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RESEARCH ARTICLE

Medicinal Attributes of *Aerva javanica* Native to Pothohar Plateau

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ABSTRACT

In this study, ethanolic, methanolic and acetone extracts of *Aerva (A.) javanica* were investigated for their phenolic compounds, antioxidant and antimicrobial activities. Antioxidant activity was determined through 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging, superoxide radical scavenging, ß-carotene linoleic acid and ferric reducing antioxidant power assays. Antimicrobial activity was assessed by disc diffusion assay. All extracts exhibited effective 2, 2-Diphenyl-1-picrylhydrazyland superoxide radical scavenging activity, inhibition of denaturation of ß-carotene and ferric reducing antioxidant power contents. Extracts also showed good antimicrobial potential with appreciable phenolic and flavonoid contents. Nevertheless, methanolic extract of *A. javanica* exhibited highest antioxidant and antimicrobial activities as compared to ethanolic and acetone extracts proving itself most suitable extraction solvent for biologically active phenolic compounds from *A. javanica*. The solvent nature significantly ($P<0.05$) affected the phenolic contents, antioxidant and antimicrobial activities. In conclusion, *A. javanica* may be a potential source of antioxidant and antimicrobial compounds.

INTRODUCTION

Plants produce a large and heterogeneous group of compounds called phenolic compounds (Mazid et al., 2011). To date, more than 8000 phenolics have been identified and are widely distributed in plant kingdom (Crozier et al., 2009). These are synthesized from carbohydrates through shikimate pathway in response to ecological and physiological factors like pathogens attack and UV radiations. Quantities of phenolic compounds vary in different tissues and also within different populations of the same plant. Their quantities depend upon plant species, soil conditions, harvest time and growing conditions. Simple phenolic acid quantities are greater in young tissues, but later on these phenolic acids combine to form polyphenolic constituents such as flavonoids (Khoddami et al., 2013). Phenolic compounds include simple phenolic acids (low molecular weight) like ferulic acid, caffeic acid, chlorogenic acid, coumaric acid, gallic acid and large, complex poly-phenols like flavonoids, quinones, coumarins, stilbenes, curcuminoids and lignans (Crozier et al., 2009). These compounds protect plants from pathogen attacks and by making food unpalatable to herbivorous predators (Apak et al., 2007). Phenolic compounds possess anti-inflammatory, anti-mutagenic, anti-carcinogenic (Stalikas, 2007), antimicrobial (Majhenic et al., 2007), antiulcerative, antiviral (Umamaheswari and Chatterjee, 2008) antioxidant, anti-coagulative, antihistaminic and anti-allergic (Hossain et al., 2013) activities.

A. javanica belongs to Amaranthacea family (Khan et al., 2012). It is a perennial herb, native to Africa, Asia and extensively scattered in various regions of the world (Judd et al., 2008). Traditionally, *A. javanica* is used for the treatment of wounds, cough, diarrhoea, ulcer and hyperglycaemia (Khan et al., 2012) and is also used as diabetic, demulcent and diuretic (Qureshi and Bhatti, 2009). Its decoction is used to remove swelling and to relieve toothache; whereas, its powder is used for ulcers in domestic animals and seeds for headache. The whole plant of *A. javanica*is is useful in treating chest pain, diarrhea and ascariasis (Samejo et al., 2012).

Considering the medicinal properties of this plant, this study was designed to evaluate the phenolic contents, antioxidant and antimicrobial attributes of *A. javanica* from Pothohar plateau. Further, scanty information related to these activities of this Pothohar plateau origin plant makes its selection as a candidate plant to explore its potential.

MATERIALS AND METHODS

Collection and extraction of plants

A. javanica was collected from different regions of Pothohar plateau and identified by expert botanist from the Department of Botany, University of Agriculture, Faisalabad, Pakistan (Voucher No. 625-14-1). After proper cleaning and drying, collected plants were powdered by a mechanical grinder. Plant powder (20g) was mixed with 200mL of each solvent system (methanol, ethanol and acetone) in conical flasks. Extraction was carried out in an orbital shaker for 6 hours at room temperature (Sultana et al., 2009), filtered and residue was extracted twice. The combined supernatants were dried in a rotary evaporator and stored in cool dry place till further analysis. Extraction yield of each solvent was calculated as %yield.

