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Evaluation of Antioxidant and Antimicrobial Activities of *Stachys* Leaves Extract

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ABSTRACT

The genus *Stachys* L. (Lamiaceae) includes about 300 species located in temperate and tropical regions across the world Mediterranean, Asia, America and southern Africa. Several species of this genus are extensively used in various traditional medicines of special pharmacological interest are considered the anti-inflammatory, antioxidant, analgesic, anxiolytic and antidepressant activity. The aim of this study was to evaluate the bioactive compounds, antioxidants and antimicrobial activity of *Stachys* leaves extract. The antioxidant activity was determined by measuring total phenolic content (TPC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). Methanol extracts of *Stachys* leaves extract and evaluated for its antimicrobial activity. The antibacterial activity was assessed using paper disc method against two bacteria namely *Staphylococcus aureus* and *E. coli*. Showed that leaves extracts presented the highest phenolic compounds content for both species. Furthermore, GC-MS analysis allowed the identification of 37 bioactive compounds regardless of *Stachys* leaves extract. Leaves extract of *Stachys* possessed the highest total phenol content and antioxidant activity (DPPH). The antimicrobial tests showed that leaves extracts of *Stachys* exhibited the highest activity against two bacterial strains (*Staphylococcus aureus* and *Escherichia coli*). The studied leaves of *Stachys* is considered an excellent source of natural bioactive molecules that could be an interesting material for medicinal and food purposes.

***Keywords*:** *Stachys*, Antioxidants, Antimicrobial, GC-MS profile.

Introduction

Medicinal herbs have been used for many decades as an alternative treatment or medicine. In recent years, the use of herbal medicine has increased due to its effectiveness, affordability and safety claims. According to the World Health Organization (WHO), about 80-90% of the population of developing countries depends on medicinal herbs as a method of primary health care.[1-2] Medicinal herbs contain very important phytochemicals, many of them are secondary metabolites such as phenols, alkaloids, flavonoids, terpenes, saponins, and others. In addition, the active compounds possess antioxidant and antimicrobial properties that are essential for pharmaceuticals due to their use as therapeutic agents. Antioxidant compounds play an indispensable role in preventing oxidation of molecules inside the cell leading to defending healthy normal cells from damage caused by free radicals. Protecting cells from free radical damage/cell death is the central mechanism to prevent many diseases.[3] *Stachys* (Lamiaceae) is a large genus that includes about 275 to 300 species.[4] This genus is mostly distributed in subtropical and tropical regions of both hemispheres [5]. *Stachys* extract is reported to have several pharmacological activities including antibacterial,[6] antioxidant,[7] anticancer,[8] anti-inflammatory,[9] anti-nephritic,[10] and anti-Helicobacter,[11] anti-anxiety.[12] In addition, *Stachys* leaf and stem are used in the preparation of food such as yoghurt to improve the taste and as spices and flavours,[13] Hence, the current study was conducted to assess the in vitro antioxidants activity, antimicrobial, phytochemical and GC-MS analysis of *Stachys* leaves extract.

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Materials and Methods

Samples collection

Fresh leaves of the Stachys L. were collected from the local market obtained from farms in the north of Nasiriyah city, Thi -Qar, Iraq, between July and August 2021. The Stachys samples were identified at the Lab of Plant Taxonomy, Iraq Natural History Research Center and Museum, University of Baghdad, Baghdad, Iraq and were authenticated with herbarium number SP. Pl.: 580 (1753). The leaves of Stachys were cleaned and then oven dried at 60 ^{o}C for 24 h. The dried sample was then pulverized using a mechanical grinder and then stored at 4 ^{o}C until use.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was performed using Agilent Technologies. GC-MS was carried out according to method.[14] Methanolic plant extracts were filtered through a purple nylon syringe filter (0.45 µm) and then 5 microliters of plant extract were injected in the split mode (10:1). Nitrogen gas was used as a carrier, at the rate of 1 mL/min. The injector temperature was 250^{0}C. Then analyses were separated on a fused silica capillary column (30m × 0.25 mm × 1µm). The oven program was set as follows: initial temperature of 110^{0}C held for 2 min, and then ramped to 200^{0}C at a rate of 10^{0}C/min without holding; 280^{0}C was maintained for 9 min with a program rate of 5^{0}C /min. For mass spectra determination, the ionization energy of 70 eV, while the mass scanning range was 10–400 m/z. Identification and interpretation of GC-MS mass spectrum were conducted using NIST library mass spectra. Furthermore, the retention index (RI), name, molecular structure and weight of the components of the test extracts were ascertained from those obtained in the literature.

