ANALYZING THE CHARACTERISTICS AND BIOLOGICAL ACTIVITIES OF METHANOLIC EXTRACTS FROM *STACHYS BYZANTINE* LEAVES

Ali Q. Khazaal **Zainab F. Mahmood** **Sanaa A. Hammood**
Lecturer **Lecturer** **Lecturer**
Dept. Biotech., Coll. Sci., University of Baghdad, Iraq.
ali.k@sc.uobaghdad.edu.iq

ABSTRACT

This study was aimed to examine the suppressive effects of *Stachys byzantine* leaf methanolic extracts on cancer cell lines and pathogenic microbes. Five concentrations of *S. byzantine* methanolic extract, from 25 to 400 µg/ml, were applied to HepG1 and HCT116 cells. *S. byzantine* methanolic extracts inhibited liver and colon cancer cell viability at different concentrations. The MTT assay results demonstrated that the extract's IC-50 values for HepG1 were 271.61µg/ml and for HCT116 was 184.86µg/ml. The methanolic extracts obtained from *S. byzantine* showed antibacterial activity against Gram-negative and Gram-positive bacteria. The extract inhibited *Staphylococcus aureus* with 15, 20, and 22 mm inhibition zones at 100, 200, and 400 mg/ml, respectively. However, the extract was more effective against *Pseudomonas aeruginosa*, with inhibitory zones of 30, 30, and 35 mm at 100, 200, and 400 mg/ml respectively. This study indicates that methanolic extracts derived from *S. byzantine* leaves have anti-cancer and antibacterial properties.

Key words: liver cancer, colon cancer, *Staphylococcus aureus,* and *Pseudomonas aeruginosa*

تحليل الخواص والفعالية الحيوية للمستخلص الميثانولي لاوراق نبات البطنج

علي قاسم خزعل زينب فرقد مختار سناء عبد حمود
مدرس مدرس مدرس
قسم التقنيات الاحيائية – كلية العلوم – جامعة بغداد – العرا ق

المستخلص

تهدف هذه الدراسة الى اختبار التأثيرات التضادية للمستخلص الميثانولي لأوراق نبات البطنج على خطوط الخلايا السرطانية والميكروبات المسببة للأمراض. تم تطبيق خمس تراكيز من مستخلص الميثانولي لأوراق نبات البطنج تتراوح من 25 إلى 400 ميكروغرام/ مل على خلايا HepG1 و HCT116. اظهر المستخلص الميثانولي لأوراق نبات البطنج إلى تثبيط نمو الخلايا سرطان الكبد والقولون وكانت قيم IC-50 لـ HepG1 هي 271.61 ميكروغرام/مل و 184.86 ميكروغرام/مل بالنسبة لخلايا HCT116. أظهر المستخلص الميثانولي لأوراق نبات البطنج نشاطًا مضادًا للبكتيريا السالبة والموجبة لصبغة كرام. وجد ان للمستخلص قابلية تثبيط المكورات العنقودية الذهبية بمناطق تثبيط تبلغ 15 و 20 و 22 ملم عند 100 و 200 و 400 ملغم / مل على التوالي. كان المستخلص أكثر فعالية ضد الزائفة الزنجارية حيث بلغت مناطق التثبيط 30، 30، و 35 ملم عند 100، 200، و 400 ملغم / مل على التوالي. تشير هذه الدراسة إلى أن المستخلص الميثانولي المشتق من أوراق نبات البطنج له خصائص مضادة لنمو الخلايا للسرطانية ومضادة للبكتريا المرضية.

الكلمات المفتاحية: سرطان الكبد، سرطان القولون، بكتريا المكورات العنقودية الذهبية، بكتريا الزائفة الزنجارية.

