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Pharmacological and biological features of ethanol extract of Salvia virgata Jacq.

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Abstract

Salvia virgata as an ethnomedicinal plant comprehends a variety of efficient active ingredients and shows diverse pharmacological actions, such as anti-proliferative, anti-inflammatory, and antimicrobial effects. In this study we aimed to determine the biological content of the ethanolic extract of *S. virgata* and to determine its possible pharmacological effects. The broth microdilution technique was carried out for determining antimicrobial activities, and the test microorganisms included *E. coli, S. aureus, B. cereus, P. aeruginosa, C. albicans*, and *C. tropicalis*. DPPH and ABTS methods were used to detect antioxidant activity. The XTT cell viability test was utilized to assess the antiproliferative activity of the ethanolic extract of *S. virgata* on L929 and MDA-MB-231 cell lines. *S. virgata* exhibited reasonable antimicrobial effects against *E. coli* (0.312 mg/mL) and *S. aureus* (0.312 mg/mL). DPPH and ABTS IC_{50} values were determined 291.58 ± 0.004 µg/mL, 16.74 ± 0.007 µg/mL respectively. *S. virgata* ethanolic extract TPC and TFC were observed 283.35 ± 10.4 mg GAE/g and 13.37 ± 1.6 mg QE/g, respectively. The extract was screened against α-amylase and α-glucosidase, AChE, and BChE enzymes, and inhibition activity was determined 75.73%, 62.72%, 67.19%, 3.18% respectively. The extracts did not significantly affect the L929 cell viability, while MDA-MB-231 remarkably reduced cell viability (IC_{50} = 0.118 mg/mL). The ethanolic extract of *S. virgata* can be considered as a potential therapeutic agent in the treatment of different pathological conditions due to its antimicrobial, antioxidant, and anticancer effects.

Keywords: *Salvia virgata* extract; antimicrobial; antioxidant; enzyme inhibition; antiproliferative

Introduction

At the beginning of human history, medicinal and aromatic plants have been utilized as flavors in foods, and for therapeutical purposes in folk medicine. The Lamiaceae family has great importance because of its aroma and nutritional value. Sage (Salvia L.) is a valuable medicinal and aromatic plant from the family of Lamiaceae. Species of the genus Salvia are generally rich in essential oils and therefore they are important pharmacologically and in the perfume industry [1]. Sage species have been the subject of many prevalent kinds of research due to their use in public health from ancient times to the present day and have taken their place in the pharmacopeia of many countries around the world [2]. Infusions obtained from these species are commonly used in traditional Turkish medicine for their wound healing, diuretic, diaphoresis, and anti-flatulence effects [3]. There are about 900 taxa of Salvia genus spreading around the world, and 99 sage species, 51 of them are endemic, naturally grown in Turkey Flora [4].

The leaves of *Salvia virgata*, also known locally as "yılancık", used externally for wound healing, while decoction can be beneficial in the treatment of leukemia [5]. *S. virgata* is a perennial species with violet-blue to lilac, rarely white flowers, and 20 to 160 cm tall, prefers very different habits such as mostly bushes, forests, meadows, empty fields, limestone, and volcanic rock cliffs, etc. It distributes up to 2300 m above sea level. It is flowering from May to September. *S. virgata* is native to Asia and South eastern Europe, and it has spread throughout Turkey [6,7].

Humankind has achieved countless successes so far, and the discovery of penicillin, the first antibiotic, is one of them. More than 90 years have passed since penicillin was discovered, and it has touched the lives of countless people to this day, saving many lives [8]. Gradually increasing antibiotic use approached its peak with an increase of 36% between 2010 and 2014 [9]. In recent

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years, scientists have drawn attention to the resistance developed by bacteria against the antibiotics used. Two main factors draw attention to the formation of resistance. The first of these is the frequent prescription against non-bacterial diseases such as viral infections and the irregular use of antibiotics with prescriptions written in non-fatal doses [10,11]. Because of the resistance to antibiotics and its possible toxicities, the importance of essential oils, and various plant extracts has increased. In other words, the return to nature has started again [12].

