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Phytochemical Analysis, Antimicrobial and Antioxidant Activity of Crude Extracts from Selected Medicinal Plants

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ABSTRACT

Background: The search for potential natural bioactive compounds has been carried out for a long time. Researchers working in the field of natural product chemistry are starting to realize the importance of ethnomedicinal knowledge.

Methodology: The aim of this study was to carry out preliminary analysis of different plants to understand their therapeutic potential.To this end, methanolic extracts were prepared from four different plant specimens. Antimicrobial, antioxidant and cytotoxicity assays were carried out at different concentrations of the extracts.

Results: The highest percentage yield by methanol extraction was achieved from *Sapindus murokosii*. Phytochemical analysis detected presence of different types of secondary metabolites present in these species. Among the plants selected, *Ficus religiosa* showed the highest quantity of phenolics and flavonoids content along with lowest IC_{50} and LC_{50} for anti-oxidant activity and cytotoxicity assay respectively.

Conclusion: The results obtained leads to the conclusion that the selected plants might be possessing strong therapeutics which can be further analyzed for isolation and identification of bioactive compounds.

INTRODUCTION

Nepal, with its rich natural resources, has been successful to certain extent in utilizing its ethnomedicinal knowledge for various ailments. People in the rural area still rely on herbal medicine. This is mainly attributed to lack of access and economic insecurity to modern drugs (1). The country claims over 2.2% of biological wealth of world's natural flowering plants. Nepal ranks 25th in biodiversity richness globally and is 9th richest Asia in terms of flowering plant (2). The search for novel drugs is accelerating, with focus now shifting towards the ethnomedicinal knowledge as the drugs from natural sources has been known to possess little side effect when compared to the synthetic one.

Relative safety of herbal medicine and emergence of drug resistant pathogens has made it imperative to explore novel

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areas for natural drug discovery. Phytochemicals (phenol, flavonoid, tannin, alkaloid etc.) are the naturally occurring bioactive chemical compounds found in different parts of the plants like roots, stem, leaves etc. (3). Phytochemical screening is one of the techniques to identify new sources of therapeutically and industrially important compounds like alkaloids, flavonoids, phenols, steroids, tannins, saponins etc. present in the plant extracts (4).

MATERIALS AND METHODS

Collection of Plant Specimens

The plant materials were collected under the project "Bioprospecting on Bioresources" (Nepal project) supported by Korean Research Institute for Bioscience and Biotechnology (KRIBB), Central Department of Biotechnology, Tribhuvan Univeresity and Ethnobotanical Society of Nepal (ESON). The collected specimens were identified and verified from Central Department of Botany, Tribhuvan University, Nepal (Table 1).

Table 1 Description of plant species under study including plant parts used for the study along with their site of location and life forms of the plants

Sample Code	Scientific Name	Life Form	Plant parts Used	Collection Site	GPS Coordinates	Elevation (meters)
BT001	*Ficus religiosa L.*	Tree	Bark	Narayansthan-7, Atherikhola, Baglung	N 28° 12.560', E 83° 38.752'	975
BT002	*Lygodium japonicum* (Thunb.) Swartz	Climber	Whole plant	Phulkharka-3 Dhading	N 28°03.840', E 84° 55.225'	1375
BT003	*Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis	Shrub	Leaf, Stem	Phulkharka-3 Dhading	N 28° 04.358', E 84° 55.172'	1670
BT004	*Sapindus murokosii* Gaertner	Tree	Leaf	Vetenary, Dhangadi	N 28° 42' 10.5", E 80° 35'00.1"	154

Preparation of the plant material

The collected plant materials were air/shade dried at 32-35°C for 6 days to remove all moisture. The dried plant material was powdered with the help of grinder, and the fine powder was collected on sterile and dry polyethylene bag for extraction. About 100 g of fine powder of each plant sample was taken separately and dissolved in 750 ml of 100 % methanol and left to percolate for 24 hrs. After percolation, these samples were subjected to ultra-sonication for 3 days (2 hrs each day). The samples were then filtered to remove the residual content and the methanolic extract was reduced using Rotatory Evaporator. Thus, obtained solid mass was weighed carefully to identify gram of extract per 100 g of the plant powder. The extracts were kept at 40°C until further analysis. A 100 mg/ml stock of each plant extract was prepared and used for antimicrobial tests, antioxidant activity, quantification of the total phenol and total flavonoids.

Qualitative phytochemical analysis

The methanolic extracts were used to screen for the presence of various secondary metabolites **(5).**

Quantitative phytochemical analysis

Total polyphenol content

The total polyphenol content in crude extract was determined using the Folin–Ciocalteu phenol reagent (6). Gallic acid was used as a standard.

Total flavonoid content

The total flavonoid content in the plant extract was estimated using the Aluminium chloride (AlCl3) colorimetric method (7). The tests were performed in triplicates with Quercetin as standard.

Antioxidant activity

The antioxidant activity of crude methanolic extracts and standard (Ascorbic acid) was assessed on the basis of the radical scavenging effect of the stable 1, 1- diphenyl-2 picryhydrazyl (DPPH) - free radical activity. The assay was performed with varying concentration of plant extracts (10-150 µg/ml) with ascorbic acid (1-200 µg/ml) used for standardization (8).

