International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 174–180

https://doi.org/10.21448/ijsm.722574

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Antimicrobial and Antiproliferative Activities of Chia (*Salvia hispanica* L.) Seeds

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Abstract: The genus *Salvia* L. (Lamiaceae) has been traditionally used for the treatment of various illnesses since ancient times. *Salvia hispanica* L., commonly known as Chia, is an annual herbaceous plant which was one of the most significant crops for pre-Columbian civilizations (Aztec and Maya) in America. Nutritional potential and beneficial effects of Chia seeds on human health have been previously reported. Therefore, this study aims to investigate anti(myco)bacterial, antifungal, and antiproliferative activities of Chia seeds. Ethanol extract of Chia seeds were tested against *Staphylococcus aureus* (ATCC 25925), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25923), *Acinetobacter baumannii* (ATCC 02026), *Aeromonas hydrophila* (ATCC 95080), *Candida albicans* (ATCC 14053), *Candida tropicalis* (ATCC 1369), and *Candida glabrata* (ATCC 15126) using broth microdilution method. Antimycobacterial activity was performed against *Mycobacterium tuberculosis* H37Rv using resazurin microtiter plate method. Ampicillin, Ethambutol, Isoniazid, and Fluconazole were chosen as reference drugs. Antiproliferative effect of the various concentrations (200, 100, 50, and 25 µg/mL) of ethanol extract was tested against A549 human lung cancer cell lines using MTT method. Ethanol extract was found to be more effective against *A. baumannii* (MIC: 62.5 µg/mL) than reference drug Ampicillin (MIC: 125 µg/mL). There was a correlation between increased doses and antiproliferative activity of extract against A549 human lung cancer cell lines (p <0.05).

ARTICLE HISTORY

Received: April 18, 2020
Revised: May 10, 2020
Accepted: June 25, 2020

KEYWORDS

Salvia hispanica L.,
Chia,
Seed,
Antimicrobial activity,
Antiproliferative activity

1. INTRODUCTION

Intake of dietary phytochemicals has been played important roles in the prevention of various illnesses including cancer, inflammatory, and cardiovascular diseases. Due to their medicinal properties plants and their metabolites are also used in different industries [1]. *Salvia* L. (sage) is the most species-rich genus of the family Lamiaceae (mint family) with approximately 1000 species [2]. It has been reported since ancient times that *Salvia* species have been traditionally used in the treatment of tuberculosis, bronchitis, and microbial infections [3]. Some species of the genus have been used worldwide on account of their beneficial effects on human health and nutritional properties [4].

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ISSN-e: 2148-6905 /© IJSM 2020

Salvia hispanica L., commonly named as Chia, is an annual herbaceous plant which is native to northern Guatemala and southern Mexico and is also cultivated in some countries including Mexico, Bolivia, Australia, Argentina, Colombia, Peru, and Guatemala [4]. In the recent years, Chia seeds which were one of the most significant crops for pre-Columbian civilizations (Aztec and Maya) in America [4,5], have been used in the food, animal feed, medical, cosmetics, and pharmaceutical industries [6]. Chia seeds have important roles as nutritional supplement and functional food. Moreover, seeds contain no toxic components and gluten, thus making Chia seeds a safe ingredient also gluten free diets [4].

According to the literature, antiproliferative activity of Chia seeds was studied against some cancer cell lines [7,8]; however, we didn't reach any available literature on antiproliferative effect of Chia seeds against A549 human lung cancer cell lines. Additionally, antimicrobial activity of Chia seeds has been investigated in few studies [9,10]. But some factors such as geographical origin and extraction procedure were changed composition of bioactive compounds in seeds. The consumption of Chia seeds has been increasing over the years due to their health benefits and uses in cooking [4]. Therefore, in this study, we aimed to investigate *in vitro* anti(myco)bacterial, antifungal, and antiproliferative activities of Bolivian Chia seeds.

2. MATERIAL and METHODS

2.1. Chemicals

Isoniazid, Fluconazole, Ethambutol, RPMI 1640 Medium, 3-(N-morpholino)-propanesulfonic acid, Resazurin sodium salt powder, Dulbecco's modified eagle's medium (DMEM), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] (MTT), and Fetal calf serum (FCS) were purchased from Sigma-Aldrich (St. Louis, MO, USA); ethanol and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany); and Middlebrook 7H9 broth, casitone, glycerol, and oleic acid-albumin-dextrose-catalase were purchased from Becton Dickinson (Sparks, MD, USA). All solutions were prepared with distilled water and freshly prepared solutions were used.