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INTRODUCTION

Liver and colon cancer are well-known and common types of cancer that cause serious health problems in many countries around the world. Liver cancer is the 6th most common type of cancer in the world and the 4th leading cause of cancer-related deaths. Colorectal cancer is the 3rd most common type of cancer identified and the 2nd most common type of cancer death (6). Pathogenic bacteria like *Staphylococcus aureus* and *Pseudomonas aeruginosa* can also cause infectious diseases that represent a health problem. *S. aureus* is a Gram-positive bacterium that causes skin diseases and toxic shock syndrome, a disease that can be fatal (24). *P. aeruginosa* is a Gram-negative bacterium that can infect people suffering from cancer, immunodeficiency, burn wounds, and cystic fibrosis (21). The aforementioned facts demonstrate that liver and colon cancers, together with infectious diseases caused by *S. aureus* and *P. aeruginosa* are huge global public health problems that are causing a huge pressure on medical resources. The development of resistance is a significant challenge in the treatment of cancer or infectious diseases. The primary obstacle in cancer treatment is the development of drug resistance in the many cell types present in cancerous tissues (23). This resistance made malignant tumors more severe. (12). Antibiotic resistance, in turn, is a major worldwide issue facing modern medicine and society. Each year, problems brought on by bacterial strains that resist antibiotics result in around 700,000 mortalities. Due to their resistance to potent antibiotics, *S. aureus* and *P. aeruginosa* are two of the six main species of bacteria that are resistant to several antibiotics (7). The probability of curing infectious illnesses and cancer is greatly reduced by these characteristics. Therefore, the need to find a novel method for treating infectious illnesses and cancer is increasing. Plants that contain medicinal properties and their chemical compounds are gaining attention as beneficial supplemental therapies (25). Recent clinical investigations have shown that different herbal medications exhibit a variety of anti-cancer and antibacterial effects (2 and 28). One of these medicinal plants is the genus of Stachys plant which comprises more than three hundred kinds, found in several locations around the world specifically in moderate zones. Many species of this genus of Stachys are a significant source of biologically active molecules, which are identified as secondary metabolites. Therefore, several Stachys species have been used in traditional medicine for their ability to treat different types of diseases (30 and 5). The current study aims to investigate the biological ingredients, antioxidant, anti-proliferative, and anti-bacterial activities of methanolic extracts from the leaves of *Stachys byzantine* (*S. byzantine*), which is readily available in Iraq. This research examined the suppressive impact of a methanolic extract from *S. byzantine* on liver and colon cancer cells as well as *S. aureus* and *P. aeruginosa* bacteria.

MATERIALS AND METHODS

Extract preparation: The dried leaves powder of the *S. byzantine* (50 g) was extracted with 500 ml of 80% methanol for 72 hours using the soxhlet apparatus. The extract was then filtered and evaporated using an oven at 40˚C for 48 hours to obtain 10 g of the methanolic crude extract. The extract was kept in a dark glass container in the refrigerator at 4 °C until further use (14).

Cell line: The human hepatocellular carcinoma cell line HepG1 and the human colorectal carcinoma cell line HCT116 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and 1% streptomycin. The cells were subjected to Trypsin-EDTA treatment, transferred to a new culture dish and placed in an incubator. The cells were incubated in a humidified incubator at 37 °C with a CO_2 concentration of 5% (22).

Determining the cytotoxicity effects: HepG1 and HCT116 cells were placed in 96-well plates with a density of 10^4 cells per well, using a final volume of 200 µL. Once a confluent monolayer was formed, cells were treated with various concentrations (0, 25, 50, 100, 200, and 400) µg/mL of the methanolic extract of *S. byzantine*. Ultimately, the absorbance was quantified using a plate reader at a wavelength of 570 nm.

Measuring anti-proliferative effects: The antiproliferative effects were assessed after a 48-hour treatment with varying doses of methanolic extract. This was done by removing the medium, adding 10 µL of MTT solution (5 mg/mL), and incubating the cells at 37 °C for 2 hours. Subsequently, the liquid fraction containing suspended particles was separated, and 100 µL of Dimethyl Sulfoxide (DMSO) was added to dissolve the blue crystals. The cell survival rate was calculated as follows: (Absorbance of the methanolic extract of *S. byzantine* – Absorbance of the blank) / (Absorbance of the control group– Absorbance of the blank) ×100 (19). The absorbance of blank refers to the absorbance of the media alone. In contrast, the absorbance of control refers to the absorbance of the cells treated with DMSO, and the absorbance of the methanolic extract of *S. byzantine* refers to the cells treated with different concentrations of extract. IC-50 value of the methanolic extract of *S. byzantine* against HepG1 and the HCT116 cell line was then calculated.

