J. Pl. Res. Vol. 17, No. 1, pp 98-103, 2019

Phytochemical Evaluation and In Vitro Antimicrobial Activity of the Roots of *Flemingia strobilifera* (L.) R. Br.

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Abstract

Ethno-medicinal uses of *Flemingia strobiifera* in the Magar communities at Kawaswoti urban municipality, Province no. 4, Nepal was surveyed. Phytochemicals present in the hexane and aqueous methanolic extracts of the roots were evaluated by chemical tests and GC-MS analysis. The antimicrobial activity of the extracts was carried out against 8 bacterial species by the agar well diffusion method. Zone of inhibition was compared with standard antibiotics ampicillin and gentamicin. The aqueous methanolic extract showed stronger antimicrobial activity against *Escherichia coli*. The lowest MIC and MBC values were 1.56 and 6.25 mg/ml, respectively. Phytochemical screening revealed the presence of polyphenols and terpenoids. The antimicrobial activity of the plant material might be due to the presence of these phytochemicals.

Keywords: *Ethno-medicine, Magar community, Phytoconstituents, Zone of inhibition*

Introduction

Flemingia strobilifera (L.) R. Br. belongs to family Leguminosae. It is known as bharkauli jhar and bhatwasi in Nepali. It is used in folkloric medicine, such as leaves and flowers for tuberculosis, and roots for ulcers, body swellings, epilepsy, insomnia, fever, indigestion, diarrhea and dysentery (Bhattarai, 1991; Manandhar, 2002; Ghalot et al., 2011; Kumar et al., 2011b). It is used as fodder by Chepang communities in mid hills of Nepal (Rijal, 2011). Root powder is applied on the body by Darai tribe of Chitwan district, Nepal for scabies (Dangol & Gurung, 2000). Madan et al. (2009) have isolated isoflavonoids from *F. strobilifera* roots and showed antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus* and *Escherichia coli*. Kumar et al. (2011a) reported a significant anthelmintic activity of the alcoholic and chloroform extracts of the leaves of *F. strobilifera*. Roots of *F. strobilifera* constituted phenols, flavonoids, steroids, flavonoids glycosides and tannins (Madan et al., 2010).

From the field studies, it was came to know that the Magar communities in Kawaswoti urban municipality, Province no. 4, Nepal use juice of *F. strobilifera* roots for the treatment of diarrhea, dysentery and gastritis. Therefore to validate ethno-medicinal knowledge, antimicrobial susceptibility test of *F. strobilifera* root extracts was evaluated in the present work.

Materials and Methods

Field visit

The study was carried out in Kawaswoti urban municipality of Nawalpur district, Province no. 4, Nepal. Ethno-medicinal data of the medicinal plants of the Magar communities were collected during field visit in April, 2016. Herbaria were prepared and confirmed through comparison with specimens at National Herbarium and Plant Laboratories, Godawari, Nepal.

Materials

Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) were purchased from HiMedia Laboratories Pvt. Ltd. Hexane and Methanols were purchased from Fisher Scientific.

Preparation of the plant extracts

Roots of *F. strobilifera* were dried in shade at room temperature. Air dried plant materials were ground. The ground plant material (100 g) was successively extracted with hexane (800 ml, 7 hours) and 70% methanol (800 ml, 22 hours) using a Soxhlet extractor. These plant extracts were concentrated by using a rotary evaporator and vacuum dried. The extracts were stored in a refrigerator at 4ºC 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 (Ciulei, 1982). Among other tests, Braymer, Dragendorff, Liebermann-Burchard and Salkowski tests were carried out to detect polyphenols, alkaloid, steroids and terpenoids, respectively.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses of the hexane and aq. methanolic extracts of *F. strobilifera* was analyzed 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 solutions of the extracts were prepared in HPLC grade hexane and methanol, and a volume of 2 μl was injected. The constituents were identified by comparing the mass spectra available in a MS database (NIST 08).

