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Phytochemical, antibacterial and DPPH free radical scavenging evaluations of the barks of *Aegle marmelos* (L.) Correa

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Abstract
The therapeutic value of the barks of *Aegle marmelos* (L.) Correa for the treatment of fever and cough has been recognized from the ethnobotanical survey in the Magar community in Hupsekot Rural Municipality, Nepal. Fruits of the plant, though, being much explored, exploitation of the uses of the barks is scarce. In this study, phytochemicals present in the hexane and aq. methanolic extracts were evaluated by chemical tests and GC-MS. These extracts constituted biologically active ingredients of alkaloids, steroids, terpenoids and polyphenols. The GC-MS analysis of the hexane extract revealed that it constituted a number of fatty acids and hydrocarbons, while the aq. methanolic extract possessed mainly steroids (γ-sitosterol 71.19% and sitostenone 10.14%). The antibacterial evaluation displayed that the extracts were effective against Ampicillin resistant *Pseudomonas aeruginosa*, and *Bacillus subtilis* and *Klebsiella pneumoniae* as well. The MIC values were ranged from 12.5 to 50 mg/ml. The presence of carbonyl compounds, fatty acid methyl esters, phenolics and steroids could be responsible for the antibacterial activity. The aq. methanolic extract also exhibited DPPH free radical scavenging capacity with IC_{50} value of 961.53 µg/ml.

Keywords: *Aegle marmelos*, antibacterial, antioxidant, DPPH assay, GC-MS, phytochemical screening

Introduction
Aegle marmelos (L.) Correa (Family: Rutaceae) is known as Bael in Nepali, Bel in Magar, Bilva in Sanskrit and Stone apple in English [1]. Different parts of Bael tree have cultural, religious and medicinal values in Nepal. The fruits, roots, leaves and branches have been used in traditional medicines [2]. The ripe fruits have been used for the treatment of chronic diarrhoea and dysentery, and used as a tonic for heart and brain. Decoction of roots and sometimes the stem barks have been used to get remedies from melancholy, intermittent fevers and palpitation of heart [1]. Several Ayurvedic preparations such as Dashmool, Bilwadi churna, Bilwadi leha, Bilwadi Ghrita, Bilva Kwath, etc. have used different parts of *A. marmelos* to treat inflammations, diarrhoea, dysentery, etc. *A. marmelos* has also been used for the treatment of diabetes in Ayurvedic, Unani and Siddha systems [3]. Medicinal importance of *A. marmelos* has been documented in a number of review articles [1-6]. Various phytoconstituents such as coumarins, polysaccharides, alkaloids, carotenoids, phenolics, tannins, flavonoids, pectins, terpenoids, etc. have been isolated from the various parts of *A. marmelos* [1-6]. Citarasu *et al.* have reported bactericidal activity of *A. marmelos* against some bacterial species [7]. The extracts of leaves, roots and fruits have been reported to be active against several bacterial strains [8-11].

In Nepal, *A. marmelos* is distributed abundantly in the Siwalik region, inner Terai and lower valley region, mostly at riverside having sandy soil at 150-1220 m altitude [12]. Juice of the ripe *A. marmelos* fruits has very good market in Nepal and India. *A. marmelos* is identified as a major high-valued, non-timber forest product (NTFP) of Nepal and possesses a great potentiality to improve the livelihood of the Community Forest User Groups (CFUGs) members in Nepal. In the recent past, a few *A. marmelos* juice factories have been established in different parts of the nation with the initiation of District Forest Offices (DFOs) and the Federation of Community Forestry Users Nepal (FECOFUN).

Despite fruits and leaves of *A. marmelos* have been much investigated, on the other hand, barks of *A. marmelos* have rarely explored [8, 13, 14]. While conducting field studies on the ethnomedicinal plants used by Magar community in Dhobadi and Deurali VDCs of Nawalparasi District, Nepal (the locality is renamed as Hupsekot Rural Municipality, Nawalpur District), we came to know that fruits of *A. marmelos* are eaten for heat sickness and gastritis [15], and decoction of the barks is used for the treatment of fever and cough by the villagers.