Antioxidant extraction

Plant materials were extracted using the methods described previously.[15] Briefly, 0.1 g dried plant powder and 10 ml 50% aqueous acetone was stirred for 1 h in a 25-mL universal bottle at 1,000 rpm using a magnetic stirrer (IKA, Staufen, Germany). Samples were then

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centrifuged at 4,750 g for 10 min using a mini centrifuge (Thermo-line, China) and the supernatants were used for further analyses.

Total phenol content (TPC)
The determination of antioxidant activity through TPC was carried out according to the method.[15] About 100 µL leaf extract was added with 0.4 mL distilled water and 0.5 mL diluted Folin-Ciocalteu reagent. The samples of *Stachys* leaves and stem extracts with Folin-Ciocalteu reagent were left for 5 min before 1 mL 7.5% sodium carbonate (w/v) was added. The absorbance was taken at 765 nm wavelength with a spectrophotometer after 2 hours. The calibration curve of gallic acid was set up to estimate the activity capacity of samples. The result was expressed as mg of gallic acid equivalents per 100 g of sample (mg GA/100 g of DW).

DPPH radical scavenging activity
The determination of antioxidant activity through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging system was carried out according to the method[15]. The stock solution was prepared by dissolving 40 mg DPPH in 100 ml methanol and kept at -20°C until used. About 350 mL stock solution was mixed with 350 ml methanol to obtain the absorbance of 0.70±0.01 unit at 516 nm wavelength by using a spectrophotometer (Epoch, Biotek, USA). About 100 µL *Stachys* leaves extracts with 1 ml methanolic DPPH solution prepared were kept overnight for scavenging reaction in the dark. The percentage of DPPH scavenging activity was determined as follows: DPPH scavenging activity (%) = [(A blank –A sample) / A blank] × 100. Where A is the absorbance.

Antibacterial assay
S. aureus and *E. coli* were used in the experiment. Mueller Hinton agar was used in the antibacterial assay. Plant extracts were dissolved in methanol and acetone to obtain a concentration of 300 mg/mL. Antibacterial assays were conducted using the disc diffusion method as previously described by [16]. Negative controls were prepared using the same solvent employed to dissolve the plant extract. Zones of inhibition around the discs were measured in mm. Gentamicin was used as positive control and DMSO as the negative control. The experiment was repeated in triplicates and the mean diameter of the inhibition zones was calculated.

Statistical analysis
Data were expressed as the means of three independent experiments. Statistical comparisons of the results were performed by one-way ANOVA using SPSS ver.23. Significant variances ($P<0.05$) among the concentrations were analysed by the Duncan'triplicates range test.

Results and Discussion

Gas chromatography-mass spectrometry (GC-MS) analysis
Gas Chromatography-Mass analysis of leaves extract of *Stachys* revealed the presence of various groups of bioactive compounds (Figure 1). The bioactive compounds with their retention time (RT), molecular formula, area, molecular weight and biological activity are exhibited in Table 1. Aluminum, triethyl, Pivalate, [(1-nitro-2-methyl-3-cyclohexenyl)methyl]ester, Dodecane, 1-fluoro-, 1,6-Octadien-3-ol, 3,7-dimethyl-, formate, D-Limonene, Linalool, Acetic acid, phenylmethyl ester, 4-Benzyloxy-6-hydroxymethyl-tetrahydropyran-2,3,5-triol, Propanoic acid, phenylmethyl ester, Methyl anthranilate, Caryophyllene, Phenol, 3,5-bis(1,1-dimethylethyl)-, Isoamyl salicylate, Diethyl Phthalate, Cinnamaldehyde, .alpha.-pentyl-, Benzoic acid, 2-(3-methylbutyl)amino-, methyl ester, Phytol, acetate, 7-Methyl-Z-tetradecen-1-ol acetate, Oxiraneundecanoic acid, 3-pentyl-, methyl ester, trans-, n-Hexadecanoic acid, 2,5-di-tert-Butyl-1,4-benzoquinone, Retinoic acid, 17-Pentatriacontene, Phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl-, Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester, 1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,5,6,7,8,9,10,10a-dodecahydro-1,4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R (1.alpha.,4a.beta., 7.beta., 10a.alpha.), 8,14-Seco-3,19-epoxyandrostane-8,14-dione,17-acetoxy-3.beta.-methoxy-4,4-dimethyl-, Nonacosane, Dotriacontane, Hentriacontane, Vitamin E, Dasycarpidan-1-methanol, acetate (ester), Ethyl iso-allocholate, Octadecane, 3-ethyl-5-(2-ethylbutyl)-, gamma.-Sitosterol, 1H-Cyclopropa[3,4] benz [1,2-e]azulene-5,7b,9,9a-tetrol, 1a,1b,4,4a,5,7a,8,9-octahydro-3-(hydroxymethyl) -1,1,6,8-tetramethyl-,5,9,9a-triacetate, [1aR-(1a.alpha.,1b.beta., 4a.beta.,5.beta., 7a.alpha., 7b.alpha.,8.alpha.,9.beta.,9a.alpha.)]-, Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5.beta.)-, Pivalate, [(1-nitro-2-methyl-3-cyclohexenyl)methyl]ester and Dodecane, 1-fluoro-. In the current study, some of the identified compounds have been reported to have several biological activities.