Total phenolic contents (TPC)

Total phenolic contents of extracts were measured according to the method of Aiyegoro and Okoh (2010). TPC were determined as milligrams of gallic acid (GAE)/g of plant extract using gallic acid as standard. Briefly, 10% Folin-Ciocalteu reagent (2.5 mL) was added to250 µg/mL extract solution (0.5 mL). Then, 2% (w/v) sodium carbonate solution (2 mL) was added to the above mixture, mixed vigorously and left for incubation (30 min; 45^{o}C). Absorbance was taken at 765 nm using UV/Vis spectrophotometer (Lambda 25, Perkin Elmer, USA).

Total flavonoid contents (TFC)

Total flavonoid contents of plant extracts were quantified by the method of Kumar and Kumar (2009). Briefly, One mL of extract solution was diluted with 4 mL of distilled water in a volumetric flask. Initially, 5% $NaNO_2$ (0.3 mL) was added in the mixture. After 5th minute 10% $AlCl_3$ (0.3 mL) and at 6th minute 2 mL of NaOH (1M) were added. The mixture was diluted by adding distilled water up to 10 mL and agitated thoroughly. Absorbance was taken at 510 nm. Quercetin was used as standard and total flavonoid contents were calculated as quercetin equivalents (mg/g of plant extract).

Anti-oxidant activity

DPPH radical scavenging assay

Free radical scavenging activity was assayed by the procedure of Kumar and Kumar (2009). Absorbance of methanolic solution of DPPH (33 mg/L) was taken at 0 min which is control absorbance. Then, 5mL of methanolic solution of DPPH was added in 1mL of each extract solution (250 µg/mL). Absorbance of above mixture was determined at 517nm after 30 minutes of incubation in dark. DPPH radical scavenging activity was determined in term of % inhibition.

Inhibition (%) = (Control Absorbance - Sample Absorbance / Control Absorbance) × 100

Superoxide radical scavenging (SOR) assay

One mL of 50 mM sodium carbonate and 0.4mL of 24mM NBT (nitro blue tetrazolium) were added in 1mL of each extract solution (250 µg/mL). Then 0.2mL of 0.1 mM EDTA solution and 0.4 mL of 1 mM hydroxylamine hydrochloride were added. After 15 minutes of incubation at 25^{o}C, absorbance was taken at 560 nm. SOR activity was calculated as % inhibition as described by Veena and Mishra (2011).

Inhibition (%) = [(Ao-At) / Ao x 100]

Where, Ao represents the absorbance of control (without extract) and at stands for the absorbance of the sample (with extract).

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed to determine reducing power of plant extracts (Chan et al., 2007). In brief, 0.2M phosphate buffer (2.5 mL, pH=6.6) was mixed in 1mL plant extract solution. Then, 1% (w/v) potassium ferricyanide (2.5 mL) was added into mixture and left for 20 minutes at 50^{o}C. After incubation, 2.5 mL of 10% (w/v) trichloroacetic acid solution was mixed in the above mixture. Afterwards, 2.5 mL of the reaction mixture was separated in a test tube and mixed with 2.5 mL distilled water. Ferric chloride solution (500 µL; 0.1% w/v) was added into diluted mixture and incubated for 30 minutes. Absorbance was taken at 700 nm. Ferric reducing power of extract was determined in milligram gallic acid equivalent/g (mg GAE/g) of plant extract.

Anti-oxidant activity determination in a ß-carotene linoleic acid system

Plant extracts were evaluated for their antioxidant activity through bleaching of ß-carotene (Chatha et al., 2011). Two mL of ß-carotene solution was added in Linoleic acid (40 mg) and Tween-40 (400 mg). ß-carotene solution was prepared in chloroform (1 mg/mL). After mixing, chloroform was removed on a water bath at 45^{o}C for few minutes. Distilled water (100 mL) was added in this semisolid material with continuous stirring to form an emulsion. Small amount of this emulsion (5 mL) was added into 0.2 mL of extract solution (500 mg/L). Absorbance was taken immediately at 470 nm. Emulsion without ß-carotene was taken as blank. Then, mixture was kept at 50^{o}C in a water bath for 120 minutes. Absorbance was taken again after 120 minutes at 470 nm. Anti-oxidant activity coefficient of extracts was calculated as

$AAC= (A_{A(120)}-A_{C(120)})/(A_{C(0)}-A_{C(120)})\times 1000$

Where, $A_{A(120)}$ and $A_{C(120)}$denotes the absorbance of the sample and control after 120 minutes and $A_{C(0)}$represents the absorbance of control at 0 minute.

