution to the biological activities of *Xanthoria* lichens.

Figure 3. Representative GC-MS profile of *Xanthoria* taxa (*Xanthoria candelaria*).



This study revealed that *Xanthoria* lichens exhibited high oxygen radical absorbance capacity, moderate radical scavenging and reducing capacities, moderate AChE and BChE and pronounced pancreatic lipase and high tyrosinase inhibitory activities. Since parietin compound primarily contributed of the total phenolics (≥ 90%) and well correlated (a range of r^2 = 0.8-0.98) with abovementioned antioxidant and enzyme inhibitory activities, it can be speculated that parietin might be the major contributor compound of biological activities of *Xanthoria* lichens with additive effects of volatile and fatty acid compounds. Therefore, bioactive rich *Xanthoria* lichens might be useful in the treatment and/or management of metabolic and/or neurological diseases as natural biotherapeutics. Additionally, parietin can also be utilized in chemotaxonomic studies of *Xanthoria* species as key phenolic compound.

Table 3. Bioactive composition of *Xanthoria* species.

	A) *Phenolic composition by HPLC-MS/MS*					
Individual phenolic compounds		MS/MS		Concentration (mg/g extract)		
		$[M+1]^+$/ $[M-1]^-$	Fragments (m/z) (+/-)	*Xanthoria elegans*	*Xanthoria parietina*	*Xanthoria candelaria*
1	Parietin	-/283	-/283	35±0.2	38±1	49±1
	B) *Volatile composition by GC-MS*					
	Retention time	Compound	Fragment ions	Relative concentration (%)		
2	15.06	Alpha-terpinene	39, 41 79, 91, 93, 105, 121,	1.3±0.1	1.3±0.0	2.2±0.1
	C) *Fatty acid composition by GC-MS*					
3	36.77	Palmitic acid	60, 73, 83, 97, 129, 143, 157, 171, 185, 199, 213, 227, 239, 256	11±1	21±1	12±1
4	40.40	Stearic acid	55, 60, 73, 87, 115, 129, 143, 157, 171, 185, 199, 213, 227, 241, 255, 267, 284	1.4±0.1	2.7±0.1	1.3±0.1
5	41.17	Oleic acid	55, 69, 83, 97, 111, 125, 151, 165, 180, 195, 207, 222, 246, 264	13.4±0.6	14±1	14±1
6	42.47	Linoleic acid	55, 67, 81, 95, 110, 123, 136, 150, 164, 185, 209, 223, 241, 262, 280	34±2	31±3	39±3
7	43.77	α-Linolenic acid	55, 67, 79, 93, 108, 121, 135, 149, 177, 191, 209, 222, 235, 249, 264, 278	20±1	14±1	20±2

All data represent the mean ± standard deviation of at least three independent experiments (n=3).

4. CONCLUSION

This study reports the chemical profile, antioxidant and enzyme inhibitory activities *Xanthoria* lichens (*X. parietina, X. elegans and X. candelaria*) naturally grown and traditionally utilized as therapeutics in North Eastern Anatolia, Turkey. Parietin rich *Xanthoria* lichens exhibited pronounced antioxidant and enzyme inhibitory (particularly pancreatic lipase) activities. *Xanthoria* lichens might be novel candidates of biotherapeutics such as: *Xanthoria candelaria* for antioxidant and antilipase, *Xanthoria elegans* for anticholinesterase, and *Xanthoria parietina* for antiamylase and antiglucosidase activities. Data obtained within this study revealed that *Xantoria* species are belong to a high-tolerated lichen taxa towards to different ecological and climatic conditions due to their parietin content.

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

Abdullah DALAR: Investigation, Methodology, Project administration, Visualization. **Muzaffer MUKEMRE**: Investigation, Methodology. **Gokhan ZENGIN**: Investigation, Methodology. **Rabia Sena TURKER**: Investigation, Methodology. **Ali ASLAN**: Funding acquisition, Investigation, Methodology, Project administration, Software, Visualization, Writing- original draft, Writing- review & editing.

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Research Article

Biological activity studies on *Klasea serratuloides* (DC) Greuter & Wagenitz subsp. *karamanica* B. Dogan & A. Duran extracts obtained with different extraction methods

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Abstract: The aim of this study was to investigate the antioxidant and enzyme inhibition activities of methanol extracts prepared from *Klasea serratuloides* (DC) Greuter & Wagenitz subsp. *karamanica* B. Dogan & A. Duran with Soxhlet, ultrasonic extraction, and maceration methods. 1,1-Diphenyl-2-picryl hydroxyl (DPPH) quenching assay and 2,2'-azinobis-3- ethylbenzothiozoline-6-sulfonic acid (ABTS) cation decolorization test were used to evaluate *in vitro* radical scavenging activity. The total phenolic content was determined with the Folin-Ciocalteu method while the total flavonoid content was evaluated by the aluminum chloride colorimetric method. According to the results, DPPH, ABTS radical scavenging activity and iron chelating activity of methanol of *Klasea serratuloides* were shown concentration-dependent manner. The extract obtained from maceration was found to be higher than the other extracts. It suggests that the maceration technique was more effective than the other extraction methods for the determination of the phenolic content. The methanol extract of KS using soxhlet (61.17 ± 3.62) and ultrasonic extraction (58.76 ± 1.46) showed higher inhibition than the extract prepared with maceration methods (34.54 ± 0.73) against BChE. All extracts displayed moderate inhibition activity against AChE. As for enzyme inhibition activity, the extract from soxhlet method was found to be more potent tyrosinase, acetylcholinesterase and butyrylcholinesterase inhibitors than the other extracts prepared by ultrasound assisted extraction and maceration methods. The present study suggests that *K. serratuloides* should be given special attention to conduct further investigation for its phytochemical constituents that attribute to their antioxidant potentials, and enzyme inhibition activities.

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1. INTRODUCTION

Klasea Cass. (Asteraceae) is represented by 46 species, and 10 sections. Although it is formerly defined as a section of the *Serratula* genus, it is sometimes accepted as an independent genus (Annales *et al*., 2012; Dogan *et al*., 2014). It is located in Himalayas, Central Asia, Mediterranean basin, SE Europe, China, southern Russia, Iran, and Turkiye. The genus *Klasea,* is distributed in Mediterranean and Irano – Turanian phytogeographic regions of Turkiye, with 15 species. Five of these species are endemic to Turkiye (Dogan *et al*., 2014). *K. serratuloides*

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(DC.) Greuter & Wagenitz is located in Van, Tunceli (Babacan *et al*., 2017; Tel *et al*., 2015). *Klasea serratuloides* subsp. *karamanica* (KS) is endemic to the Ayrancı, Ermenek, Ereğli and Ulukışla, Anatolia. It is morphologically similar to *K. serratuloides* subsp. *serratuloides* (Dogan *et al*., 2014). There is a limited number of biological activity studies on both species and genus. Flowering part of *Klasea pusilla* (Labill.) Greuter &Wagenitz is traditionally used treatment of insect bites in Mount Hermon-Lebanon (Baydoun *et al*., 2015).

Klasea serratuloides (KS), perennial, 8-42 cm tall, arachnoid, 5-8 mm in diameter below. Stout, unbranched stem that emerges from thick fleshy roots. Leaves are obovate in shape, coriaceous, petiolate, conspicuously veined, typically simple, rarely lyrate-pinnatifid, 10–30 (–38) 5-12 (16) cm in length, with sinuate-lobulate margins. Capitula solitary on peduncles ranging in length from 7 to 12 cm, with a diameter of 3.0 to 6.0 cm and a height of 3.5 to 5.5 cm. 3.0–5.0 3.5–4.5 cm subglobose involucre Phyllaries are 8-seriate, greenish with a few dark striations, ovate acuminate with a strongly reflexed, pale-colored apical section; Outer phyllaries are 8-10 5-7 mm, sparsely arachnoid; inner phyllaries are 20-25 6- 8 mm, glabrous. Petals are pale and straight, about 15-20 mm in length. 5-lobed mauve corolla with glandular hairs on the lobes; tubes 30-35 mm long. Achenes are 10-13 mm in length. Pappus plumose, dirty-white, persistent, 15–18 mm. Flowers in May-July, and fruits in July –August (Doğan *et al*., 2014).

Oxidative stress caused by reactive oxygen species plays a role in the pathogenesis of many chronic and degenerative diseases such as multiple sclerosis, immune disorders, cardiovascular disease, Alzheimer, Parkinson, cancer, diabetes mellitus, skin disorder, and dementia (Mao *et al*., 2013; Szymanska *et al*., 2018; Eruygur *et al*., 2020). The antioxidants obtained from medicinal plants, and dietary supplements can prevent the formation of possible diseases by inhibiting free radicals. In recent years, the natural antioxidants have been preferred as a priority, therefore the investigation on the discovering of new phytochemicals with antioxidant activity has still been maintaining by researchers. According to the literature, phenolic compounds, which are common in plants, are the most important components with their high antioxidant activity and free radical scavenging effect among the secondary metabolites (Rice-Evans *et al*., 1997; Gan *et al*., 2010). Therefore, determining of total phenolic contents of the medicinal plants is mainly remarkable for the antioxidant capacities of the plants.

Alzheimer's disease is characterized by social behavioral disorder, loss of memory, and cognitive performances. It is known that more than one mechanism may be effective in the pathogenesis of this disease. Cholinergic hypothesis, which is one of these, has been suggested that in Alzheimer's patients the deficits in cholinergic function may contribute to the cognitive decline associated with delirium. When acetylcholinesterase that hydrolyzes acetylcholine is inhibited by the chemical agents, they can be most promising to take place in the treatment of the disease (Orhan *et al*., 2009; Senol *et al*., 2013; Eruygur *et al*., 2019). The discovery of novel molecules or extracts originated from plants that inhibit acetylcholinesterase and butyrylcholinesterase could be model for the development of new drug candidates for the treatment.

Polyphenol oxidase, or tyrosinase, is a copper-containing enzyme. This enzyme is found in bacteria, fungi, plants, and mammals. It catalyzes the hydroxylation of tyrosine (monophenol) to 3,4-dihydroxyphenylalanine or DOPA (o-diphenol) and the oxidation of DOPA to dopaquinone (o-quinone) in the human body. Melanin is a color pigment that protects our skin from UV light damage. But excessive production of melanin causes pigmentation disorders. The purpose on usage of tyrosinase inhibitors is to prevent melanin production by eliminating the enzyme function (Likhitwitayawuid, 2008; Liyanaarachchi *et al*., 2018). Tyrosinase inhibitors are used in the treatment of hyperpigmentation, as well as included in the cosmetic preparations as skin whitening agents.

In this study, we have evaluated the enzyme inhibitory, and antioxidant activities of the methanol (MeOH) extracts which were prepared using different methods from aerial parts of *Klasea serratuloides* (DC) Greuter & Wagenitz subsp. *karamanica* B. Dogan & A. Duran *(*KS). We have also reported that total phenol and flavonoid contents, as well as the biological activities of the MeOH extracts differed from each other depending on the using extraction techniques, such as maceration, Soxhlet extraction, and ultrasound assisted extraction.

2. MATERIAL and METHODS

2.1. Plant Material

KS aerial parts were taken from the natural flora of Karaman, Southern Turkiye (C4: Karaman, Ermenek, on slopes of Tepebaşı village, 1100 m, 21.06.2017, S. Doğu 3456 Y. Bağcı). The plant material was identified by botanist Yavuz Bağcı and Süleyman Doğu. The voucher specimen was held at Selcuk University's Herbarium in Konya (KNYA) Turkiye. In the year 2020, the antioxidant and enzyme inhibition activities were carried out in the laboratories of Selcuk University's Pharmacy Faculty in Konya.

2.2. Chemicals and Equipment

2-2′-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), EDTA (Ethylenediaminetetraacetic acid), sodium carbonate, quercetin, gallic acid, ferrozin, AChE, BChE, tyrosinase, L-tyrosine, galantamine, and kojic acid were purchased from Sigma–Aldrich (St. Louis, MO). Folin–Ciocalteu's phenol reagent, hydrochloric acid, and methanol were obtained from Merck (Darmstadt, Germany). All chemicals used in the experiments were of analytical grade. To evaporate the combined solvents using Rotary evaporator (Buchi R-300, Switzerland). Spectrophotometric measurements were performed by a microplate reader (Multiskan Go; Thermo Scientific Inc.).

2.3. Preparation of The Extracts

The aerial parts of KS were dried in the shade until they reached a constant weight, then pulverized in a blender. In separate bottles, 20 g of dried plant material was extracted for 6 h with 180 mL of methanol using the Soxhlet, ultrasonic extraction, and maceration techniques. The extract was then filtered via Whatman No. 1 filter paper. A rotating evaporator at 40 ° C was used to concentrate the filtrate under reduced pressure. Table 1 shows the yield of KS extracts obtained by Soxhlet, Ultrasonic extraction, and maceration techniques.

Table 1. % Yield of the extracts from *Klasea serratuloides.*

Sample	Extraction Methods	%Yield of the extacts (w/w)
		MeOH
Klasea serratuloides	Soxhlet	37.28
	Ultrasonic extraction	22.57
	Maceration	20.05

2.4. Determination of Total Phenolic Content (TPC)

For the measurement of the TPC in the extracts of KS, the spectrophotometric Folin-Ciocalteu (F-C) method was used according to the method of Clarke *et al*., (2013) with slight modification. 10 µL of extract, suitably diluted with DMSO, were combined with 100 µL of freshly 10-fold diluted F–C reagent in distilled water. After 5 min, 100 µL of 7.5% Na_2CO_3 was added, and the absorbance was measured at 650 nm with a microplate reader after 60 min of incubation (Multiscan Sky). In parallel, appropriate blanks (DMSO) were run. All analyses were done in triplicate, and the results were represented by means of standard deviation (SD).

The TPC was estimated as mg GAE (gallic acid equivalent)/ g extract using a calibration curve of concentration vs absorbance.

2.5. Determination of Total Flavonoid Content (TFC)

The total flavonoid concentration of KS extracts was determined using the aluminum chloride colorimetric technique (Yang *et al*., 2011). On a 96-well plate, the test solution (150µL, 0.3 mg/mL) produced with ethanol was combined with 2% $AlCl_3$. In a microplate reader, the absorbance was measured at 435 nm after 15 m of room temperature incubation. The total flavonoid content was calculated as mg QE (quercetin equivalent)/ g extract dry weight basis.

