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Elucidation of Apigenin Derivatives from Ethyl Acetate Fraction of *Stachys byzantina* with Potent Antioxidant and Anti-*Staphylococcus aureus* Properties

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Abstract

The *Stachys* genus is a medicinal plant, with 34 different species, from Lamiaceae family that grows in Irano-Anatolian plateau. There are 12 species of *Stachys* native to Iran. Some species of Stachys have been used in traditional medicine of Iran, Turkey, Italy, Greece, China, and Japan for the treatment of various diseases like inflammation and common cold. Some researchers have noted many biological effects, such as cytotoxic, antioxidant, immunomodulator, anti-inflammatory and antibacterial effects for the members of this genus. There are a few evidences on phytochemical constituents of *S. byzanthina*. The aim of this study was phytochemical investigation of various extracts of this plant to obtain the most efficient fractions and compound(s) to study the antimicrobial effects of this plant. Separation and isolation of compounds was performed by various methods of chromatography. The structure of each compound was identified by spectroscopic methods. Antimicrobial studies were performed on different fractions of S. byzantina by microdilution method and to determine the Minimum Inhibitory Concentration (MIC), agar well diffusion was done. Ethyl acetate fraction has the highest total phenol contents (1082.05 mg GAE/g fraction) and antioxidant capacity (IC50= 1.23 µg/mL). Also, ethyl acetate fraction, the most potential fraction of *S. byzanthina*, demonstrated the largest inhibition zone (17.5 ± 0.7 mm) with 5 mg/mL in MIC against *Staphylococcus auresus*. Apigenin and its derivatives were isolated from this fraction and according to literature; they can be responsible for antibacterial activity of this fraction of *S. byzanthina*.

Keywords: *Stachys byzantine*; Antibacterial agents; Ethyl acetate fraction; *Staphylococcus aureus*; Minimum Inhibitory Concentration (MIC); Apigenin

Introduction

Stachys byzantina with common name of Lamb's-ear is a member of the Lamiaceae (Labiatae) family. This medicinal plant is a perennial herb, covered with silver-colored hair; the leaves are without stipule; flowers are spike like with pink color, and the corolla completely surrounded by calyx. These characters are mentioned specifically for the genus of Stachys which contain S. byzantina as native species of Irano-Anatolian [1].

The phytochemical compounds identified in Stachys genus can be classified into saponin triterpenoids [2], clerodane diterpenoids [3], kaurenoides [4], iridoids [5], flavonoids and caffeic acid derivatives [6]. In 1991, El-Ansari *et al*. isolated 24 flavonoids, including apigenin glycosides, luteolin glycosides, and

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chrysoeriol glycosides, from aerial parts of *S. aegyptica* [7]. Xanthomicrol and five flavonoids were isolated from extract of *S. schtschegleevii* by Nazmiyeh et al. in 2006 [8]. Delazar et al. isolated and identified two acylated flavonoids glycosides (chrysoeriol 7-*O*-(6-*O*-acetyl-*β*-D-allopyranosyl)-(1→2)-*β*-*D*-glucopyranoside and apigenin 7-*O*-*β*-D-(6-*ρ* coumaroyl)-glucopyranoside) from *S. bombycina* in 2005 [9].

Another category of constituents of *Stachys* is phenylethanoid glycosides with caffeoyl esters. These compounds were isolated from *S. officinalis* [10], *S. macrantha* [2], *S. sieboldin* [11], and *S. riederi* [3].

The species of *Stachys* were extensively used in traditional medicine of several countries such as Iran, Turkey, Italy, Greece, China and Japan [6,12]. *Stachys* is mostly consumed as decoction (herbal tea) to improve common cold and symptoms of asthma, inflammation, sclerosis of spleen, genital tumors, and cancerous ulcers [6,13]. *S. byzantina*, as a well-known species, is traditionally used to treat rheumatic disorders, digestive problems such as cramp, dysentery, and abdominal pains, epilepsy and neuropathy, and also it is used as a diuretic and sedative agent [12,14]. For example, from 37 species of *Stachys* in Russia, 12 of them have medicinal uses such as sedative, anti-hypertension, wound healing, antitussive, and treatment of urinary tract infections [15]. *S. infalata* is used for treatment of rheumatic pain, diarrhea, and wound healing [16]. Also, *S. sieboldii* is used for wound healing, other species of this genus are used to treatment of *Helicobacter pylori* infection in Persian medicine [17].

