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Biological Activities of Three Different Medicinal Plants from Himalayan Region of Nepal

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Abstract

Crude chloroform/methanol and methanol extracts of leaves and twigs of three medicinal plants collected from different altitudes of Nepal, *Rhododendron lepidotum, Hippophae rhamnoids* and *Cornus capitata* were examined for their antibacterial, cytotoxicity and antiproliferative activities. The antimicrobial activity shown by methanol extracts of *R. lepidotum* and *C. capitata* against *Staphylococcus aureus* was comparable with that of streptomycin antibiotic whereas the activity of the extract of *H. rhamnoids* was very low. Cytoxicity (LC_{50}) values ranged from 46.4 to 111.6 µg/ml with *R. lepidotum* and 129.4 to 464.1 µg/ml with *C. capitata* indicating high cytoxic property in the extract of *R. lepidotum* and medium in *C. capitata*. Similarly, anti-proliferative activity of *R. lepidotum* against HeLa cell line was relatively high activity in comparison to *C. capitata* and *H. rhamnoids.* Phytochemical screening revealed the presence of flavonoids, tannins, terpenoids, steroids, glycosides and coumarins, which could be responsible for the bioactivities shown by these plants.

Key words: antibacterial, antiproliferative, microtitration, HeLa cells

Introduction

The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and disease preventive properties. These phytochemicals, often-secondary metabolites present in smaller quantities in higher plants, include alkaloids, steroids, flavonoids, terpenoids, tannins, and many others. Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been assayed so far. Current research has focused on phytochemical investigation of higher plants which have ethnobotanical information associated with them. The phytochemicals isolated are then screened for different types of biological activity (Harborne 1998).

In this part of the research we have selected three medicinally important plants *Rhododendron lepidotum, Hippophae rhamnoids* and *Cornus capitata* which are abundantly found in Nepal and are used by local people as medicines and insecticides purpose. The phytochemical and biological screening was carried out on all these plants that showed high potency of their importance.

R. lepidotum (family Ericaceae) is a highly variable shrub, up to 2m tall with white, yellow, pink or purple color flowers. Leaves are elliptic with upper surface dark green and lower surface large brownish scales. Distribution of this plant is ranging from 2400 to 4900 m altitudes. Traditionally, leaf and flower powders of *R. lepidotum* are used as a snuff in headache (Khan *et al*. 2008). Some species of the genus *Rhododendron* have a long history of use as folk medicine in various countries. Essential oils, ursolic and oleanolic acids,

phenolcarboxylic acids, coumarins, flavonoids and other compounds extracted from the aerial parts of *R. adamsii* have been observed among the biologically active compounds.

H. rhamnoids (Sea buckthorn), a member of the family Elaeagnaceae, is a deciduous spiny shrub or small tree between 2-4m high, widely distributed throughout the temperate zone of Asia and Europe (Lu 1992, Li & Schroeder 1996). The plant is hard, drought and usually cold tolerant, useful for the land reclamation and farmstead protection (Zhang 2000). Plants bear foliage from April to November, flowers in June - July and red, yellow and orange colored berries from mid August to October. Sea buckthorn has been shown to have a potent antioxidant activity, mainly attributed to its flavonoids and vitamin C content (Rosche 2004). Extract of this plant is used to protect the bone marrow from damage due to radiation, and faster recovery of bone marrow cells (Agrawal & Goel 2002). The branches and leaves contain bioactive substances, which are used to produce oil that is quite distinct from the oil produced from the fruit and is used to treat skin diseases, such as atopic dermatitis (Yang 1999).

C. capitata is an evergreen shrubby or a soft-textured small tree, flowering from June to July and the seeds ripen from September to November. The bark is a source of tannin that is used as an astringent. In China, its bark is used medically as folk remedies to treat arthritis and injuries (Li & Schroeder 1996). Native from the Himalayas to Indochina, this species was introduced from Nepal to England in 1825 (Nathaniel). It varies considerably in hardiness but cannot tolerate regions with prolonged freezes in winter. It is notably thirsty, so it may require a sound irrigation system.

Methodology

Plant materials

The plant materials, leaves and twigs of *R. lepidotum* and twigs of *H. rhamnoids* were collected from Godha Tabala of Langtang valley and bark of *C. capitata* from Syabru Besi, altitude varying from 1700 to 3800 m, Rasuwa district, Nepal, during November 2008. A senior botanist helped to identify these plants.

Plant extraction

The plant materials were dried, chopped, and grinded and extracted for several hours with cold chloroform/ methanol (1:1), and methanol alone. Organic solvents were removed separately from each of these extracts under reduced pressure using rota vapour. Percentage yield of each extract was calculated, by the following formula.

$$\% \text{ Yield} = \frac{\text{Weight of extract obtained}}{\text{Total weight of the samples loaded}} \text{ X } 100$$

Phytochemical screening

Phytochemical screening of the crude chloroform/ methanol (1:1) and methanol extract of the plants was carried out using standard phytochemical methods (Harborne 1998 Aguinaldo *et al*. 2005).