S. virgata is used against wound healing, skin diseases, and blood cancer, because of that, it is an important and high-value plant [13,14]. Besides, it has known various biological activities such as antimicrobial, antioxidant, antidiabetic, hypoglycemic, antinociceptive peroxidase, oxidase, anti-inflammatory, and antibacterial [15,16]. Whereas, within the scope of the study we have presented, anticancer and enzyme inhibitor effect screening of S. virgata is being investigated for the first time.

In the present study, we aimed to define the biological activities of 80% ethanol extracts of *S. virgata*. For this, the antiproliferative, antimicrobial, antioxidant, and various enzyme inhibitory activities of the extract were analyzed.

Materials and Methods

Plant materials

The aerial parts of the plants in full flowering periods were collected from the natural area (Yozgat-3446408 E, 3948346 N, 1216 m) on 05.07.2017. Collected fresh aerial parts were dried at 21°C. Species identification of the collected plants was made in Yozgat Bozok University Biology Department. All experimental stages of the study were carried out in the laboratories of Sivas Cumhuriyet Faculty of Pharmacy. Because, all our experimental researches were conducted in vitro conditions, no ethical committee approval was obtained.

Preparation of extracts

The aerial parts of the plants firstly were dried on shadow, and then dried parts of the plant were milled with a blender (Bluehouse). 10 grams of plant powder was soaked in 50 mL of 80% ethanol-distilled water with 48 hours of intermittent shaking, then filtered from inside Whatman qualitative filter paper, Grade 1. The filtrate was condensed to complete dryness in a rotary evaporator at reduced pressure and 40°C. Analysis of the extracts obtained was done using GC-MS.

Gas chromatography-mass spectrometry (GC/MS) and GC analysis of extracts

The Gas Chromatography/Mass Spectrometer was exploited to identify the components of the extracts and determine the relative percentages [17]. GC-MS analyses were worked with a mass spectrometer detector. The technique, in which helium gas at 1.5 ml constant flow rate was used as the carrier gas, was programmed in split mode with an injection volume of 1 µl and a rate of 5 per minute among 80-300. Post-run was set at 300°C for 2 min. The total run time was 60 minutes [18]. The chemical composition of the obtained extract was searched with three different libraries (W9N11.L, NIST05a.L, and wiley7n.I).

In *vitro* antioxidant activity

The DPPH radical scavenging activity of the extract was evaluated in accordance with the Blois method with minor modification [19]. ABTS radical scavenging activity was evaluated by the method of Re and others with minor modifications [20]. Total phenolic content was determined with spectrophotometric method [21], and expressed as gallic acid equivalents and flavonoid content was determined with the aluminum chloride colorimetric method of Molan and Mahdy [22]. The total ingredient of flavonids was defined as milligram catechin equivalent corresponding to one gram of the dry weight of the extract.

In *vitro* enzyme inhibition assay

The *acetylcholinesterase/butyrylcholinesterase* inhibition analysis was carried out in accordance with the Ellman method as described by our previous study [23]. The α-glucosidase and α-amylase enzyme inhibitor activity analysis was performed with the technique presented by Kumar and others [24]. Acarbose was defined as a positive control for both methods [25].