Antimicrobial activity: The agar well diffusion method was used to evaluate the antimicrobial activity of the plant crude extract. The bacterial suspension was prepared from fresh colonies and the concentration was adjusted to 1.5×10^8 CFU/ml (McFarland turbidity). The bacterial strain was inoculated by streaking it on a Mueller-Hinton agar plate. After that, a sterile cork borer was used to make a 5 mm diameter well. Different concentrations of the methanolic extract including 100, 200, and 400 mg/ml were prepared by serial dilution. Then, 50 µl of each extract was then added to each well. After that, the plates were left at room temperature for 15 minutes to enhance the diffusion of the extracts into the agar and then the dishes were incubated at 37°C for 24 hours. Antimicrobial activity was detected by measuring the inhibition zone that appeared after the incubation period (1).

High-performance liquid chromatography (HPLC): The active compounds in the crude extract of *S. byzantine* were examined using HPLC depending on the conditions of the chromatograph (Shimadzu LC-2010 A HT). The column of separation resolves C18-DB, 3µm particle size (50 mm * 2.0 mm I.D) column, the detector wave-length λ 370 nm, and mobile phase that used was (Acetonitrile 40%: 60% 2% Acetic acid) at pH 2.6. The flow rate was 0.8 ml/min and the injection volume was 20 µl. The test was done by dissolving the methanolic extract of *S. byzantine* isolated in methanol alcohol so that the concentration of the methanolic extract of *S. byzantine* solution becomes 40 µg /mL (15).

Gas chromatography-mass spectrometry (GC–MS): a gas chromatograph Agelint Technologies 7820A, USA was used to detect the availability of various compounds in the crude extracts isolated from leaves of S. *byzantine*. The conditions of GC were as follows: Analytical Column: Agelint HP-5ms Ultra lneit (30 m length x 250 µm inner diameter x 0.25 µm film thickness). The volume of injection was 1µl, Pressure was about 12 psi, and the line Temperature was 250 ˚C. in contrast, the Aux heaters temperature was 300 ˚C, and the Carrier Gas: He 99.99%. The injector temperature was 250 ˚C. The scan Range: m/z 25-1000, and the injection type was Spitless. The temperature of the oven was programmed as follows: first ramp: 60 ˚C for 3 min; second ramp: 60 ˚C to 180 ˚C for 7 ˚C/min; third ramp 3: 180˚C-280˚C, 8 ˚C/min; fourth ramp 4:280˚C for 5 min (31).

Antioxidant Assay: Free radical scavenging activity of the methanolic extract of the *S. byzantine* was assessed using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) technique, as described in a study by (13). Briefly, 1mL DPPH solution at 0.1mM was mixed with 3 mL of the extract solution at 270 µg/mL concentration. The mixtures were rapidly stirred and placed in the dark at 25 ˚C for 30 minutes. After that, a spectrophotometer measured the absorbance at a wavelength of 517 nm. The following formula was used to determine the DPPH radical.: Antioxidant activity = ((Absorbance of control – Absorbance of the sample) / Absorbance of control)) x 100%. Absorbance of control refers to the absorbance of the DPPH radical alone, and Absorbance of sample refers to the absorbance of the DPPH radical when combined with the sample extract.

Statistical analysis: The collected data were subjected to statistical analysis by utilizing an unpaired t-test. The values were reported as

the means ± standard error of the mean (SEM) of three or four measurements. A p-value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Determining the cytotoxicity effects: To determine the cytotoxicity effects of methanolic extract of *S. byzantine* on the liver cancer cell HepG1 and colon cancer cell HCT116, six different concentrations of the methanolic extract of *S. byzantine* were applied to the cells, including (0, 25, 50, 100, 200, and 400)µg/ml. The results showed that the methanolic extract of *S. byzantine* had different effects on the cytotoxicity of the HepG1 and HCT116 lines. (Figure 1 A and B). The extract exhibited a dose-dependent inhibitory effect on the colon and liver cancer cell line. These results demonstrate the strong and concentration-dependent anti-cancer effects of *S. byzantine* extract on colon and liver cancer cells.

Determining the cell viability: This test is important because it shows how well the extract inhibits the growth of the cancer and the percentage of inhibition at each concentration. The results of this experiment showed that the methanolic extract of S. byzantine may inhibit liver and colon cancer cell growth at different concentrations (Figure 2 A and B). The extract showed that the highest concentration had the highest percentage of inhibition, while the lowest amount had the lowest percentage of inhibition. The survival percentage for liver cancer cells was 97.03, 88.58, 78.08, 64.10, and 35.02, and for colon cancer cells was 84.94, 79.59, 61.71, 51.16, and 45.95 for the extract concentration of 25, 50, 100, 200, and 400 µg/ml respectively. MTT assay determined the IC-50 of methanolic extract of *S. byzantine* against liver cancer cell HepG1 and colon cancer cell HCT116. According to the calculations, the IC-50 for liver cancer cell HepG1 was 271.61 µg/ml and for colon cancer cell HCT116 was 184.86 µg/ml.