Brine shrimp lethality assay

The brine shrimp lethality assay (BSLA) was carried out based on the protocol of Krishnaraju *et al*. (2005) with slight modifications. Brine shrimp eggs, 50 mg were sprinkled in a beaker with 300 ml of artificial sea water and incubated at 32–35 ^{o}C for 24h. Stock solution of crude extract was prepared by dissolving 100 mg of extract in 25 ml of dimethyl sulphoxide (DMSO). The stock solution was diluted to 10, 100 and 1000 ppm. After 24 h of incubation, the nauplii were transferred with a pipette and 10 brine shrimps were taken to test in triplicate for each concentration. Each set of brine shrimps containing tubes were incubated at 32–35°C for 24h. Survivors were counted and the percentage mortality at each vial was determined using the equation:

$$\% \text{ Mortality} = \frac{\text{No. of dead neuplli}}{\text{Initial no. of live neupalli}} \text{ X } 100$$

Antimicrobial screening

Zone of inhibition method

The crude extracts of *R. lepidatum, H. rhamnoids* and *C. capitata* were tested for their antibacterial property against three pathogenic bacteria: *Staphylococcus aureus, Escherichia coli* and *Salmonella paratyphii.* These microbes were collected from the Kathmandu University Teaching Hospital (KUTH), Dhulikhel. The zone of inhibition was measured by filter disc method (6mm diameter) using nutrient agar. The effects of methnol extracts of all the samples on microorganisms were then studied and their zone of inhibition was compared taking Streptomycin antibiotic as standard.

Microtitration method

Methanol extracts of *R. lepidotum, H. rhamnoides* and *C. capitata* were tested for their property to inhibit the growth pattern of pathogenic bacteria. Streptomycin solution was prepared in phosphate buffer saline (PBS) (Sigma, US) and filtered through 0.45µm syringe filter and stored at -20°C. Standardized broth 500µl was transferred into 5ml of freshly prepared sterile Brain Heart Infusion Broth (BHIB) which was then dispensed into 96 well plates (Tarsons, India) along with filter sterilized solution of the plants with final volume of 200µl in each well. Absorbance was taken at 630nm using BioElisa Reader and data were retrieved using KC Junior® software for 10 h at an interval of 1 h. Freshly prepared sterile broth was also dispensed in some wells as a control.

In vitro growth inhibition assay

Tetrazolium dye uptake method was carried out as described by Alley *et al.* (1988) and Mosmann (1983). Briefly, HeLa cell line (Everest Biotech, Nepal) was cultured in RPMI-1640 (HiMedia, India) supplemented with 10% FBS (GIBCO, Invitrogene Corporation, UK) and 1% antibiotics (HiMedia, India) in T-75 cell culture flasks (Greiner bio-one, Germany). Cells were harvested by trypsinized followed by cell counting by trypan blue (HiMedia, India) and then diluted to 10^5 cells/ml. 100µl of diluted cell was dispensed in each well of sterile 96 well plate (Tarsons, India) so as to maintain 10,000 cells/well and incubated for 12h at 5% CO_2 incubator. On the next day, the used media from the wells were removed and different concentrations of two pure fractions, obtained from methanol extract, prepared on fresh sterile media were added on the wells and fresh media was added on control wells and incubated for 48h at 5% CO_2 incubator. Sterile stock solution of MTT dye (HiMedia, India) prepared on PBS solution (Sigma, USA) at 5mg/ml was diluted at a ratio of 1:10 on fresh sterile media and 100µl of this solution was added on each well including one set of well with MTT dye but no cells to balance the absorbance value and was incubated for 3.5h in dark condition. The media with MTT solution was taken out from the well and 100µl of Sorensen's glycine buffer was added on each well and incubated for 30min to dissolve formazan crystal. Absorbance was taken at 490nm with reference wavelength 630nm on BioElisa® reader, and the data were retrieved using KC Junior®. A graph was plotted as absorbance versus concentration of extracts/ subfractions.

Results and Discussion

Extraction yields of the plants extract

Significant amount of yields were obtained from the plants extracted in methanol and a mixture of chloroform/methanol (Table 1). Yield percentage of methanol extract was higher compared to chloroform/ methanol indicating the presence of more polar or compounds with high molecular weight.

Table1. Extraction values of three plants in different solvents

Plants	Solvents	Extract yield (%)
Rhododendron lepidotum	Chloroform/methanol(1:1)	4.397
	Methanol	6.070
Hippophae rhamnoids	Chloroform/methanol(1:1)	4.647
	Methanol	7.02
Cornus capitata	Chloroform/methanol(1:1)	3.05
	Methanol	10.90

Phytochemical screening

The result of phytochemical screening on the crude chloroform/methanol and methanol extracts of the three plants revealed the presence of secondary metabolites (Table 2). No alkaloids were detected in all of the extracts whereas flavonoids and reducing sugars were observed in all cases indicating their antioxidant, anti-cancer properties that protect them from invading bacteria, fungi or yeast.