Therefore, we evaluated the antimicrobial activity of the barks of *A. marmelos* to validate ethnomedicinal knowledge in the present research.

Materials and Methods

Field visit

Magar communities of Dhobadi, Jugepani, Giruwari and Jhyalbas Villages of Hupsekot Rural Municipality, Nawalpur District, Province no. 4, Nepal (altitude 150-1800 m) were visited in December 2015. Ethnomedicinal data were collected from the local healers and knowledgeable informants by structural interviews. Plants were collected during transect walks through the vegetation. Herbaria were made, identified and confirmed at the National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal with a voucher specimen no. 9495051. The barks were air dried at room temperature for laboratory tests.

Materials

Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) were purchased from HiMedia Laboratories Pvt. Ltd. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich. Spectrophotometry was carried out using an Elisa microplate reader (EPOCH2, BioTek Instruments).

Extraction of the plant material

Air dried bark of *A. marmelos* was ground. The plant material (100 g) was successively extracted with hexane (800 ml, 7 h) and 70% methanol (800 ml, 22 h) using a Soxhlet extractor. These plant extracts were concentrated using a rotary evaporator under reduced pressure. The concentrated extracts were stored in refrigerator at 4 ºC until further use.

Phytochemical screening

Phytochemical screening of the hexane and aq. methanolic extracts was performed using different specific reagents to find out different phytoconstituents present in the plant extracts [16]. Braymer, Dragendorff, Sodium carbonate, Ammonium hydroxide, Shinoda, Kedde, Froth, Biuret, Sulfuric acid, Liebermann-Burchard, Salkowski, Fluorescence, and Borntiger tests were carried out to detect tannins and polyphenols, alkaloid, anthocyanosides, anthocyanosides, flavonoids, cardiac glycosides, saponin, proteins and amino acid, carotenoids, steroids, terpenoids, coumarins, and anthraquinones respectively.

Gas Chromatography-Mass Spectrometry

The gas chromatography-mass spectrometry (GC-MS) analyses of the hexane and aq. methanolic extracts of the barks of *A. marmelos* was analysed using an Agilent 7890A GC system coupled with an Agilent 5975 C mass selective detector, equipped with a HP-5MS GC column (5% phenyl methyl siloxane, Agilent 19091S-433, 30 m × 250 µm internal diameter, 0.25 µm film thickness). Helium was used as a carrier gas at flow rate of 1.21 ml/min. The instrument was operated in the electron impact (EI) mode at 70 eV and ion source temperature 230 °C in the scan range of 50-500 m/z. The initial column temperature was set at 40 °C held for 2 min, ramped at a rate of 4 °C/min to 270 °C and held for 5.5 min (total run time 65 min). A dilute sample solution of the extracts was prepared either in chloroform or methanol, and a volume of 2 µl was injected in split injection technique. The constituents were identified by comparing the mass spectra available in a MS database (NIST 08).

Antibacterial Susceptibility Assay

The bacterial strains *Bacillus subtilis* (ATCC 6051), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27263), and *Salmonella enterica* subsp. *enterica serovar typhi* were used.

The Agar well diffusion method was used to evaluate antimicrobial activity [17, 18]. First, inoculum was prepared by the Colony suspension method. The isolates were transferred to MHB and the suspension was adjusted to McFarland standards 0.5, equivalent to 1×10^8 CFU. The bacterial inoculum were swabbed on sterile MHA plates. The concentrated hexane and aq. methanolic extracts were dissolved in dimethyl sulfoxide (0.1 g/ml concentration). Wells of 6 mm diameter bored on the MHA plates were loaded with 50 µl of the samples prepared. Ampicillin and Gentamicin of 10 µg per disc (Mast Diagnostics) were used as standards. Dimethyl sulfoxide was used as a negative control. The loaded MHA plates were incubated at 35±2 °C for 18-20 hours. Tests were performed in triplicates. Zone of inhibition (ZOI) was measured. The extracts displaying antibacterial activity were further used to evaluate the minimum inhibitory concentration (MIC) by using the two-fold macrodilution broth methodology and subsequently the minimum bactericidal concentration (MBC) values were determined.