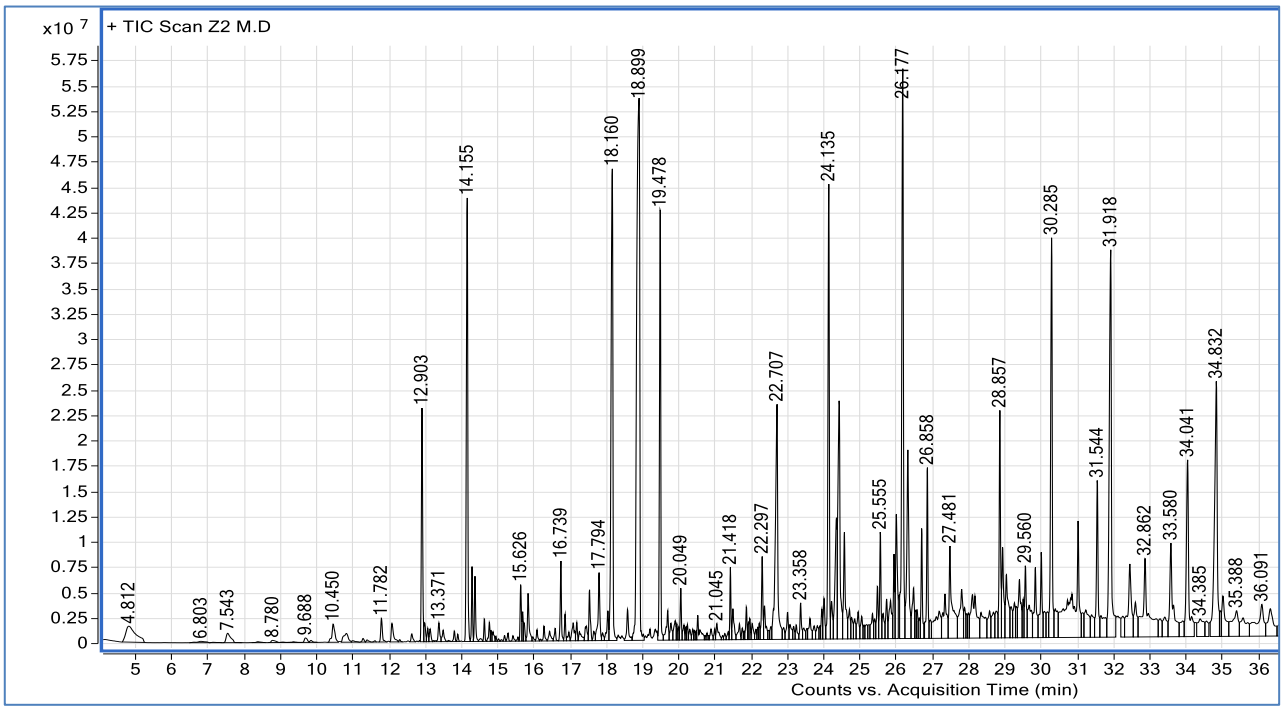

Figure 1: Gas chromatogram of methanolic *Stachys* leaves extract

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Table 1: List of compounds from methanol *Stachys* leaves extract observed in GC-MS with their retention time and biological activity