Antimicrobial activity

Four bacterial strains (*Escherichia coli* (*E.coli*), *Pasturella multocida* (*P. multocida*), *Bacillus subtilis* (*B. subtillus*) and *Staphylococcus aureus* (*S. aureus*)) and three pathogenic fungi (*Aspergillus flavus* (*A. flavus*), *Aspergillus niger* (*A. niger*) and *Fusarium*

solani (*F. solani*)) were used to estimate the antimicrobial activity of extracts of *A. javanica.* The bacterial and fungal strains were obtained from Protein and Molecular Biology Laboratory (PMBL), Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan. Antimicrobial activity of extracts of *A. javanica* was evaluated by disc diffusion assay (Mehmood et al., 2012). Sterilized petri plates were inoculated with bacteria/fungi. Each extract solution (100 µL/disc) at a concentration of 10 mg/mL was poured on the disc (10 mm diameter) and placed the disc on inoculated agar plate. Rifampicin (100 µL/disc) and Terbinafine (100 µL/disc) were used as standards for bacteria and fungi, respectively. Anti-microbial activity was reported in terms of the diameter of inhibition zone (mm).

High pressure liquid chromatography (HPLC) analysis for Phenolic acids

Pre-treatment of samples for HPLC analysis was performed (Dek et al., 2011). Briefly, 50 mg of extracts was dissolved in 24 mL methanol and homogenized. Then, 16 mL distilled water and 10 mL of 6 M HCl were added and kept in an oven for 2 hrs at 95ºC. The final solution was filtered through a 0.45µm filter paper. HPLC analysis was conducted using LC-10A, Shimadzu (Japan) equipped with UV-Vis detector. Separations were performed using reverse-phase (RP) ODS C18 column (25cm×4.6mm, 5µm) at room temperature. Gradient elution was carried out by using deionized water with AA (pH 2.27) as solvent A and ACN (99.99%) as solvent B. The gradient used was: 100 to 50% solvent A (0 to 15 min), 45% solvent B (15 to 30 min) and 100% solvent B (30 to 45 min). Mobile phase flow rate was set at 1.0 mL/min. Detector was carried out at 280 nm. Peak identification and quantification were accomplished on comparison of retention time and area of standards, respectively.

Statistical analysis

All the assays for phytochemical studies, antioxidant and antimicrobial activities were performed in triplicate and results was presented as mean±SD. Data was further subjected to analysis of variance (ANOVA) using Minitab 2000 version 13.2 statistical software at 5% significance level (Sultana et al., 2009).

RESULTS

Extraction efficiencies (% yield) of three solvents viz., methanol, ethanol and acetone are shown in Fig. 1. The results indicated significant differences (P<0.05) among extraction yields of different solvents being maximum extraction with methanol (6.49%) followed by ethanol (4.63%) and acetone (2.98%). It indicated that phenolic contents of *A. javanica* were solvent dependent (Table 1). Decreasing trend of total phenolic contents was in the order of methanol>ethanol>acetone. Highest total flavonoid contents have been obtained from methanolic extract (68.69 mgQE/g), followed by ethanol (61.29 mgQE/g) and acetone (57.27 mgQE/g). It was observed that extraction solvent significantly (P<0.05) affected the total phenolic and flavonoids contents.

Table 1: Total phenolic and flavonoid contents of *Aerva javanica*

Solvent extracts	TPC(mg GAE/g)	TFC(mg QE/g)
Ethanol	80.18±4.0[b]	61.29±3.5[b]
Methanol	82.31±4.1[a]	68.69±4.5[a]
Acetone	74.06±3.7[c]	57.27±2.9[c]

Values are means ± SD, Values with different letters in superscripts are significantly different (P<0.05). TPC = Total phenolic contents; TFC = Total flavonoid contents; GAE = Gallic acid equivalent; QE = Quercetin equivalent