2.6. *In vitro* Antioxidant Activity

2.6.1. *DPPH radical scavenging activity*

The radical scavenging activity of extracts was determined using the DPPH radical scavenging assay. After mixing 20 µL of test solution with 180 µL of DPPH solution in a 96-well plate. The plate was measured at 540 nm using an Elisa reader (Multiscan Sky, USA) after 15 m of incubation in the dark (Eruygur *et al*., 2019). Ascorbic acid was used as positive control. All analyses were done in triplicate, and the results were represented by means of standard deviation (SD). The following equation (1) was used to calculate the percent DPPH scavenging effect:

$$\% \text{ DPPH Scavenging Effect} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} x100 \quad (1)$$

2.6.2. *ABTS radical scavenging activity*

According to Re *et al*., the extracts' ABTS cation radical decolorization activity was performed with minor modifications (Re *et al*., 1999). Allowing 15 mL of 7 mM ABTS and 264 µL of 140 mM potassium persulfate solution to stand in the dark at room temperature for 16 h before the experiment yielded the ABTS•+ radical stock solution. The ABTS•+ working solution was made fresh by diluting the stock solution with 80%MeOH and measuring the absorbance at 734 nm to get 0.70 ± 0.02. 50 µL of sample solution were combined with 100µL of ABTS•+ working solution in a 96-well plate. The mixture was then allowed to stand at room temperature for 10 minutes, after which the absorbance was also measured at 734 nm. All of the activities were repeated three times, with the results reported as a means of standard deviation. For comparison of the ABTS+ scavenging activity, ascorbic acid was utilized as an antioxidant standard. The following equation (2) was used to calculate the percent ABTS scavenging effect:

$$\% \text{ ABTS Scavenging Effect} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} x100 \quad (2)$$

2.6.3. *Iron Chelating Activity*

The iron chelating activity of the extracts was determined based on the interaction of ferrozin-Fe^{2+}-complex (Chai *et al*., 2014). In summary, a mixture of 0.4 mL of 0.2 mM ferrozine, 0.2 mL of 0.1 mM $FeSO_4$ and 0.2 mL of extract was incubated at room temperature. The absorbance was measured at 562 nm after a 10-minute incubation period. As a positive control, EDTA was utilized.

2.7. Enzyme Inhibition Activity

2.7.1. *Acetylcholinesterase / butyrylcholinesterase inhibition assay*

This experiment was carried out according to the Ellman al., method with some modifications (Ellman *et al*.,1961). A mixture of 20 µL of test sample/reference standard at various concentrations, 140µL of 200 mM phosphate buffer (pH 7.7), 10 µL of 5,5-dithio-bis-(2-nitrobenzoic acid (DTNB) and 20µL of enzyme (0.22 U/mL for acetylcholinesterase/0.1 U/mL

for butyrylcholinesterase prepared in PBS buffer) was incubated for 15 min at 25 °C. Following the addition of 10 µL of 0.5 mM DTNB, 10 µL of substrate (0.71 mM acetylthiocholine iodide / 0.2 mM butyrylthiocholine iodide) was mixed and incubated for another 5 min. When substrate was added, the absorbance of the plate was measured at 0 and 5 min, yielding a yellow color at 412 nm. As a positive control, galantamine was used. The following equation (3) was used to express the results:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} x100 \quad (3)$$

2.7.2. *Tyrosinase enzyme inhibition activity*

Tyrosinase inhibitory activity of the extracts was evaluated according to the method previously reported (Yang *et al*., 2012). 20µL of sample solution diluted with buffer, 100µL of phosphate buffer, and 20µL of tyrosinase (250 U/mL) were combined in each well of a 96 well plate and incubated for about 10 min at 25 °C. The mixture was incubated for 30 min at 25 °C after adding 20µL of 3 mM L-tyrosine as a substrate. After incubation period, the absorbance was read at 492 nm. In place of the sample, kojic acid and phosphate buffer (100 mM PBS, pH 6.8) were employed as positive and negative controls, respectively. The equation (3) was used to calculate the extracts' inhibitory effects on tyrosinase.

3. RESULTS / FINDINGS

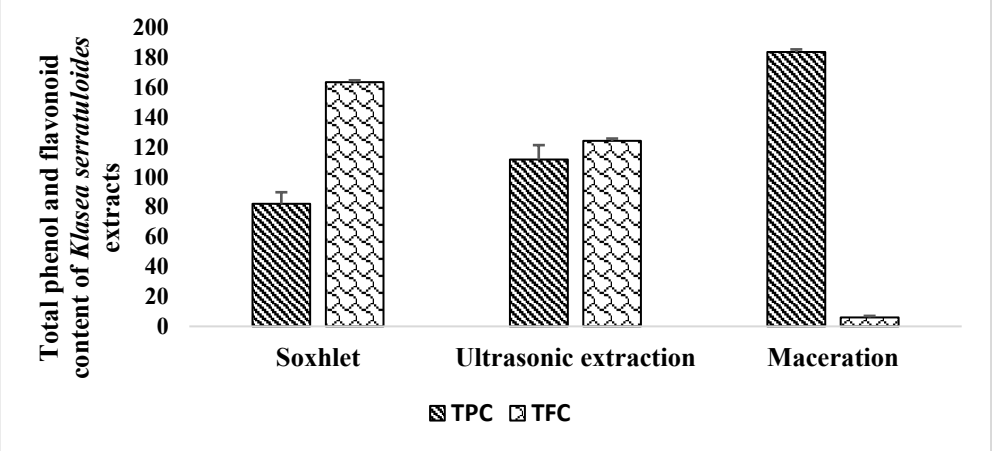
3.1. Total Phenolic and Flavonoid Content

Many chemical components in medicinal plants have various biological activities. Flavonoid and phenolic compounds are among the most important classes. Flavonoids such as kaempferol, quercetin, and rutin are polyphenolic compounds and they are known for their potent free radical scavenging effects (Rice-Evans *et al*., 1997; Atanassova *et al*., 2011). Total phenol content of extracts was calculated as mg gallic acid equivalent /g extract with the calibration equation of y=0.0027x+0.0084 (r^2= 0.9966). Total flavonoid content of extracts calculated as mg Quercetin equivalent (QE)/g extract equivalent as y=0.0346x+ 0.2221 (r^2= 0.9773).

When the total phenol and total flavonoid contents were evaluated in this study, the total phenol content of extracts prepared from KS with Soxhlet, ultrasonic extraction and maceration methods were determined as 82.19±7.71, 111.80±9.69, and 183.87±1.76 mg, respectively.

According to the results, total phenol content was found to be the highest in the extract prepared with maceration, it was followed by ultrasonic extraction and the least in Soxhlet extract. Total flavonoid content was expressed as quercetin equivalent (QE). The total flavonoid content of methanol extracts prepared from *KS* with Soxhlet, ultrasonic extraction and maceration methods was determined as 163.65±1.29, 124.33±1.55, and 6.07±0.94 mg QE/g, respectively (Figure 1). It can be thought to be that the maceration technique was more effective for extraction of phenolic compounds, the Soxhlet technique was more effective for extraction of flavonoids compounds in this plant.

Figure 1. Total phenol and total flavonoid content of KS extracts prepared with Soxhlet, ultrasonic extract and maceration methods.



3.2. Antioxidant Activity

The *in vitro* antioxidant activities have been determined by DPPH, ABTS and iron chelating activity methods. Radical scavenging activities of KS were compared with synthetic antioxidant compounds. All of extracts exerted high radical scavenging activity with both DPPH and ABTS assay and showing scavenging activity over 50%. The scavenging ability of these samples showed a concentration dependent activity profile. The best antioxidant activity was observed in the extract of KS with maceration methods in DPPH (IC_{50}: 24.32 ± 1.02 µg/mL) and ABTS (IC_{50}: 13.44 ± 0.29 µg/mL). It was observed to have higher antioxidant activity compared to positive control. The extract of KS obtained with soxhlet (39.15 ± 2.44%) was more active than other extract with ultrasonic extraction (36.15±0.61%) and maceration (30.56±2.28%) in terms of iron-chelating activity. As a result, KS has antioxidant activity in all three methods as given in the Table 2. In a previous study, *K. serratuloides* (DC.) Greuter &Wagenitz were found to have antioxidant activity in DPPH, CUPRAC, and ferrous ion-chelating methods (Tel *et al*., 2015).

Table 2. ABTS and DPPH radical scavenging effects, and iron chelating activities of the extracts of *K. serratuloides*.

Extraction Methods	ABTS (percentage± S.D.[a]) 83.33 µg/mL[b]	DPPH (percentage± S.D.[a]) 50 µg/mL[b]	Iron-chelating activities (percentage± S.D.[a]) 250 µg/mL[b]
Soxhlet	80.93 ± 3,71 (IC_{50}: 45.79 ± 0.67 µg/mL)	60.87 ± 4.9 (IC_{50}: 19.81 ± 0.69 µg/mL)	39.15 ± 2.44
Ultrasonic extraction	54.63 ± 3.07 (IC_{50}: 47.27 ± 2.21 µg/mL)	72.21 ± 2.85 (IC_{50}: 40.78 ± 2.17 µg/mL)	36.15±0.61
Maceration	82.05 ± 0.90 (IC_{50}: 13.44 ± 0.29 µg/mL)	87.51 ± 0.33 (IC_{50}: 24.32 ± 1.02 µg/mL)	30.56 ± 2.28
Reference	87.51 ± 0.17[c]	93.91 ± 0.14[c]	87.06 ± 0.34[d]

a: Standard deviation, b: Final concentration, c: Ascorbic acid (2 mg/mL) d: EDTA (2 mg/mL)

3.3. Enzyme Inhibition Activity

The methanol extracts obtained from the aerial parts of KS were tested for their acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) tyrosinase (TYR) inhibitory activity at the concentration of 50, 100, and 200 µg/mL. Inhibitory activity of extracts given at different concentration in Table 3. All extracts displayed moderate inhibition activity against AChE, while all extracts exhibited high inhibition activity against BChE. The methanol extract of KS using soxhlet (61.17 ± 3.62) and ultrasonic extraction (58.76 ± 1.46) showed higher inhibition than the extract prepared with maceration methods (34.54 ± 0.73) against BChE at 50µg/mL of concentration. As can be seen from the Table 3, the extract prepared with soxhlet showed 45.57% inhibition against AChE and 61.17% inhibition against BChE at 50 µg/mL. All of them have moderate inhibition activity against TYR at the concentration of 125µg/mL and 250 µg/mL. The methanol extracts of KS prepared with soxhlet, ultrasonic extraction and maceration technique was showed 29.49%, 25.12%, and 52.80% inhibition against TYR at concentration of 125 µg/mL. According to Tel *et al*. (2015), methanol extract of *K. serratuloides* showed completely good anti-tyrosinase activity at 50 µg/mL (31.1%) and hexane extract showed high anti-acetylcholinesterase activity (IC_{50}: 134.7 ± 2.1 µg/mL) (Tel *et al*., 2015).

Table 3. Enzyme inhibition effects of the extracts of *K. serratuloides*.

Inhibitory activity	Concentration	Soxhlet extraction	Ultrasonic extraction	Maceration	Reference [d.e]
AChE (percentage ± S.D.[a])	50 µg/mL[b]	45.57±0.96	27.44±0.13	41.39±0.73	99.10±1.18[d]
	100 µg/mL[b]	75.19±1.63	50.84±0.37	46.40±4.00	
	200 µg/mL[b]	93.86±3.26	86.63±2.67	90.07±2.83	
BChE (percentage ±S.D.[a])	50 µg/mL[b]	61.17±3.62	58.76±1.46	34.54±0.73	84.34±4.85[d]
	100 µg/mL[b]	69.59±2.19	-[c]	-[c]	
	200 µg/mL[b]	-[c]	-[c]	-[c]	
TYR (percentage ± S.D.[a])	62.5 µg/mL[b]	20.53±3.34	7.27±4.68	15.52±1.18	80.96±0.51[e]
	125 µg/mL[b]	29.49±2.09	25.12±2.65	52.80±2.35	
	250 µg/mL[b]	38.04±1.50	30.52±2.68	-[c]	

KS: *Klasea serratuloides* [a]Standard deviation, [b]Final concentration [c]Not detected [d]Galanthamine hydrobromide [e]Kojic acid

When the results of this study are evaluated, in DPPH and ABTS methods, the antioxidant activity of the KS extracts prepared by maceration technique was higher than the others. The maceration technique is more suitable for the extraction of phenolic compounds that play a role in antioxidant activity.

The highest iron chelation activity was observed extract of KS with Soxhlet. For enzyme inhibition, the extract from Soxhlet was found to be more effective against both cholinesterase and tyrosinase. The yield of KS extract obtained by Soxhlet was higher than the yield of other extracts. Therefore, it can be said that the natural compounds with strong cholinesterase and TYR inhibitory activity can be extract with soxhlet technique from KS.

4. CONCLUSION

As a conclusion, antioxidant activity was found to be high in all extracts of KS prepared by different methods. Especially the extract prepared with soxhlet demonstrated the highest inhibitory activity against all enzymes. Although all the extracts show good inhibition against tested enzymes, the chemical composition analysis of the extracts should be conducted to determine which compound is responsible for the activity. It has been seen as promising for the related diseases in the future.

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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.

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Authors are expected to present author contributions statement to their manuscript such as. **Nuraniye Eruygur:** Investigation, Supervision, Arrangement and Analysis, reviewing and correcting the original draft. **Fatma Ayaz:** Investigation, Arrangement and Analysis. **Yavuz Bağcı:** Plant collection and Diagnosis. **Süleyman Doğu:** Plant collection and Diagnosis. **Tuğsen Doğru:** Writing, Analysis, creating of Table and Graphics. **Merve Koçak:** Writing, Arrangement, and Analysis.

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Research Article

Antidiabetic Potential and Chemical Constituents of *Haloxylon scoparium* Aerial Part, An Endemic Plant from Southeastern Algeria

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Abstract: Diabetes mellitus is a chronic metabolic disease. Traditional medicines are currently still popular as an alternative in the treatment of this disease. However, the mechanism of action in lowering blood sugar of most folk recipes remains unproven. The objective of this study is to evaluate the antidiabetic potential of an Algerian halophyte in regulating postprandial hyperglycemia via α-amylase inhibitory activity. For this, methanolic and aqueous crude extracts were prepared from the aerial part of *Haloxylon scoparium* Pomel and analyzed by HPLC/ UV method. Total flavonoids, total tannins and total alkaloids as specific extracts were also prepared from the same part. The *in vitro* α-amylase inhibition assay using starch-iodine was performed. As results, the methanolic crude extract seems to be the best with 29 phenolic compounds of which the most abundant is gallic acid. All tested extracts showed better α-amylase inhibitory activities. Among these extracts and compared to acarbose (IC_{50} = 17.96 µg/ mL), the methanolic crude extract had the highest inhibitory activity (IC_{50} = 22.9 µg/ mL), followed by total flavonoids and alkaloids. Finally, we conclude that *Haloxylon scoparium* aerial part had displayed maximum inhibition against α-amylase enzyme especially with its methanolic crude extract. It can be used for management of postprandial hyperglycemia with lesser side effects and provide a strong rationale for further animal and clinical studies.