Cell viability assay

MDA-MB-231 cell line (human breast carcinoma, ATCC, HTB-26) and L929 cell line (mouse fibroblast cells, ATCC) were cultured in DMEM including 10% (v/v) FBS and 1% penicillin/streptomycin, at 37°C in a 5% CO_2 humidified atmosphere until they reached approximately an 85-95% confluence. The cell viability of the ethanolic extract of *Salvia virgata* was assessed using the XTT cell viability assay (Roche Diagnostic, Germany) against the MDA-MB-231 and L929 cell lines. The extract was dissolved in Dimethyl sulfoxide (DMSO) and diluted with DMEM prior to exposure. Cells were seeded in 96-well plates at the density of 1.5 × 10^4 cells per well and were allowed to attach for nearly 6-8 h. After attachment, the cells were exposed to several concentrations (0.0625, 0.125, 0.25, 0.5, 1 mg/mL) of ethanolic extract of *S. virgata* for 24 h. Besides, non-treated cells and cells exposed to DMSO (0.5%) were used as negative control and solvent control respectively. At the end of the exposure time to the extract, an XTT labeling reagent (50 µL) was mixed into all well for the assignment of metabolically active cells and then the plates were incubated at cell culture conditions for 4 h. After mixing, the absorbance of each well was measured using a plate spectrophotometer (Thermo, Germany) at 450 nm against the control. All experiments were managed in three independent and different experiments, and the cell proliferation was clarified in % related to control (100% of viability).

Antimicrobial activity

The antimicrobial properties of the extracts were investigated by means of *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), and *Candida albicans* (ATCC 10231) strains. The extracts whose minimum inhibitory concentration (MIC) was specified by using the broth microdilution method were dissolved in 50% DMSO (50 mg/mL) and applied to the microplate with two-fold dilution. Mueller Hinton Broth was used for bacteria and Sabouraud Dextrose Broth for *C. albicans*. The final inoculum size was determined as 5 × 10^5 CFU/mL in bacteria wells and 0.5-2.5 × 10^3

CFU/mL in *C. albicans* wells [26,27]. Final extract concentration in wells was ranged from 2.5 to 0.004 mg/mL. At the end of 24 hours' incubation at 37°C, the lowest extract concentration that entirely inhibits microbial growth was defined as the MIC value of the extracts.

Statistical analysis

Each experiment was repeated at least three times and the data are reported as the mean ± SD. One-way analysis of variance (ANOVA) was used to compare the results from different treatments and control cells. P values less than or equal to 0.05 were considered to be statistically significant. Data were analyzed using the GraphPad Software (San Diego, CA, USA).

Results

Chemical components of 80% ethanol extracts of *Salvia virgata*

The GC-MS analysis were implemented to identify the chemical composition of 80% ethanol extracts of Salvia virgata. According to Table 1, the major component "Olean-12-en-3-ol, acetate, (3.beta)- (CAS) (21.41%)" was determined, and it was followed by "Caparratriene" with 14.54% and "Cyclododecane" with 6.95%.

DPPH radical scavenging activity (%)

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical shows absorption at 517 nm. Because it can readily undergo reduction by an antioxidant, it uses in the free radical-scavenging for activity determine [30]. In this study, the results of the percentage DPPH Radical Scavenging Activity that the *Salvia virgata* had significant antioxidant activity. The IC_{50} values for DPPH and gallic acid (reference matter) were determined as 291.58 ± 0.004 µg/mL and 0.39 ± 0.01 µg/mL. According to the obtained data in this study, the curve of *S. virgata* had a value close to the standard gallic acid curve (Figure 1).

Table 1. Chemical components of 80% ethanol extracts of *Salvia virgate*

Chemical Components	RT*	Relative Content (%)
2-Methoxy-4-vinylphenol	22.604	2.95
1-Dodecanol (CAS)	28.269	3.94
MEGASTIGMATRIENONE 2	32.160	1.35
Cyclododecane	33.470	6.95
Phytol	40.737	4.57
beta.-Amyrin	46.030	2.65
Viminalol	48.748	1.65
.alpha.-Amyrin	48.800	1.41
Olean-12-en-3-ol, acetate, (3.beta)- (CAS)	52.038	21.41
Caparratriene	53.635	14.54
Hop-22(29)-en-3.beta.-ol	56.026	5.25
Total		66.67