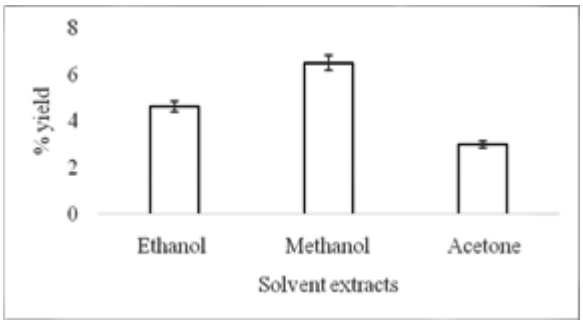

Fig. 1: Extraction yield of *Aerva javanica* using different solvents

Different solvent extracts of *A. javanica* showed the ability to donate H^+ to DPPH free radical showing anti-oxidant activity. DPPH radical scavenging activity of *A. javanica* extracts ranges from 51.99 to 64.45%. Methanolic extract (64.45%) showed highest activity indicating that increase in the polarity of extracting solvent resulted in higher recovery of total phenolic, thus exhibiting greater DPPH radical scavenging activity (Table 2). These results revealed that extraction solvent was the significant factor affecting the DPPH radical scavenging activity (P<0.05).

All extracts of *A. javanica* reflected highly significant (P<0.05) variable SOR scavenging activity (Table 2). Ethanolic, methanolic and acetone extracts showed 65.22, 67.23 and 55.15% SOR scavenging activities, respectively. Like radical scavenging activities, FRAP contents were also significantly (P<0.05) dependent upon extraction solvent type. Highest FRAP contents have been found in methanolic extract with more reducing power as compared to other solvent extracts (Table 2). Plant extracts demonstrated anti-oxidant activity by preventing the discoloration ß-carotene and expressed as antioxidant activity coefficient (AAC). Different extracts of *A. javanica* revealed significant (P<0.05) antioxidant activities in the order of methanol>ethanol>acetone (Table 2).

Table 2: Anti-oxidant activities of different solvent extracts of *Aerva javanica*

Solvent extracts	DPPH free radical scavenging (% inhibition)	SOR scavenging (% inhibition)	FRAP contents (mg GAE/g)	AAC
Ethanol	59.05±2.9[b]	65.22±3.4[b]	20.42±1.6[b]	250.47±12.5[b]
Methanol	64.45±3.2[a]	67.23±3.3[a]	21.09±1.1[a]	323.12±16.2[a]
Acetone	51.99±2.7[c]	55.15±2.8[c]	18.42±0.9[c]	243.05±12.2[c]

Values are means ± SD, Values with different letters in superscripts are significantly different (P<0.05). DPPH = 2,2-diphenyl-1-picrylhydrazyl; Anti-oxidant activity coefficient; SOR = Superoxide radical; FRAP = Ferric reducing antioxidant power; AAC = GAE = Gallic acid equivalent

Table 3: Antimicrobial activities of different solvent extracts of *A. javanica*

Microbial strains	Solvent extracts			Rifampicin/
	Ethanol	Methanol	Acetone	Terbinafine
Escherichia coli	14±0.7[d]	22±1.1[b]	20±1.0[c]	34±1.9[a]
Pasteurellamultocida	12±0.5[c]	14±0.7[b]	14±0.7[b]	28±1.6[a]
Bacillus subtillus	19±0.9[b]	19±0.7[b]	18±0.9[b]	38±2.0[a]
Staphylococcus aureus	15±0.8[b]	16±0.8[b]	12±0.6[c]	34±1.9[a]
Aspergillusflavus	13±0.7[b]	13±0.9[b]	13±0.7[b]	45±4.5[a]
Aspergillusniger	11±0.6[b]	15±0.8[b]	14±0.7[b]	50±4.2[a]
Fusariumsolani	12±0.6[b]	15±0.7[b]	13±0.6[b]	48±4.0[a]

Values are means ± SD; Values with different letters in superscripts within rows are significantly different (P0.05)

Table 4: Phenolic compounds in different solvent extracts of *A. javanica*

Phenolic acids (mg/g of plant extract)	Solvent extracts		
	Ethanol	Methanol	Acetone
Gallic acid	0.67	---	---
Caffeic acid	1.63	1.29	1.33
Vanillic acid	---	2.41	---
4-hydroxy-3-methoxy benzoic acid	2.10	---	2.46
Syringic acid	---	1.63	---
p-coumaric acid	5.41	---	---
m-coumaric acid	---	4.70	2.56
Ferulic acid	1.27	---	---

Antimicrobial activity (Table 3) of different solvent extracts of *A. javanica* against all microbial strains differ significantly (P<0.05) from the standard used. Highest activity has been shown by methanolic and acetone extract against *Escherichia coli* with inhibition zones of 22 and 20 mm. Ethanolic extract exhibited maximum activity against *Bacillus subtillus* with inhibition zone of 19 mm and least activity by ethanolic extract against *Aspergillus niger* with inhibition zone of 11 mm. All extracts showed prominent activity against bacterial strains as compared to fungal strains.

