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Antimicrobial Studies of Stem of Different *Berberis* Species

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Abstract – *Berberis* is an important medicinal plant, of the family Berberidaceae. Different *Berberis* species and their parts are very common in herbal drug markets of India and world over as an adulterant/substitute to '*Daruharidra*' i.e. *B. aristata* DC. Antimicrobial activity of 50% hydroalcoholic extracts of stem of four *Berberis* species *viz. B. aristata* DC., *B. asiatica* Roxb. ex DC., *B. chitria* Lindl. and *B. lycium* Royle and the isolated alkaloid berberine were tested against eleven bacterial and eight fungal strains. The extracts with the strongest antibacterial activity was obtained from *B. lycium* followed by *B. aristata, B. asiatica* and *B. chitria.* Based on these results it is possible to conclude that the hydroalcoholic extract and alkaloid (berberine) has stronger and broader spectrum against bacterial strains as compared to fungal strains. The result obtained in the present study authenticates and support the use of these plants in folklore medicine for treatment of various infectious diseases caused by the bacterial pathogens. However, an attempt has been made to explore the possibilities of utilizing stem part rather than roots of these species with the aim to conserve this species which is over exploited due to diverse use of its root. These findings will stimulate the search for novel, natural products as new antibacterial/ antifungal agents which may be useful to pharmaceutical industries.
Keywords – *Berberis*, Antimicrobial activity, Berberine, HPTLC

Introduction

Berberis (Berberidaceae) has been found an important place in Traditional as well as modern systems of medicine for their efficacious medicinal properties. The root, stem, and bark are used for treating a variety of ailments such as eye and ear diseases, rheumatism, jaundice, diabetes, malarial fever, stomach disorders, skin disease and as tonic (Watt, 1883; Kirtikar & Basu, 1933; Chopra *et al*., 1958). Its use in the management of infected wounds has also been described in Ayurvedic classical texts (Shusurut Samhita, 1963). Alkaloid berberine occurs most frequently with high percentage in various genera of family Berberidaceae, which is located chiefly in the cortical tissue of the roots and stems. The major alkaloid from *Berberis* species is berberine, which is reported for various infectious diseases *viz*. Cholera (Dutta & Panse, 1962), acute diarrhea (Lahiri & Dutta 1967), amoebiasis, latent malaria, oriental, sore and skin infections (Anonymous, 1988). Although a detailed pharmacognostic study of *B. aristata* DC., *B. asiatica* Roxb. Ex DC. and *B. chitria* Lindl. is reported by Srivastava *et al.* (2001, 2004, 2006), but till date no such claims has been validated on its antimicrobial activities except on stem bark of *B. asiatica* (Bhandari *et al*., 2000).

In Ayurvedic Pharmacopoeia of India (Anonymous, 1965) roots of *Berberis aristata* is mentioned as official part of the drug, *Daruharidra.* However, an attempt has been made to explore the possibilities of utilizing stem part rather than roots of these species with the aim to conserve this species which is over exploited due to diverse use of its root.

Therefore, the present study in under taken to evaluate the hydroalcoholic (50%) extract of stem of four species along with its isolated alkaloid i.e. berberine as antimicrobial agents against 11 bacterial and 8 fungal strains.

Experimental

Plant material and extraction – Four *Berberis* species were collected in 1998 from the Dhanaulti (Uttaranchal) region of Western Himalayas, India, identified by Dr. A.K.S. Rawat, Scientist and lodged in National Herbarium of the institute with the following voucher numbers [LWG 221239, 1998; **BAr**], [LWG 221240, 1998; **BAs**], [LWG 221241, 1998; **BC**], [LWG 221238, 1998; **BL**].