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1. INTRODUCTION

Diabetes mellitus is defined as a chronic disease characterized by an increase in fasting blood sugar (WHO, 2016). Among all cases of diabetes in the world, about 90% are type II. Postprandial hyperglycemia (PPHG) is a very important risk factor in the onset and development of type II diabetes (Dong *et al*., 2012). Dietary carbohydrates such as starch give after hydrolysis several molecules of glucose which are the main source in PPHG. With α-amylase enzyme, dietary polysaccharides are hydrolyzed to oligosaccharides and disaccharides

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and then to monosaccharides by α-glucosidase (Bischoff *et al*., 1985). To date, the most effective strategy in the management of post-prandial hyperglycemia is the reduction of glucose intake by decreasing or blocking the digestion of carbohydrates into monosaccharides in the gastrointestinal tract. α-amylase and α-glucosidase are digestive enzymes released in the intestinal lumen to digest carbohydrates into simple glucose molecules (Ortiz-Andrade *et al*., 2007; Shang *et al*., 2012).

Inhibition of α-amylase enzyme may slow and delay the digestion by hampering breakdown of starch and may be considered an effective strategy in reducing the intensity of metabolic disturbances and regulating the hyperglycemic condition (Ashok Kumar *et al*., 2011). This endocrine problem is certainly due to impaired insulin secretion and insulin sensitivity (Ceriello, 2005; Nyenwe *et al*., 2011). Delayed secretion of this hormone, after eating something, can cause a persistent increase in postprandial blood sugar (PPG) in the range of 140 to 190 mg/ dl, which further increases to 200 mg/ dl and in extreme cases up to 400 mg/ dl (Bachhawat *et al*., 2011; Mohamed Sham Shihabudeen *et al*., 2011). The first hours of the postprandial phase of diabetes, which follow the strong insulin secretion, are very particular due to the increase in the level of glycated hemoglobin (HbA1c) leading to several vascular complications (neuropathy, retinopathy, etc.) and an increased risk of cardiovascular problems (Bonora & Muggeo, 2001; Aryangat & Gerich, 2010; Campos, 2012).

In recent decades, certain synthetic inhibitors, such as acarbose, miglitol and voglibose, have been developed and received considerable and particular attention for the management of type II diabetes (Toeller, 1994). These synthetic inhibitors or α-amylase inhibitor drugs are generally used with other oral hypoglycemic drugs (sulfonylurea, metformin) to reduce the level of HbA1c. However, the unwanted side effects of these synthetic inhibitors like diarrhea, flatulence, abdominal distension, meteorism, bloating, nausea and intestinal cystoid pneumatosis (Hollander, 1992; Puls, 1996; Kojima *et al*., 2010) have attracted scientists' interest in medicinal plants. Plant-based α-amylase inhibitors may present a particularly credible alternative for controlling PPHG (Horii *et al*., 1986; Kwon *et al*., 2006).

Since ancient times, people have used plants for self-care and defense against many chronic and infectious diseases. Among these plants, *Haloxylon scoparium* Pomel which is distributed in temperate salt habitats, especially in the coastal regions of the Mediterranean Sea, arid steppes, and deserts (Mulas, 2004), is widely used as a remedy for the treatment of eye and vision disorders, hypertension and skin diseases (Allali *et al*., 2008), and also for the treatment of cancer, hepatitis, inflammation and obesity (Eddouks *et al*., 2002).

This study aims to evaluate the antidiabetic potential of this Algerian halophyte through the *in vitro* evaluation of the α-amylase inhibitory activities of its aerial part extracts.

2. MATERIAL and METHODS

2.1. Plant Material

From a random samples of *Haloxylon scoparium* Pomel of Amaranthaceae family (Täckholm, 1974; Boulos, 1999) collected in 2016 in southeastern Algeria (Figure 1), aerial parts were isolated, cleaned of microorganisms and residual soil and air dried at room temperature for 15 to 20 days. These parts are then ground and a voucher specimen has been deposited in the Herbarium of the Plant Biology and Environment Laboratory of the Faculty of Sciences for future reference.

Figure 1. General habitus of the studied species.

2.2. Phytochemical Screening

A phytochemical screening was carried out following standard procedures for the highlighting of the presence or absence of bioactive compounds of the secondary metabolism of *Haloxylon scoparium* (Ronchetti & Russo, 1971; Fadeyi *et al*., 1989; Odebiyi & Sofowora, 1990; Harborne, 1998; Hagerman *et al*., 2000; Abulude *et al*., 2004; Abulude, 2007).

2.3. Plant Extracts and Total Polyphenols Contents

2.3.1. *Crude extracts*

According to Falleh *et al*. (2008) and by cold maceration of 2.5g of dry plant matter in 25 mL of absolute solvents (methanol and distilled water), methanolic and aqueous crude extracts of the studied plant were prepared and then stored at 4°C for later use.

2.3.2. *Specific extracts*

2.3.2.1. Total Flavonoids: According to Foungbe *et al*. (1976), total flavonoids extraction was carried out according to the CHARAUX-PARIS standard method (Paris, 1954). It's a universal method which consists in stabilizing a mass of dried herbal drug equal to 10 g for one hour in 200 mL of ethanol at a temperature of 96 °C. After filtration and drying, the drug was pulverized and then depleted using a Soxhlet with 200 mL of absolute ethanol brought to a temperature of 96 °C for 4 hours. After maceration for 12 to 24 hours, the two ethanolic solutions were combined and evaporated under reduced pressure. The residue was taken up in 20 mL of boiling water, the resulting aqueous solution was left to stand for 24 hours. Finally, the liquor was exhausted in a separating funnel in three successive stages with ether, ethyl acetate and n-butanol. According to Solfo (1973), flavonoid compounds don't pass in ether, they are in trace amounts in ethyl acetate and only the butanol compound contains an amount that can be studied. The butanol extract was then evaporated using a rotary evaporator at a temperature of 30 °C. The residue was then collected in little of methanol and stored at a temperature of 4 °C for future use.

2.3.2.2. Total Tannins: To extract the total tannins existing in the aerial part of our plant, we followed the method of Sowunmi *et al*. (2000) which consists in macerating 50 g of dried plant material for 24 hours with magnetic stirring in a mixture of ethanol and boiling water (200 mL/ 500 mL). After filtration, the resulting liquor was depleted in a separating funnel several times with chloroform. After evaporating the organic phase using a rotary evaporator, the residue was taken up in 10 mL of methanol (1%) and then stored in the refrigerator at a temperature of 4 °C.

2.3.2.3. Total Alkaloids: The extraction of total alkaloids was carried out according to Fattorusso & Taglialatela-Scafati (2007): 100 g of herbal drug was degreased with 300 mL of petroleum ether. This step lasts 24 hours to ensure proper elimination of fats and pigments. Filtration was subsequently carried out; the filtrate was discarded. The marc was left in the open air for 30 minutes to be dried and to remove the organic solvent. The marc undergoes maceration for 24 hours in 200 mL of an alkalinized chloroform solution by adding a few drops of ammonia until a basic pH (pH = 9). Filtration was then carried out, the filtrate was collected. In order to allow good depletion of the marc and to improve the extraction yield, this maceration was done in triplicate. The collected filtrates were grouped together and partially concentrated using a rotary evaporator. The recovered concentrate is extracted with 200 mL of 3% aqueous sulfuric acid (H_2SO_4) solution. The acid phase was then basified with an ammonia solution to pH = 9. Then, liquid-liquid extraction was carried out using a separating funnel with 20 mL of chloroform. It was mixed by stirring up and down for a few minutes. After standing for about 30 minutes, two phases were observed, the aqueous phase above and the organic phase below. The organic phase (organic solution of total alkaloids) was recovered and then concentrated until the chloroform was completely eliminated in a rotary evaporator at 40 °C. The concentrate

thus obtained is a brown paste of alkaloids, to which 20 mL of acetone or very little of methanol has been added. Finally, the mixture was stored in the refrigerator at a temperature of 4 °C.

2.3.3. *Total polyphenols contents*

To evaluate the total polyphenols content, we followed the standard method of Singleton & Rossi (1965) using the Folin-Ciocalteu reagent. The content was expressed in mg gallic acid equivalent per gram of dry weight (mg GAE/ g DW).

2.4. Phytochemical Analysis (HPLC determination of phenolic compounds)

HPLC/ UV analysis was carried out at the Chromatographic Analysis Laboratory of the Center for Research and Physico-Chemical Analysis (CRAPC) in Algiers (Capital of Algeria) using a Young Line 9100 brand device equipped a high pressure liquid chromatography pump fitted with a UV deuterium detector. The chromatographic separations were carried out in reverse phase with a column Zorbax Eclipse XDB-C18 (stationary phase: 150 mm x 4.6 mm, 5 µm) and a mobile phase formed from two different solvents: A: Acidified water with 1% of acetic acid and B: absolute methanol. The temperature was maintained at 25°C and the chosen injection volume was 20 µL. The mobile phase solvents were of HPLC grade and the flow rate was set at 1mL / min. The chromatographic conditions consist of a gradient mode: Initial A 95%; B 5%, at 55min: A 5%; B 95%, at 60min: A 95%; B 5%. The UV-visible detector was tuned to signals 254 and 280 nanometers.

2.5. Antidiabetic Activity (α-amylase inhibitory activity)

In order to evaluate the antidiabetic activity of different extracts of *Haloxylon scoparium* in Algerian northern Sahara, the α-amylase inhibition test was performed using a modified method of Kusano *et al*. (2011). The undigested starch due to enzyme inhibition was detected at 630 nm (blue, starch-iodine complex). To do that, 200 mg of starch were dissolved in 25 mL of NaOH (0.4 M). The substrate solution was heated at 100°C for 5 minutes. After cooling, the pH was adjusted to 7.0 and the final volume was made up to 100 mL with distilled water. Acarbose was used as a positive control. 40 µL of substrate solution was pre-incubated at 37°C for 3 minutes with 20 µL of acarbose or plant extract in increasing concentrations. 20 µL of 3 U/mL of α-amylase were then added (20 mM phosphate buffer with 6.7 mM NaCl, pH 6.9), and the mixture was further incubated at 37°C for 15 min. Termination of the reaction was done by adding 80 µL of HCl (0.1 M). Then, 100 µL of iodine reagent (2.5 mM) were added, and the absorbance was measured at 630 nm. Percentage of inhibition of α-amylase enzyme was calculated using the formula:

$$\% \text{ Inhibition} = (1-[Abs_2-Abs_1/\ Abs_4-Abs_3]) \times 100$$

Where, Abs_1 is the absorbance of the incubated mixture containing plant sample, starch, and amylase; Abs_2 is the absorbance of incubated mixture of sample and starch; Abs_3 is the absorbance of the incubated mixture of starch and amylase; Abs_4 is the absorbance of incubated solution containing starch. Results were expressed as IC_{50}. IC_{50} value represents the concentration of inhibitor required to achieve 50% enzyme inhibition. In the case of significant inhibition, IC_{50} values were determined through nonlinear regression by fitting to a sigmoid dose-response equation with variable slope using GraphPad Prism version 7.00 for Windows, GraphPad Software, Inc. La Jolla California USA.

2.6. Statistical Analysis

Experimental tests were realized in triplicate and all results were expressed as mean ± standard error of the mean (SEM). All values of α-amylase inhibitory activity of *H. scoparium* aerial extracts were statistically compared by ANOVA test (analysis of variance) using MINITAB version 16.0 program (Values were considered significant at $p < 0.05$).

3. RESULTS

3.1. Phytochemical screening

The results of phytochemical tests of the different compounds of *H. scoparium* aerial part are grouped in Table 1.

Table 1. Phytochemical compounds of *H. scoparium* aerial part.

Flavonoids	Tannins	Saponins	Cardinolids	Anthocyanins	Leuco anthocyanins	Alkaloids	Terpenes and Sterols
+	+	+	-	+	-	+	+

(+) : Detected, (-) : Not detected

These tests revealed the presence of six major compounds of secondary metabolism (flavonoids, tannins, saponins, anthocyanins, alkaloids, terpenes and sterols) and the absence of two other important compounds: Cardinolids and leucoanthocyanins. The presence of these secondary metabolites suggests that the plant might be of medicinal importance.

3.2. Yields and total polyphenols contents

Yields of different extracts and results of the colorimetric analysis of total polyphenoFls of the studied species are represented in Table 2.

Table 2. Yields and different compound contents in *H. scoparium* extracts.

Plant extract	Yield (%)	Total polyphenols content (mg GAE/ g DW)
Methanolic Crude Extract (MCE)	20.66	228.582 ± 0.689
Aqueous Crude Extract (ACE)	44.44	336.756 ± 0.855
Total flavonoids (T. Flav)	04.36	-
Total Tannins (T. Tan)	26.27	-
Total Alkaloids (T. Alc)	0.53	-

From this Table 2; *H. scoparium* is found to be richer in aqueous crude extract with 44.44% in its aerial part. Concerning total polyphenols which are known by their several biological activities, the obtained results of colorimetric assays show a very high content of total polyphenols (336.756 ± 0.855 mg GAE/ g DW) in the aqueous crude extract (ACE) of *H. scoparium* aerial part, and 228.582 ± 0.689 mg GAE/ g DW in crude methanol extract (MCE).

3.3. Phytochemical analysis

HPLC/ UV analysis showed the richness of *H. scoparium* crude extracts in phenolic compounds. The obtained chromatogram profiles are shown in Figures 2 and 3.

Figure 2. HPLC/ UV chromatogram profile of methanolic crude extract.