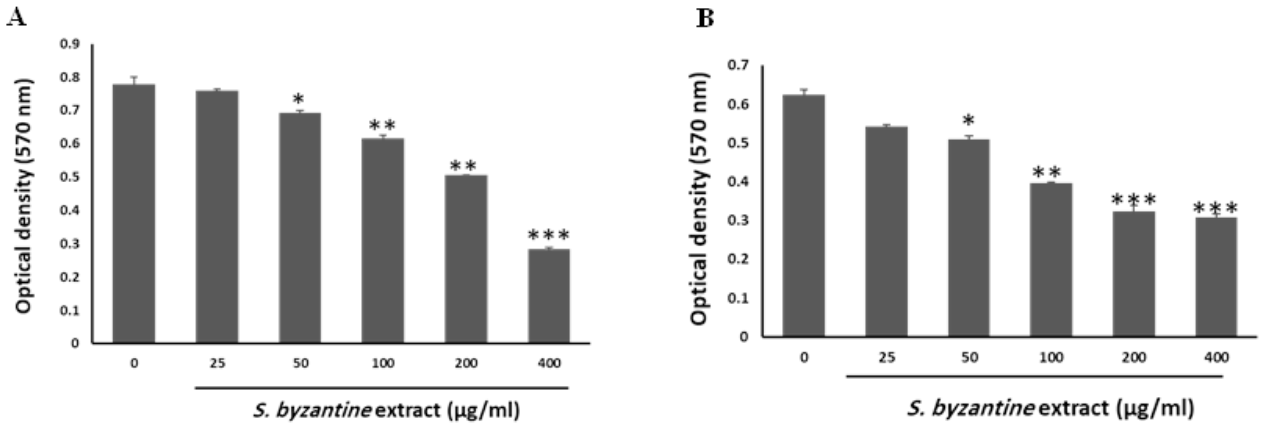

Figure 1. Growth of cancerous cell lines after treatment with different concentrations of methanolic extract of *S. byzantine*. (A) Liver cancer cell HepG1, and (B) colon cancer cell HCT116. *P < 0.05, **P < 0.01, ***P < 0.001.

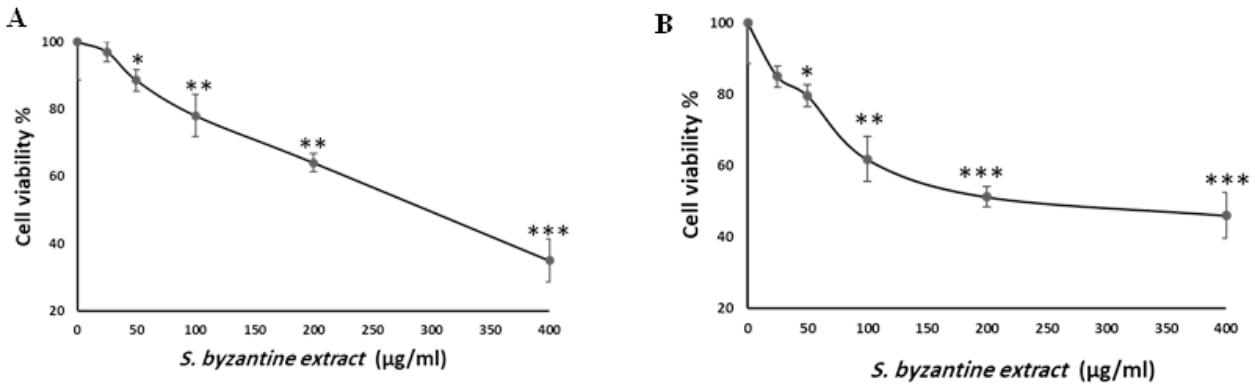

Figure 2. Inhibitory effects of different concentrations of the methanolic extract of *S. byzantine* on cancerous cell lines. (A) liver cancer cell HepG1, and (B) colon cancer cell HCT116. *P < 0.05, **P < 0.01, ***P < 0.001