Air-dried materials were grinded with a mechanical grinder, and sifted through a wire screen (mesh size, 2 × 2

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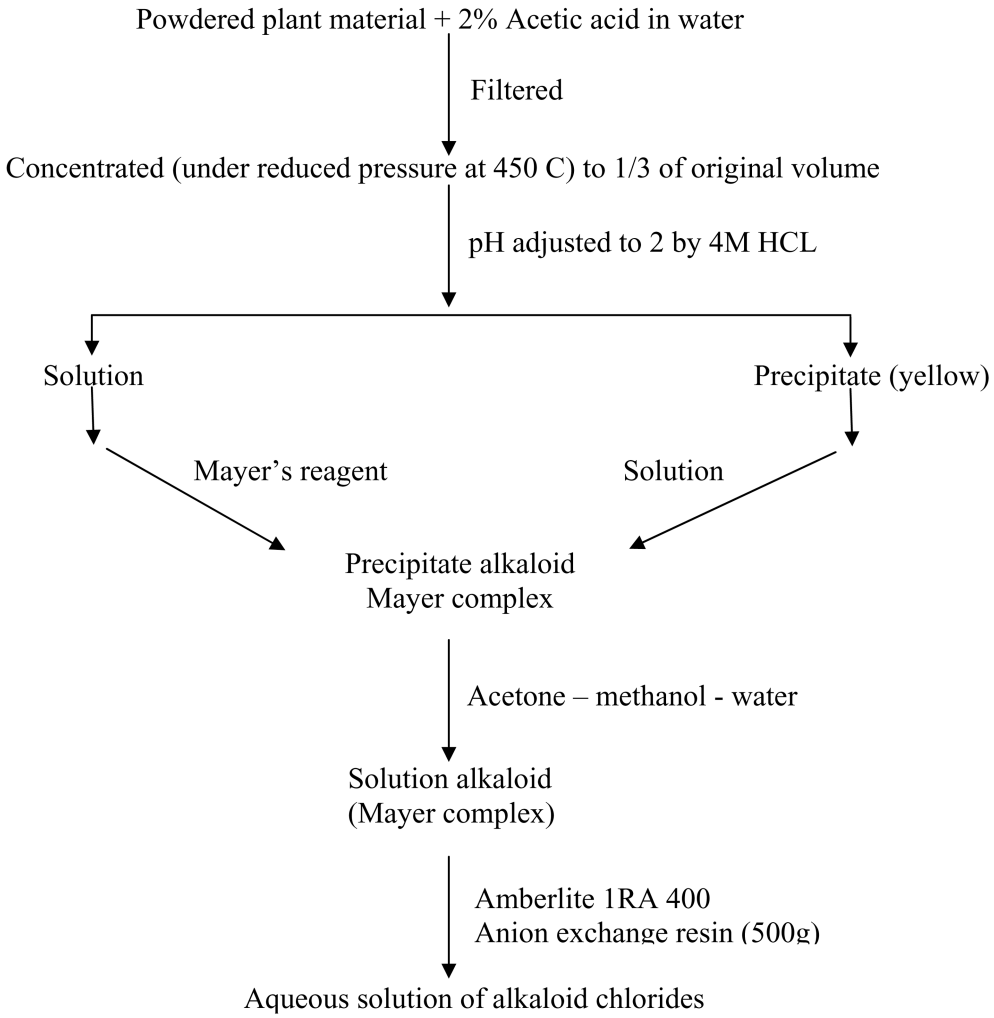

mm). The materials (100 g each) were then cold percolated with 50% ethanol (w/v) at room temperature for 24 h. The extracts were decanted, filtered with Whatman No. 1 filter paper and concentrated in reduced pressure at below 40°C on rotary evaporator for biological screening against microorganism.

Quantitative estimation of alkaloids – The quantitative estimation of berberine alkaloid as berberine hydrochloride was estimated according to Siwon *et al*. (1980) with some modification and flow chart is described above. This was also used for the screening against microorganisms.