No	Compound name	Formula	RT	Area	M.W	Biological Activity
1	Aluminum, triethyl	$C_6H_{15}Al$	4.812	29843221	114.16	Antimicrobial
2	Pivalate, [(1-nitro-2-methyl-3-cyclohexenyl)methyl]ester	$C_{13}H_{21}NO_4$	6.803	2289626	255.31	Antimicrobial
3	Dodecane, 1-fluoro-	$C_{12}H_{25}F$	7.543	8167600	188.32	Antimicrobial
4	1,6-Octadien-3-ol, 3,7-dimethyl-, formate	$C_{11}H_{18}O_2$	9.688	2548591	182.25	Antimicrobial
5	D-Limonene	$C_{10}H_{16}$	10.45	10355563	136.23	Antimicrobial
6	Linalool	$C_{10}H_{18}O$	11.782	7393288	154.25	Antimicrobial, anti-oxidant Anti-inflammatory, anticancer
7	Acetic acid, phenylmethyl ester	$C_9H_{10}O_2$	12.903	56846826	150.17	Antimicrobial
8	4-Benzyloxy-6-hydroxymethyl-tetrahydropyran-2,3,5-triol	$C_{13}H_{18}O_6$	13.232	374736	270.28	Antimicrobial
9	Propanoic acid, phenylmethyl ester	$C_{10}H_{12}O_2$	14.367	15078767	192.25	Anticancer
10	Methyl anthranilate	$C_8H_9NO_2$	15.626	13246416	204.35	Antimicrobial
11	Caryophyllene	$C_{15}H_{24}$	16.739	17670030	204.35	Neurodegenerative Diseases
12	Phenol, 3,5-bis(1,1-dimethylethyl)-	$C_{27}H_{50}OP_2$	17.794	22833620	452.6	Antimicrobial
13	Isoamyl salicylate	$C_{12}H_{16}O_3$	18.16	163418818	208.25	Antimicrobial
14	Diethyl Phthalate	$C_{12}H_{14}O_4$	18.899	311632085	222.24	
15	Cinnamaldehyde, .alpha.-pentyl-	$C_{14}H_{18}O$	19.478	104562315	202.29	Anticancer
16	Benzoic acid, 2-(3-methylbutyl)amino-, methyl ester	$C_{13}H_{19}NO_2$	20.049	3409705	221.29	Antimicrobial
17	Phytol, acetate	$C_{22}H_{42}O_2$	21.045	14301220	338.6	Antitubercular
18	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	21.418	6532588	268.4	
19	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, trans-	$C_{19}H_{36}O_3$	22.297	21361403	312.5	Antioxidants
20	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	22.707	109377828	256.42	Antioxidants
21	2,5-di-tert-Butyl-1,4-benzoquinone	$C_{14}H_{20}O_2$	23.358	10191588	220.31	Antioxidants
22	Retinoic acid	$C_{20}H_{28}O_2$	25.555	26704129	300.40	Antioxidants
23	17-Pentatriacontene	$C_{35}H_{70}$	26.177	6537193	490.90	Antimicrobial
24	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	$C_{69}H_{93}O_6P$	26.858	42873229	1049.4 0	Antimicrobial, anti-oxidant
25	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O5$	27.481	53897193	568.90	Anticancer
26	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,5,6,7,8,9,10, 10a-dodecahydro-1,4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R (1.alpha.,4a.beta., 7.beta., 10a.alpha.)	$C_{21}H_{32}O_2$	28.857	58431553	316.47	
27	8,14-Seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3.beta.-methoxy-4,4-dimethyl-	$C_{24}H_{36}O_6$	29.56	30540480	420.50	Antimicrobial
28	Nonacosane	$C_{29}H_{60}$	30.285	123928451	408.80	Antimicrobial
29	Dotriacontane	$C_{32}H_{66}$	31.544	50372635	408.78	Antimicrobial
30	Hentriacontane	$C_{31}H_{64}$	31.918	164086992	436.80	Antimicrobial
31	Vitamin E	$C_{29}H_{50}O_2$	32.862	47467189	430.71	Antioxidants
32	Dasycarpidan-1-methanol, acetate (ester)	$C_{20}H_{26}N_2O_2$	33.58	16352763	326.40	Antioxidants
33	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	34.041	56669781	436.60	Antimicrobial
34	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	34.385	82104375	366.70	Antibacterial
35	.gamma.-Sitosterol	$C_{29}H_{50}O$	34.832	134576940	414.70	Antidiabetic
36	1H-Cyclopropa[3,4]benz[1,2-e]azulene-5,7b,9,9a-tetrol, 1a,1b,4,4a,5,7a,8,9-octahydro-3-(hydroxymethyl)-1,1,6,8-tetramethyl-, 5,9,9a-triacetate, [1aR (1a.alpha.,1b.beta.,4a.beta.,5.beta.,7a.alpha., 7b.alpha.,8.alpha.,9.beta.,9a.alpha.)]-	$C_{26}H_{36}O_8$	35.388	7568816	476.60	Antioxidants
37	Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5.beta.)-	$C_{29}H_{50}O_2$	36.091	29994893	430.70	Antioxidants