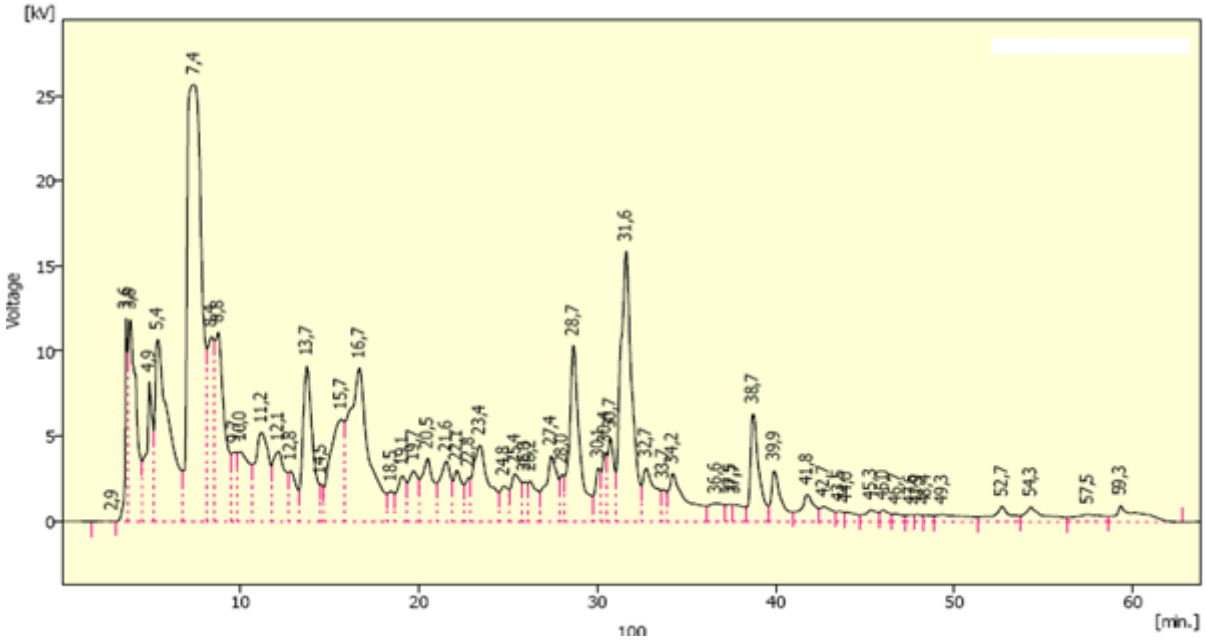
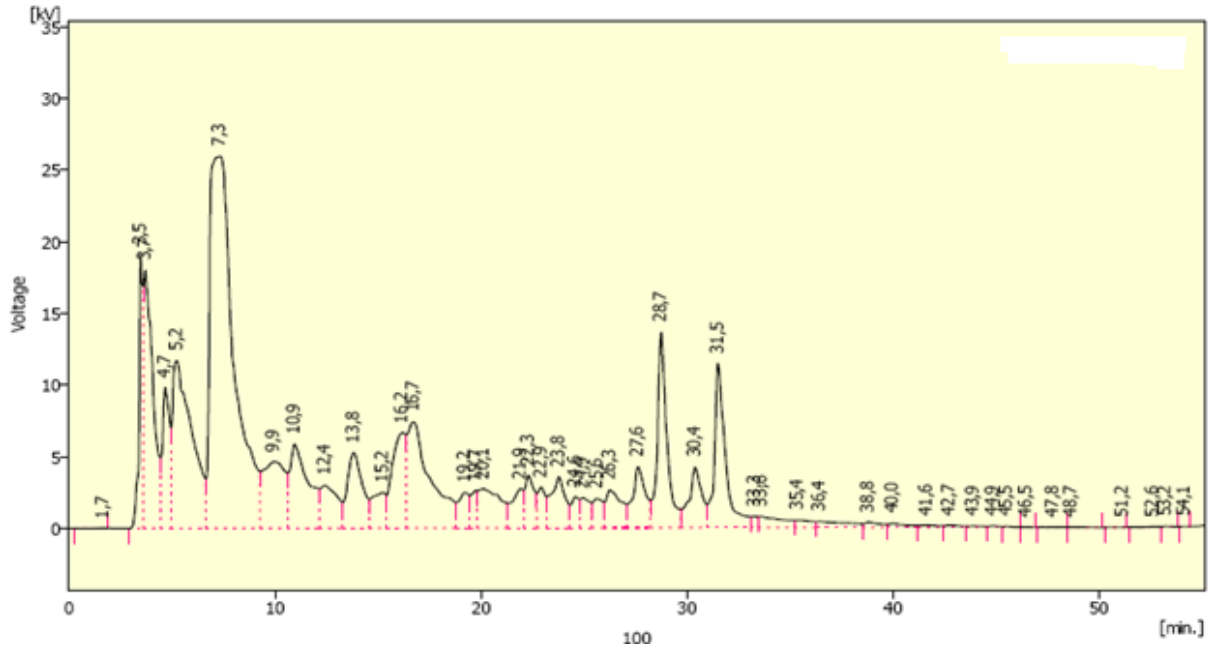


Figure 3. HPLC/ UV chromatogram profile of aqueous crude extract.



Based on the retention time, the sample peak area and the standard peak area, several compounds were identified (Table 3). From this table, 29 phenolic compounds were identified in MCE solution of which the most abundant is gallic acid (14.4%) followed by catechic acid (7.0%), rutin (7.0%) and trans-cinnamic acid (4.2%). However, ACE analysis revealed only 18 phenolic compounds with a predominance of gallic acid (24.5%) and catechic acid (6.6%).

Table 3. Phenolic compounds of methanolic and aqueous crude extract.

P	MCE			ACE		
	RT	A	Compounds	RT	A	Compounds
1	3.837	3.7	Ascorbic acid	3.477	2.8	Ascorbic acid
2	7.420	14.4	Gallic acid	7.327	24.5	Gallic acid
3	11.203	2.8	Resorcinol	10.943	4.2	Resorcinol
4	13.737	3.6	Catechin	13.827	3.1	Catechin
5	15.653	3.2	Gentisic acid (2,5-dihydroxy benzoïc acid)	16.177	3.4	Gentisic acid (2,5-dihydroxy benzoïc acid)
6	16.687	7.0	Catechic acid	16.727	6.6	Catechic acid
7	20.503	1.6	Cafeic acid	20.127	2.4	Cafeic acid
8	21.553	1.6	Isovanillic acid	21.927	1.2	Isovanillic acid
9	22.120	1.0	Syringic acid	22.310	1.3	Syringic acid
10	22.820	0.5	Vanillic acid	22.927	2.8	Vanillic acid
11	23.420	2.8	Epicatechin	23.777	2.0	Epicatechin
12	24.787	0.7	Benzoïc acid	24.593	0.6	Benzoïc acid
13	25.387	1.1	Para-coumaric acid	25.643	0.8	Para-coumaric acid
14	26.237	0.8	Scopoletin	26.260	1.6	Scopoletin
15	27.420	1.8	Ferulic acid (3-hydroxy 4-methoxycinnamic)	27.627	2.1	Ferulic acid
16	28.670	4.2	Trans-cinnamic acid	28.743	5.1	Trans-cinnamic acid
17	30.053	0.7	Berberin	30.393	2.1	Robinin
18	30.437	0.8	Robinin	31.510	4.9	Rutin
19	30.737	1.2	Salicylic acid	/	/	/
20	31.620	7.0	Rutin	/	/	/
21	32.737	1.6	Euleropein	/	/	/
22	33.687	0.4	M-anisic acid	/	/	/
23	34.220	2.0	Myricetin	/	/	/
24	38.720	2.1	Quercetin	/	/	/
25	39.903	1.2	Naringenin	/	/	/
26	41.753	0.8	Apigenin	/	/	/
27	52.670	0.6	Kaempferol	/	/	/
28	54.287	0.7	Tangiritin	/	/	/
29	59.303	0.9	3-hydroxy-flavone	/	/	/
1	3.837	3.7	Ascorbic acid	3.477	2.8	Ascorbic acid
2	7.420	14.4	Gallic acid	7.327	24.5	Gallic acid

P: Peak, RT: Retention time (min), A: Area (%)

3.4. α-amylase inhibitory activity

Results of α-amylase inhibitory activity of different plant extracts of *H. scoparium* are represented in the table below (Table 4). From these results, all the extracts and acarbose showed dose-dependent inhibition of α-amylase enzyme. Enzyme inhibition is directly proportional to the concentration of the tested sample. When the concentration is high, the inhibition is strong. A very large percentages of inhibition were recorded that could exceed 90% with the exception of the aqueous crude extract (ACE) which showed only moderately low inhibition values.

Table 4. α-amylase inhibitory activity results.

Sample	Concentration (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Methanolic Crude Extract (MCE)	5	22.158±0.005	22.9±0.009[b]
	10	46.884±0.009	
	25	58.431±0.008	
	50	62.095±0.015	
	100	71.395±0.006	
	250	78.251±0.005	
	500	80.387±0.018	
	1000	88.355±0.135	
Aqueous Crude Extract (ACE)	5	21.326±0.014	45.244±0.027[e]
	10	30.930±0.005	
	25	49.723±0.005	
	50	52.758±0.005	
	100	66.880±0.005	
	250	68.649±0.006	
	500	72.401±0.045	
	1000	79.306±0.005	
Total Flavonoids (T. Flav)	5	20.010±0.002	24.337±0.005[c]
	10	39.150±0.002	
	25	45.337±0.004	
	50	66.787±0.002	
	100	82.788±0.001	
	250	83.060±0.002	
	500	85.095±0.006	
	1000	88.259±0.002	
Total Tannins (T. Tan)	5	18.993±0.004	28.957±0.005[d]
	10	29.029±0.002	
	25	39.340±0.002	
	50	65.720±0.002	
	100	80.514±0.005	
	250	87.060±0.001	
	500	91.725±0.006	
	1000	92.609±0.047	
Total Alkaloids (T. Alc)	5	21.772±0.005	25.787±0.005[c]
	10	37.754±0.005	
	25	44.978±0.001	
	50	59.621±0.005	
	100	76.372±0.005	
	250	92.494±0.004	
	500	92.656±0.005	
	1000	94.015±0.003	

Table 4. Continues.

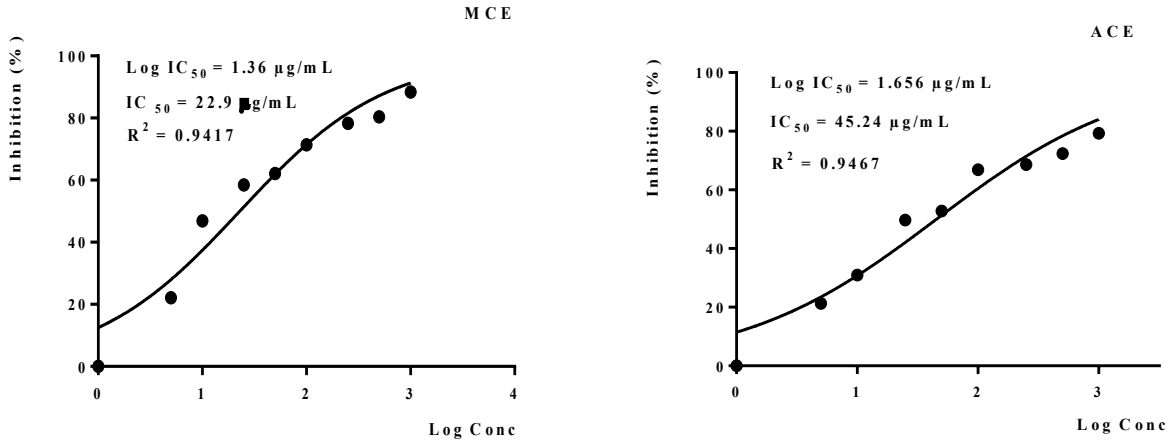
Acarbose (Positive control)	2	18.74±0.017	17.96±0.014[a]
	4	24.594±0.013	
	8	49.050±0.030	
	16	70.314±0.013	
	32	83.104±0.042	
	64	87.064±0.042	
	128	95.20±0.022	

In the last column, means followed by different letters are significantly different ($p < 0.05$)

In general and concerning concentrations which are less than 100 µg/ mL of plant samples (Table 4), the ACE recorded the lowest percentages of inhibition with a maximum of 66.880 ± 0.005%. As for the crude methanolic extract (MCE) and by comparison with the other plant extracts which are found to be more or less similar, a remarkable and very particular increase in inhibition percentages is observed from the concentration 5 to 25 µg/ mL.

Concerning the positive control (acarbose), more or less similar results are recorded compared to those obtained with MCE, especially at low concentration. For concentrations above 50 µg/ mL, acarbose appears to be better with very strong inhibition values.

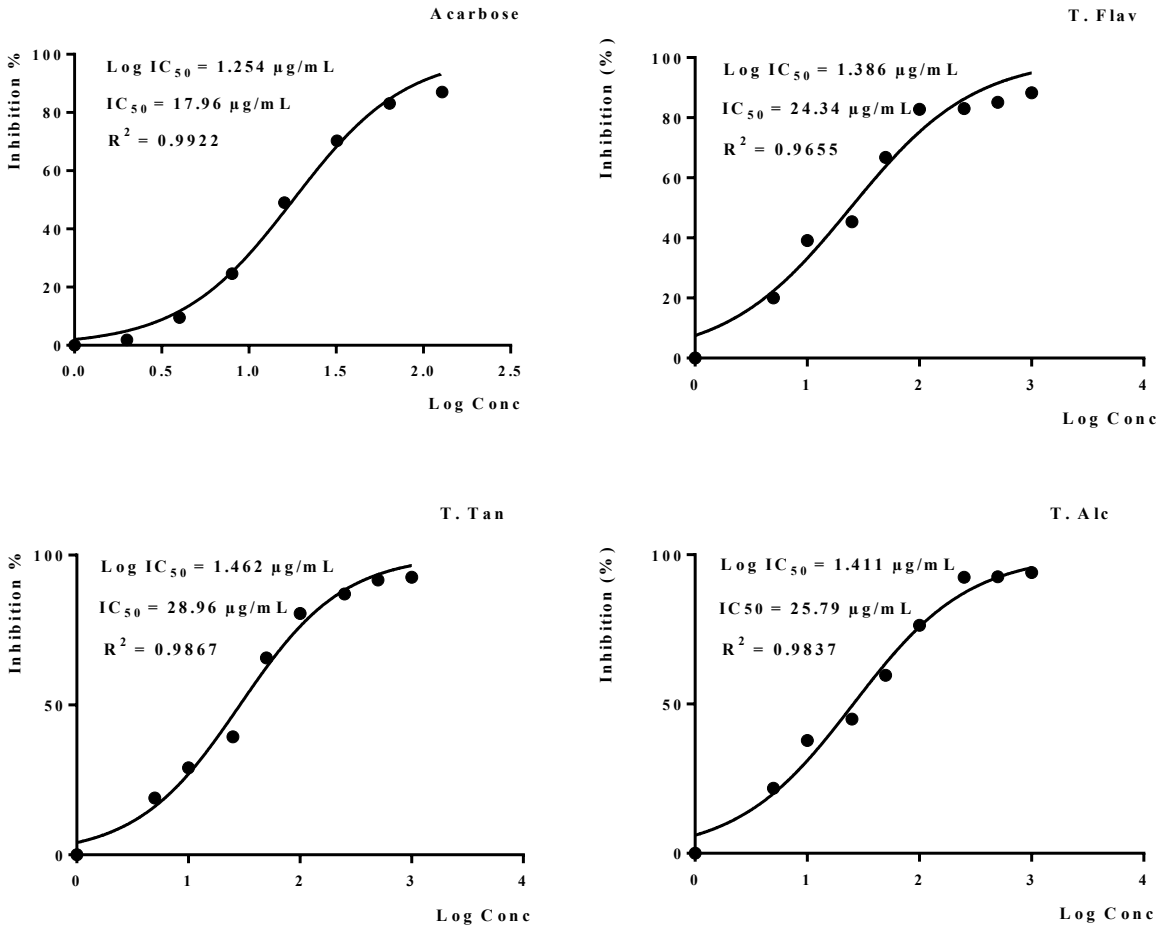
Figure 4. α-amylase inhibition of methanolic and aqueous crude extracts.