HPTLC studies – A densitometric HPTLC analysis was also performed for the development of characteristic fingerprint profile, which may be used as markers for quality evaluation and standardization of the drug. In addition the study also explores the possibilities for using stem part of these species as a substitute of root. For this, 1 g powdered root was refluxed for 5 min on water bath with 5 ml methanol consequently three times, filtered and filtrate taken as test solution along with reference berberine (7 µl of each) and was applied on HPTLC precoated silicagel G60 F_{254} Merck glass plates of 20 × 10 cm with the help of Camag Linomat-IV applicator and eluted the plate to a distance of 6.20 cm at room temperature (19°C) in solvent system *n*-propanol : water : formic acid (90 : 8.0 : 0.4). Berberine was identified at R_f 0.32 which was more or less similar in percentage among all the four species (Fig. 1 & 2). Calibration curves for berberine was linear and within range of 100-1000 µg (Table 1).

Microbial strains and growth conditions – *Micrococcus luteus* MTCC (106), *Bacillus subtilis* MTCC (121), *Bacillus cereus* MTCC (430), *Enterobacter aerogenes* MTCC (111), *Escherichia coli* MTCC (443), *Klebsiella pneumoniae* MTCC (109), *Proteus mirabilis* MTCC (1429), *Pseudomonas aeruginosa* MTCC (424), *Staphylococcus aureus* MTCC (96), *Salmonella typhimurium* MTCC (98) and *Streptococcus pneumoniae* MTCC

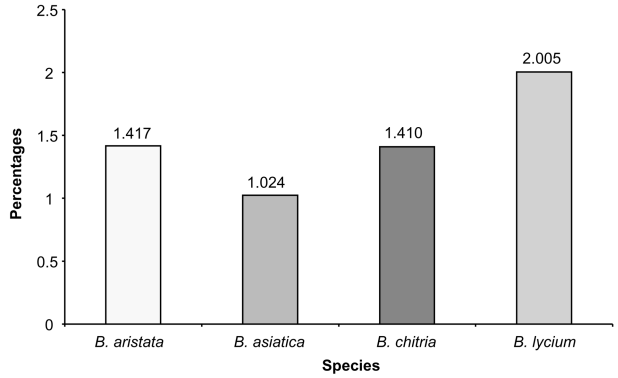

Fig. 1. Berberine percentage in stem of different *Berberis* species.

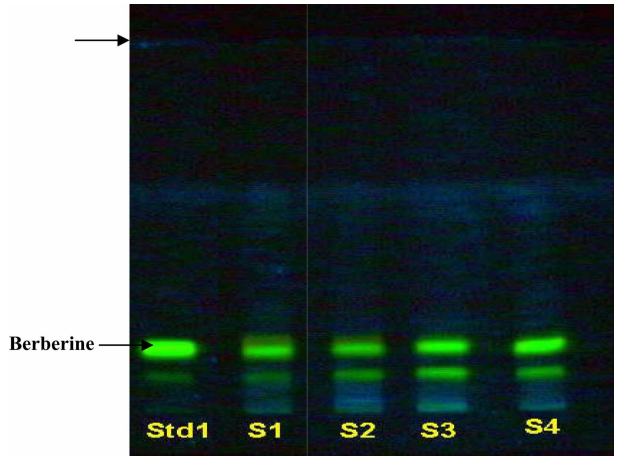

Fig. 2. HPTLC profile of stem of four *Berberis* species (under UV 366).
(S1: *Berberis aristata*; S2: *Berberis asiatica*; S3: *Berberis chitria*; S4: *Berberis lycium*; Std1: Berberine standard.