Eight phenolic acids i.e. gallic acid, caffeic acid, 4-hydroxy-3-methoxy benzoic acid, vanillic acid, *P*-coumaric acid, syringic acid, *m*-coumaric acid and ferulic acid were used as standards. Identified phenolic compounds in different solvent extracts of *A. javanica* have been depicted in Table 4. Caffeic acid has been recovered from all extracts of *A. javanica*. In ethanolic extract, *p*-coumaric acid shows highest quantity (5.41mg/g). In case of methanol and acetone extracts *m*-coumaric acid has been recovered in highest quantities (4.70; 2.56 mg/g).

DISCUSSION

The trend of using plants based drugs has remarkably increased during last few decades (Menghani et al., 2012). Natural botanics have been analyzed for medicinal activities against infections and also for antioxidant activity. These activities formed the basis of plants utilization in pharmaceuticals and natural therapy (Muthukumaran et al., 2011). Extraction is the separation of biologically active compounds from plant tissues by using appropriate solvents and extraction procedures (Tiwari et al., 2011). We employed three extraction solvents (ethanol, methanol and acetone) and methanol proved to be efficient solvent for the extraction of biologically active compounds from *A. javanica.* Results also indicated that nature and quantities of phenolic compounds depend upon solvent nature used for extraction. Methanol is polar and is best solvent for the extraction of variety of phenolic compounds (Srinivas and Reddy, 2012). James et al. (2014) also concluded that alcoholic solvents are efficient for the maximum recovery of phenolic compounds from *Vitex doniana* as compared to acetone and water.

Results showed that highest total phenolic contents (TPC) were obtained in methanolic (82.31 mg GAE/g) extract of *A. javanica*, followed by ethanolic (80.18 mg GAE/g) and acetone (74.06 mg GAE/g) extracts. Sethi and Sharma (2011) also reported that maximum total phenolic contents (34.5 mg GAE/g) in *A. tomentosa* was obtained from methanolic extract as compared to petroleum ether, dichloromethane, ethylacetate and aqueous solvents through soxhlet apparatus. Tiwari et al. (2011) described that alcohols such as ethanol and methanol are best solvents for the maximum recovery of phenolic compounds from plant matrix. Mechanism behind this property is the degradation of cell walls and seeds which have non-polar character, releasing phenolic compounds from cells. They also reported that all the antimicrobial compounds of plants are saturated or aromatic organic compounds and can be easily extracted through methanol or ethanol.

Different solvent extracts of *A. javanica* were analyzed for total flavonoid contents (TFC). Highest TFC were found in methanolic extract (68.69 mg QE/g) followed by ethanolic extract (61.29 mg QE/g) and least in acetone extract (57.27 mg QE/g). Ragavendran et al.,

(2012) measured 11.83 mg of catechin/g in the ethanolic extract of *A. lanata*. Differences in TFC in the ethanolic extracts might be due to different plant species.

All the solvent extracts of *A. javanica* showed DPPH free radical scavenging activity to a varying degree, with methanol (64.45%), ethanol (59.05%) and acetone (51.99%) at an extract concentration of 250µg/mL. Fatimi et al., (2007) reported 91.6% DPPH radical scavenging activities of methanolic extract of *A. javanica* at a concentration of 500 µg/mL, working on medicinal plants from Yemen. Ragavendran et al., (2012) reported 62.8% DPPH radical scavenging activity of *A. lanata* plant extract (2.5mg/mL), which is far less than our results. DPPH assay is widely used because of its simplicity, feasibility, sensitivity and has a good stability (Prabhune et al., 2013). During the reaction, its purple colour changes to yellow on receiving proton from the plant bio-actives especially phenolics. This change in colour is measured as decrease in absorbance, which indicates free radical scavenging (antioxidant) activity of the plant extracts (Melendez et al., 2014).