There are quite few studies on application of *S. byzantina* in literature. Acetone and methanol extract of *S. byzantina* have shown anti-inflammatory and analgesic effect in formalin-test and carrageenan-induced rat paw edema model [18]. In another study on antifungal effect of 35 medicinal plants in Brazil by Durante MCT *et al*., *S. byzantina*, Achillea millefolium and Mikania glomerata essential oils of the leaves and roots of plants have shown best inhibitory effect on *Candida albicans* [19].

One of the most important effects of *Stachys* genus is antibacterial effect. Antibacterial effects of essential oils of S. cadina and *S. chrysantha* on Gram positive bacteria such as *Staphylococcus aureus*, Staphylococcus epidermidis and Gram negative bacteria such as Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa have been proved in 1999 by Skaltsa HD et al. in Greece [20]. The results of another antibacterial study about essential oil of aerial parts of *S. officinalis* in Serbia indicated Minimum Inhibitory Concentrations (MICs) on Gram positive bacteria are lesser than MICs against other group [21]. Saeedi et al. studied antimicrobial activity of methanol extract of *S. byzantina, S. inflata, S. lavandulifolia* and *S. laxa* again some infectious microorganisms. The results of this study showed flavonoids, as one of the main components of *Stachys* spp., led to antibacterial effects especially against Gram positive bacteria [22]. Another study by Mahboubi *et al*. on ethanol and chloroform extracts of *S. byzantina* confirmed the role of polar components such as flavonoids on growth inhibition of Gram positive bacteria [23].

A great number of medicinal plants are source of natural antioxidant. There are some studies on investigation of antioxidant effects of different species of *Stachys* genus [24]. The valuable effect of some species of *Stachys* and *Phlomis* genera to control peroxide production in sunflower oil was demonstrated [25]. Also, the cytotoxic and antioxidant activity of nine species of *Stachys* against HL-60, K562, and MCF-7 cells has been proved [26].

Many studies confirmed that there is a close relationship between total phenol content and antioxidant capacity of medicinal plants. In a study on some species of *Stachys*, the results indicated that *Stachys* has no effect on lipid peroxidation and xanthine oxidase inhibition, but showed very powerful effect on free radical scavenging [27].

Since *S. byzanthina* demonstrated antibacterial effects in hospital-acquired infection, and also its antibacterial and antioxidant properties were confirmed. The aim of this study was isolation and identification of secondary metabolites of effective antioxidant and antibacterial fraction of *S. byzantina* against *S. aureus*.

Materials and Methods

Plant material

The aerial parts of *S. byzantina* C. Koch were collected in September from Mazandaran province (Roudbarak-Kelardasht) of Iran. The scientific name of this plant was confirmed by botanist Dr. Y. Ajani (Research Institute of Forests and Rangelands (RIFR), Tehran, Iran) and the herbarium specimen stored in herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (8564-TEH).

Extraction and fractionation

Aerial parts of plant were dried, away from moisture and direct sunlight. Four hundred seventy grams of dried plant was powdered and extracted using methanol (80%) by maceration method. After concentrating the extract by rotary evaporator, total weight of extract was 105 g, so the yield of extraction was 22.3% approximately. In order to fractionation of *S. byzantina* extract were dispersed in water and liquid-liquid extraction was performed by separatory funnel to yield hexane (16 g), chloroform (2.8 g), and ethyl acetate (12.3 g) fractions. At the end of this step, the residue was dried and then fractionated with methanol to achieve methanol fraction (36.6 g) and the latest

residue named water fraction (37.3 g).

Total phenol contents

Folin-Ciocalteau assay is used to determine presence of total phenolic compounds in each fraction. 2 mL of folin-ciocalteau reagent (1 mL of folin-ciocalteau solution diluted with 9 mL deionized distillated water) was added to 200 µl of sample (200 µg/mL). This mixture was stored at 37 °C. After 5 min, 1.5 mL of saturated sodium bicarbonate solution (6 g of $NaHCO_3$ dissolved in 100 mL deionized distillated water) added. This combination was stored for 90 min in dark place at room temperature and absorbance measured at 725 nm by Shimadzu, UV/VIS model 160A spectrophotometer. The blank was a cell without sample with all previous steps was done on it. All tests were repeated three times [28].