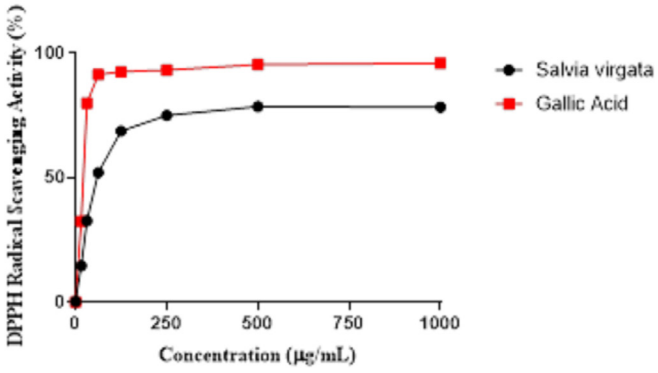


Figure 1. DPPH radical scavenging activity of 80% ethanol extracts from *Salvia virgata*

ABTS radical scavenging activity (%)

According to the ABTS radical scavenging activity method, *S. virgata* plant had a strong antioxidant activity. The scavenging effect of the extract on ABTS radical increased in a linear manner with increasing concentration from 0.1 to 2.0 mg/mL. Gallic acid was evaluated as a positive control in ABTS radical scavenging activity test and ABTS radical scavenging activity curve gave approximately the same value as the gallic acid curve. The IC_{50} values for ABTS and gallic acid were determined as 16.74 ± 0.007 µg/mL and 0.39 ± 0.01 µg/mL (Figure 2).

TFC (Total Flavonoid Content) and TPC (Total Phenol Content)

Many plant extracts have phenolics and flavonoid compounds. These compounds have antioxidant properties and they can prevent fatal disease formation such as cancer, etc. [36-39]. In this study, it was obtained that the total phenol content is higher than total flavonoid content (283.35 ± 10.4 mg GAE/g and 13.37 ± 1.6 mg QE/g, respectively) (Figure 3). The methanol extract of Salvia virgata enriched in phenolic compounds [32].

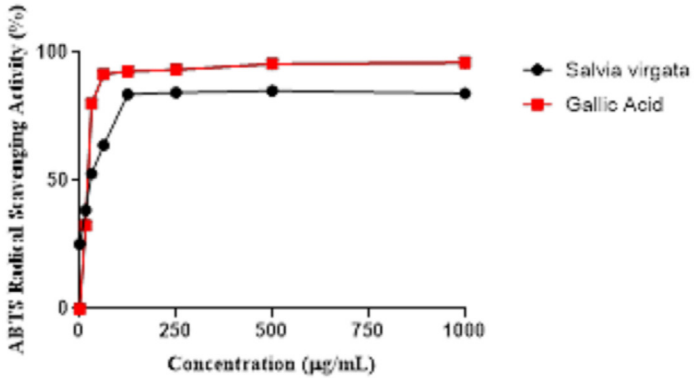


Figure 2. DPPH radical scavenging activity of 80% ethanol extracts from *Salvia virgata*

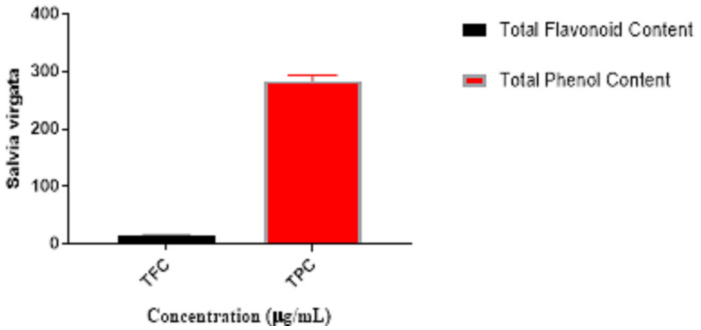


Figure 3. Total flavonoid (mg QE/g) and total phenolic content (mg GAE/g) of *Salvia virgata*

Enzyme inhibition activity

In this study, the aqueous ethanol extract of *Salvia virgata* was screened against α-amylase and α-glucosidase, AChE, and BChE enzymes. The enzyme inhibition activity results are present in Table 2. The ethanol extract of *S. virgata* exhibited 67.19% and 3.18% inhibition of AChE and BChE, which is lower than the reference drug galanthamine (93.87% for AChE and 89.89% for BChE). However, the extract demonstrated higher α-glucosidase and α-amylase inhibitor activity (75.73% and 62.72%, respectively) than reference drug acarbose (57.56% and 58.40%, respectively).