As for IC_{50} values, the MCE comes first compared to other plant extracts with 22.9 µg/ mL (Figure 4). By comparison with acarbose (IC_{50} = 17.96 µg/ mL) (Figure 5), this extract was found to be the best in inhibiting the enzymatic activity of α-amylase.

The other plant extracts also seem to be very strong in inhibiting the activity of the α-amylase enzyme, with values more or less near to that of acarbose (positive control) and more particularly flavonoids and alkaloids (Figure 5). The crude aqueous extract comes last with a large IC_{50} value of around 45.24 µg/ mL (Figure 4).

Figure 5. α-amylase inhibition of specific extracts and positive control (acarbose).



4. DISCUSSION and CONCLUSION

First of all and from the obtained results of yields, it seems that the phytochemicals existing in the aerial part of *H. scoparium* have a big affinity for water and are more extractable with water than with absolute methanol. By referring to qualitative tests results (Table 1), the recorded yields confirm the intensity of the results of preliminary phytochemical tests and therefore it can said that the crude extract of aerial part is composed essentially of polyphenols. These results seems to be similar to those of Mezghani-Jarraya *et al*. (2009) with 15.10% of methanolic extract of *H. scoparium* aerial part from Sfax in Tunisia and better than those of Bouaziz *et al*. (2016) which obtained only 6.15% as yield of methanolic extract of the same plant species in the same region of Sfax in Tunisia. Furthermore, and by comparison with the results of Bourogaa *et al*. (2014) on the same plant species in southern Tunisia (only 11%), a very big difference is recorded in this study with more than 44% of yield of aqueous crude extract of the aerial part of *H. scoparium* in southeastern Algeria. However, this low yield (11%) is also reported in the results of Taîr *et al*. (2016) with the same plant species in the region of Naâma in northwestern Algeria.

In addition, a similarity is recorded by comparison with some previous results of Allaoui *et al*. (2016) obtained on the same part of *H. scoparium* of Ghardaïa in Algerian northern Sahara, where a very high contents of total polyphenols (397.743 mg GAE/ g of extract) and flavonoids (82.835 mg QE/ g of extract) were obtained in ethyl acetate extracts which is less polar than water and methanol. This confirms the richness of this plant on polyphenols and also the effect

of the extraction solvent on secondary metabolites contents. However, the obtained results are completely better than those of Tahar *et al*. (2015) carried out on crude extracts of *H. scoparium* aerial part from Laghouat in the North of the Algerian Sahara (prepared in different solvents of increasing polarity: ethyl acetate, dichloromethane and n-butanol); very low levels of total polyphenols were recorded, ranging from 2.416 to 18.666 mg GAE/ g DW.

Concerning specific extracts yields (total flavonoids, tannins and total alkaloids), we can affirm that the recorded differences strongly depend on the quality and the polarity of the solvent or the mixture of extraction solvents. These results confirm the intensity of the phytochemical screening results and justify the high extractability of polar solvents and the high affinity of alkaloids and phenolic compounds for solvents of increasing polarities. Thus, the obtained results from quantitative and qualitative tests show the superior biochemical quality of the studied plant.

Moreover and concerning the biochemical composition of both of crude extracts of this halophyte which has not been the subject of many publications, few researchers have studied its phenolic composition. The only reported studies were those of Ben Salah *et al*. (2002) & Jarraya *et al*. (2005) on *H. scoparium* of Sfax in east-central Tunisia. If we refer to their results, we can affirm that there is good agreement in the Chemotypes which are revealed from chromatographic analyzes. They reported the presence of isorhamnetin and 1-methylsalsolinol and few of triglycoside flavonols such as Quercetin and Quercetin-3-o-robinobioside. In addition, Chao *et al*. (2013) showed the presence of some phenol acids (Coumaric acid, Cinnamic acid and Caffeoylquinic acid), simple phenols such as Catechol and a Chrysoeriol which is considered to be a flavone. In general, this difference in yields, in phenolic compounds or any other secondary metabolite contents and also in phytochemical composition of *H. scoparium* aerial part extracts or even underground part of the same plant species in different regions; can be attributed to many operating conditions of the experiment like polar or non-polar extraction solvent, quantity of plant material, dry or fresh, temperature and extraction time, and even extraction methods (Lee *et al*., 2003) and to several another factors as environmental and climatic factors (geographical area, drought, soil, microclimate type and bioclimatic stages, some aggressions and diseases) (Atmani, 2009); Genetic heritage, period and time of harvest, stage of development of the plant and also quantification methods (Miliauskas *et al*., 2004).

The intensity and severity of diabetes mellitus, characterized by chronic hyperglycemia with metabolic disturbances (Puavilai *et al*., 1999) as a result of abnormal elevation of blood glucose due to insufficient insulin secretion by the β cells of the pancreas and/ or a resistance to the insulin exploit associated with disturbances in the metabolism of carbohydrates (lipids and proteins too) which lead to long-term complications (Khavandi *et al*., 2013), can be reduced by the inhibition of the α-amylase enzyme which allows the 'elimination of undigested carbohydrates thus slowing the absorption of D-glucose into the bloodstream.

Universal scientific communities are trying to adopt a therapy that not only targets the average glucose concentration and the HbA1c indicator, but at the same time tries to treat the instability of the glucose level (Monnier & Colette, 2006). Initial management of PPHG is necessary in the early prevention of diabetic complications, especially in type II diabetes (Ratner, 2001). Drugs such as acarbose, voglibose and miglitol have been shown to be effective in inhibiting α-amylase activity and controlling type 2 diabetes by suppressing carbohydrate hydrolysis (Kim *et al*., 1999). As reported in other studies, phenolic compounds and alkaloids exhibited a wide range of biological activities (Arunachalam *et al*., 2014a; Arunachalam *et al*., 2014b). The obtained results of the antidiabetic activity evaluated by the *in vitro* tests clearly demonstrated that MCE, with the other specific extracts but to a lesser extent, of *H. scoparium* species had a strong inhibitory activity against α-amylase enzyme. They may therefore be

potentially useful in managing glucose-induced hyperglycemia and merit further phytochemical and preclinical studies.

In conclusion, we can say that *H. scoparium* is very rich in phenolic compounds with a Chemotype of 29 identified compounds especially in its crude methanolic extract of its aerial part with predominance of gallic acid. Our *in vitro* results indicate also that among these plant extracts, the potential top three extracts that have shown an effective α-amylase inhibition are Methanolic crude extract, total Flavonoids and total Alcaloïds respectively. The aqueous crude extract did not show a strong α-amylase inhibitory potential but might have some other mode of inhibitory mechanism. This work is the first to report the isolated compounds from the aerial part of *H. scoparium* which has potential α-amylase inhibitory activity. The main advantage of these extracts is their non-toxicity, but they have not gained medicinal importance and worldwide acceptance due to absence of scientific validation. In perspective, this study merits to be carried out *in vivo* using animal models to know the extent of emulation with *in vitro* results.

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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

Authors are expected to present author contributions statement to their manuscript such as; **Salah Benkherara**: Plant collection and performing the *in vitro* experimentation. **Ouahiba Bordjiba**: Methodology, Supervision and Validation. **Samiha Harrat**: Manuscript review and analysis. **Ali Boutlelis Djahra**: Investigation, resources and Visualization. All authors have read and approved the final manuscript.

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Research Article

Structure-Activity and Antioxidant Properties of Quercetin and Its Co^{2+} Chelate

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Abstract: Quercetin and its metal complexes have anti-oxidation, anti-bacterial, anti-tumor, and kinds of enzymatic activities. Studies in recent years, these activities are very important for health and pharmaceutics. The purpose of this manuscript is to determine the structure-activity relations and antioxidant properties of the Quercetin and Quercetin-Co^{2+} chelate from a theoretical view and to be used these compounds in the treatment of the diseases. We found that Quercetin is more stable than Quercetin-Co^{2+} chelate but Quercetin-Co^{2+} chelate is more conductive and the O22-H bond of the Quercetin molecule has the highest antioxidant activity. The remarkable electron delocalization occurred between the donor (C17-C19) anti bond and acceptor (C13-C15) anti bond with 319.62 kcal/mol stabilization energy in Quercetin.

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1. INTRODUCTION

Flavonoids are considerably available plant-derived polyphenol compounds with several biological and chemical activities. (Kasprzak *et al.*,2015). Due to the hydroxyl groups in their structure flavonoids are demonstrated antioxidant properties and with the help of these groups, they can coordinate metal ions and form complexes (Symonowicz *et al.*, 2012). Metal complexes of flavonoids have several stimulating features: they are colored, often fluorescent, anti-oxidant or pro-oxidant, antimicrobial, antiproliferative, and biologically ascendant in numerous different manners.

Generally, the free radical scavenging properties of flavone compounds constitute their antioxidant mechanism. The other mechanism for antioxidant behavior may occur from interactions between flavonoids and transition metal ions, preventing the participation of metal ions in free radical formation processes (Mira *et al.*, 2002). Recent studies have shown that metal-flavonoid complexes have favorable biological and pharmacological activities and some of them have been used successfully in clinical applications (Grazul *et al.*, 2009; Afanas'eva *et al.*, 2001).

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Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the most bioactive and prevalent nutritional flavonoids, which is widely found in the flowers, leaves, and fruits of many plants (Chen *et al.*, 2009; Bravo *et al.*, 2001). It has been notified that quercetin coincides with complexes with transition metal ions, such as Cu^{2+}, Mn^{2+}, and Fe^{2+}. Quercetin and its chelating activity with Cu^{2+}, Mn^{2+}, and Fe^{2+} reveal wide biological activities with increasing bioavailability, such as anti-oxidation, anti-bacterial, anti-tumor, and the talent to impress many kinds of enzymatic activities (Zhou *et al.*, 2001; Mendoza *et al.*, 2011). These quercetin/metallic ion complexes not only improve its bioavailability and alter the in vivo delivery route of quercetin but also encourage new pharmacological activity (Malesev *et al.*, 2007; Cornard *et al.*, 2002; Yamashita *et al.*, 1999).

In the literature, many papers cover specific aspects of the activity of flavonoid metal complexes, e.g. their antioxidant properties, enzyme-mimicking behavior, therapeutic potential, or use in chemical analysis. Furthermore, we need to know the physical properties of metal complexes of flavonoids as well as chemical properties. There are five antioxidant mechanisms we have explained previously in our article (Kiraz 2019; Torreggiani *et al.*, 2005; El Hajji *et al.*, 2006; Leopoldini *et al.*, 2006; Xu *et al.*, 2007; Jurasekova *et al.*, 2009; Dehghan *et al.*, 2011; Dolatabadi, 2011; Zhang *et al.*, 2011).

In this study, we have utilized the B3LYP/6-31++G(d,p) method to explore the antioxidant effects and structure-activity relationships of quercetin chelate with Co^{2+}. The electronic properties and various molecular descriptors such as the bond dissociation enthalpy (BDE), adiabatic ionization potential (AIP) of the chelate complexes have also been obtained and studied, which are relevant to show evidence of antioxidant activity.

2. MATERIAL and METHODS

Hybrid density functional theory (DFT) is one of the most successful quantum chemistry tools in describing the ground state and excited properties of metal complexes of flavonoids. The most popular DFT method is B3LYP (Becke3-Parameter method for calculating that part of the molecular energy due to overlapping orbitals plus the Lee-Yang-Parr method of accounting for correlation) (Tachibana *et al.*, 2002; Pai *et al.*, 2006; Zade *et al.*, 2006).

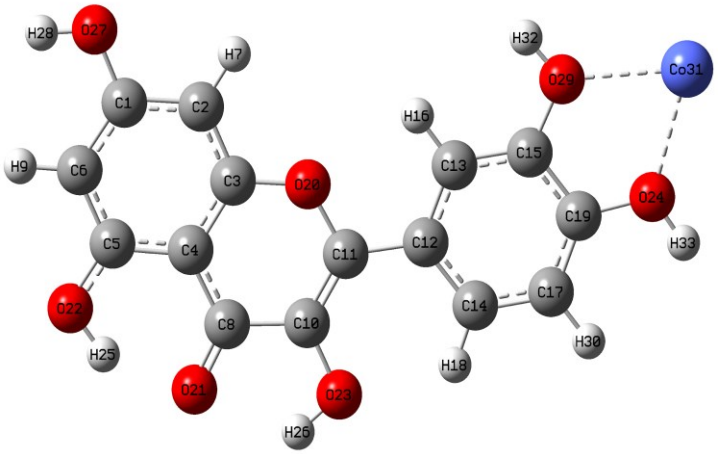
The molecular structure of the compound was optimized to get the global minima by using the B3LYP/6-31++G(d,p) level. In this study, geometric parameters (bond lengths and bond angles), the highest occupied molecular orbital (HOMO) energies, the lowest unoccupied molecular orbital (LUMO) energies, the electronic properties (total energy, dipole moment, electronegativity, chemical hardness, and softness), optical properties of a compound formed by Co^{2+} and flavonoid have been performed by using Gaussian 16 (Frisch *et al.*, 2016) and GaussView 6.0 (Dennigton *et al.*, 2016) was used for visualization of the structure. We calculated the probability of transitions between a ground state and excited states by using time-dependent density functional theory (TD-DFT). The major contribution rate of HOMO–LUMO orbitals is determined by using the GaussSum 2.2 program (Joseph *et al.*, 2014).