The standard curve of gallic acid solution in different concentrations (0, 25, 50, 100 mg/mL) was drawn and total phenol content of each fraction was reported as mg of gallic acid equivalents (GAE)/g of fraction.

Antioxidant activity determinations

Free radical scavenging (DPPH) Assay

Free radical scavenging activity of total extract and different fractions were investigated by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method [29]. Two mL of DPPH (0.04 mg/mL) added to 1 mL of each sample (0.8, 0.5, 0.25, 0.1, 0.05, 0.005, 0.0005 mg/mL). The blank contained 2 mL DPPH solution with 1 mL methanol without sample. The control included 1 mL sample with 2 mL methanol. The absorbance was measured at 517 nm with Shimadzu, UV/VIS model 160A spectrophotometer at several times (0, 5, 10, 15, 20, 25 and 30 min). The percentage of Antioxidant Activity (AA %) was calculated using the following formula at 30 min:

$$AA\% = (1-(Abs_{-Sample}-Abs_{-control})/Abs_{-blank}))\times 100$$

All tests were repeated three times. The IC_{50} of each fraction was reported according to AA% and equation of trend line.

Reduction antioxidant power (FRAP) Assay

Ferric reducing antioxidant power of total extract and different fractions were determined by FRAP method [30]. Two point five mL of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl added to 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6, as named FRAP reagent. 50 µL of each fraction (100 µg/mL) was added to 1.5 mL of FRAP reagent. This mixture was incubated for 10 min at 37 °C, and after that absorbance of samples measured by Shimadzu, UV/VIS model 160A spectrophotometer at 593 nm. The blank sample was 1.5 mL of FRAP reagent plus 50 µL methanol.

All tests were repeated three times. The calibration curve of aqueous solution of $FeSO_4.7\ H_2O$ in different concentration (125, 250, 500, 1000 mmol/L) was plotted and antioxidant activity of each fraction was reported as Fe^{2+} mmol /g of fraction.

Antibacterial assay

Agar well diffusion method

Antibacterial activity of total extract, and different fractions was measured by agar well diffusion method. The petri dishes were filled with 25 mL sterile Mueller-Hinton-Agar. Then, 1.5×108 CFU/mL (equal to 0.5 McFarland) of standard *S. aureus* (ATCC: 6538), inoculated to growth medium. Seven wells with 6 mm diameter were punched. Ten mg of each sample dissolved in 1 mL DMSO and after that 90 µl of this solution were loaded into surrounding wells. In center well DMSO was loaded alone as blank. Gentamicin disk was used as positive control. Plates were incubated in 37°C for 24 h and then diameter of zone of inhibition was measured and reported.

MIC determination

Minimum Inhibitory Concentration (MIC) is defined as minimum concentration of antibiotic that can prevent visible growth of microorganism. In microdilution method for achieving MIC, 2 mg of each sample was dissolved in 100 mL of DMSO and 900 mL of Mueller-Hinton Broth (MHB) was added. A half of milliliter of this suspension was diluted with 0.5 mL of sterile MHB and serial dilution was continued up to 10 times. One hundred microliter of each prepared suspension was dispensed in 96-well plate, and 100 µL of sterile MHB and also DMSO: MHB (9:1), both used as blank. *S. aureus* (ATCC: 6538, 1.5×108 CFU/mL equal to 0.5 McFarland) was poured in each well at a rate of 100 µL. It is clear that the concentration of each well was halved after adding 100 µL of bacteria suspension. The 96-well plate was incubated at 37 °C for 24 h, and after that the results were investigated. According to results of microdilution method, the concentration below 1 mg/mL was not effective for growth inhibition. So MIC determination was considered by Agar well diffusion. Six concentration of each sample (10, 5, 2.5, 1.25, 0.625, and 0.312 mg/mL) were tested in this step.