Antimicrobial susceptibility test

The hexane and aq. methanolic extracts were screened against a total of 8 bacterial strains namely *Pseudomonas aeruginosa* (ATCC 27263), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6051), *Shigella dysenteriae* (ATCC 13313) and *Salmonella enteric* subsp. *enteric serovar typhi*.

Inoculums were prepared to McFarland standard 0.5 as described in Nemkul et al., (2018). The inoculums were used within 30 minutes.

The antibacterial screening of these extracts was evaluated by using the agar well diffusion technique (Perez et al., 1990). The standardized bacterial inoculums were uniformly spread on the respective sterile MHA agar Petri dishes using sterile cotton swabs. The wells were punched on the agar gel using sterile borer of 6 mm diameter. The wells were filled with 50 il of plant extracts of 0.1g/ml concentration dissolved in dimethyl sulfoxide (DMSO). Ampicillin and gentamicin (Mast dagnostics) of 10 μg per disc were used as standard references. DMSO was used as control. The plates were incubated at 37°C for 18-24 hours. Tests were performed in triplicate. Zone of inhibition (ZOI) was measured in mm.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Broth dilution technique was used to determine MIC values of the extracts which displayed antimicrobial property following (Wiegand et al. 2008). The final inoculum size for broth dilution was 5×10^5 colony-forming units (cfu) ml^{-1}.

Microplates were used for MIC determination. The sterility control wells were filled with 100 μl of MHB, and the growth control wells and wells labeled for different concentration were filled with 50 μl of MHB. 50 μl of stock solution of the extract (0.1g/ml) was added and series of dilutions of the extracts were adjusted by double dilution method. The bacterial suspension adjusted to 1×10^8 cfu ml^{-1} was diluted to 1:100 and vortexed. Each well containing the extract dilutions and the growth control was filled with 50 μl of the bacterial suspension. This results in the final desired inoculum of 5×10^5 cfu ml^{-1}.

After incubation for 18-24 hours at 37°C, the MIC was taken as the lowest concentration of the antimicrobial agent that inhibited visible growth of the tested bacteria as observed with the unaided eye. MBC values were then determined by directly streaking the content of the wells inhibiting bacterial growth on MHA plates.

Results and Discussion

Magars in the study sites use root juice of *F. strobilifa* in gastritis, dysentery and diarrhea, hence, the plant material was chosen in this work. Upon successive Soxhlet extractions of the root of *F. strobilifera* (100 g) using hexane and 70% methanol yielded hexane extract (0.44g, 0.44%, light yellow) and aq. methanolic extract (10.52 g, 10.52%, reddish black). Phytochemical screening revealed that the hexane extract constituted steroids, terpenoids, and the aq. methanolic extract constituted polyphenols.

GC-MS analysis of the hexane extract led to identify 27 compounds accounting 99.37% of the total constituents (Table 1). Out of 27 compounds, 19 hydrocarbons (60.74%), 4 fatty acids (13.18%), 1 acid ester (24.29%), 1 ester (0.55%), 1 alcohol (0.36%) and 1 ketone (0.25%) were identified. Octadecanoic acid was reported to be antimicrobial (Mujeeb et al., 2014). n-Hexadecanoic acid was reported to have antioxidant activity (Kumar et al., 2010). (Z,Z)-9,12-Octadecadienoic acid and oleic acid are cancer preventive and anti-inflammatory agents (Alagammal, 2011). From the aq. methanolic extract, 5 compounds were identified (Table 2). Phthalic andydride (36.62%), n-hexadecanoic acid (20.67%), 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (11.36%), 1-heptadecene (8.76%) and octadecanoic acid (5.28%) were the main constituents accounting 82.69% of the total constituents. 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one was reported to be antimicrobial agent (Kumar et al., 2010).