DPPH Free Radical Scavenging Assay

Only the aq. methanolic extract was used to evaluate antioxidant capacity using the DPPH free radical scavenging assay [19]. Briefly, DPPH• radical solution was prepared by overnight stirring of DPPH (3.94 mg) in distilled methanol (100 ml) at 0 ºC to obtain a solution of 0.1 mM concentration. A stock methanolic solution of the aq. methanolic extract was prepared (concentration 2000 µg/ml). Appropriate amounts of the stock solution were filled in the respective wells of a 96 well plate and were further diluted with methanol to obtain 1500, 1000, 750, 500, 250, 100, 50 and 25 µg/ml concentrations (total volume of 50 µl). Next, as a positive control, 50 µl of gallic acid solutions of concentrations 20, 10 and 5 µg/ml were used to obtain a linear curve. To each well was added 250 µl of DPPH• solution. For blank, 300 µl of distilled methanol was used. For control, 250 µL of DPPH• solution and 50 µl of distilled methanol was used. The microplate was shaken at room temperature for 30 min in the dark and the absorbance was determined at 517 nm wavelength. The DPPH• radical scavenging ability was calculated according to the equation:

$$\text{DPPH• scavenging rate (\%)} = [1 - (A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$$

Where, A_{sample}, A_{blank} and $A_{control}$ are the absorbances measured for the sample, blank and control, respectively. Concentration of the extract for 50% inhibition of DPPH• radical was calculated using formula $IC_{50} = (50 - c) / m$, where c is the intercept and m is the slope of the linear curve.

Statistical Analysis

Statistical analysis was done using Microsoft excel program. Antimicrobial susceptibility assay were carried out in triplicates (n = 3) and presented as average±SEM (standard error mean).

Results and Discussion

Upon successive Soxhlet extractions of the stem bark of *A. marmelos* (100 g) yielded the hexane extract (2.7 g, 2.7%, yellowish brown) and aq. methanolic extract (8.25 g, 8.25%, reddish brown). Phytochemical screening revealed that the hexane extract constituted alkaloids, steroids and terpenoids, whereas the aq. methanolic extract constituted polyphenols, alkaloids and steroids.

As indicated in Table 1, the hexane extract of *A. marmelos* constituted a number of fatty acids. Out of 16 compounds identified, 7 are free fatty acids, 3 of them unsaturated, and 3 are fatty acids methyl esters accounting 29.28% of the total extract. The presence of palmitic acid (10.48%), stearic acid (6.34%) as well as 3-pentadecylphenol (4.59%) may be responsible for the antibacterial activity of the extract [20,21]. Alkanes such as tetracosane (6.61%) and heptacosane (9.78%) were also identified as major ingredients.

Table 1: Phytoconstituents identified from the hexane extract of *A. marmelos* barks

S. N.	R. T.	Compound	Molecular Formula	Molecular Weight	Peak Area %	Compound nature
1	34.399	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	0.27	Fatty acid
2	38.457	Methyl palmitate	$C_{17}H_{34}O_2$	270.46	0.69	Ester
3	39.439	Palmitic acid	$C_{16}H_{32}O_2$	256.43	10.48	Fatty acid
4	40.672	Octadecanal	$C_{18}H_{36}O$	268.48	0.57	Ketone
5	42.352	Methyl octadeca-9,12-dienoate	$C_{19}H_{34}O_2$	294.45	0.36	Ester
6	43.122	Methyl 16-methylheptadecanoate	$C_{19}H_{38}O_2$	298.51	0.40	Ester
7	43.280	Octadeca-9,12-dienoic acid	$C_{18}H_{32}O_2$	280.45	2.60	Fatty acid
8	43.411	Vaccenic acid	$C_{18}H_{34}O_2$	282.47	5.08	Fatty acid
9	43.716	Octadec-13-enoic acid	$C_{18}H_{34}O_2$	282.47	2.46	Fatty acid
10	43.967	Stearic acid	$C_{18}H_{36}O_2$	284.48	6.34	Fatty acid
11	48.146	Icosanoic acid	$C_{20}H_{40}O_2$	312.54	0.60	Fatty acid
12	49.035	2-Hydroxycyclopentadecan-1-one	$C_{15}H_{28}O_2$	240.39	0.93	Ketone
13	50.628	3-Pentadecylphenol	$C_{21}H_{36}O$	304.52	4.59	Phenol
14	54.463	Tetracosane	$C_{24}H_{50}$	338.66	6.61	Alkane
16	57.916	Heptacosane	$C_{27}H_{56}$	380.74	9.78	Alkane