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Our study findings showed that the methanolic extract significantly inhibited and caused apoptosis in liver and colorectal cell lines. Our findings support some other studies that found that Satchys species have anti-proliferative effects against a variety of cancer types. The methanolic extract of *S. pilfera* exhibited anti-proliferative action against HT29 colon cancer (18). Furthermore, the crude extract of *S. parviflora* induced DNA fragmentation leading to significant inhibitory effects against prostate and breast cancer cells (27). Another study indicated that the dichloromethane extract of *S. circinata* possesses anti-inflammatory and anti-proliferative activities against MCF7 and HepG2 cells (29). These data strongly suggest that Satchys has strong anti-proliferative effects against several types of cancer.

Antimicrobial activity: testing the in vitro activity demonstrated that the methanolic extract has antimicrobial properties against *S. aureus* (Figure 4) and *P. aeruginosa* (Figure 5). The methanolic extract was tested at three different concentrations including 100, 200, and 400 mg/ml. It exhibited antibacterial efficacy against *S. aureus*, with inhibition zones of 15, 20, and 22 mm respectively (Table 1). However, the activity of the extract against *P. aeruginosa* was higher, resulting in an inhibitory zone of 30, 30, and 35 mm for extract concentrations of 100, 200, and 400 mg/ml respectively. The current results are consistent with those of previous studies that have shown the antibacterial properties of several Stachys species. An investigation discovered that ethanolic extracts from *Stachys spp.* had in vitro antibacterial activity and antibiofilm characteristics against *Candida albicans*, as well as both Gram-positive and Gram-negative strains (3). In addition, the essential oil extracted from *S. rupestris* displayed efficacy against a wide range of bacteria commonly associated with skin diseases, such as *Candida tropicalis*, *E. coli*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *S. aureus*, and *C. albicans* (9). In addition, the ethanol extracts obtained from the leaves of *S. sylvatica* were tested against human ocular infectious agents. The extract showed antibacterial activity against *S. capitis*, *Moraxella nonliquefaciens*, and *Cutibacterium acnes*. The extracts also showed a mild impact on some bacterial strains including *Bacillus cereus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *S.aureus*, and *S. epidermidis* (8). Altogether, these data suggest that the extract of Stachys has effective antimicrobial properties.

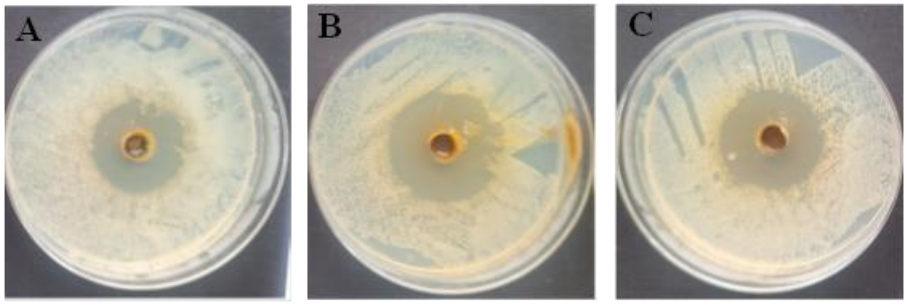

Figure 4. The antibacterial activity of the methanolic extract of *S. byzantine* against *S. aureus* (A) 100 (B) 200, and (C) 300 mg/ml.

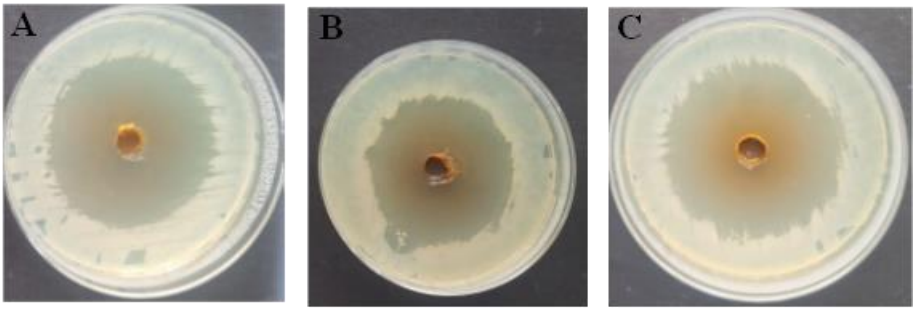

Figure 5. The antibacterial activity of the methanolic extract of *S. byzantine* against *P. aeruginosa* (A) 100 (B) 200, and (C) 300 mg/ml.