2.2. Plant Material and Extraction Procedure

Commercially available Chia seeds from Bolivia (2019 harvest) were purchased from a local market. Powdered seeds were extracted twice with ethanol (20 mL solvent per 1 g seed; 96%) by stirring overnight at room temperature then filtered using Whatman Grade No.1 filter paper. Solvent was evaporated via a vacuum evaporator (Heidolph Instruments, Germany) and obtained extract was kept in the dark at 4 °C.

2.3. Antimicrobial Activity

Gram-negative bacterial strains [*Acinetobacter baumannii* (ATCC 02026), *Escherichia coli* (ATCC 25923), *Aeromonas hydrophila* (ATCC 95080)]; gram-positive bacterial strains [*Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25925)]; *Mycobacterium tuberculosis* H37Rv; and fungal strains [*Candida albicans* (ATCC 14053), *Candida tropicalis* (ATCC 1369), *Candida glabrata* (ATCC 15126)] were procured from Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey.

2.3.1. Antibacterial Activity

Antibacterial activity was studied using a broth microdilution method [11]. Ampicillin was used as a reference drug. Sample was dissolved in DMSO for preparing initial concentration (2000 µg/mL). The mixture was used for having stock solution which was diluted in Mueller-Hinton broth. Further dilutions of reference drug and extract were prepared (1000-0.9 µg/mL). Standard strain working suspensions were made in sterile tubes. Turbidity adjusted to match McFarland standard No: 0.5. Further dilutions (1:20) of suspensions were prepared in

distilled water and added to each plate (10 µL). Thus each plate's bacterial concentration was adjusted to $5x10^5$ CFU/mL. Effect of DMSO was tested. The minimal inhibitory concentration (MIC) values were determined in duplicate tests.

2.3.2. Antimycobacterial Activity

Antimycobacterial activity was tested using the resazurin microtiter assay [11]. Isoniazid and Ethambutol were used as reference drugs. Resazurin reagent was prepared using Resazurin sodium salt powder. Middlebrook 7H9 broth containing 0.1% casitone, 0.5% glycerol, and 10% oleic acid-albumin-dextrose-catalase and 7H9-S medium were used for preparing culture medium. A resazurin working solution (0.01% (w/v)) made in distilled water and stock solutions (1000 µg/mL) of extract and reference drugs prepared in DMSO were filtered through 0.22 µm membrane filter (Ministar, Goettingen, Germany). A two-fold dilution series were performed using 7H9-S medium (100 µL) in a 96-well microtiter plate. 0.12-250 µg/mL concentration ranges were detected. A growth control and a sterility control were added to each plate. The bacterial inoculum was prepared in a tube which was containing 7H9-S medium (5 mL) via resuspending a loopful of Lowenstein-Jensen culture medium. During 2 min the tube was mixed then waited to allow sediment. After supernatant was added in sterile tube, the turbidity adjusted to match McFarland standard No: 1. 7H9-S medium was used to prepare dilutions (1:20) of these suspensions. Plates were inoculated with diluted suspension (100 µL) then put into plastic bags. After incubation period (37 °C, 7 days) Resazurin working solution (30 µL) was added to each well then plates were incubated (37 °C, 24 h) and results were visually recorded. The lowest concentration that prevents complete color change of resazurin from blue to pink was determined as MIC value. Experiments were done in duplicate.

2.3.3. Antifungal Activity

Antifungal activity was studied using a broth microdilution method of NCCLS [12]'s standard document (M27-A2) with minor modifications [11]. RPMI 1640 medium which buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propanesulfonic acid was used. Fluconazole was used as a reference drug. Working suspensions of standard strains were made as a 1:100 dilution followed by a 1:20 dilution of the stock suspensions using RPMI 1640 medium. Stock solutions (1000 µg/mL) of extract and reference drug dissolved in DMSO were filtered through membrane filters. Two-fold dilution series were added in a 96-well microtiter plate using RPMI 1640 medium (100 µL). 250-0.12 µg/mL concentration ranges were tested. A growth control and a sterility control were added to each plate. 100 µL of working inoculum suspension was added to each plate and plates were incubated (48 h, 35 °C). MIC values were visually determined in duplicate tests.

