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Research article

Effect of total phenolic and flavonoid contents of *Ampelocissus martini* on radical scavenging and antibacterial activities

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Abstract

Ampelocissus martini Planch., an edible plant belonging to the Vitaceae family, is used in traditional medicine to cure certain conditions. However, the biological activities of the vine and rhizome extracts of *A. martini* have not been investigated; hence, the objective of this work was to extract and investigate the bioactive substances of the vine and rhizome of *A. martini.* Three organic solvents (hexane, ethyl acetate, methanol) were used and all the extracts were analyzed to determine the total phenolic and flavonoid contents, using a Folin-Ciocalteu assay and the colorimetric aluminum chloride method, respectively. The results showed that the ethyl acetate and methanol extracts of vine and rhizome had significantly higher total phenolic and flavonoid contents than the hexane extracts. The correlation coefficients between total phenol and flavonoid contents (r = 0.96) were positive. In addition, the ethyl acetate and methanolic extracts of vine and rhizome had a higher percentage of radical scavenging activity than the hexane extracts when measured using the 1,1-diphenyl-2-picrylhydrazyl method and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate free radical scavenging assay. The antibacterial screening was carried out using disc diffusion with Gram-positive and Gram-negative bacterial strains. Good antibacterial activity was indicated with the ethyl acetate and methanol extracts of vine and rhizome and was supported by the analysis of the total phenolic and flavonoid contents. The present study provided evidence that the ethyl acetate and methanol extracts of vine and rhizome of *A. martini* contained extremely important medicinal bioactive substances, such as phenolics and flavonoids, in large quantities that had good biological activity.

Introduction

Phenolic compounds are substances that aid the growth and expansion of plants, with the structure of the phenolic compounds in each plant being different (Lin et al., 2016). At present, there are more than 8,000 known phenolic compounds, from simply structured groups like phenolic acid to polymeric structures such as lignin (Butkhup, 2012). Phenolic compounds can be found in seeds (soybeans, peanuts, rice, sesame), fruits (grapes, oranges), leaves (tea) and other parts of plants, with phenolic acids and flavonoids being common secondary metabolites in the phenolic compound groups.They are found in almost all plants and fulfill physiological functions (Han et al., 2007; Butkhup, 2012). They can also be beneficial for human health due to their antioxidant and antibacterial activities (Mocan et al.,

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2014; Mahmoudi et al., 2016). These compounds are central to the involvement of free radical scavenger and metal ions in accelerating oxidation reactions occurring with fat and other molecules (Han et al., 2007; Szydłowska-Czerniak et al., 2008; Mohamed et al., 2010;). Free radicals are defined as atoms or molecules possessing unpaired electrons generated as by-products of normal metabolism and cause several chronic diseases such as cancer, heart attacks and diabetes (Basma et al., 2011). Therefore, new research for traces of these compounds in various parts of plants is ongoing.

Ampelocissus martini Planch., locally known as wild grape, belongs to the Vitaceae family and is widely distributed in Thailand (Yardpiroon et al., 2014). Its leaf is a traditional herbal ingredient used to cure asthma, while the vine is used as a mild laxative and cough remedy and the root is used to cure bruises and coughs (Zongo et al., 2010; Wongnarat and Srihanam, 2016). In the folk medicine of the Isan region, Northeast Thailand, the root and bark of *A. martini* are used to cure swelling (Srisuwan et al., 2014). Previous research reported that the phenolic components of different-colored wild grape fruits showed good antioxidant and antibacterial activities (Zongo et al., 2010; Jirum et al., 2013; Yardpiroon et al., 2014). However, some activities of the vine and rhizome of *A. martini* have not yet been reported. Hence, the current study aimed to expand the same research by screening vine and rhizome parts for antibacterial activity and preliminary free radical scavenging properties. The phenolic and flavonoid components were extracted using hexane, ethyl acetate and methanol, with the objective to discover a new source of a valuable material that could be used as a therapeutic agent.