Table 1. Diameter of the inhibition zone of the methanolic extract of *S. byzantine*. The results are the mean of four separate experiments. The results are expressed as mean ± SEM

Concentration of the extract (mg/ml)	Diameter of inhibition zone (mm)	
	S. aureus	*P. aeruginosa*
100	15±1.73	30±2.08
200	20±2.30	30±2.30
400	22±1.15	35±1.52

HPLC analysis: HPLC analysis revealed that the methanolic extract includes many bioactive constituents (Figure 6). A critical limitation of our HPLC testing is our inability to identify the structure and nomenclature of the active compounds present in our extract. The reason for this is a lack of standardized materials for comparison with each peak in the HPLC analysis. Consequently, we employed gas chromatography (GC) to identify the active components existing in our sample.

GC-MS Analysis: the analysis revealed that the methanolic extract includes numerous biologically active components (Figure 7). Unlike HPLC, GC possesses a library that allows us to determine the chemical composition and taxonomy of the active ingredients found in our extract (Table 2). The GC analysis in the current study supports earlier research that revealed the presence of many active constituents in *S. byzantine*. For example, research has recorded the existence of flavonoids, terpenoids, phenolic acids, and other secondary metabolites that have been proven to possess anti-inflammatory, and antioxidant properties (30). The GC experiment showed that peak#15, which stands for hexadecanoic acid, had the highest concentration. A compound called octadecanal was found as peak #17 and was the second most common in our GC study. Peak #1, which was third in terms of content, was found to be carbamic acid. Finally, peak#10, which stands for 4,6-Dimethoxy-5-nitropyrimidine, was the fourth most concentrated chemical in the GC study. Previous research also found that Hexadecanoic acid was present in *S. byzantine* using GC analysis, which agrees with our results. (17). The results of our GC study agree with those of Gad et al., who found that Octadecanal is a vital component of the GC analysis of *S. byzantine* (10). These data suggest that *S. byzantine* has several chemicals that show potential as anticancer and antimicrobials.