2.4. Antiproliferative Activity

Determination of cell viability was studied by MTT method. A549 human lung cancer cell lines were procured from ATCC (American Type Culture Collection, VA, USA). DMEM which was supplemented with FCS (10%) was used for cell cultivation. Cells were kept in suitable culture conditions (95% air; 5% CO_2; 37 °C). After reaching 70-80% confluency cells were detached with Trypsin-EDTA solution (3.0 mL) and settled to 96-well plates (10^4 cells per well). After 24 h, various concentrations (200, 100, 50, and 25 µg/mL) of ethanol extract dissolved in DMSO were applied and cells were incubated (24 h). Cells treated with growth medium containing no FCS were used as a control. After incubation, supernatants were replaced with MTT (1 mg/mL) dissolved in growth medium then incubated (37 °C) until purple precipitate was visually detected. The supernatants were removed; cells which absorbed MTT were dissolved in DMSO. Plates were detected using a spectrophotometer (Epoch, Winooski, USA) at a 550 nm. Effect of DMSO was tested. Experiments were done in four replicates [13].

2.5. Statistical Analysis

SPSS 25.0 (IBM, NY, USA) was used for statistical analyses. The data are provided as the mean ± SD. Kruskal Wallis H and one-way analysis of variance (ANOVA) with Tukey's post hoc test were used. P values < 0.05 were considered as significant.

3. RESULTS and DISCUSSION

In the present study antimicrobial and antiproliferative activities of ethanol extract (yield 20.67% (w/w)) of Chia seeds were evaluated. Antimicrobial activity results are provided in Table 1. When compared to reference drug Ampicillin (MIC: 125 µg/mL) seed extract had greater activity against *A. baumannii* (MIC: 62.5 µg/mL). Extract showed antimycobacterial activity against *M. tuberculosis* H37Rv (MIC: 62.5 µg/mL); however, the efficiency of the extract was not found as strong as Isoniazid and Ethambutol (MIC values: 0.97 µg/mL and 1.95 µg/mL, respectively). Extract showed the highest antifungal activity against *C. glabrata*; but result was not found as high as Fluconazole (MIC values: 31.25 µg/mL and 3.90 µg/mL, respectively).

Table 1. Minimum inhibitory concentrations of Chia seeds, and reference drugs against bacterial and fungal strains (µg/mL).

Microorganisms	*S. hispanica*	Reference drugs			
		A	I	E	F
Bacterial strains					
Staphylococcus aureus ATCC 25925	250	31.25	-	-	-
Bacillus subtilis ATCC 6633	250	0.9	-	-	-
Escherichia coli ATCC 25923	250	15.62	-	-	-
Acinetobacter baumannii ATCC 02026	62.5	125	-	-	-
Aeromonas hydrophila ATCC 95080	125	31.25	-	-	-
Mycobacterium tuberculosis H37Rv	62.5	-	0.97	1.95	-
Fungal strains					
Candida albicans ATCC 14053	62.5	-	-	-	31.25
Candida tropicalis ATCC 1369	62.5	-	-	-	15.62
Candida glabrata ATCC 15126	31.25	-	-	-	3.90

Values determined in duplicate with deviations within one two-fold dilution. -: Not tested. (A: Ampicillin; I: Isoniazid; E: Ethambutol; F: Fluconazol)

Antimicrobial effect of Chia seeds was investigated against several microorganisms including *E. coli, A. baumanii, S. aureus,* and *C. albicans*; and aqueous and aqueous-ethanol extracts exhibited antimicrobial activity against *E. coli* [10]. Despite these results, protein hydrolysates of Chia seeds were not showed antimicrobial activity against *E. coli, Salmonella typhi, Shigella flexneri, Klebsiella pneumoniae, S. aureus, B. subtilis*, and *Streptococcus agalactiae* [9]. In our study, seeds exhibited antibacterial activity against both tested gram-negative and gram-positive bacterial strains. According to the literature, composition and concentration of bioactive compounds in Chia seeds vary depending on some factors like geographical origin, climatic conditions, agricultural practices, and extraction procedures [4]. These factors also change the effectiveness of the tested extract. This may explain different results in our study and in previous studies. Due to their hydrophilic cell wall structure which contains lipopolysaccharides inhibits accumulation of hydrophobic oils and extracts, and penetrations of these substances through the target cell membrane, gram-negative bacteria are more resistant against natural components than gram-positive bacteria [14]. When compared to Ampicillin, seeds showed greater activity against gram-negative nosocomial pathogen *A.*

baumannii which is one of the important healthcare problems worldwide because of its ability to gain resistance to all classes of antimicrobial agents used against it [15]. According to the our results, Chia seeds might be promising sources in the development of novel therapeutic agents against infections caused by *A. baumannii*.