Materials and Methods

Plant material and extraction of A. martini vine and rhizome

Fresh vine and rhizome samples of *A. martini* were collected during January 2015 from Tha Sae district, Chumphon province, Thailand. The voucher specimen was deposited at the Herbarium, Department of Biology, Faculty of Science, Chang Mai University, Thailand as S. Aiamyang 1. Dried small pieces of both sampled parts (1,000 g) were macerated for 1 wk with sequentially polar organic solvents (6 L of hexane, ethyl acetate and methanol). The resulting extracts were filtered and the filtrates were concentrated under reduced pressure using a rotary evaporator to produce six crude extracts of vine and rhizome. The percentage yields of the hexane, ethyl acetate and methanol crude vine extracts were 0.14%, 0.51% and 4.29%, respectively, and 0.19%, 0.42% and 22.56%, respectively, for the rhizome extracts. All extracts were then analyzed for the determination of their phenols and flavonoids contents and for their radical scavenging and preliminary antibacterial activities.

Chemicals

Folin-Ciocalteu reagent and potassium persulfate ($K_2S_2O_8$) were purchased from Loba Chemie (India). Gallic acid, rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate radical cation (ABTS), butylated hydroxytoluene (BHT), ascorbic acid, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminum trichloride ($AlCl_3$), sodium hydroxide (NaOH) and all culture media were purchased from Sigma and Himedia (India). All other reagents used were of analytical grade and the highest purity.

Total phenolic content

The total phenolic compounds in the vine and rhizome crude extracts were determined using the Folin-Ciocalteu method of Vittaya and Chalad (2016) with gallic acid as the standard. A sample of 10 mg of each extract and standard were diluted with methanol to a final volume of 10 mL. An aliquot of 0.1 mL from each diluted sample was then pipetted into a vial containing 2.5 mL of water and 0.2 mL of Folin-Ciocalteu reagent. The reaction mixture was mixed and 1.25 mL of Na_2CO_3 solution (20%) was finally added in each tube. The tubes were vigorously vortexed and, after being left to stand for 60 min in the dark, the absorbance values of all samples and the standard were measured at 765 nm using an ultraviolet-visible spectrum spectrophotometer. The total phenolic content was expressed as gallic acid equivalent (GAE) calculated from the calibration curve per gram of crude extract (CE).

Total flavonoid content

The flavonoid content of *A. martini* extracts was determined using the aluminum chloride assay according to Srisuwan et al. (2014) with slight modification. An amount of 100 µ L of 1 mg/mL concentration of each sample was placed in a centrifuge tube and 0.5 mL of 5% $NaNO_2$ was added. The reaction mixture was left to stand for 6 min at room temperature and then 0.2 mL $AlCl_3$ was added. After 5 min, 0.5 mL 1 M NaOH was added and the total was made up to 1.5 mL with distilled water. The solution was mixed well again and the absorbance was measured against a blank at 510 nm using an ultraviolet-visible spectrum spectrophotometer. The total flavonoid contents were measured by plotting the calibration curve of the rutin standard, determined as milligrams of rutin equivalent (RU) per gram of CE.

1,1-Diphenyl-2-picrylhydrazyl free radical scavenging activity

The DPPH free radical scavenging activity of the extracts was determined by slightly modifying the method of Vittaya et al. (2017). An amount of 2 mL of each sample (1 mg/mL) and the standard were mixed with 2 mL of 0.15 mM methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The control was prepared as above without the extract. The absorbance of the solution was measured at 517 nm against the blank. BHT and ascorbic acid were used as standard reagents for comparison with these extracts. The percentage of free radical scavenging activity of each concentration was calculated using Equation 1:

DPPH scavenging effect (%) =

$$(1 - (A_{sample} - A_{sample\ blank}) / A_{control})) \times 100 \quad (1)$$

where A_{sample} is the absorbance of the test sample with DPPH solution, $A_{sample\ blank}$ is the absorbance of the test sample only and $A_{control}$ is the absorbance of DPPH solution. All measurements were performed in triplicate and expressed as average values.

2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonate free radical scavenging activity

The ABTS free radical scavenging assay followed the method of Re et al. (1999). The ABTS radical cation ($ABTS^+$) was prepared by mixing 50 mL (7 mM) of ABTS with 880 µL (140 mM) of potassium persulfate. This mixture was then kept away from light for 16 hr at room temperature. It was then diluted with methanol to give an absorbance of 0.700 ± 0.025 units at 734 nm using a spectrophotometer. An aliquot of 0.1 mL of sample extract was added to 0.9 mL of diluted $ABTS^+$ solution. The reaction mixture was shaken and left for 6 min at room temperature. Absorbance was measured at 734 nm, after the incubation time. The free radical scavenging assay was carried out in triplicate and the ABTS radical scavenging capacity was calculated using Equation 2:

$$\% \text{ Inhibition} = [(A_{control} - A_{sample)/}A_{control}] \times 100 \quad (2)$$

where $A_{control}$ is the absorbance of the extract without $ABTS^+$ solution and A_{sample} is the absorbance of the extract with $ABTS^+$ solution.