Table 2 reveals that the aq. methanolic extract of *A. marmelos* barks mainly constituted steroids *viz*. 3-deoxyestradiol (1.05%), ethyl *iso*-allocholate (0.44%), γ-sitosterol (71.19%), sitostenone (10.14%) and cholest-1-eno[2,1-α]naphthalene, 3',4'-dihydro (0.90%) accounting 83.72% of the total extract. The mass spectrum obtained for γ-sitosterol in GC-MS analysis was carefully compared with the available library spectra of β-sitosterol and γ-sitosterol. A peak appeared at m/z 303 in the spectrum of γ-sitosterol was comparably intense leading us to identify the constituent. γ-Sitosterol exhibits strong antifungal, antibacterial and anti-angiogenic activities [22]. It is being used in traditional medicine to treat ulcers, bronchitis, diabetes and heart diseases.

Table 2: Phytoconstituents identified from the aq. methanolic extract of *A. marmelos* barks

S. N.	R. T.	Compound	Molecular Formula	Molecular Weight	Peak Area %	Compound Nature
1	21.116	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.18	1.26	Phenol
2	28.925	1-(3,5-Dimethoxyphenyl)ethan-1-one	$C_{10}H_{12}O_3$	180.20	2.41	Ketone
3	33.766	(*E*)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180.20	0.38	Phenol
4	39.349	3-Deoxyestradiol	$C_{18}H_{24}O$	256.39	1.05	Steroid
5	42.498	Methyl 7,10,13-hexadecatrienoate	$C_{17}H_{28}O_2$	264.41	0.34	Ester
6	43.369	Oleic acid	$C_{18}H_{34}O_2$	282.47	0.76	Fatty acid
7	46.769	Ethyl *iso*-allocholate	$C_{26}H_{44}O_5$	436.63	0.44	Steroid
8	49.349	γ-Sitosterol	$C_{29}H_{50}O$	414.39	71.19	Steroid
9	58.226	Sitostenone	$C_{29}H_{48}O$	412.70	10.14	Steroid
10	60.892	Cholest-1-eno[2,1-α]naphthalene, 3',4'-dihydro	$C_{35}H_{52}$	472.80	0.90	Steroid

The result of antibacterial susceptibility assay is presented in Table 3. The aq. methanolic extract displayed antimicrobial activity against Gram-positive bacteria (*B. subtilis*, *E. faecalis* and *S. aureus*) as well as Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa* and *S. typhi*, except *E. coli*). The hexane extract was found effective against *B. subtilis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. The hexane extract showed a greater ZOI (12±0 mm) as compared to the aq. methanolic extract (9.4±0.6 mm) against *B. subtilis*. The hexane extract also inhibited the growth of *E. coli* in some extent, while the aq. methanolic extract was found completely ineffective. The result also showed that *S. aureus* was the most susceptible (ZOI = 33 mm), on the other hand, *B. subtilis* (ZOI = 8 mm), *K. pneumoniae* (ZOI = 9 mm) and *P. aeruginosa* (no ZOI) were found resistant to the standard drug Ampicillin. Both the hexane and aq. methanolic extracts showed same potentiality against *K. pneumoniae* as compared to the standards Ampicillin and Gentamicin. Interestingly, these extracts were effective against *P. aeruginosa* as well as *B. subtilis*, which were resistant to Ampicillin. The aq. methanolic extract was more potent than the hexane extract against *S. aureus*. Poonkothai and Saravanan also observed antibacterial efficacy of the methanolic extract of the *A. marmelos* barks against *S. aureus* [8].