Table 1. R_f value by HPTLC and linear regression equation for the determination of berberine

Compound	R_f value	Regression equation	r^2
Berberine	0.32	y = 4412.97 x + 32719.09	0.986

(2672), and 8 fungi *Candida albicans* MTCC (183) and *Cryptococcus albidus* MTCC (2661), *Trichophyton rubrum* MTCC (296), *Aspergillus niger* MTCC (16404), *A. flavus* MTCC (1973), *A. spinulosue* MTCC (16919), *A. terreus* MTCC (1782) and *A. nidulans* MTCC (11267). These organisms were procured from Institute of Microbial Technology (IMTECH-CSIR) Chandigarh, India. Cultures of bacteria were grown on Nutrient broth (Hi-Media) at 37°C for 18-24 hrs and on fungus on Potato dextrose broth (Hi-Media) at 28°C for 48 - 72 hrs and were maintained on respective agar slant at 4°C.

Antimicrobial assay

Disc-diffusion assay – The dried extracts and berberine were dissolved in hydro alcoholic (50%) and distilled water to a final concentration of 50, 1, 3, and 5 mg/ml and sterilized by filtration through 0.45 µm Millipore filters. The agar diffusion method (Murray *et al.*, 1995) was used to evaluate the antimicrobial activity. A final inoculum, using 100 µl of suspension containing 10^8cfu/ml of bacteria and 10^4spore/ml of fungi spread on Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) medium respectively.

The disc (6 mm in diameter-Hi-Media) was impregnated with 10 µl of 50 mg/ml crude extract and 1, 3, and 5 mg/ml berberine extracts placed on seeded agar. Gentamicin (10, 30, and 50 µg/disc) were used as positive controls for bacteria and Ketoconzole (10 µg/disc) for fungi. Ethanol (50%) was used as negative control. The experiments were conducted in triplicate and test plates were incubated at 37°C for 18 - 24 hrs for bacteria and 28°C for 3 to 5 days for fungi depending on incubation time required for visible growth. Antimicrobial activity was evaluated by measuring the zone of inhibition against test organisms with the help of Hi Antibiotic zone scale™ (Hi-Media).

Standard microdilution assay (NCCLS) – The minimum inhibitory concentration (MIC) was determined for extracts, which showed high (above 50%) antimicrobial activity with the disc diffusion method.

Inoculum preparation – Stock bacterial inoculum suspensions were obtained from 6 - 12 hrs culture on Mueller-Hinton broth at 37°C. Those final suspensions served for the inoculum preparation. The cell density of each suspensions was determined by NCCLS guidelines (NCCLS 1999) using a counting chamber and then adjusted to 0.5 McFarland turbidity at the concentration of 10^5 - 10^6 CFU (Colony forming units)/ml by dilution with MHB.

The fungi were grown on Potato dextrose agar at 28 - 30°C for 3 - 7 days, to induce conidia formations. Then the culture was washed with 2 ml of peptone water (Saline solution) and the suspension was transferred to a sterile tube, where the heavy particles were allowed to settle for 5 min. The upper homogenous suspensions were transferred in a new sterile tube and filled with Mycological peptone (Hi-Media). This suspension is considered as initial inoculums. After dilutions in MP, the inoculums were adjusted microscopically to about 10^4 CFU/ml.

Antibacterial activity – Antibacterial activity was performed according to NCCLS guidelines (1999) and Zgoda and Porter, (2001) with some slight modifications. Briefly extract was dissolved in DMSO and filtered through 0.2 micron non- pyrogenic filter and serially diluted (two fold) with 2.5% DMSO to give a dilution

range - 1000 - 1.95 µg/ml Test are performed in sterile U. bottom 96 - well by dispensing in to each well 95 µl of MHB broth and 5 µl of inoculum (0.5 McFarland Tub.) 100 µl of test extract was finally added to each appropriate well. The final volume in each well was 200 µl. The broth without extracts and DMSO 2.5% were used as positive control stand and antibiotic - Gentamicin (Sigma) was used as positive control. Concentration ranged of the antibiotic from 1000 - 0.97 µg/ml. The plates were covered with sterile sealer and incubated at 37°C for 18 - 24 h to indicate bacterial growth 40 µl of 0.2 mg/ml P-iodonitro terazolium violet (INT) Sigma solution was added to each well and incubated for further 30 minutes. Inhibition of bacterial growth was visible as a clear well and the presence of growth detected by the presence of pink red colour.