Elucidation of compounds

According to results, the ethyl acetate fraction with potent antioxidant and antibacterial effects was selected for isolation and identification of its components. Eight gram of ethyl acetate fraction was subjected on sephadex LH-20 (5.5 × 45 cm), eluting with methanol to yield 5 sub-fractions. Sub-fraction 5 was nominat-

ed for another sephadex LH-20 (2 × 70 cm) column and was chromatographed with 100% methanol. Compounds 1 (9.6 mg), and 2 (28.6 mg) were purified from this column. The spots on TLC had purple color under UV366, and yellow visible color after anisaldehyde reagent was sprayed. Then, sub-fraction 3 with 38 mg was re-chromatographed on silica gel column (1 × 45 cm) eluted with gradient of chloroform to methanol solvent to achieve pure compound 3 (15.3 mg). Color of compound 3 under UV_{366} and anisaldehye reagent was as same as other compounds.

The structures of elucidated compounds were identified by ^{1}H-NMR spectroscopic method.

Results and Discussion

Total phenol content

Table 1 showed the amount of phenolic compounds in ethyl acetate fraction is higher than other samples and the content of total phenol of ethyl acetate fraction was the most in all fractions, equal to 1082.05 mg GAE/g fraction.

Antioxidant activity

The results of two antioxidant DPPH and FRAP assays of hydroalcoholic extract and different fractions of *S. byzanthina*, as antioxidant activity tests, were summarized in table 1.

Antibacterial assay

Agar well diffusion

Ethyl acetate fraction of *S. byzantina* demonstrated the largest inhibition zone diameter on *S. aureus* growth medium among other fractions of this medicinal plant at 10 mg/mL concentration (Table 2.)

MIC determination

Turbidity in all wells demonstrated all samples unable to inhibit bacterial growth in any concentration below 1.0 mg/mL. Also, turbidity in DMSO wells indicated DMSO has no antibacterial activity.

The lowest concentration could create zone of inhibition around each well was determined as MIC. MIC was 5 mg/mL in methanol and ethyl acetate fractions, and 10 mg/mL in other samples, which was the first concentration that inhibited bacterial growth.

Data analysis of isolated compounds

Three compounds were isolated and identified from ethyl acetate fraction.

Compound 1: 4-Methoxycinnamic acid, ^{1}H-NMR (500 MHz, DMSO-*d6*): δ 7.55 (1H, d, *J*= 16 Hz, H-*β*), 7.53 (2H, *d*, *J*= 8 Hz, H-3, H-5), 6.77 (2H, *d*, *J*= 8 Hz, H-2,

Table 1. Total phenol content an antioxidant activities in different fraction of Stachys byzanthina

Sample	Total Phenol (mg GAE/g fraction)	FRAP (mmol Fe^{2+}/g fraction)	DPPH (IC_{50}) (µg/mL)
Hydroalcoholic extract	402.9	1194	15.72
Hexane fraction	65.4	803.4	128.46
Chloroform fraction	282.05	1235	109.81
Ethyl acetate fraction	1082.05	1459	1.23
Methanol fraction	586.25	1350	12.37
Water fraction	627.9	1052	3.48
Vitamin E	-	313	14.1
Butylated hydroxyanisole (BHA)	-	880	7.8

Table 2. Antibacterial activity of S. byzantina in Agar well diffusion method against S. aureus

Sample	Average of inhibition zone diameter (mm) (± SD)
Hydroalcoholic extract	11.0 (± 0.00)
Hexane fraction	9.0 (± 1.41)
Chloroform fraction	10.0 (± 1.41)
Ethyl acetate fraction	17.5 (± 0.70)
Methanol fraction	12.5 (± 0.70)
Water fraction	10.0 (± 0.00)
Gentamicin	25.5 (± 0.50)