3. RESULTS / FINDINGS

To examine the electronic and antioxidant properties, it is necessary to know the most stable state of Quercetin and Quercetin-Co^{2+} chelate. These calculated structures (Yalçın, 2019) are shown in Figure 1 and Figure 2.

Figure 1. Optimized geometry of Quercetin.

Figure 2. Optimized geometry of Quercetin chelate with Co^{2+}.



3.1. Electronic Properties

Electronic parameters of Quercetin and Quercetin-Co^{2+} chelate are presented in Table 1 (Yalçın, 2019). Since HOMO-LUMO energy difference (ΔE) represents the chemical reactivity of a molecule.

Table 1. Molecular descriptors of Quercetin and Quercetin-Co^{2+} chelate calculated at B3LYP/6-31++G(d,p) level.

Parameters	Quercetin	Quercetin−Co^{2+}
E_{LUMO} (eV)	-2.29	-2.55
E_{HOMO} (eV)	-5.93	-5.29
$\Delta E = E_{LUMO} - E_{HOMO}$ (eV)	3.64	2.74
I (ionization potential) (eV)	5.93	5.29
A (electron affinity) (eV)	2.29	2.55
χ (electronegativity) (eV)	4.11	3.92
η (global hardness) (eV)	1.82	1.37
S (global softness) (eV^{-1})	0.55	0.73
μ (electronic chemical potential) (eV)	-4.11	-3.92
ω (global electrophilicity index) (eV)	4.64	5.60

As seen from Table 1, the energy difference between HOMO and LUMO orbitals of Quercetin and Quercetin-Co^{2+} chelate are 3.64 and 2.74 eV, respectively. According to these ΔE values, Quercetin -Co^{2+} chelate is more conductive than Quercetin but the other parameters are quite close to each other. Especially, when we examine the global hardness and softness values of the molecules, the higher global hardness values are the evidence of the low reactivity of the molecule.

Figure 3. Molecular orbitals compositions of Quercetin.

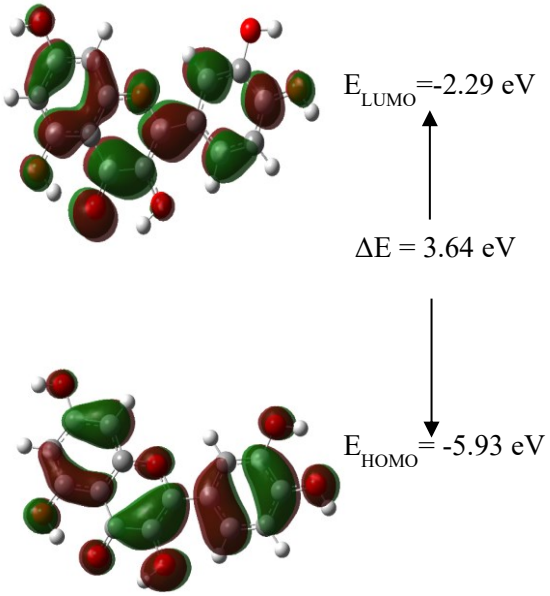
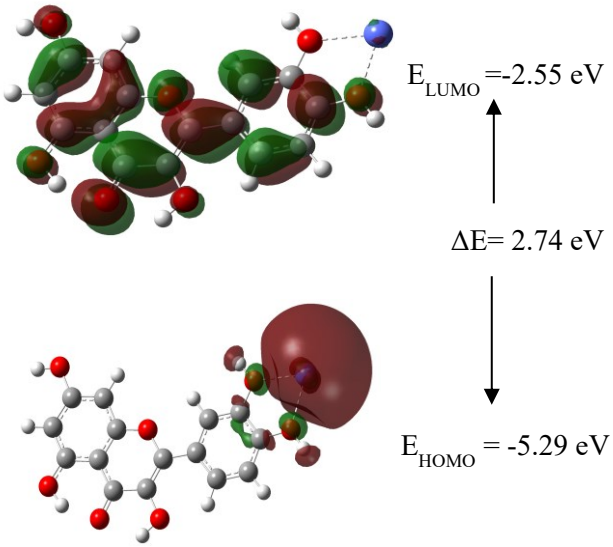


Figure 4. Molecular orbitals compositions of Quercetin-Co^{2+} chelate.



As seen from Figure 3; HOMO and LUMO orbitals are distributed throughout the Quercetin molecule. The LUMO orbitals are spread over the whole molecule while the HOMO orbitals are on Co^{2+} seen in Figure 4.

TD-DFT calculations in the gas phase were carried out on Quercetin and Quercetin-Co^{2+} put account B3LYP/6-31++G(d,p) functional to understand the electronic transitions of the compounds. Table 2 shows the electronic transitions, major contributions, calculated absorption peaks (λ_{max}'s), excitation energies, oscillator strengths (f), and assignments of the transitions of the Quercetin and Quercetin-Co^{2+} molecules.

The electronic absorption peak (at 373 nm for Quercetin and 4565 nm for Quercetin-Co^{2+}) corresponds to the transition from the ground state to the first excited state, which corresponds to HOMO to LUMO excitation with high oscillator strengths. This band arises from an $n \rightarrow \pi^*$ transition (Kiraz, 2020). The second absorption band at 330 nm arises from HOMO-1 to LUMO transition for Quercetin and at 4193 nm arise from HOMO-2 to LUMO+12 for Quercetin-Co^{2+}. However, the oscillator strength for the second transition is lower than the first transition for Quercetin and 0 for all the transitions of the Quercetin-Co^{2+}. The third absorption peak at 305 nm for Quercetin arises from HOMO-2 to LUMO and the 2810 nm peak for Quercetin-Co^{2+} arises from HOMO to LUMO excitation. Also, for the Quercetin, the oscillator strength for the second transition is lower than the third transition.

Table 2. Calculated absorption wavelengths, energies, and oscillator strengths of quercetin and quercetin-Co+2 using the TD-DFT method at the B3LYP/6-31++G(d,p) level.

Quercetin				
Excitation Major Cont.	CI expansion coefficient	WL Calc. gas phase (nm)	Excitation Energy (eV)	Osc. Str. (f)
Excited State 1	Singlet-A			
77 → 79 (3%) (HOMO-1 → LUMO)	0.12996	373.16	4.99	0.4253
78 → 79 (93%) (HOMO →LUMO)	0.68078			
Excited State 2				
77 → 79 (94%) (HOMO-1 → LUMO)	0.68399	330.26	4.06	0.0853
78 → 79 (3%) (HOMO → LUMO)	-0.13194			
Excited State 3				
76 → 79 (90%) HOMO-2 → LUMO	0.66933	304.89	4.07	0.0980
78 → 82 (5%) HOMO → LUMO + 3	0.16101			

Quercetin-Co^{+2}				
Excitation	CI expansion coefficient	WL Calc. Water (nm)	Excitation Energy (eV)	Osc. Str. (f)
Excited State 1	Singlet-A			
90B → 104B (50%) HOMO-1 → LUMO + 12	-0.70437	4565.32	0.27	0.0000
90B → 108B (29%) HOMO-1 → LUMO + 16	-0.05248			
Excited State 2				
89B → 104 (47%) HOMO-2 → LUMO + 12	0.68673	4193.09	0.30	0.0000
89B → 105B (27%) HOMO-2 → LUMO + 16	-0.22732			
Excited State 3				
91B → 92B (99%) HOMO → LUMO	0.99553	2809.92	0.44	0.0000

3.2. Antioxidant Properties

For the antioxidant property; the significant parameters are bond dissociation enthalpy (BDE) which is associated with hydrogen atom transfer (HAT) mechanism and adiabatic ionization potential (AIP) which is related to the single electron transfer (SET) mechanism. These parameters are acquired by donating a hydrogen atom or single electron from the OH bonds (Leopoldini *et al.*, 2011). The lower BDE value remarks the better antioxidant activity of the molecule (Urbaniak *et al.*, 2012).

Table 3. Antioxidant Parameters of Quercetin and Quercetin-Co^{+2} chelate.

Antioxidant Parameters	Quercetin		Quercetin-Co^{+2}	
	O22 − H	O23 − H	O22 − H	O23 − H
BDE (kcal/mol)	393.78	398.31	442.82	429.51
AIP (kcal/mol)	166.36	166.36	158.68	158.68
PDE (kcal/mol)	-32.15	-27.61	24.58	11.28
PA (kcal/mol)	72.17	62.51	183.26	65.37
ETE (kcal/mol)	62.05	76.23	109.90	104.58

According to Table 3, calculated BDE values in the gas phase specify that the O22-H bond of the Quercetin molecule has the highest antioxidant activity while the O22-H bond of the Quercetin-Co^{2+} chelate is the lowest antioxidant capacity.

Proton dissociation enthalpy (PDE) with the AIP are also important parameters for the antioxidant capacity. The low value of the PDE parameter express that a single electron transfer followed by a proton transfer (SET-PT) mechanism is energetically chosen for the antioxidant activity (Kiraz, 2019). For the calculated values of PDE, the O22-H bond of the Quercetin has much more antioxidant activity than the O22-H bond of the Quercetin-Co^{2+} chelate.

The sequential proton loss electron transfer (SPLET) mechanism is another significant antioxidant mechanism in which antioxidants grab free radicals and also the radical scavenging activity of a molecule can be assayed with this mechanism (Urbaniak *et al.*, 2012). For this mechanism, the proton affinity (PA) and electron transfer enthalpy (ETE) parameters are very substantial. The O23-H bond of the Quercetin has the lowest PA value and the O22-H bond of the Quercetin has the lowest ETE value. Therefore, the SPLET mechanism is not preferred for the antioxidant activity for Quercetin and Quercetin-Co^{2+} chelate.

3.3. NBO Analysis

Natural Bond Orbitals (NBO) analysis is a remarkable method to determine intra and intermolecular bonding and charge a transfer or conjugative interaction in molecular systems (Tomasi *et al.*, 2005). The second-order Fock matrix was executed to utilize the donor-acceptor interactions (Snehalatha *et al.*, 2009). The significant element of the Fock matrix is the stabilization energy $E^{(2)}$ associated with the delocalization and a greater $E^{(2)}$ value presented the donating inclination from electron donors to electron acceptors.

The NBO analysis expresses conspicuous donor-acceptor type delocalization from lone-pair (LP) of oxygen orbitals with anti-lone-pair (LP*) of a metal orbital. The delocalization effects because of the LP-LP* interactions in the molecule play an excessively conspicuous task on the coordination environments of the Co^{2+} ion (Kiraz, 2017). As seen from Table 4; the significant

interactions of Quercetin compound are π*(C8-O21) → π*(C3-C4), π*(C10-C11) → π*(C12-C14), π*(C3-C4) → π*(C5-C6), π*(C17-C19) → π*(C12-C14), π*(C17-C19) → π*(C13-C15) which have stabilization energies 214.33, 201.67, 313.25, 319.62 and 298.96 kcal/mol, respectively (Yalçın, 2019).

Table 4. Second-order perturbation energies E^2 (kcal/mol) corresponding to the most important charge transfer interaction (donor-acceptor) in Quercetin by DFT/B3LYP/6-31++G(d,p) method.

Donor	Type	ED/e	Acceptor	Type	ED/e	E^2 (kcal/mol)
C1-C2	π	1.66161	C3-C4	π*	0.49121	29.19
			C5-C6	π*	0.36994	12.49
C3-C4	π	1.62687	C1-C2	π*	0.40740	12.42
			C5-C6	π*	0.36994	24.69
			C8-O21	π*	0.41495	34.97
C5-C6	π	1.69283	C1-C2	π*	0.40740	27.93
			C3-C4	π*	0.49121	12.20
C10-C11	π	1.76811	C8-O21	π*	0.31363	24.61
			C12-C14	π*	0.40886	10.63
C12-C14	π	1.64600	C10-C11	π*	0.31363	17.65
			C13-C15	π*	0.35887	19.64
			C17-C19	π*	0.02505	19.56
C13-C15	π	1.67621	C12-C14	π*	0.40886	18.30
			C17-C19	π*	0.40455	20.52
C17-C19	π	1.68530	C12-C14	π*	0.40886	19.45
			C13-C15	π*	0.35887	18.05
O20	LP2	1.75006	C3-C4	π*	0.49121	30.68
			C10-C11	π*	0.31363	27.91
O21	LP2	1.85643	C4-C8	σ*	0.04909	12.13
			C8-C10	σ*	0.06078	15.98
			O22-H25	σ*	0.05798	17.20
O22	LP2	1.81463	C5-C6	π*	0.36994	39.67
O23	LP2	1.86352	C10-C11	π*	0.31363	31.40
O24	LP2	1.88412	C17-C19	π*	0.40455	25.89
O28	LP2	1.85642	C1-C2	π*	0.40740	30.75
C3-C4	**π***	**0.49121**	**C5-C6**	**π***	**0.36994**	**313.25**
C8-O21	**π***	**0.41495**	**C3-C4**	**π***	**0.49121**	**214.33**
			C10-C11	π*	0.31363	107.82
C10-C11	**π***	**0.31363**	**C12-C14**	**π***	**0.40886**	**201.67**
C17-C19	**π***	**0.40455**	**C12-C14**	**π***	**0.40886**	**319.62**
			C13-C15	**π***	**0.35887**	**298.96**

Table 5. Second-order perturbation energies E^2 (kcal/mol) corresponding to the most important charge transfer interaction (donor-acceptor) in Quercetin-Co^{2+} by DFT/B3LYP/6-31++G(d,p) method.

Donor	Type	ED/e	Acceptor	Type	ED/e	E^2 (kcal/mol)
C1-C2	π	0.83323	C3-C4	π*	0.24413	14.39
C3-C4	π	0.81404	C5-C6	π*	0.18601	12.45
			C8-O21	π*	0.20990	17.29
C5-C6	π	0.84687	C1-C2	π*	0.20380	13.98
C10-C11	π	0.88069	C8-O21	π*	0.20990	12.59
C19	**LP1**	**0.50954**	**C14-C17**	**σ***	**0.00631**	**31.33**
O20	LP2	0.87479	C3-C4	π*	0.24413	15.48
			C10-C11	π*	0.15911	13.80
O22	LP1	0.90750	C5-C6	π*	0.18601	19.85
O23	LP2	0.93154	C10-C11	π*	0.15911	15.67
O27	LP2	0.92933	C1-C2	π*	0.20380	15.23
Co31	LP1	0.99873	C13-C15	σ*	0.01370	0.03
			C17-C19	σ*	0.01456	0.03
			C19-O24	σ*	0.01315	0.11
	LP3	0.99508	C13-C15	σ*	0.01370	0.15
			C15-O29	σ*	0.01164	0.14
			C17-C19	σ*	0.01456	0.15
			C19-O24	σ*	0.01315	0.40

The most important interaction for the Quercetin-Co^{2+} chelate is the LP1(C19) → σ*(C14-C17) with the stabilization energy of 31.33 kcal/mol. The other interactions are generally π* orbitals between C-C bonds. From Table 5; LP2 (20) → π*(C3-C4), LP2 (O22) → π*(C5-C6), and LP (O23) → π*(C10-C11) have stabilization energies are 15.48, 19.85, and 15.67 kcal/mol, respectively.