Antibacterial activity

The antibacterial activity of methanol extracts of different plant species was tested against ATCC cultures of *Staphylococcus aureus* (25923), *Escherichia coli* (25922) and Methicillin Resistant *Staphylococcus aureus*. The test organisms were cultured at 37°C and their turbidity was adjusted to 0.5 McFarland standards. The cultures were then plated onto the MHA plates to perform antimicrobial assay by modified agar well diffusion method. Antibiotic ampicillin was taken as a positive control and methanol was taken as a negative control. Different concentrations of plant extract (100 mg/ml, 50mg/ml, 25mg/ml, 12.5 mg/ml and 6.25 mg/ml) were tested against bacterial strains. Twenty microliters were dispensed from each of the concentration into the wells. The zone of the inhibition was measured after overnight incubation.

Antifungal activity

Two fungal strains S. cerevisae and Pichia sp. were tested using fluconazole as standard drug against different concentrations of plant extracts (same as aforementioned concentrations). The assay was done in the PDA plates swabbed with respective fungal culture and the zone of the inhibition was measured after 24 hrs.

Brine shrimp lethality test

Brine Shrimp toxicity assay was employed to find out potential toxicity of the plant extracts (9). The toxicity was carried out in a 24 well plates. 1 g of brine shrimp (Artemis salina) eggs were allowed to hatch in one liter of artificial sea water prepared in laboratory. The temperature condition was maintained at 22-28oC with continuous aeration for 48 hrs. Soon after hatching, the larvae were harvested using glass pipette into small vial. Each of the larvae was picked from the vial and placed into 24 well plates. A total of ten larvae were placed into each well. Then after the crude plant extracts were prepared at three different concentrations of 1000 ppm, 100 ppm and 10 ppm and poured into each wells. The plate was then incubated at 22-28°C for 24 hrs. Potassium dichromate (1 mg/ml) was used as positive control while methanol was used as solvent control. Salt water was taken as negative control. After incubation, the viability of the larvae was observed with naked eyes under lamp light and the number of live larvae was counted.

Statistical analysis

All the experiments were performed in triplicates for each sample and the values were reported as mean ± SEM. The obtained data were also subjected to the analysis of variance and mean values were compared. All the statistical analysis was done using Graph pad Prism5 and Excel software.

RESULTS

Percentage yield of plant extracts

All the plant samples collected were subjected to methanol extraction. The total amount of methanol plant extract isolated from 100 g of the finely powdered whole plant material is shown in Fig. 1. The highest and lowest yield of extract was found in Sapindus murokosii Gaertner and Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis respectively.

Qualitative test for secondary metabolites

BT001 and BT004 were positive for all of the tests except Copper Acetate Test indicating absence of terpenoids.

BT002 was negative for Tannin as demonstrated by Gelatin Test while BT003 was devoid of Glycosides. (Table 2)

Table 2 Qualitative Phytochemical Analysis for Plant Specimens.

	Alkaloid	Phenol	Flavonoid	Glycoside	Terpenoid	Tannin	Sterol
Plant Extracts	**Mayer's Test**	**Ferric Chloride Test**	**Alkaline Reagent Test**	**Modified Brontrager's Test**	**Copper Acetate Test**	**Gelatin Test**	**Salkowski's Test**
BT001	+	+	+	+	-	+	+
BT002	+	+	+	+	+	-	+
BT003	+	+	+	-	+	+	+
BT004	+	+	+	+	-	+	+

(+) = present; (-) = absent

Quantitative test for Phenolics and Flavonoids

Highest phenolics and flavonoids content were observed for BT001 while lowest concentrations were observed for BT002. (Table 3)

Total DPPH antioxidant activity

Determination of IC_{50} values

The IC50 value for ascorbic acid was found to be 6.75±2.13 µg/ml. In case of extracts the highest and lowest IC50 values were observed in Sapindus murokosii Gaertner (91.83±.089 µg/ml) and Ficus religiosa L. (13.87±0.53 µg/ml). The species with lower IC50 are considered as better antioxidants. So, Ficus religiosa L. has the best antioxidant activity among the other plant species. The IC50 value of the other extracts was observed in between these two extremes. (Table 3)

Brine shrimp lethality test

The presence of toxic compounds from the extracts was analyzed using Brine Shrimp. BT004 had the highest LC50 value while BT001 had the lowest amongst the tested plant extracts. (Table 3)

Table 3 Total phenolic and flavonoid content, IC_{50} value for Anti-oxidant assay and LC_{50} value for cytotoxicity assay.

Code	Total Phenolic content mg GA equivalent/g of extract	Total Flavonoid content mg QE equivalent/g dry weight	Anti – oxidant IC_{50} values (µg/ml)	Cytotoxicity Assay (LC_{50} ppm±SEM)
BT001	136.84±1.24	57.49±0.60	13.87±0.53	4.26±0.00
BT002	35.95±4.65	24.38±0.50	43.81±1.01	69.40±0.00
BT003	40.11±2.44	50.01±0.20	84.77±1.74	17.81±0.00
BT004	41.22±2.68	34.30±4.45	91.83±.089	561.15±0.00

All the results are expressed as mean ± SEM and all the tests have been performed in triplicates.