Screening of antibacterial activity

The preliminary screening of the antibacterial activity of all extracts was performed three times using the paper disc diffusion method (Villanova, 1993). Seven species—*Bacillus cereus* TISTR 687, *Staphylococcus aureus* TISTR 1466, *Escherichia coli* TISTR 780, *Salmonella typhi* TISTR 292, *Klebsiella pneumoniae* TISTR 1843, *Staphylococcus epidermidis* TISTR 518, *Pseudomonas aeruginosa* TISTR 781—were studied having been obtained from the National Center for Genetic Engineering and Biotechnology, Thailand. For the disc diffusion assay, the bacteria were incubated in tryptic soy broth at 35°C for 24 hr and the turbidity was measured at 0.5 McFarland standards (1×10^8 colony forming units/mL). The bacteria were swabbed with a sterile cotton swab over the surface of the media (tryptic soy agar) and allowed to solidify. As a negative control, samples of the six crude extracts were dissolved in dimethyl sulfoxide to give stock solutions of 100 mg/ mL and were used as 10 µL of tested extracts on sterile filter paper discs (6 mm in diameter, Whatman No. 1 filter paper). After overnight incubation at 35°C for 24 hr, the zones of inhibition were measured in millimeters. Gentamicin and penicillin were used as positive controls.

Statistical data analysis

The data were presented as the mean ± SD of three independent experiments (n = 3). The phenolic and flavonoid contents as well as the free radical scavenging DPPH and ABTS were analyzed using one-way ANOVA. Means were compared using Duncan's multiple range test. Pearson correlations (r) were also calculated between the following parameters, TPC, TFC, DPPH and ABTS. The critical probability of significance was 0.01 or 0.05.

Results and Discussion

Total phenolic and flavonoid contents

Phenolic compounds are very important plant components which can scavenge free radicals and show strong inhibitory effects, with the antioxidant and antibacterial properties of these compounds being due to the number of hydroxyl groups attached to the ring structure and their relative position (Rice Evans et al., 1996). In the current study, three solvents (hexane, ethyl acetate, methanol) were used to extract the phenolic compounds and their contents are shown in Table 1. The total phenols of the *A. martini* extracts were calculated according to the equation y = 0.0018x + 0.0088 (r^2 = 0.999) expressed as gallic acid equivalent (GAE mg/g CE). The total phenolic contents (TPC) in the various extracts were in the range 0.01–6.80 mg GAE/g CE. The methanolic extracts of vine and rhizome had the highest phenolic contents (3.68 ± 0.13 mg GAE/g CE and 6.80 ± 0.33 mg GAE/g CE), followed by the ethyl acetate extracts (1.28 mg GAE/g CE and 1.89 mg GAE/g CE) and hexane extracts (0.02 ± 0.01 mg GAE/g CE and 0.01 ± 0.00 mg GAE/g CE). It has been hypothesized that the phenolic compounds present in vine and rhizome extracts of *A. martini* may play an important role in their biological activity (Abed et al., 2013; Srisuwan et al., 2014). In addition, the total flavonoid content (TFC) of each vine and rhizome extract from *A. martini* was also determined and the results showed the same trend as the total phenolic contents, presented in Table 1. This may have been due to the fact that greater amounts of phenolic and flavonoid compounds are often extracted using polar solvents such as ethyl acetate and methanol (Sultana et al., 2007). Thus, the difference in polarity of the extracting solvents could result in a wide variation in the phenolic and flavonoid contents extracted. The discovery of these phytochemicals would be therapeutically beneficial and also beneficial to human health (Butkhup, 2012). However, little information has so far been made available about the phytochemical composition of vine and rhizome of the wild grape. The results of this work revealed that the vine and rhizome of *A. martini* are rich sources of polyphenolic substances with high levels of TPC and TFC, like its fruit (Yardpiroon et al., 2014). The higher amounts of total phenolic and flavonoid contents of *A. martini* might be related directly to its biological activities, especially its antioxidant activity (Jirum et al., 2013).