4. DISCUSSION and CONCLUSION

In this study, we have compared the electronic and antioxidant properties of the Quercetin and Quercetin-Co^{2+} chelate. The energy values of HOMO-LUMO orbitals present that the Quercetin-Co^{2+} chelate is more kinetically reactive than the Quercetin molecule. The absorption peaks with non-negligible oscillator strengths for Quercetin at a longer wavelength of 305~373 nm compared with those for the Quercetin-Co^{2+} chelate at 4600~2800 nm. For the antioxidant capacity, the O22-H bond of the Quercetin molecule has the highest antioxidant activity which prefers the SET-PT antioxidant mechanism. The important electron delocalization was observed between the donor (C17-C19) anti bond and acceptor (C13-C15) anti bond with the stabilization energy 319.62 kcal/mol in Quercetin. The highest antioxidant property was observed in the Quercetin molecule where the highest electron delocalizations occurred.

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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

Authors are expected to present author contributions statement to their manuscript such as; **Fatih Yalcin**: Investigation, Resources, Software, Formal Analysis, Writing Original Draft. **Asli Ozturk Kiraz**: Methodology, Supervision, Visualization, Formal Analysis, Writing Original Draft, and Validation.

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Research Article

A Study on the Possible Mutagenicity of Different Types of Plant Growth Regulators

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Abstract: It is known that most chemicals, which have been increasingly used since ancient times, have carcinogenic and mutagenic effects. As an alternative to these chemicals, natural compounds, such as plant growth regulators (PGRs) have been used. However, are the PGRs used to obtain efficient plants in agriculture as harmless as it is thought today when it is essential to access natural foods in the food and agriculture sectors? Therefore, this study investigates the mutagenic activity of two plant growth regulators (Kinetin, Chlormequat chloride (CCC)) using the Ames/*Salmonella* short-time test system. Experiments were performed in the presence (+ S9) or absence (-S9) of metabolic activation enzymes using TA 98 and TA 100 strains of *Salmonella typhimurium*. 5 non-cytotoxic doses for each test substance were investigated. The results were evaluated by comparing them with spontaneous control plates. According to the results, a 2500 µg/plate dose of Kinetin was to be found mutagenic on the strain TA 98 in the presence and in the absence of S9 enzyme, and on the strain TA 100 in the absence of the S9 enzyme. The other substance CCC did not cause mutagenic effects on the bacterial strains.

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1. INTRODUCTION

Parallel to the advancement of technology, the number of chemicals used in agriculture and food is increasing (Tomatis, 1979; Öncül, 2009). Therefore, the identification of substances capable of inducing mutations has become an important procedure in safety assessment (Mortelmans & Zeiger, 2000).

Plant growth regulators (PGRs) are organic compounds, other than nutrients, that modify plant physiological processes. Specific PGRs are used to modify crop growth rate and growth patterns during the various stages of development, from germination through harvest and post-harvest preservation (Harms, 1988). Concerns about the unconscious use of plant growth regulators are increasingly being expressed. These are plant growth regulators that act as synthetic auxins or plant hormones that alter plant metabolism. It is widely used as a herbicide in agriculture and forestry to control broadleaf weeds (Uysal, 2010).

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PGRs fall into five groups: auxins, gibberellins, cytokines, abscisic acid, and ethylene. In addition to these, brassinosteroids, polyamines, jasmonates, oligosaccharin, and salicylates are also considered in this class by a number of researchers (Basra, 2000; Morsünbül *et al*., 2010).

The cytokines discovered during the investigation of the factors that stimulate the division of plant cells (cytokinesis) in subsequent studies are effective in many physiological and developmental events, such as the transport of nutrients, apical dormancy, formation and activity of the stem apical meristem, leaf development, breakage of bud dormancy and seed germination. In addition, it has been determined that cytokines play a role in many light-controlled developments, such as chloroplast differentiation, autotrophic metabolism development, leaf, and cotyledon expansion (Akman & Darıcı, 1998; Tiaz & Zeiger, 2008).

Kinetin is a synthetic cytokine which is first discovered (Özen & Onay, 2007; Tiaz and Zeiger, 2008). The big difference with this substance compared to others is that it is essentially a by-product of DNA degradation by heat, and it affects growth, especially by promoting cell division. In this by-product, the adenosine is displaced from position 9 to position 6 on the ring (Tiaz & Zeiger, 2008). Kinetin also maintains the synthesis of protein and nucleic acid, thereby ensuring the longevity of cut flowers (Güleryüz, 1982; Westwood, 1993; Kaynak & Ersoy, 1997).

Chlormequat chloride (CCC) is a synthetic PGR that prevents the growth of plant height. Although CCC prevents elongation in grains, it is widely used to prevent lying in grains. It is also applied to increase the fruit set in grapes. Due to a belief that it has a carcinogenic effect, its use has been limited to ornamental plants (Sağlam, 1991; Kaynak & Ersoy, 1997).

The Ames Test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs because there is a high predictive value for carcinogenicity when a mutagenic response is obtained (McCann *et al.,* 1975; Zeiger *et al.,* 1990). In this work bacterial reverse mutation assay (Ames test) was used to evaluate the mutagenic potential of the two-plant regulator (Kinetin, Chlormequat Chloride) widely used as plant growth regulators.

2. MATERIAL and METHODS

2.1. Material

The Kinetin that tested for mutagenicity is in the cytokinins group. The second substance is Chlormequat chloride (CCC), the other regulator that inhibits plant growth for the determination of mutagenicity. As the positive control mutagen, while 4-Nitro-o-phenylenediamine was used in the absence of S9 mix, 2-aminofluorene (2AF) was used in the presence of S9 mix for *S. typhimurium* TA 98 strains. As the positive control mutagen for *S. typhimurium* TA 100 strains, sodium azide (SA) in the absence of S9 mix, and 2AF was used in the presence of S9 mix (Table 1).

Table 1. Some chemical properties of the plant growth regulators and positive control examined.

Common Name	Chemical name	Chemical Formula	Purity (%)	Usage as	Reference
Kinetin	6-furfuryl-aminopurine	$C_{10}H_9N_5O$	≥98%	Plant growth regulator	Merck Inc. (2021a)
CCC	Chlormequat chloride	$C_5H_{13}Cl_2N$	100 %	Plant growth regulator	Merck Inc. (2021b)
SA	Sodium Azide	NaN_3	99.99%	Potent mutagen	Merck Inc. (2021c)
2AF	2-aminofluorene	$C_{13}H_{11}N$	98%	Potent mutagen	Merck Inc. (2021d)
4-NOP	4-Nitro-o-phenylenediamine	$C_6H_7N_3O_2$	98%	Potent mutagen	Merck Inc. (2021e)

2.1.1. *The test strains*

In the study were used TA 98 (his D 3052, rfa, Δuvr B, +R) and TA 100 (his G 46, rfa, Δuvr B, +R) strains were used, having been developed by in vitro mutations from LT2 wild strains of *S. typhimurium* by Ames et al. TA 98 strains determined the frameshift mutations and TA 100 strains determined the base-pair substitution mutations.

2.1.2. *The dose of test substance and preparation*

The plant growth regulators were weighed in a proportion and were dissolved in dimethyl sulfoxide (DMSO) and stored at room temperature. Doses of all the substances were prepared individually, and cytotoxic doses were determined. After determining the cytotoxic dose, 5 doses were selected for each item and mutagenesis experiments were applied to these doses. Non-cytotoxic doses of 2500-1000-250-25-2.5 µg/petri were chosen for the Kinetin and 1600-800-400-200-100 µg/petri for the CCC.

2.2. Methods

Preparation of stock cultures of *S. typhimurium* TA 98 and TA 100 strains, controlling the genetics of the bacteria, and an Ames/*Salmonella*/microsome test were conducted by the petri incorporation method, according to the procedure of Maron and Ames (Maron & Ames, 1983). In this method, two different agars, as Minimal Glucose Agar (MGA) and top agar, are used. The basis of the method is the counting of revertant colonies that grow between the upper layer (top agar) and the lower layer (MGA).

The metabolites of chemicals may also have mutagenic effects. Therefore, the S9 microsomal enzyme is also used to measure the mutagenic effects of the metabolites. In this manner, the determination of direct and indirect effects of mutagenic substances were studied. Experiments were performed both with and without an S9 mix in the two groups. Each dose tested using three Petri dishes and independent experiments were carried out at different times. The tests were repeated when needed. Positive controls, solvent control (DMSO), and spontaneous controls were also performed as parallel experiments. Spontaneous control petri dishes that only containing bacteria and histidine/biotin solution were used to calculate the number of colonies that automatically return from each strain. After incubation for 48-72 hours, histidine-dependent bacterial colony counts were taken from the average value of the results obtained. Standard deviation values were calculated for these results.

In the mutation testing, a rotating force of the test substance is measured from mutant strain to wild strains, whether with the original mutation. Therefore, the strains are controlled to see whether they have the genotype in terms of mutant characters.

2.2.1. *Determination of the cytotoxic effects of the test substances*

Before starting the mutagenicity assay, the cytotoxic effect (LD_{50}) should be determined. The importance of determining the LD_{50} doses that can cause inhibition on test bacteria is that it will not result in a mutagenicity test. Primarily, containing the appropriate number of bacteria ($1\text{-}2x10^9$) of the overnight culture was made up to 10^{-6} dilution using a broth. To determine the cytotoxic effect it is always used in dilution 10^{-6} of culture. 0.1 ml overnight bacterial culture and 0.1 ml of various concentrations of the test substances were added to the top agar. After this, the mixture was spread over Nutrient Agar (NA) petri dishes, and the petri dishes were incubated at 37 ºC for 24 hours. At the end of incubation, the colonies were counted (Dean *et al.,* 1985). A comparison with the control dishes without the addition of chemicals toxic and nontoxic doses was determined. Less than half of the number of colonies in the control plates were considered of a cytotoxic dose.

2.2.2. *The Ames Test*

In the assay without S9, 0.3 ml of histidine/biotin solution, 0.1 ml of various doses of the test substance, and 0.1 ml of bacterial cultures that had been incubated for 12-16 hours were added to tubes containing 3 ml top agar at 45^0C. The tubes spread to the MGA Petri dishes. After allowing to freeze, the petri dishes were reversed and located in the incubator at 37 ^{o}C for incubation over 48-72 hours. After incubation a count of colonies was counted.

In spontaneous control, histidine/biotin solution and the bacterial culture were added in the top agar tubes, 0.1 ml of DMSO was added to the DMSO control, in addition to the histidine/biotin solution and the bacterial culture. In the positive control experiment, the histidine/biotin solution, bacteria culture, and the absolute mutagenic substances specific to the strain were added. Experiments were performed in five doses for the Kinetin and CCC substances, and for each dose three petri dishes were used.

An S9 mix was prepared fresh immediately before the S9 (+) experiment. After adding the 3 ml of top agar, the histidine-biotin solution, the bacterial culture, and the tested substances as mentioned in the S9(-) experiments, then 0.5 ml of ice-held S9 mix was added. The mixture uniformly spread on MGA petri dishes. When the agar solid was incubated in an incubator for 72 hours at 37 ^{o}C. Spontaneous, positive, and dimethyl sulfoxide controls were prepared by adding 0.5 ml of the S9 mix in the same manner. After incubation, the colonies were counted manually, and the results were analysed.

3. RESULTS / FINDINGS

For a substance to be called a mutagenic agent, the number of colonies counted in mutagenicity test results must be at least twice the number of spontaneous revertant colonies. 2-3 times that of spontaneous revertant number of suspected mutagen colonies, and three times more than those which are mutagenic. Which is less than twice the number of spontaneous revertant with the results being evaluated as non-mutagenic (Maron & Ames, 1983, Emig *et al.,* 1996).

3.1. Results of Salmonella/Microsome Mutagenicity Test

The plate incorporation test results for *S. typhimurium* TA 98 and TA 100 in the presence and absence of the S9 enzyme are given in Table 2. Considering Table 2, a 2500 µg/petri dose of Kinetin was found to be mutagen in tests conducted without the S9 on the TA 98 strain. Other doses of Kinetin were found to be non-mutagenic.

Table 2. The mean and standard deviation of the results of mutagenicity testing of 5 dose of the Kinetin.

KINETIN	TA 98		TA 100	
	S9(-)	S9(+)	S9 (-)	S9(+)
Spontaneous Control (SC)	32.5±2.5	56.5±3.5	168±26	188±13
DMSO Control (DC)	32.5±2.5	58.5±7.5	189±9	176.5±5.5
2,5 µg/ petri	23±0[a]	64.5±0.5 [a]	231±20 [a]	169±37 [a]
25 µg/ petri	38.5±0.5 [a]	75±3 [a]	235.5±31 .5 [a]	215.5±34.5[a]
250 µg/ petri	43.5±5.5 [a]	88.5±4.5 [a]	248±16 [a]	290.5±46.5[a]
1000 µg/ petri	56±1 [a]	86.5±4.5 [a]	252±10 [a]	328±70 [a]
2500 µg/ petri	694±437 [b]	788±352 [b]	247.5±17.5 [a]	834±206 [b]

Table 2. *Continues*

	Positive Controls (PC)			
4-Nitro-o- phenylenediamine (100 µg/ petri)	1457±497	-	-	-
Sodium Azide (100 µg/ petri)	-	-	862±216	-
2AF (100 µg/ petri)	-	658±39	-	688±13

a: negative, b: positive, c: suspected mutagen

As can be seen in the results in Table 3, all doses of the CCC are determined to be non-mutagenic in the presence and absence of the S9 enzyme. Chemical dose-response curves of bacteria were tested with the test substances shown in the following chart.