Antimicrobial activity

The antimicrobial activities of *S. virgata* ethanol extract against *E. coli*, *S. aureus*, *P. aeruginosa*, *B. cereus*, *C. albicans*, and *C. tropicalis* were determined by the microdilution technique at the increasing concentrations (0.312 to>2.5mg/mL) (Table 3). Holetz and others defined the antimicrobial activity as good (MIC<100 µg/mL), moderate (MIC; 100 to 500 µg/mL), weak (MIC; 500 to 1000 µg/mL) and inactive (MIC>1000 µg/mL) based on the MIC values [41].

Table 2. Enzyme inhibition activity of 80% ethanol extract of *Salvia virgata* at 2000 µg/ml concentration

Plant extract / Reference drug	Inhibition activity (% ± SD[a])			
	AChE	BChE	α – glucosidase	α -amylase
Salvia virgata	67.19 ± 1.78	3.18 ± 0.84	75.73 ± 1.48	62.72 ± 1.79
Reference Drug	93.87 ± 0.56[b]	89.89 ± 0.01[b]	57.56 ± 0.52[c]	58.40 ± 0.63[c]

[a]Standard Deviation (n=3), [b]Galanthamine hydrobromide, [c]Acarbose; [d]Kojic acid

Table 3. The antimicrobial activity values of 80% ethanol extracts of *Salvia virgata*

	Microorganisms and MIC values (mg/mL)					
	E.coli	***S.aureus***	***P.aeruginosa***	***B.cereus***	***C.albicans***	***C.tropicalis***
	ATCC 25922	ATCC 29213	ATCC 27853	ATCC 11778	ATCC 10231	DSM 11953
Salvia virgata	0.312	0.312	1.25	0.625	>2.5	1.25

According to the proposed activity alignment, the ethanol extract of *S. virgata* exhibited moderate antimicrobial activity towards *E. coli* (0.312 mg/mL) and *S. aureus* (0.312 mg/mL), while weak antimicrobial activity towards *B. cereus* (0.625 mg/mL). The ethanol extract of *S. virgata* did not show antimicrobial activities on *P. aeruginosa*, *C. albicans*, and *C. tropicalis*. The previous study has demonstrated that the essential oil of *S. virgata* displayed moderate antimicrobial activity towards *S. aureus* and *C. albicans* [15].

Cell viability assay

As a result of the XTT cell proliferation experiment, the ethanol extract at all concentrations remarkably decreased MDA-MB-231 cell viability (P<0.05) in a dose-dependent manner (IC_{50} = 0.118 mg/mL), as seen in Figure 4. Additionally, the extract displayed no considerable cytotoxicity on the L929 cell line (0.0625-1 mg/mL). As a result of the cell viability experiment, we determined that the ethanol extract of *S. virgata* exhibited an effective cytotoxic activity at all concentrations. It is thought that the antioxidant activity caused by the quantity of the polyphenolic components of the extract also causes the anti-cancer effect. It remains the first study to indicate the cytotoxic effect of *S. virgata* leaf extracts on MDA-MB-231.