SOR scavenging potential of different extracts of *A. javanica* was analyzed using nitroblue tetrazolium. Highest activity was shown by methanolic extract (67.23%), followed by ethanolic (65.22%) and acetone (55.15%) extract. Battu and Kumar (2012) also evaluated different solvent extracts (ethanol, chloroform and hexane) for antioxidant activity through superoxide radical scavenging assay and found highest SOR scavenging activity in their ethanolic extract. Superoxide anions are one of the most damaging free radicals that attack on biomolecules directly or indirectly by forming OH, H_2O_2, peroxynitrite or singlet oxygen during pathological diseases. In the oxidation-reduction reactions of cells, superoxide radicals are formed normally, later on their effects can be magnified as they produce many types of cell destroying free radicals other oxidizing agents (Kumar et al., 2014).

Results showed that reducing power of plant extracts also vary with the solvent type. Alcoholic (ethanol and methanol) extracts showed more FRAP contents than acetone extract. FRAP assay is a simple, inexpensive assay involving reduction of ferric ions to ferrous ions. In this assay antioxidants from test samples reduce Fe^{+3} in the reaction mixture to Fe^{+2} (blue colour), which is measured by spectrophotometer. Reducing power of different compounds shows that they can donate electron (Vinodhini and Lokeswari, 2014).

All the solvent extracts of *A. javanica* showed antioxidant activity in ß-carotene linoleic acid assay. Methanolic extract exhibited highest activity (323.12AAC) as compared to ethanol (250.47AAC) and acetone (243.05AAC) extracts. In this assay, linoleic acid is an unsaturated fatty acid undergoes oxidation by reactive oxygen species (hydroperoxides) produced from halogenated water. These hydroperoxides in the absence of antioxidants oxidize ß-carotene molecules, which lose their double bonds and characteristic orange colour. This change in colour was measured by a spectrophotometer. Antioxidants prevent the oxidation of ß-carotene molecules by neutralizing hydroperoxides and other free radicals produced in the reaction mixture (Lu et al., 2014).

Medicinal plants produce nutritive and non-nutritive compounds which showed antimicrobial activities and act as sheild against pathogens. Thus it is important to characterize medicinal plants for their antimicrobial activities (Sengul et al., 2009). Disc diffusion assay was performed to determine antimicrobial activity of different solvent extracts of *A. javanica.* All the extracts showed antimicrobial activity less than respective standards used. In case of bacterial strains highest activity was shown by methanolic and acetone extracts. While against fungal strains highest activity was shown by methanolic and ethanolic extracts. Overall, methanolic extract exhibited highest inhibition against all the tested microbes. Srinivas and Reddy (2012) also reported the antibacterial activity of methanolic extract of *A. javanica* leaf against *E.coli, B. subtillus* and *S. aureus,* obtained inhibition zones 14, 12 and 12mm, respectively at an extract concentration of 100mg/mL. The activity in these results is far less than our findings which are 22, 19 and 16mm against *E.coli, B. subtillus* and *S. aureus.* These variations might be due to different geographical and agro-climatic conditions of both the areas under study. Sen and Batra (2012) evaluated different solvent extracts of *Melia azedarach* for their antimicrobial activity and found that its alcoholic (ethanol and methanol) extracts showed greater inhibition against pathogenic micro-organisms as compared to its petroleum ether and aqueous extracts.

Saikia and Handique (2013) concluded while working on methanol, chloroform, acetone and petroleum ether extracts of H*ippophae salicifolia* that methanol was the most efficient solvent for extraction of biologically active compounds which gave good antioxidant and antibacterial activities.

In our results acetone extract of *A. javanica* also showed good antimicrobial activities which is in accordance with Das et al., (2010) who described that acetone has the ability to solubilize hydrophilic and lipophilic compounds from plants. It is volatile and less toxic and very useful for the extraction of antimicrobial compounds.

CONCLUSION

A. javanica conclusively exhibited good antioxidant and antimicrobial activities. This plant also harbors appreciable quantities of phenolic and flavonoids. Among different extraction solvents, methanol was the

best solvent for the maximum recovery of phenolic compounds from *A. javanica*. It is recommended that this plant from Pothohar plateau may contribute towards the development of new drugs against various disease conditions.

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