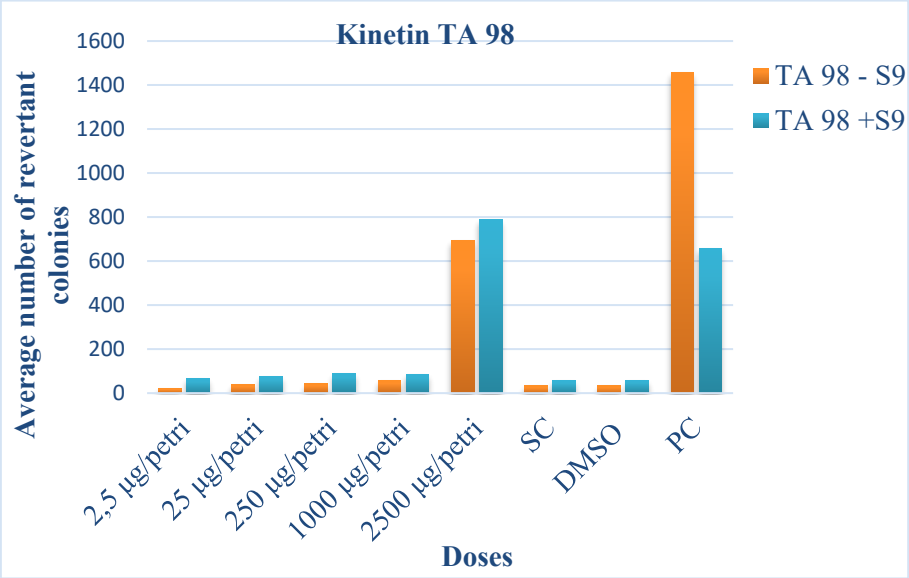
Table 3. The mean and standard deviation of the results of mutagenicity testing of 5 dose of CCC.

CCC	TA 98		TA 100	
	S9 (-)	S9(+)	S9 (-)	S9(+)
Spontaneous Control (SC)	32±2	59±8	130.5±7.5	142±13
DMSO Control (DC)	27.5±2.5	60.5±0.5	143±7	154.5±32.5
100 µg/ petri	38±7[a]	35.5±4.5 [a]	136±24 [a]	128.5±2.5 [a]
200 µg/ petri	28.5±9.5 [a]	67.5±1.5[a]	103.5±4.5 [a]	150.5±3.5[a]
400 µg/ petri	38.5±18.5 [a]	65±22 [a]	155.5±4.5 [a]	183.5±24.5[a]
800 µg/ petri	36±2 [a]	87±2 [a]	136±8 [a]	186±33 [a]
1600 µg/ petri	49±1 [a]	86.5±3.5 [a]	166.5±16.5[a]	182±21 [a]
	Positive Controls (PC)			
4 Nitro-o- phenylenediamine (100 µg/ petri)	643±317	-	-	-
Sodium azide (100 µg/ petri)	-	-	646.5±22.5	-
2AF (100 µg/ petri)	-	535±25	-	434±30

a: negative, b: positive, c: suspected mutagen

As can be seen in Figure 1, when the mutagenic activity of the Kinetin was investigated in the presence and absence of the S9 enzyme on the TA 98 strain, it was found that the number of colonies obtained was more than twice the number of spontaneous colonies, and it was determined that a 2500 µg/petri dose of Kinetin was mutagenic against the TA98 strain.

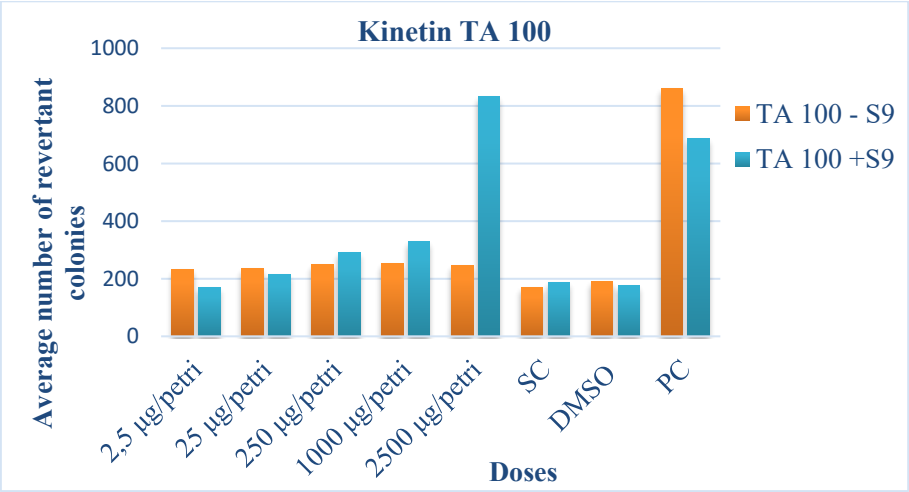
Figure 1. The dose-response chart of Kinetin in the experiments with or without S9 on the TA 98.



*Doses of SC,PC and DMSO controls are 100 µg/petri

As can be seen in Figure 2, when the mutagenic activity of the Kinetin substance was investigated in the presence and absence of the S9 enzyme on the the TA100 strain, since the number of colonies obtained in the presence of the S9 enzyme was determined to be more than twice the number of spontaneous colonies, it was determined that a 2500 µg/petri dose of Kinetin was mutagenic against the TA100 strain.

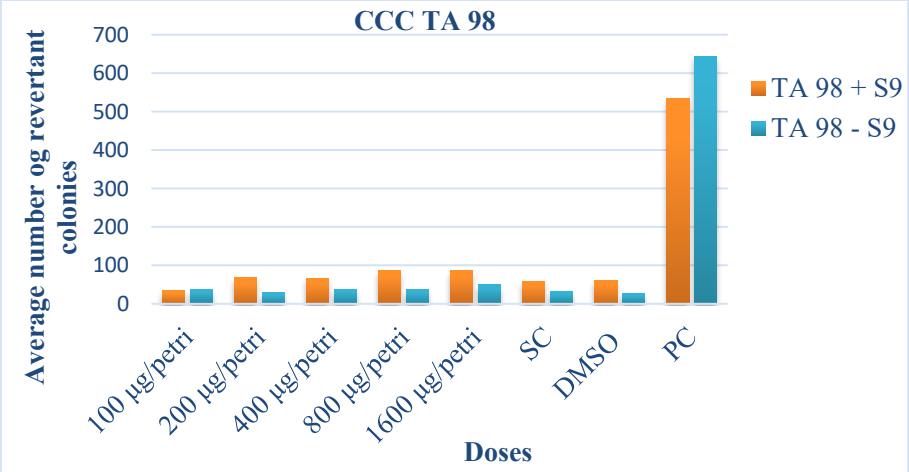
Figure 2. The dose-response chart of Kinetin in the experiments with or without S9 on the TA 100.



*Doses of SC,PC and DMSO controls are 100 µg/petri

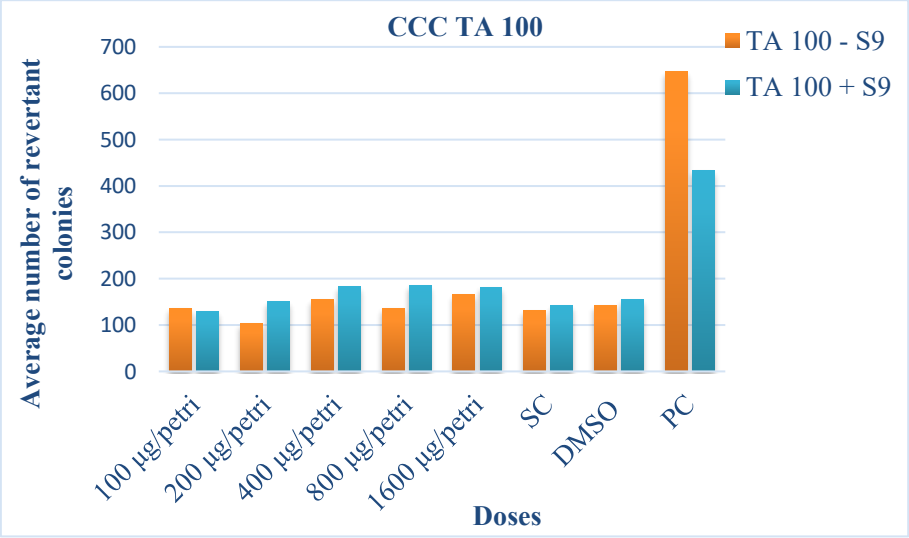
When Figures 3-4 are examined, the count of colonies of the experiments of the CCC in the presence of and absence of the S9 enzyme were two times less or similar to the number of colonies of spontaneous control. Therefore CCC was evaulated as a "non-mutagenic agent". According to the numbers achieved, the CCC was not mutagenic on the TA 98 and TA 100 strains. 2500 µg/petri of Kinetin was identified as mutagenic on the TA 98 and TA 100 strains.

Figure 3. The dose-response chart of the CCC in the experiments with or without S9 on the TA 98.



*Doses of SC,PC and DMSO controls are 100 µg/petri

Figure 4. The dose-response chart of the CCC in the experiments with or without S9 on the TA 100.



*Doses of SC,PC and DMSO controls are 100 µg/petri

4. DISCUSSION and CONCLUSION

There are several of synthetic and natural substances with unknown biological effects in our environment (Bağcı, 1985; Forman & Ames, 1991). In fact, any chemicals that do not show directly can create genetic damage in humans (Ames, 1985). Small amounts of various chemicals in our environment can cause mutations or cancer today (Dökmeci, 1988; Vural, 1984). One of the most important issues in the application of PGR is the user level that will not harm the humans, the environment, or plants and harvest before a certain time of these substances (Halloran & Kasım, 2002). In terms of acute toxicity, the effects of PGR are not immediately lethal, but the experimental studies show that they may cause harmful effects for carcinogenic, immune-toxic, reproductive, and so on, if there is long-term exposure to these substances (Çetinkaya & Baydan, 2006). For all these reasons, in our research, we aim to investigate two plant growth regulators frequently used in agriculture that have potential mutagenic effects and whether they are carcinogenic.

The Ames Test is a widely accepted and simple test that can be used to determine the mutagenic and antimutagenic potential of various chemicals, pesticides and hormones, foods,

drugs, and so on that can cause gene mutations and which have a high predictive value for in vitro carcinogenicity (Zeiger *et al.*, 1990; Pillco & de la Peña, 2014). Therefore, in this study, the incorporation the Ames Test using *S. typhimurium* TA98 and TA100 was employed to assess the mutagenic activity of Kinetin and CCC in the presence or absence of metabolic activation with an S9 fraction.

As stated by Ames (1973), spontaneous conversion of bacterial strains from a his- to a his+ state is possible within certain limits (20-50 revertant/plate for TA 98; 75-200 revertant/plate for TA 100). As a result of our research, for the TA98 strain, the mean number of spontaneous colonies was found to be 56.5±3.5 and a 59±8 revertant/plate in the presence of the S9 enzyme; the other hand spontaneous colonies were found to be 32.5±2.5 revertant/plate, 32±2 revertant/plate, and 41.5±6.5 revertant/plate were found in the absence of the S9 enzyme. For the TA 100 strain, the mean number of spontaneous colonies was 188±13 revertant/plate and 142±13 revertant/plate in the presence of the S9 enzyme and, on the other hand 134±4 revertant/plate, 168±26 revertant/plate, 130.5±7.5 revertant/plate, and 125.5±13.5 revertant/plate were found in the absence of the S9 enzyme.

At the start of our research, cytotoxic doses of Kinetin were determined. Since the number of colonies obtained from the petri dishes in which kinetin was applied at a dose of 10000 µg/petri, was below half the number of colonies obtained in the control petri dish, it was determined as cytotoxic. Therefore, 2.5-25-250-1000-2500 µg/petri doses of the kinetin substance were started in the study.

According to data obtained, a 2500 µg/ petri dose of Kinetin was evaluated as mutagenic for TA 98 strains in the presence and absence of the S9 enzyme. In experiments conducted in the presence of the S9 enzyme, a 2500 µg/petri dose of Kinetin for TA 100 strains was evaluated as mutagenic. Therefore, Kinetin was seen to be effective as mutagenic as frameshift and point mutations. The mutagenic effect that was initially not determined, but determined after the addition of the S9 enzyme for TA 100 strains, can be formed due to the resulting intermediate compound of the tested substance being metabolized by enzymes.

Yeşilada (2000) investigated the effect of Kinetin on mutant wing spots induced by mutagen EMS using the somatic mutation and recombination test of *Drosophila melanogaster* in a study. The 10^{-3} M concentration of Kinetin decreased the number of double spots, while the 10^{-4} M concentration caused an increase in the number of all types of spots. The change in the effect of Kinetin at high concentrations may be due to its mutagenic effect. In our study, while no mutagenic effect was observed at 2.5, 25, 250, 1000 µg/Petri doses of Kinetin, a significant difference was observed in terms of mutagenicity at a 2500 µg/Petri dose.

Kappas (1983) investigated the genotoxic activity of Kinetin (6-furfuryl-aminopurine) on *Aspergillus nidulans*. According to the test results, Kinetin did not increase the somatic separation frequency of *A. nidulans*. He also stated that S9 did not increase the effect of Kinetin in tests using the metabolic activation technique. However, in our study, Kinetin was not found to be mutagenic in the absence of the S9 enzyme on the TA 100 strain, while it was observed to be mutagenic in the presence of the S9 enzyme.

All the doses of CCC were evaluated as non-mutagenic in the presence and absence of the S9 enzyme in our research. When a literature review is conducted, mutagenicity data related to the the CCC substance, no genotoxic or mutagenic effect was found. However, in a study conducted by Sussmuth and Lingens (1976), while CCC did not show mutagenic effects on the *E. coli* K12 strain, valine sensitive in 0.2 to 0.5 M concentration and of a pH between 5 and 8, showed mutagenic effects at pH 9. In addition, it was found that maternal fertility may be affected at a high dosage of chlormequat in certain rodents (EFSA, 2008). For these reasons, it

could be thought that CCC does not cause frameshift and base-pair change mutations, while living things can produce different effects.

In terms of human health, the use of a combination of several short-time test systems instead of a single test system makes the results more reliable in determining the mutagenic properties of chemical compounds (Ennever & Rosenkranz, F 1986; Hofnung & Quillardet, 1986; McDaniels, 1990; Quillardet & Hofnung, 1988). In order for the test substances to be specified as mutagen or non-mutagen with certainty, these substances should be evaluated using different test systems, the results should be compared, and a reliable conclusion should be reached in this way.

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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

Authors are expected to present author contributions statement to their manuscript such as. **Sevilay Yapici**: Investigation, Resources, Visualization, Formal Analysis, and Writing - original draft. **Guven Uraz**: Methodology, Supervision, and Validation. **Ebru Beyzi**: Investigation, Methodology, Supervision, and Validation.

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