Table 1 Total phenolic and flavonoid contents of vine and rhizome extracts of *A. martini*

Plant	Part used	Solvent	Total phenolic content (mg GAE/g CE)	Total flavonoid content (mg RU/g CE)
A. martini	Vine	Hexane	0.02 ± 0.01[a]	2.84 ± 0.42[a]
		Ethyl acetate	1.28 ± 0.06[b]	3.93 ± 0.16[b]
		Methanol	3.68 ± 0.13[d]	5.76 ± 0.17[c]
	Rhizome	Hexane	0.01 ± 0.00[a]	2.49 ± 0.28[a]
		Ethyl acetate	1.89 ± 0.08[c]	6.12 ± 0.10[c]
		Methanol	6.80 ± 0.33[e]	13.52 ± 0.21[d]

GAE = gallic acid equivalent; RU = rutin equivalent; CE = crude extract;
Data presented as mean ± SD from analysis of three samples, in triplicate
Different lowercase superscript letters in the same column for each part indicate a statistical difference at $p < 0.05$.

Antioxidant activity

To determine the relationship between the level of phenolic compounds and antioxidant activity, the percentage of free radical scavenging activity was analyzed in different extracts of *A. martini* using the DPPH method and ABTS assay. Generally, the DPPH method is used to measure free radical scavenging activity by testing the oxidation reaction of crude extracts when reacted with DPPH, which is a stable, purple, free radical at room temperature and when DPPH accepts electrons or hydrogen free radicals, it changes from purple to the yellow color of DPPH-H (Vittaya et al., 2017). This ability of the DPPH free radical to undertake reduction by an antioxidant was measured in terms of the reduction in its absorbance at 517 nm. In the current study, the percentage of free radical scavenging of DPPH was in the range 16.20–96.13% across extracts of the two parts (Table 2). The ethyl acetate and methanol extracts of *A. martini* vine and rhizome had very high levels of inhibitory activity (>90%). Excellent scavenging ability was clearly exhibited by the ethyl acetate and methanol extracts, as shown in Fig. 1 Moreover, a moderate correlation was observed between the DPPH scavenging activity of all extracts and their phenolic (r = 0.63) and flavonoid (r = 0.54) contents.

ABTS, a cation free radical, is a well-known synthetic compound widely used to evaluate the antioxidant activity of the ABTS free radical which is produced by the oxidation of ABTS with potassium persulfate converting to the non-radical form when exposed to antioxidants (Seethalaxmi et al., 2012; Huang et al., 2014). The percentage of free radical scavenging of ABTS was in the range 4.53–99.01% in both parts. The results in Figure 1 show high levels in the ethyl acetate and methanol extracts. The trend of the free radical scavenging ability in this assay was similar to that observed in the DPPH method. A good correlation was observed between the ABTS scavenging activity of all extracts and their phenolic (r = 0.83) and flavonoid (r = 0.73) contents, as presented in Table 3. The antioxidant activities increased with increased phenolic and flavonoid contents in the ethyl acetate and methanol extracts. Determination of the bioactive constituents present in ethyl acetate and methanol in the vine and rhizome would be interesting and deserves further study.

Table 2 Percentage radical scavenging activity of vine and rhizome extracts of *A. martini*

Plant	Part used	Solvent	Free radical scavenging percentage (%)	
			DPPH assay	ABTS assay
A. martini	Vine	Hexane	16.20 ± 0.33[a]	4.53 ± 0.42[a]
		Ethyl acetate	94.71 ± 0.33[bc]	62.60 ± 0.16[b]
		Methanol	94.71 ± 1.08[bc]	98.22 ± 0.77[c]
	Rhizome	Hexane	58.36 ± 0.00[a]	12.00 ± 0.80[a]
		Ethyl acetate	94.15 ± 1.24[b]	81.03 ± 1.33[b]
		Methanol	96.13 ± 0.94[c]	99.01 ± 0.91[c]
BHT			95.56 ± 0.08[c]	98.90 ± 0.57[c]
Ascorbic acid			96.27 ± 1.55[c]	99.42 ± 0.45[c]

DPPH = 1,1-diphenyl-2-picrylhydrazyl; ABTS = 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate radical cation; BHT = butylated hydroxytoluene.
data presented as mean ± SD from analysis of three samples, in triplicate.
Different lowercase superscript letters in the same column for each table block indicate a statistical difference at $p < 0.05$.