The identified compounds from fruit extract have been reported to have therapeutic properties by several researchers. For example, 1,6-Octadien-3-ol, 3,7-dimethyl-, Linalool, D-Limonene, Phenol, 3,5-bis(1,1-dimethylethyl)-, gamma-Sitosterol, antimicrobial, anticancer, antioxidants , antidiabetic, antimicrobial and anti-inflammatory.[17-20]

Antioxidant activity

Antioxidant activity of *Stachys* leaves extract was evaluated by two methods, total phenol content and DPPH. The DPPH assesses the potential of antioxidants to normalise free radicals through reduction mechanisms, which decolourises the stable free radical DPPH from violet colour to yellow.[21] It is well known that DPPH scavengers have a wide range of biological functions such as lipid peroxidation inhibitory action and radioprotective.[22] There is scientific evidence that shows antioxidant and pharmacological properties of plants are usually associated with the presence of phenolic compounds and electron donor agents.[23] In the current study, the strong antioxidant activity of *Stachys leaves extract* is perhaps due to the phenolic contents.[24] In our results, methanol extract had greater chelating action which was due to its higher phenol contents. As indicated in Table 2, the total phenolic contents of *Stachys leaves* ranged from 227.64 to 182.92 mg GAE/100 g DW. The results showed that the methanolic extract has significantly

(P<0.05) higher scavenging activity compared to acetone extract. The results explained that antioxidant activity was sensitive to extraction solvents; generally, methanol gave the highest extraction recovery (88.12%). The varied scavenging activity in different parts of the plants could be attributed to the presence of bioactive compounds such as phenolics, flavonoids and tannins. Previously, a strong correlation has been observed between the radical scavenging power of plant extract with total phenolics and flavonoid contents.[25] The different results obtained from the previous studies may be attributed to different cultivars, growing conditions, maturity stage at harvest, or the storage conditions and time elapsed before the fruits were analyzed.

Antibacterial activity

Results of the antibacterial susceptibility assay of both the acetone and methanol extracts are presented in Table 3 showing zone inhibition. The methanol extract showed strong antibacterial activity against the resistant strains of *S. aureus* and *E. coli*. This extract showed variance in antibacterial potentiality as standard antibiotics used against *S. aureus* and *E. coli*. Antimicrobial test of methanol extract of *Stachys leaves extract* has presented moderate sensitivity to the *S. aureus* and *E. coli*. The highest zone of inhibition (11.27 mm) was recorded against *S. aureus*. Gentamicin was found more effective against the two bacterial species. Compared to acetone and methanol extract, the acetone extract was also found effective to inhibit the growth of *S. aureus* and *E. coli*. The antibacterial activity observed was mainly because of the presence of phenolic compounds. The extraction yield was affected by the extraction method, polarity, solubility, concentration, pH, temperature and extraction solvents.[26].Other studies reported the occurrence of phenolics, alkaloids and tannins in plant extracts and they are related to antimicrobial activity.[27]

Table 2: Antioxidants activity of the extracts of *Stachys* leaves

Samples	TPC (mg GAE/100 g DW)	DPPH (%)
Methanolic extract	227.64 ± 3.53^{a}	88.12 ± 3.85^{a}
Acetone extract	182.91 ± 2.67^{b}	76.40 ± 2.06^{b}

[a–c] Different letters within each column indicate significant difference ($p < 0.05$)

Table 3: Antibacterial activity of the extracts of *Stachys* leaves

Samples	Minimum inhibitory concentration (mg/ml)	
	Staph. aureus	***E. coli***
Methanolic extract	11.27 ± 0.51^{b}	9.37 ± 1.85^{b}
Acetone extract	9.81 ± 1.33^{c}	7.51 ± 0.28^{c}
Gentamicin	17.25 ± 0.33^{a}	16.86 ± 0.50^{a}

[a–c] Different letters within each column indicate significant difference ($p < 0.05$)

Conclusion

In this current study, a comprehensive study on in vitro GC-MS profile of *Stachys leaves extracts, and their* antioxidant and antimicrobial activities were investigated for the first time. The results of this work supported the original premise. The leaves extracts possess various groups of phytochemicals with high content of total phenolic content in addition to possible antioxidant properties. The extracts were able to inhibit the antibacterial activity in high concentrations. The GC-MS analysis of *Stachys leaves extract* revealed the presence of many phytoconstituents that have been known to possess therapeutic properties. From these results, it could be concluded that *Stachys leaves extract has* various bioactive compounds, total phenol content, and strong antioxidant and antibacterial activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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