Antibacterial assay

Zone of inhibition could be observed against E. coli from BT004 only while BT002 and BT004 extracts showed relative antibacterial activity against gram positive bacterium S. au*reus* at a concentration of 100 mg/ml. MRSA displayed susceptibility to BT003 extracts at 100 mg/ml. (Table 4)

Antifungal assay

No substantial antifungal activity could be perceived from any of the plant extracts.

Table 4 Antimicrobial Activity of Plant Extracts

Code	Zone of Inhibition (mm)																															
	100 mg/ml					50 mg/ml					25 mg/ml					12.5 mg/ml					6.25 mg/ml					+ve Control					-ve Control				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A-	B	C	D	E	A	B	C	D	E
BT001	-	5	-	-	-	-	4	-	-	-	-	3	-	-	-	-	3	-	-	-	-	3	-	-	-	25	29	-	#	-	6	6	6	6	6
BT002	-	8	-	4	-	-	5	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	25	29	-	#	-	6	6	6	6	6
BT003	-	3	7	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25	29	-	#	-	6	6	6	6	6
BT004	9	7	-	4	-	6	7	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25	29	-	#	-	6	6	6	6	6

A – *Escherichia coli*; B – *Staphylococcus aureus*; C – Methicillin Resistant *Staphylococcus aureus*; D – *Saccharomyces cerevisiae*; E – *Pischia* spp. +ve Control for A, B and C: Ampicillin; +ve Control for D and E: Fuconazole. –ve Control: Methanol.

DISCUSSION

Potent bioactive compounds contained in plants can elicit both poisonous and medicinal value. Whether it causes a beneficial or an adverse result may depend on the amount eaten and the context of intake. Alkaloids, glycosides, flavonoids, saponins, tannins, steroids are important bioactive constituents present in plants. Alkaloids, in particular, are toxic against micro-organisms (anti-bacterial and anti-fungal), and insects, so it ensures protection to humans and animal health (10).

Lygodium japonicum (Thunb.) occupies a broad scope for therapeutic potential. It has been used as a diuretic, expectorant as well as to treat colds, inflammation, snakebites, and physical ill-manisfestations (11). The plant possesses strong antioxidant properties along with antimicrobial and hepatoprotective ability (12). Our study also showed the antimicrobial potential of Lygopodium japnicum against the tested gram positive bacteria while cytotoxicity assay indicated the presence of potent toxic compounds.

The extracts of Randia tetrasperma (Roxb.) Benth. & Hook. F. ex Brandis was the only one to show activity against MRSA indicating it as a possible source of antimicrobial compound againt the drug resistant bacterium. The fruits of the plant have been found to be irritating and emetic and used as fish poison. Pulp of fruit has been used for dysentery, anthelmintic, abortifacient treatment (13). The plant contains various phytoconstituents such as alkaloids, saponin, tannins, flavonoids, glycosides and phenols. The amount of flavonoids we obtained was similar to previous studies while the polyphenol quantity varied which may be due to difference in the solvent used as well as various environmental parameters and genetic predispositions (14). Sapindus mukorossi Gaertner was the only plant whose extract showed positive antibacterial activity against both E. coli and S. aureus. In northern India, its fruit and seeds

of were regarded as a cure for epilepsy. A decoction of the fruit was used as an expectorant while seeds were used in China to stop dental caries (15). Leaves were used in baths to relieve joint pain and the roots were used in the treatment of gout and rheumatism. Other pharmacological effects of Sapindus mukorossi (Gaertn) were antimicrobial, insecticidal, spermicidal, anti-trichomonas, anti-tumor, hepatoprotective, anxiolytic, molluscicidal, anti-inflammatory and piscicidal (16).

Ficus religiosa L. leaf juice has been used for the treatment of asthma, cough, sexual disorders, diarrhoea, haematuria, earache and toothache, migrane, eye troubles, gastric problems and scabies (17). The plant has been used to cure different diseases like dysentery, diarrhea, nervous disorders, tonic, astringent and many more. Each part of the plant has its own significance and was used separately to cure distinct disorders. The plant possesses many pharmacological properties due to the presence of the phytochemicals like tannins, saponins, flavonoids, steroids, terpenoids and cardiac glycosides. According to Ayurvedic system of medicine, F.religiosa L. was well known to be useful in diabetes (18). These studies also correlates with our data as the plant showed the lowest IC50 value for anti – oxidant activity that shows it's potential for utilization in various radical mediated ailments.

The selected plants showed promising potential for further research as a probable source of natural therapeutic agents. The combined research findings of this paper as well as those done by other researchers show that plants can be a great source of bioactive compounds whose side effects are sidelined by their benefits. However, due to changing global climate many of these natural treasures are on the brink of extinction whose long term harmful effects outweighs the current superficial profits we are making. An in depth research needs to be carried out to understand the medicinal properties of these plant resources such that humans and animals in general can profit from the nature.

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