H-6), 6.38 (1H, *d*, *J*= 16, H-*α*), 3.68 (1H, *s*, -OCH3).
Compound 2: Apigenin-7-O-β-D-(6″-trans-*ρ*-coumaroyl) glucoside, 1H-NMR (500 MHz, DMSO-*d6*): δ 7.91 (2H, d, *J*= 8 Hz, H-2′, H-6′), 7.47 (1H, d, *J*= 16 Hz, H-*β*), 7.35 (2H, d, *J*= 8 Hz, H-2″, H-6″), 6.88 (2H, d, *J*= 8 Hz, H-3′, H-5′), 6.82 (1H, *s*, H-3), 6.81 (1H, *s*, H-8), 6.65 (2H, d, *J*= 8 Hz, H-3″, H-5″), 6.46 (1H, *s*, H-6), 6.30 (1H, *d*, *J*= 16, H-*α*), 5.15 (1H, d, *J*= 7.25, H-1‴).
Compound 3: Apigenin-7-*O*-glucoside, 1H-NMR (500 MHz, DMSO-*d6*): δ 7.92 (2H, d, *J*= 8 Hz, H-2′, H-6′), 6.89 (2H, *d*, *J*= 8 Hz, H-3′, H-5′), 6.84 (1H, *s*, H-3), 6.82 (1H, s, H-8), 6.43 (1H, s, H-6), 5.05 (1H, *d*, *J*= 7, H-1‴), 3.5- 4.5 protons of sugar).
The structure of these three compounds was shown in figure 1.

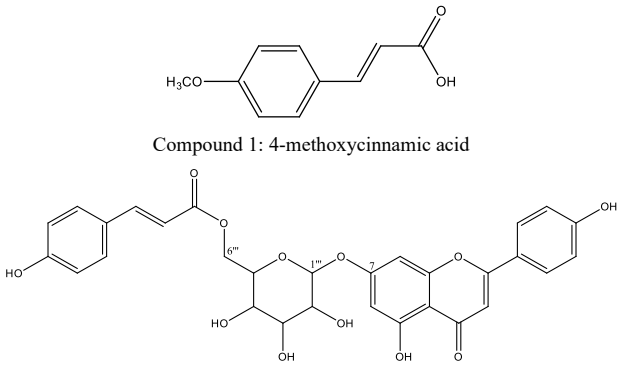

Figure 1. Elucidated compounds from ethyl acetate fraction of Stachys byzantina

Discussion

Different species of *Stachys* have wide applications in traditional medicine of several countries including Iran [31], Turkey [32], Greece [33], Italy [34], China [35], and other Mediterranean countries such as anti-inflammatory, antioxidant and antimicrobial effects. In the present study, the antioxidant, antibacterial effects, and total phenol content of total extract and different fractions of *S. byzantina* were investigated to find potent fraction of plant, and its phytochemical compounds were elucidated.
The results of previous investigation showed *S. byzantina* had lowest antioxidant activity by DPPH method (135.1±4.5 µg/mL) and the 3rd position in total content of phenolic compounds by Folin-Ciocalteau method (23.9±0.8 mg gallic acid/ g dry extract) among other species [26], while in our study, the hydroalcoholic extract and ethyl acetate fraction of *S. byzanthina* demonstrated potent inhibition on both free radical oxidation-reduction process. It is maybe because place and time of plant collection which effect on plant phytochemical compounds and biological effects. The most potential antioxidant sample was ethyl acetate fraction with 1459 mmol Fe^{2+}/g fraction in FRAP test and IC_{50} equal to 1.23 µg/mL in DPPH method. The antioxidant effect of ethyl acetate faction was comparable with vitamin E with IC_{50} of 14.1 in DPPH test.
Also a last study showed that the total phenol and total flavonoid content of *S. byzantina* in methanol and water fractions were more than ethyl acetate fraction, but total flavonols content in ethyl acetate fraction was more than other fraction. The results of antioxidant activity of this research, contrary to the present study, represented potential of methanol and water fractions are higher than ethyl acetate fraction [36]. Asnaashari *et al*. was isolated apigenin 7-*O*-glucoside and apigenin 7-*O*-(6-*ρ*-cis-coumaroyl)-glucoside from methanol fraction of aerial parts of *S. byzantina* and these compounds had antioxidant activity with IC_{50} equal to 450 and 310 µg/mL respectively, in DPPH method [37]. The results of this study shows which only this flavonoid is not responsible for powerful antioxidant activity of *S. byzantina* and likely other flavonoids and phenolic compounds or combination of these second metabolites can play an important role in the occurrence of this effect. Other studies confirmed relevance between existence of phenolic compounds and antioxidant activity of *Stachys* genus.
Also, antioxidant effects of some species of *Stachys* have been mentioned in various studies [18]. For example, Vundać *et al*. in 2007 investigated the antioxidant effect of *S. taxa*. The relation between free radical scavenging activity and total flavonoid content showed responsibility of phenolic compounds in antioxidant effects [27].
The antibacterial effect of *S. byzantina* was revealed in other studies [22,26]. Methanol extract of *S. byzantina* had shown antibacterial effect against Gram positive bacteria (*S. aureus* and *S. sanguis*) and Gram negative bacteria (*E. coli*, *P. aeruginosa*, and *K. pneumoniae*) by Saeedi *et al*. in 2008. They declared flavonoids are responsible for antibacterial effects of this species [22]. In 2003, Stamatis *et al*. demonstrated hydroalcoholic extract of another species of *Stachys*, S. alopecuros (Synonym of *Betonica alopecuros*) has antibacterial effect against *H. pylori* [38]. All the results of previous research were aligned with our antibacterial results in this study.In 2010, Abichandani *et al*. attributed antibacterial effects of *S. schtschegleevii* to phenolic compounds that present in methanol extract [39]. The antibacterial activity of different fractions