Table 3: Antimicrobial susceptibility assay of the extracts of *A. marmelos* barks

S. N.	Bacteria used	ZOI (in mm diameter)				
		Hexane extract	Aq. methanolic extract	Ampicillin (10 µg/disc)	Gentamicin (10 µg/disc)	Dimethyl sulfoxide
1	*B. subtilis*	12±0	9.4±0.6	8	15	–
2	*E. faecalis*	–	12±0	13	18	–
3	*S. aureus*	–	12±0	33	17	–
4	*E. coli*	8±0	–	26	17	–
5	*K. pneumoniae*	10±0	10.3±0.33	9	10	–
6	*P. aeruginosa*	10±0	11±0.43	–	15	–
7	*S. typhi*	–	10±1	15	13	–
(–) sign indicates no ZOI was observed.						

The MIC value displayed by the hexane extract against *B. subtilis*, *K. pneumonia* and *P. aeruginosa* was 25 mg/ml, whereas the MBC values obtained were 50, 25 and 50 mg/ml respectively. The MIC values of the aq. methanolic extract were 25, 25, 12.5, 25, 25 and 50 mg/ml against *B. subtilis*, *E. faecalis*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *S. typhi* respectively, whereas the MBC values obtained were 50 mg/ml against *B. subtilis*, *S. aureus*, *K. pneumonia* and *P. aeruginosa*, and >50 mg/ml against *E. faecalis* and *S. typhi*.

To evaluate DPPH free radical scavenging activity, first we obtained a linear curve for the standard gallic acid using the data obtained for % inhibition *versus* concentration ($y = 3.0431x + 12.032$, $R^2 = 0.998$) and found the IC_{50} value of 12.48 µg/ml, which was comparable to previous reports [23]. In a similar way, IC_{50} = 961.53 µg/ml value for the aq. methanolic extract of the barks of *A. marmelos* was computed ($y = 0.043x + 8.6542$, $R^2 = 0.934$). Generally, the total polyphenols content in the sample is responsible for a high antioxidant activity. Compared to the polyphenols content, steroids were found to be major constituents in the aq. methanolic extract of *A. marmelos* barks in this study (Table 2). This could be a reason for exhibiting of a moderate antioxidant activity of the extract. There are some reports which claimed that the fruits and leaves extracts of *A. marmelos* possessed a high antioxidant activity. Patra *et al.* [24] have reported 81.10% inhibition of DPPH radical by fermented juice of *A. marmelos* at 15 µl/ml concentration and Karumaran *et al.* [25] have claimed that the ethanolic extract of the leaves exhibited IC_{50} value of 63.84±0.05 µg/ml in DPPH free radical scavenging assay.

Conclusion

Knowledge on antibacterial potentiality of the barks of *A. marmelos* was derived from the ethnobotanical survey in the Magar community of Hupsekot Rural Muncipality, central Nepal. The community uses decoction of barks to treat fever and cough. Compared to the leaves and fruits, investigation on the barks of *A. marmelos* is rare. This work showed that the hexane and aq. methanolic extracts of barks of *A. marmelos* exhibit antibacterial activity in the support of traditional knowledge. Gratifyingly, the extracts showed a significant antibacterial activity against *B. subtilis*, *K. pneumoniae* and *P. aeruginosa*, which were found resistant to Ampicillin. Displaying of antibacterial activity by the hexane extract was mainly due to the presence of fatty acids, while steroids present in the aq. methanolic extract have major role for the activity. The aq. methanolic extract also displayed a moderate antioxidant activity by scavenging DPPH free radical with IC_{50} value of 961.53 µg/ml. These results have encouraged us to isolate active components and the works are under progress in our laboratories.

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