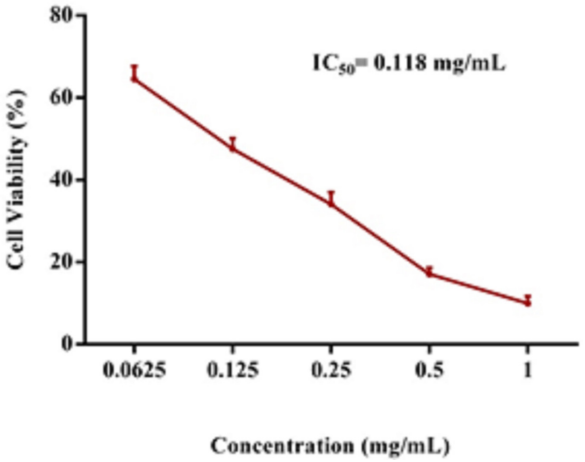


Figure 4. Effects of the ethanol extract from *Salvia virgata* on the viability of MDA-MB-231 cell line, after treatment with various concentrations (range: 0.065-1 mg/mL) for 24 h. The extract shows no toxicity in normal cells

Discussion

Traditional and herbal medicines play important role in primary health care in many low- and middle-income countries. Medicinal plants frequently contain numerous bioactive components that have various biological and physiological activities [42]. Salvia is the most spacious genus of the Lamiaceae family, with in excess of 900 species around the world which about 60 species. The folklore uses of Salvia species are endorsed by pharmacological and phytochemical inquiries [43].

This study was handled in order to detect the content of the ethanol extract of *Salvia virgata*. In this context, it was collected when the plant was in full flowering periods. Then, the ethanolic extract was obtained from the aerial parts of the plant and Gas Chromatography-Mass Spectrometry (GC/MS) analysis was performed. The components of the extracts and the relative percentages were determined by scanning three different libraries (W9N11.L, NIST05a.L, and wiley7n.I) through GC/MS analysis [18]. In line with the results we obtained, we determined that the major content of the ethanol extract of *S. virgata* was "Olean-12-en-3-ol, acetate" with (21.41%). We also found that this is followed by "Caparratriene" and "Cyclododecane" with 14.54% and 6.95% ratios, respectively. The study done in Turkey by Koşar and others, they were extracted with different solvents (hexane, ethyl acetate, methanol, and 50% aqueous) of *S. virgata* [2]. According to that, *S. virgata* has rosmarinic acid, caffeic acid, and lutelin-7-O-glycoside as a phenolic component. According to the report of Alizadeh in 2005, the main components of *S. virgata* were determined as β-caryophyllene (24.58-42.54%), caryophyllene oxide (10.25-19.88%), sabinene (8.64-19.58%), 1-Octen -3-Ol (7.54-8.59%), terpinene-4-ol (4.25-6.64%), and α-thujene (3.74-6.46%) by GC-MS [15]. As can be seen in previous results, secondary metabolites are synthesized from primary metabolites, and itis formed as a result of the responses of plants to environmental conditions. It also acts as a chemical intermediary between the plant and its environment [28]. The same plant species, which are growing in different environmental conditions, can accumulate significantly different secondary metabolites [29]. For these reasons, secondary metabolites production should be done in culture condition. Also, it creates opportunities for obtaining different plant bioactive compounds.

We have studied DPPH [30], ABTS [20] radical scavenging activity tests, as well as TFC [22] and TPC [21] tests, which are usable for the search for flavonoids and phenols that show antioxidant activity, in the extract that we think, has high antioxidant capacity. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, which absorbs at 517 nm and can be easily degraded by an antioxidant, is used in free radical scavenging to determine activity. As a result of our findings, the ethanol extract of *S. virgata* had a strong antioxidant activity with IC_{50} values for DPPH (291.58 ± 0.004 μg/mL) and gallic acid (reference matter; 0.39 ± 0.01 μg/mL). Another marker that supports our DPPH findings is our analysis results of ABTS radical scavenging activity. The previous studies have indicated that the methanol extract of *S. virgata* showed moderate antioxidant activity [31,32], on the other hand, Tosun and others in 2009 [33], and Şenol and others in 2010 [34] found *S. virgata* extracts to high free radical scavenging capacity. Our results support that the IC_{50} values for ABTS (16.74 ± 0.007 μg/mL) and gallic acid (0.39 ± 0.01 μg/mL) the strong antioxidant activity of *S. virgata* extract, as with DPPH. According to the report by Karatoprak and others [35], the water extract of *Salvia virgata* had antioxidant activity by the ABTS method. DPPH and ABTS assay put forward *Salvia virgata* antioxidant activities.