of *S. byzantina* which collected from Turkey were demonstrated MIC greater than 2500 µg/mL against *S. aureus* [40]. In another study, ethanol: water (5:1) extract of *S. byzantina* with MIC equal to 12.5 mg/mL shown acceptable anti-*S. aureus* activity [41], while methanol: water (8:2) extract of this plant was more effective against *S. aureus* in this study (MIC= 10 mg/mL).

There are several investigations about isolation of phenolic compounds especially flavonoids from *Stachys* genus. In our study, apigenin and cinnamic acid derivatives were isolated from ethyl acetate fraction of *S. byzanthina*. Apigenin derivatives demonstrated antioxidant and antibacterial properties. According to the Hirano *et al.* study, the half maximal inhibitory concentration of synthetic apigenin was 5.93 µM by DPPH method [42], but the isolated apigenin from aerial parts of Teucrium polium demonstrated IC_{50} = 30.3±2.2 µg/mL [43]. In another study, ferric reducing antioxidant power of apigenin isolated from Teucrium ramosissimum was 0.205 µM equivalent of Trolox [44].

Several studies investigated the antibacterial activity of apigenin as a member of flavonoid class and flavone sub-class, especially on *S. aureus* in literature. In 2015, Morimoto *et al.* introduced apigenin as an antibiotic against methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) with MIC equal to 4 µg/mL and 128 µg/mL, respectively [45]. Other studies demonstrated the MIC equal to 11.1 µg/mL and 7.8 µg/mL for apigenin which was synthesized in laboratory and isolated from Scutellaria barbata, respectively [46,47]. Also, apigenin can have significant antibacterial effect against Gram positive bacteria such as *Streptococcus ratti*, *Streptococcus gordonii*, and *Prevotella intermedia* [48]. In a study the presence of lipopolysaccharides in outer membrane of Gram negative bacteria, is introduced as causes of lower antibacterial effect of apigenin against them [49]. But there is a research by Nayaka *et al.*, demonstrated the significant antibacterial activity of apigenin on *Salmonella typhimurium* and *Proteus mirabilis* than other bacteria [50]. In 2013, apigenin-7-*O*-*β*-D- (6″-trans-*ρ*-coumaroyl) glucoside was isolated from *S.byzantina*. This compound demonstrated weak antiproliferative activity against Vero cell line, as kidney cancer cell line, with 0.5 mg/mL [51]. Also, the interaction of apigenin and its derivatives with inhibition of peptidoglycans synthesis, beta-lactamase, and DNA gyrase activity of bacteria can cause antibacterial effects of *S. byzantina* [45].

Conclusion

Stachys byzantina is a valuable medicinal plant that showed different biological effects including antioxidant and antibacterial effects aginst *S. aureus* in our study. Due to presence of phenolic compounds, this medicinal plant introduced as a source of antioxidant components such as flavonoids. Ethyl acetate fraction of *S. byzantina*, as an intermediate fraction in term of polarity, contains polar and non-polar compounds like derivatives of cinnamic acid and maybe the additive or synergistic effects of these compounds lead this fraction as significant antibacterial candidate.

Ethics Consideration

Tehran University of Medical Sciences (TUMS) research ethics committee approved all ethics considerations of this research under IR.TUMS.PSRC.REC.1396.3100 code.

Conflict of Interests

The authors declare that there is no conflict of interest.

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