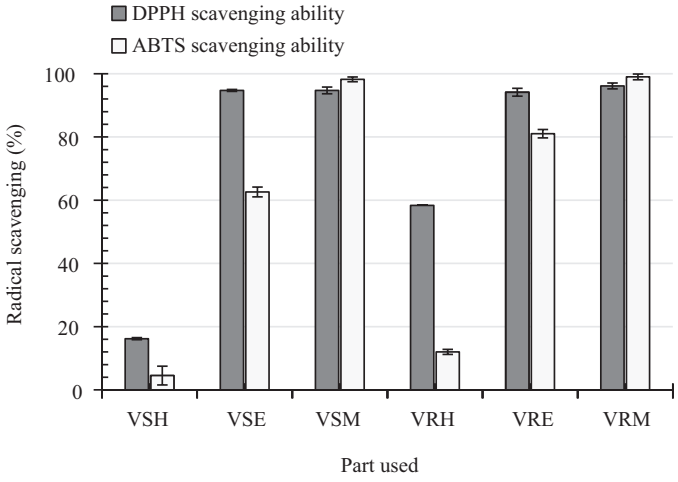

Fig. 1 Percentage of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2/-azino-bis-3-ethylbenzothiazoline-6-sulfonate radical cation (ABTS) radical scavenging activity of vine and rhizome extracts of A. martini, where VSH, VSE and VSM are vine samples extracted using hexane, ethyl acetate and methanol, respectively, and VRH, VRE and VRM are rhizome samples extracted using hexane, ethyl acetate and methanol, respectively, and error bars show ± SD.

Table 3 Correlation (R^2 values) between the TPC and TFC values of *A. martini* extracts and their radical scavenging DPPH and ABTS values

Values compared	Correlation R^2 ($p < 0.05$)
TPC and TFC	0.96
TPC and %DPPH	0.63
TFC and %DPPH	0.54
TPC and %ABTS	0.83
TFC and %ABTS	0.73

TPC = total phenolic content; TFC = total flavonoid content; DPPH = 1,1-diphenyl-2-picrylhydrazyl; ABTS = 2,2/-azino-bis-3-ethylbenzothiazoline-6-sulfonate radical cation.

Antibacterial activity

All crude extracts were preliminarily tested for antibacterial activity using the paper disc diffusion method with *B. cereus* and *Staphylococcus aureus* (Gram-positive) as well as with *E. coli*, *Salmonella typhi*, *K. pneumoniae*, *Salmonella epidermidis* and *P. aeruginosa* (Gram-negative). The results are shown in Table 4 and Fig. 2, in which there are some clearly visible inhibition zones of the pathogenic bacteria. Most crude extracts were active against Gram-positive and Gram-negative bacteria, producing inhibition zones with diameters in the range 6.18–16.40 mm, with the largest diameters produced by the ethyl acetate and methanolic extracts. Notably, the ethyl acetate rhizome extract produced inhibition zones of *B. cereus* and *Salmonella* that measured 14.55 ± 0.81 and 16.40 ± 0.45 mm, respectively. It was surprising that the extract of this part of *A. martini* was more active for Gram-positive strains than the Gram-negative bacteria which having thicker cell walls and an outer membrane comprising a high content of lipid-polysaccharide sheeting (Abed et al., 2013), which may protect against the passage of hydrophobic groups. In summary, the different extracts were effective antibacterial agents, with the ethyl acetate and methanol extracts having the greatest activity. The activity against both the Gram-positive and Gram-negative bacteria may have been due to the presence of phenolic substances (Bazzaz et al., 2011; Mouafi et al., 2014). To increase the biological activity of the ethyl acetate and methanolic extracts of vine and rhizome of *A. martini*, it would seem important to analyze and purify their phenolic compounds in further studies.

The results indicated that the extracts of both vine and rhizome from *A. martini* had different total phenolic and flavonoid contents. The ethyl acetate and methanolic extracts of vine and rhizome had higher total phenolic contents than the hexane extracts. This result correlated with the ability of the extracts to scavenge free radicals *in vitro* and it is believed that this plant is a potential useful source of natural antioxidants. In addition, both parts of *A. martini* in the various crude extracts gave fractions which demonstrated varied levels of broad-spectrum antibacterial activity. Good activity was exhibited by the ethyl acetate and methanol fractions of vine and rhizome against Gram-positive and Gram-negative bacteria. Hence, the current findings suggested that the extracts of vine and rhizome of *A. martini* can be used as alternative sources of natural antioxidants for health supplements and pharmaceutical purposes. Furthermore, isolation of the chemical components of the extracts may lead to a better understanding of the mechanism of their antioxidant and antibacterial activity, which will help develop their therapeutic application.