Table 1: Phytoconstituents identified in the hexane extract of *F. strobilifera*

S. N.	RT	Compounds	Area %	Nature of compound
1	26.962	4-(4-Methoxyphenyl)-2-butanone	0.25	Ketone
2	28.054	5-Phenyldecane	1.02	Hydrocarbon
3	28.299	4-Phenyldecane	0.88	Hydrocarbon
4	28.807	3-Phenyldecane	0.95	Hydrocarbon
5	29.843	2-Phenyldecane	1.43	Hydrocarbon
6	30.580	1,4a-dimethyl-7-(propan-2-ylidene)decahydronaphthalen-1-ol (Juniper camphor)	0.36	Alcohol
7	30.760	6-Phenylundecane	2.10	Hydrocarbon
8	30.858	5-Phenylundecane	4.42	Hydrocarbon
9	31.125	4-Phenylundecane	4.54	Hydrocarbon
10	31.671	3-Phenylundecane	3.27	Hydrocarbon
11	32.675	2-Phenylundecane	4.36	Hydrocarbon
12	33.422	6-Phenyldodecane	4.93	Hydrocarbon
13	33.547	5-Phenyldodecane	4.79	Hydrocarbon
14	33.853	4-Phenyldodecane	3.61	Hydrocarbon
15	34.404	3-Phenyldodecane	3.38	Hydrocarbon
16	35.391	2-Phenyldodecane	3.98	Hydrocarbon
17	35.986	6-Phenyltridecane	5.53	Hydrocarbon
18	36.150	5-Phenyltridecane	3.40	Hydrocarbon
19	36.455	4-Phenyltridecane	2.63	Hydrocarbon
20	37.012	3-Phenyltridecane	2.70	Hydrocarbon
21	37.977	2-Phenyltridecane	2.82	Hydrocarbon
22	39.286	Butyl octyl phthalate	0.55	Ester
23	39.423	n-Hexadecanoic acid	8.32	Fatty acid
24	43.247	(Z,Z)-9,12-Octadecadienoic acid	0.68	Fatty acid
25	43.394	Oleic acid	2.01	Fatty acid
26	43.929	Octadecanoic acid	2.17	Fatty acid
27	51.670	2-(((2-ethylhexyl)oxy)carbonyl)benzoic acid	24.29	Acid ester

Table 2: Phytoconstituents identified in the methanolic extract of *F. strobilifera*

S. N.	RT	Compounds	Area %	Nature of compound
1	15.234	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	11.36	Flavonoid fraction
2	21.033	Phthalic andydride	36.62	Anhydride
3	39.325	n-Hexadecanoic acid	20.67	Fatty acid
4	43.356	1-Heptadecene	8.76	Hydrocarbon
5	43.896	Octadecanoic acid	5.28	Fatty acid

The results of antimicrobial susceptibility tests are shown in Figures 1-3 and Table 3. The aq. methanolic extract of the roots of *F. strobilifera* showed an equal antimicrobial efficacy as gentamicin against *S. typhi*. The extract exhibited potential antimicrobial activity against *E. coli* (ZOI = 16.5±0.67 mm) and *S. aureus* (ZOI = 15.66±0.33 mm). The extract also showed antimicrobial activity against *B. subtilis*, *K. pneumoniae* and *P. aeruginosa* which were resistant to standard antibiotic ampicillin. *S. dysenteriae*, causal bacteria of shigellosis, was also inhibited. Madan et al. (2009) have reported antimicrobial activity of some isoflavonoids isolated from the roots of *F. strobilifera* against gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli* and *P. aeruginosa*). The hexane extract showed antimicrobial activity against *S. aureus, B. subtilis, E. coli* and *P. aeruginosa*. The synergistic effect of various constituents present in the extracts is responsible for the antimicrobial activity.

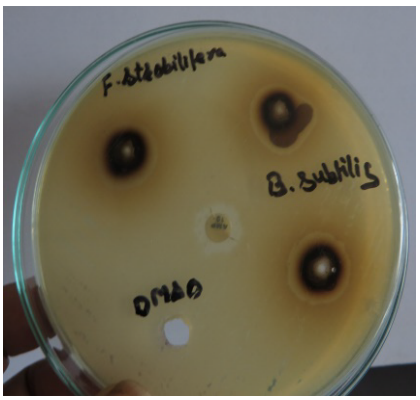

Figure 1: The aq. methanolic extract showing antibacterial activity against *B. subtilis*. Ampicillin as +ve control.