Antiproliferative activity results are shown in Table 2. Significantly lower cell viability levels were observed in 100 µg/mL concentration applied group than control group (0.963 ± 0.036 and 1.092 ± 0.012, respectively) and in 200 µg/mL concentration applied group than control and DMSO groups (0.936 ± 0.036; 1.092 ± 0.012; and 1.085 ± 0.009, respectively) ($p < 0.05$). However, there were no significantly differences found between the other groups ($p > 0.05$).

Table 2. Antiproliferative effect of Chia seeds on A549 human lung cancer cell lines in MTT cell viability assay.

Groups	Control	DMSO	*S. hispanica*			
			Studied concentrations of the extract (µg/mL)			
			25	50	100	200
Results	1.092 ±0.012 (1.083-1.109)	1.085 ± 0.009 (1.073-1.095)	1.021 ± 0.025 (1.000-1.057)	0.983 ± 0.031 (0.953-1.025)	0.963 ± 0.036[a] (0.938-0.974)	0.936 ± 0.036[ab] (0.896-0.971)

Measuring the average ± SD. Min-Max value intervals are in parenthesis. Kruskal Wallis H and ANOVA with Tukey's *post hoc* test were used. n = 4. A P value less than 0.05 was considered to be significant. [a] Significantly different from control group. [b] Significantly different from DMSO group. (Control: the group was not exposed any chemical, just incubated only with medium; DMSO: the group was treated medium with DMSO).

Chia oil reduced tumor growth, metastasis, and cell mitosis in neoplastic tissue and increased apoptosis. However; in Walker 256 model Chia flour supplementation didn't prevent tumor bearing effects [8]. Effects of mucilage compounds on some cancer lines (HeLa, HCT-15, HCT-116, MCF7, MDA-MB-231, MCF7/Vin, MCF7/Vin^+, MCF7/Vin^- cells, Vero, and HepG2 cells) were evaluated and significant inhibition on proliferation of MCF7, HeLa, and HepG2 cells with low toxicity were determined [7]. However, we didn't reach any available literature on antiproliferative effect of Chia seeds against A549 human lung cancer cell lines. The lung cancer which is the most common type of malignant tumors with high mortality is expected to cause over than 3 million deaths for the year 2035 [16]. A549 human lung cancer cell lines have been widely studied for cancer research since 1976 [17]. In our study, ethanol extract of Chia seeds were found to be more effective against A549 human lung cancer cell lines at a dose of 200 µg/mL and there was a correlation between increased doses and activity.

Phenolic acids (caffeic acid and its derivatives, ferulic acid, rosmarinic acid [4], chlorogenic acid [4, 6, 10]), flavonoids (myricetin, quercetin, kaempferol [4, 6, 10], rutin [10], daidzin, genistein, genistin, glycitein, glycitin [4]), fatty acids (α-linolenic acid, linoleic acid, palmitic acid, stearic acid, oleic acid), tocopherols (α-, δ-, γ-tocopherol), dietary fiber, proteins, carbohydrate, vitamins, amino acids [4, 6], minerals [4, 18], mucilage [6,7], and phytosterols [19] have been reported as chemical constituents of Chia seeds in previous studies. Ethanol was a suitable solvent for phenolics, sterols [20] and broad range of polar constituents [21]. Increased uses of phenolic compounds as antimicrobial agents, and food stabilizers in food technology have been reported. Because of food safety, ethanol and water are the most suitable solvents for extraction of phenolics [22]. Therefore, in the current study, ethanol was preferred as extraction solvent. Antimicrobial properties of flavonoids, phenolic acids [20], polysaccharides and sterols [14] and antiproliferative activity of phenolic compounds [16] have been reported previously.

4. CONCLUSION

The consumption of Chia seeds has been increasing over the years and its health benefits related to chronic diseases like cardiovascular diseases, obesity, cancer, and diabetes. In the current study, we investigated some biological properties of Bolivian Chia seeds. Chia seeds might be promising sources in the development of novel therapeutic agents against *A. baumannii*. Antiproliferative effect of Chia seeds against A549 human lung cancer cell lines was determined for the first time. In the future, this study may be the basis for further studies on the effects of seeds against lung cancer and in addition to its nutritional potential this might be a new topic to support Chia consumption.

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).

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