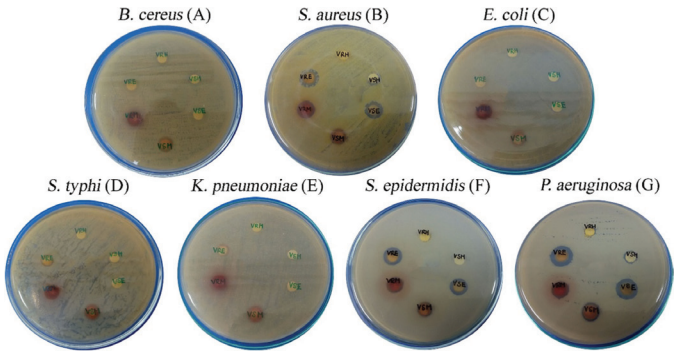

Fig.2 Antibacterial activity based on the disc diffusion method of vine and rhizome extracts of *A. martini* to: (A) *B. cereus*; (B) *S. aureus*; (C) *E. coli*; (D) *S. typhi*; (E), *K. pneumoniae*; (F) *S. epidermidis*; (G), *P. aeruginosa*, where VSH, VSE and VSM are vine samples extracted using hexane, ethyl acetate and methanol respectively, and VRH, VRE and VRM are rhizome samples extracted using hexane, ethyl acetate and methanol, respectively

Table 4 Antimicrobial activities of vine and rhizome extracts of A. martini

Plant	Part used	Solvent used	Zone of inhibition diameter (mm)						
			Gram-positive bacteria		Gram-negative bacteria				
			B. cereus	*S. aureus*	*E. coli*	*S. typhi*	*K. pneumoniae*	*S. epidermidis*	*P. aeruginosa*
A. martini	Vine	Hexane	7.43 ± 0.32^{a}	0.00 ± 0.00^{a}	6.56 ± 0.53^{a}	6.43 ± 0.11^{a}	6.32 ± 0.28^{bc}	6.46 ± 0.45^{a}	6.72 ± 0.32^{a}
		Ethyl acetate	10.92 ± 1.64^{bc}	7.86 ± 0.46^{c}	6.88 ± 0.40^{a}	12.85 ± 1.25^{b}	6.18 ± 0.31^{b}	11.68 ± 0.84^{d}	13.78 ± 0.39^{d}
		Methanol	9.94 ± 0.36^{b}	10.79 ± 0.21^{e}	6.55 ± 0.48^{a}	12.03 ± 1.19^{b}	8.64 ± 1.02^{d}	9.50 ± 0.16^{c}	9.86 ± 0.84^{b}
	Rhizome	Hexane	7.87 ± 0.84^{a}	6.51 ± 0.27^{b}	6.48 ± 0.58^{a}	6.86 ± 0.17^{a}	0.00 ± 0.00^{a}	6.26 ± 0.23^{a}	7.29 ± 0.38^{a}
		Ethyl acetate	14.55 ± 0.81^{d}	9.48 ± 0.56^{d}	7.36 ± 0.22^{a}	16.40 ± 0.45^{c}	7.04 ± 0.10^{c}	9.57 ± 0.74^{e}	11.55 ± 0.83^{c}
		Methanol	11.83 ± 0.81^{c}	8.40 ± 0.62^{c}	8.86 ± 0.88^{b}	12.75 ± 0.55^{b}	9.21 ± 0.14^{d}	8.21 ± 0.57^{b}	10.18 ± 0.59^{b}
Gentamycin			18.79 ± 0.40^{e}	22.01 ± 0.16^{f}	18.75 ± 0.34^{c}	16.41 ± 0.48^{c}	23.24 ± 0.73^{e}	18.96 ± 0.10^{e}	18.03 ± 0.23^{e}
Penicillin			10.70 ± 0.06^{bc}	25.61 ± 0.57^{g}	28.70 ± 0.10^{b}	16.91 ± 0.71^{c}	6.01 ± 0.01^{b}	9.99 ± 0.84^{c}	14.38 ± 0.53^{d}

data presented as mean ± SD from analysis of three samples, in triplicate. Different lowercase superscript letters within a column in each table block indicate a statistical difference at $p < 0.05$.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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