Figure 2: The aq. methanolic extract showing antibacterial activity against *S. typhi*. Ampicillin as +ve control.

Figure 3: The aq. methanolic extract showing antibacterial activity against *S. aureus*. Ampicillin as +ve control.

Table 3: Antimicrobial activity of *F. strobilifera*

S. N	Sample	Diameter of inhibition zone (mm)±standard error mean (SEM)							
		Gram positive bacteria			Gram negative bacteria				
		SA	*BS*	*EF*	*EC*	*ST*	*KP*	*PA*	*SD*
1	HE	11.25±0.47	13.3±0.33	-	11.66±0.33	-	-	12.33±0.66	-
2	ME	15.66±0.33	13.33±0.88	11.66±0.33	16.5±0.67	12.66±0.33	11.66±0.33	11.8±0.33	10.33±0.33
3	Ampicillin	32.5±0.5	8.5±0.5	17.75±0.25	25±1	15.5±0.5	8.5±0.5	-	23.75±0.25
4	Gentamicin	16.75±0.25	15.5±0.5	18.5±0.5	17.5±0.5	12.66±0.33	11.33±0.88	14.66±0.33	18.66±0.66
5	DMSO	-	-	-	-	-	-	-	-

HE = Hexane extract, ME = Aq. methanolic extract, *SA = S. aureus*, *BS = B. subtilis*, *EF = E. faecalis*, *EC = E. coli*, *ST = S. typhi*, *KP = K. pneumoniae*, *PA = P. aeruginosa*, *SD = S. dysenteriae*

MIC and MBC values are shown in Table 4. Lowest MIC was found to be 1.56 mg/ml for the aq. methnolic extract against *E. coli*. The extract showed bactericidal effect on *B. subtilis, E. faecalis, K. pneumoniae* and *S. dysenteriae*. It showed bactericidal effect on higher concentration against *S. aureus, E. coli, S. typhi* and *P. aeruginosa.* The bacterial viability was gradually decreased at high concentration of the extract in a dose-dependent manner.

Table 4: MIC and MBC of *F. strobilifera*

S. N.	Bacteria	Hexane extract		Aq. methanolic extract	
		MIC	MBC	MIC	MBC
1	*S. aureus*	12.5	25	3.12	6.25
2	*B. subtilis*	50	50	12.5	12.5
3	*E. faecalis*	-	-	3.12	3.12
4	*E. coli*	6.25	6.25	1.56	6.25
5	*S. typhi*	-	-	12.5	25
6	*K. pneumoniae*	-	-	6.25	6.25
7	*P. aeruginosa*	12.5	25	3.12	6.25
8	*S. dysenteriae*	-	-	50	50

Conclusion

The people of Magar communities of Kawaswoti rural municipality, Nawalpur district, Province no. 4, Nepal use juice from the roots of *F. strobilifera* for the treatment of gastritis, diarrhea and dysentery. This work showed that aq. methanolic extract of *F. strobilifera* roots exhihbit significant antimicrobial activity against *E. coli* (ZOI = 16.5±0.67) and moderately against *S. dysenteriae* (ZOI = 10.33±0.33) in the support of traditional knowledge. The extract also displayed antimicrobial activity against ampicillin-resistant *B. subtilis, K. pneumoniae* and *P. aeruginosa*.

Acknowledgements

We are grateful to Tri-Chandra Multiple Campus for forwarding necessary administrative efforts for this research study. We would like to express our thanks to the University Grant Commission (UGC) for providing grant for the research. We are grateful to Nepal Academy of Science and Technology (NAST) for providing necessary laboratory facilities for the research. Our special thanks go to local villagers, healers and informants for their cooperation. We are deeply indebted to the National Herbarium and Plant Laboratories, Godavari, Lalitpur for plant identification.

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