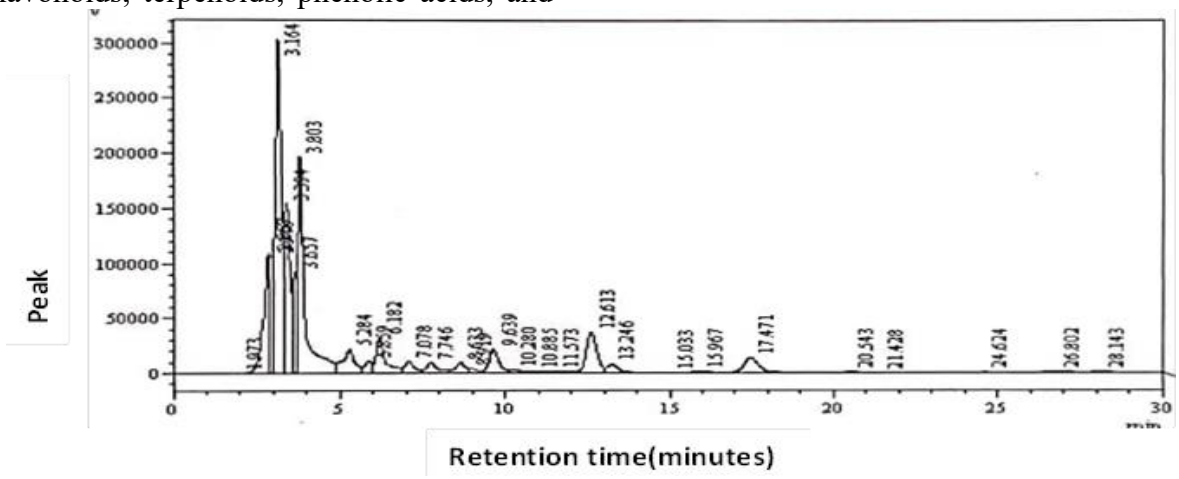

Figure 6. HPLC analysis of the extract of *S. byzantine* leaves

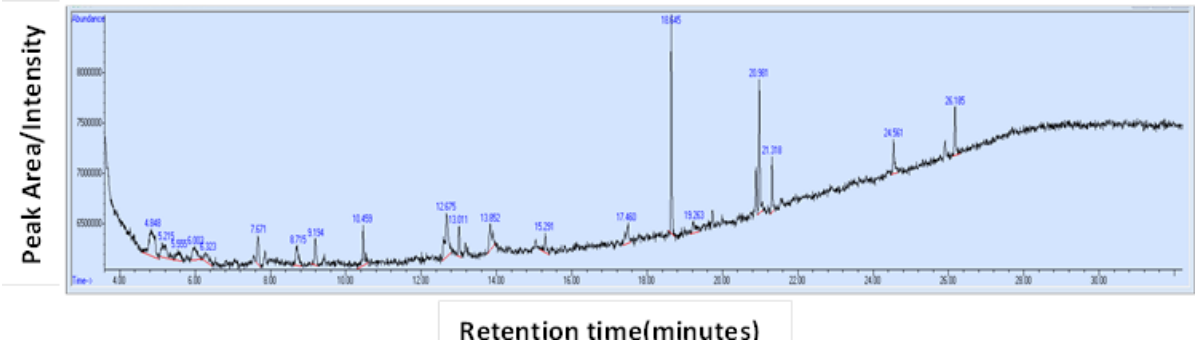

Figure 7. GC-MS chromatogram of the methanolic extract of *S. byzantine* leaves

Antioxidant assay: In our investigation, the DPPH test was used to find out if the methanolic extract contained any antioxidant components. The radical scavenging activity of the extract was determined using the formula described in the methods. The results showed that the activity of radical scavenging had a value of 0.02023. Several studies have demonstrated the presence of antioxidant compounds in the extract of various Stachys species (3). These results suggest that chemicals with antioxidant qualities are present in the S. byzantine methanolic extract. The observed anti-cancer and antibacterial activities of our extract may be due to the fact that it contains many active ingredients. Hexadecanoic acid, for example, demonstrates antioxidant, anticancer, and antibacterial properties. A study found that Hexadecanoic acid exhibited cytotoxic properties and demonstrated antitumor efficacy against colon (4) breast cancer (26), and human oral squamous cell carcinoma (20). Another study demonstrated that Hexadecanoic acid had inhibitory effects on the growth of some bacteria such as *Xanthomonas campestris* and some types of fungus such as *Fusarium oxysporum* (16). Moreover, it exhibited antibacterial efficacy against several types of pathogenic bacteria (11). All these findings have verified that the extract of *S. byzantine* has biologically active compounds that might have a role in the anticancer and antibacterial effects shown in our investigation. Our data suggest that *S. byzantine* extracts have a large chance for the development of phytopharmaceuticals.

Table 2. Bioactive compounds identified in the extract of *S. byzantine* via GC-MS analysis

Peak#	Retention time (minutes)	Area%	Compounds nomenclature
1	4.850	9.98	Carbamic acid
2	5.214	4.80	1,2-Hydrazinedicarboxylic acid
3	5.552	2.35	2,2'-(Piperazine-1,4-diyl) bis(N-(4-aminophenyl)-N-methyl acetamide)
4	6.002	3.52	3-Chloro-N-[2-methyl-4(3H)-oxo-3-q
5	6.322	2.67	3-Butyn-1-ol
6	7.672	3.23	cis-Aconitic anhydride
7	8.711	3.58	2,2'-(1,4-Piperazinediyl) bis[N-(4-methoxyphenyl) succinimide
8	9.195	2.65	Phthalic acid
9	10.459	4.79	2-[5-(3,4-Dimethoxyphenyl)-tetrazol-2-yl]-N-phenethyl-acetamide
10	12.675	9.05	4,6-Dimethoxy-5-nitropyrimidine
11	13.012	2.69	trans-β-Ionone
12	13.852	4.81	n-Pentacosane
13	15.289	2.50	9,12,15-Octadecatrienoic acid
14	17.461	3.44	Nonanoic acid
15	18.647	14.75	Hexadecenoic acid
16	19.262	2.67	3-butynol
17	20.984	10.10	Octadecanal
18	21.322	4.29	Heptadecanoic acid
19	24.559	3.63	n-butyl octadecenoate
20	26.186	4.51	Bis(2-ethylhexyl) phthalate

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