Antifungal activity – We used the same method for antifungal test. The culture medium was Mycological peptone (Hi- media) Ketoconezole (Sigma) was used as standards. Concentration ranged 1000 - 0.48 µg/ml. After inoculation, the plates were incubated at 28° - 30°C for 24 - 72 h The MIC (minimum inhibitory concentration) was considered as lowest drug concentration of antifungal agent inhibiting the growth of microorganisms. MIC was detected by lack of visual turbidity (matching the negative growth control) subcultures were made from the clear wells which did not show any growth incubation.

Results and Discussion

Antimicrobial activity of four important species of *Berberis* (*B. aristata, B. asiatica, B. chitria* and *B. lycium)* extracts (stem) and their isolated compound berberine (alkaloid) against five Gram-positive, six Gram-negative, including multi-resistant strain and eight fungal strains has been carried out. The result of antimicrobial activity with their MIC values of the tested extract (hydro alcoholic 50%) is shown in Table 2 and 3.

The isolated compound berberine was also tested against selected microorganisms and compared with standard potential antibiotic drugs gentamicin. (Hi-media) at concentration (10 µg/disc 30; µg/disc and 50 µg/disc) against bacterial strain whereas ketoconzole (10 µg/disc)

Table 2. Percent inhibition and MIC values (µg/ml) of crude extracts (hydro alcoholic 50%) of *Berberis* species (stem) with standard antibiotic gentamicin and ketokonazole (conc. 50 mg/ml).

S. No	Bacterial strain	B. aristata		B. asiatica		B. chitria		B. lycium	
	Gram positive	%	MIC	%	MIC	%	MIC	%	MIC
1.	*Micrococcus luteus*	65.75	0.62	46.30	ND	47.22	ND	62.20	0.31
2.	*Bacillus subtilis*	36.11	ND	46.30	ND	41.66	ND	57.41	0.62
3.	*B. cereus*	64.80	0.62	42.50	ND	50.00	1.25	66.67	0.31
4.	*Enterobactor aerogenes*	31.54	ND	28.83	ND	27.91	ND	30.62	ND
5.	*Escherichia coli*	52.00	1.25	42.68	ND	44.00	ND	58.68	0.62
	Gram negative								
6.	*Klebsiella pneumoniae*	80.00	0.31	68.00	0.62	49.17	ND	86.68	0.31
7.	*Proteus mirabilis*	44.02	ND	58.62	0.62	71.27	0.62	82.75	0.31
8.	*Pseudomanas aeruginosa*	41.17	ND	47.05	ND	50.00	1.25	60.75	0.31
9.	*Staphylococcus aureus*	61.46	0.62	82.09	1.25	52.09	0.62	60.40	0.31
10.	*Salmonella typhimurium*	--	--	--	--	--	--	47.62	ND
11.	*Streptococcus pneumoniae*	74.43	0.31	85.56	0.31	78.90	0.31	74.43	0.31
	Fungal Strains								
12.	*Aspergillus niger*	--	--	--	--	--	--	--	--
13.	*A. nidulans*	--	--	--	--	36.89	ND	--	--
14.	*A. terreus*	42.85	ND	--	--	--	--	64.28	0.31
15.	*A. spinulosue*	--	--	57.14	1.25	--	--	--	--
16.	*A. flavus*	--	--	85.00	0.31	--	--	--	--
17.	*Trichophyton rubrum*	--	--	--	--	--	--	--	--
18.	*Cryptococcus albidus*	--	--	--	--	57.59	1.25	--	--
19.	*Candida albicans*	--	--	--	--	--	--	--	--

-- (No inhibition); ND: Not determined; MIC: Minimum Inhibitory Concentration
%: Percentage inhibition

Table 3. Antimicrobial activities of berberine (alkaloid) compared with standard drugs gentamicin and ketoconezole (conc.: μg/disc and μg) by disc diffusion and microdilution method