We studied total flavonoid content (TFC) and total phenol content (TPC) analyses to investigate whether the detected high antioxidant activity could come from phenolic and flavonoid compounds. In this context, we observed that the ethanol extract of *S. virgata* has a rich infrastructure in terms of both phenolic and flavonoid content. In addition, as in the methanolic extract of *S. virgata*, we found that the ethanolic extract of *S. virgata*, the main subject of our study, had a higher phenolic content than the flavonoid content.

In the present study, the activity of aqueous ethanol extract of *Salvia virgata* against α-amylase and α-glucosidase, AChE, and BChE enzymes was investigated. The extract indicated higher α-glucosidase and α-amylase inhibition activity (75.73% and 62.72%, respectively) than reference drug acarbose (57.56% and 58.40%, respectively). According to the report by Ekin and others *S. virgata* extract showed 61.15% inhibition of α-glucosidase and 8.93% inhibition of α-amylase, while no inhibition activity against AChE and BChE [40]. The α-glucosidase inhibition activity results of the study are inconsistent with our results.

The antimicrobial activity of the ethanolic extract of *Salvia virgata* against *E. coli, S. aureus, P. aeruginosa, B. cereus, C. Albicans*, and *C. tropicalis* was analyzed by microdilution technique according to the cutoff defined in 2002 by Holetz and others [41]. According to the proposed activity alignment, the ethanol extract of *S. virgata* exhibited moderate antimicrobial activity towards *E. coli* (0.312 mg/mL) and *S. aureus* (0.312 mg/mL), while weak antimicrobial activity towards *B. cereus* (0.625 mg/mL). The ethanol extract of *S. virgata* did not show antimicrobial activities on *P. aeruginosa, C. albicans*, and *C. tropicalis*. Our results are in close agreement with Alizadeh who studied the antimicrobial of essential oil of *S. virgata* and reported that the essential oil of *S. virgata* displayed moderate antimicrobial activity towards *S. aureus* [15]. The results of the study indicated that the ethanol extract of *S. virgata* has shown moderate inhibitory effects against AChE and higher α-glucosidase and α-amylase inhibitory activity.

The non-radioactive, colorimetric XTT test, first described by

Scudiero et al. and later evolved by many researchers, is based on the principle of reducing a yellow tetrazolium salt to orange-colored formazan dye by living cells that have not lost its metabolic activity [44]. The test is utilized to determine the viability and proliferation rates of cells that have not lost their metabolic activity [45-46]. As a result of the XTT cell proliferation experiment, the ethanol extract at all concentrations remarkably inhibited MDA-MB-231 cell viability ($P<0.05$) in a dose-dependent manner (IC_{50} = 0.118 mg/mL).

Conclusion

The existence of a significant correlation between cytotoxic and antioxidant activity and total phenolic content suggested that the ethanolic extract of *S. virgata* may have significant anticancer activity. On the other hand, the extract has been demonstrated higher α-glucosidase and α-amylase inhibitor activity. These findings suggest that the extract may be an important product in the treatment of diabetes. Further investigations should be carried on the α-glucosidase and α-amylase inhibitory activity of *S. virgata* in order to find potential active compounds by activity guided isolation technique. As a striking result of our study, the ethanolic extract of *S. virgata* can be considered as a potential therapeutic agent in cancer and diabet.

Conflict of interests
The authors declare that they have no competing interests.

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Ethical approval
The author confirms that this article content is not required to have consent of ethics.

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