S. No	Bacterial strains	Disc Diffusion Method						Microdilution Method	
		Berberine			Gentamicin				
		B^{10}	B^{30}	B^{50}	G^{10}	G^{30}	G^{50}	Ber	Gen
1.	*Micrococcus luteus*	18	25	30	19	22	25	1.25	0.24
2.	*Bacillus subtilis*	21	26	32	22	25	30	0.31	0.48
3.	*B. cereus*	19	20	27	24	25	29	0.62	0.97
4.	*Enterobactor aerogenes*	17	24	32	20	25	30	1.25	0.24
5.	*Escherichia coli*	19	20	24	24	26	30	0.15	0.24
6.	*Klebsiela pneumoniae*	17	19	21	25	28	30	0.62	0.48
7.	*Proteus mirabilis*	21	24	27	19	26	28	0.31	0.48
8.	*Pseudomanas aeruginosa*	18	20	24	20	22	25	0.15	1.95
9.	*Staphylococcus aureus*	15	17	22	15	18	20	0.62	0.97
10.	*Salmonella typhimurium*	15	19	21	20	23	25	0.31	1.95
11.	*Streptococcus pneumoniae*	19	21	28	15	17	19	1.25	0.97
	Fungal Strain					Kt^{10}			**Kt**
12.	*Aspergillus niger*	17					20	1.25	1.95
13.	*A. nidulans*	14					24	ND	1.95
14.	*A. terreus*	12					28	3.0	15.62
15.	*A. spinulosue*	13					28	15.0	3.90
16.	*A. flavus*	10					32	3.0	1.95
17.	*Trichophyton rubrum*	10					22	7.80	7.81
18.	*Cryptococcus albidus*	12					23	6.20	0.48
19.	*Candida albicans*	08					30	6.20	0.48

Experiments were in triplicate (No variation)

G^{10} Gentamicin (10 μg/disc); G^{30} Gentamicin (30 μg /disc); G^{50} Gentamicin (50 μg/disc) Hi-Media; B^{10} Berberine (10 μg/disc); B^{30} Berberine (30 μg/disc); B^{50} Berberine (50 μg/disc); K^{10} Ketoconezole (10 μg/disc) Hi-Media; Kt: Ketoconezole (Sigma)

ND: Not determined

against fungal strains by disc diffusion and micro dilution method. (Table 3)

Extracts with the strongest antibacterial activity was obtained from *B. lycium* followed by *B. aristata, B. asiatica* and *B. chitria.* Sensitivity of test strains was in decreasing order; *B. subtilis* > *E. aerogenes* > *M. luteus* > *S. pneumoniae* > *B. cereus* > *P. mirabilis* > *P. aeruginosa* > *E. coli* > *S. aureus* > *S. typhimurium* > *K. pneumoniae*. In the case of test bacteria and their differences in susceptibility might be due to the differences in the cell wall composition of Gram positive and Gram negative bacteria (Grosvenor *et al* 1995). *B. asiatica* showing remarkable antifungal activity (85%) against *A. flavus with* the MIC value 0.31 μg/ml. Percentage of crude alkaloid (berberine) was also estimated in stem of different species and it was found that it varied from species to species i.e. maximum in stem of *B. lycium* (Fig. 1). Based on these results it is possible to conclude that the hydro alcoholic extract and alkaloid (berberine) has stronger and broader spectrum against bacterial strains as compared to fungal strains.

Conclusion

The result obtained in the present studies authenticates and support the use of these plants in folklore medicine for treatment of various infectious diseases caused by the bacterial pathogens. The potential antibacterial activity of crude extract of selected *Berberis* species is mainly due to presence of major alkaloid berberine. These findings will stimulate the search for novel natural products as antibacterial/antifungal agents by the pharmaceutical industries and also supports usage of stem parts as substitute to root of these species to industries.

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