Brine shrimp lethality assay

The brine shrimp lethality assay has been used routinely in the crude extracts of the plants to assess the toxicity towards brine shrimp, which provides an indication of possible cytotoxic properties of the test materials (Peteros & Yu 2010). The variation in BSLA results (Table 3) may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins,

flavonoids, triterpenoids, or coumarins) present in the crude extracts. Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 250 ppm or ìg/ml) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds (Riser & Cortes 1996).

Table 2. Result of the Phytochemicals present in different samples at different solvent systems.

Phytochemicals	*R. lepidotum*		*H. rhamnoid*		*C. capitata*	
	Chl:Met	Methanol	Chl:Met	Methanol	Chl:Met	Methanol
Alkaloids	-	-	-	-	-	-
Glycosides	+	+	+	+	-	-
Terpenoids	+	+	+	+	-	+
Steroids	+	+	+	+	-	-
Flavonoids	+	+	+	+	+	+
Reducing sugars	+	+	+	+	+	+
Coumarins	+	+	-	-	+	+
Tannins	-	-	+	+	-	+
Saponins	+	+	-	-	+	+

Chl: Chloroform, Met: Methanol. (+) and (-) sign indicates the presence and absence of phytochemicals respectively.

Table 3. Results of brine shrimp lethality assay on crude methanol extracts

Samples	Solvent	LC_{50} calculation						Death	% Died	LC_{50} (ppm)
		Extract con. (ppm)	Log (con)	Initial nauplii	Nauplii survival 1st	Nauplii survival 2nd	Mean survival			
R. lepidotum	MeOH	10	1	10	9	10	9.5	0.5	5	**111.59**
	100	2	10	3	4	3.5	6.5	65		
	1000	3	10	2	3	2.5	7.5	75		
	Con.		10	10	10	10	0	0		
H.rhamnoides	MeOH	10	1	10	10	10	10	0	0	**1584.89**
	100	2	10	9	9	9	1	10		
	1000	3	10	5	5	5	5	50		
	Con.		10	10	10	10	0	0		
C. capitata	MeOH	10	1	10	9	9	9	1	10	**129.15**
	100	2	10	7	8	7.5	2.5	25		
	1000	3	10	0	0	0	10	100		
	Con.		10	10	10	10	0	0		

Antibacterial properties

The zone of inhibition shown by methanol extracts of *R. lepidotum* against *S. aureus* is comparable to that of streptomycin antibiotic but is moderate against *S. paratyphii* and *E. coli*. Similar type of pattern was also observed for *H. rhamnoids* (Table 4, Fig. 1a-e). The sub-fractions obtained from *R. lepidotum* did not show significant zone of inhibition against aforementioned bacteria proving their zero bacteriocidal property but alteration of growth pattern of *S. aureus* by sub-fractions B, D, E, F (Fig 2a-d) must be due to their bacteriostatic property. Among these fractions, water insoluble fraction B shows relatively high bacteriostatic property compared with other that may be due to presence of non-polar or medium polar compounds like terpenoids, flavonoids and coumarins.

Table 4. Zone of inhibitions of methanol extracts on different pathogenic microbes

Antimicrobial activity test

Bacterial strains	Plant extracts	Antibiotic		Solvent
		Streptomycin (0.1 mg/ml) (mm)	Streptomycin (0.01 mg/ml) (mm)	Methanol (mm)
S. aureus				
	R. lepidotum	14	6	13
	H. rhamnoides	12	-	11
	C. capitata	12	-	14
S. paratyphae				
	R. lepidotum	8	-	6
	H. rhamnoides	9	-	10
	C. capitata	-	-	12
E. coli				
	R. lepidotum	12	7	7
	H. rhamnoides	13	7	7
	C. capitata	15	-	8

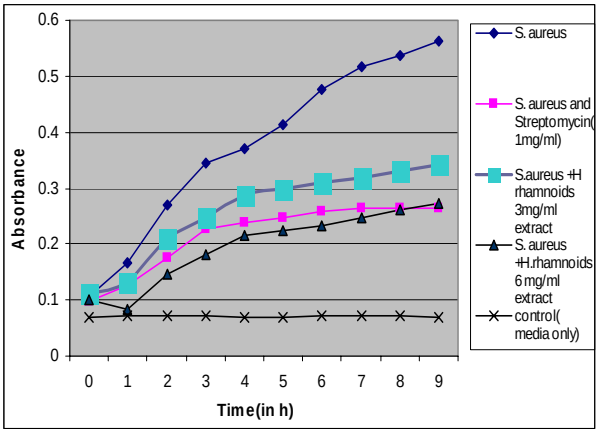

Fig. 1a. Effect of H. *rhamnoides* methanol extract on *S. aureus*

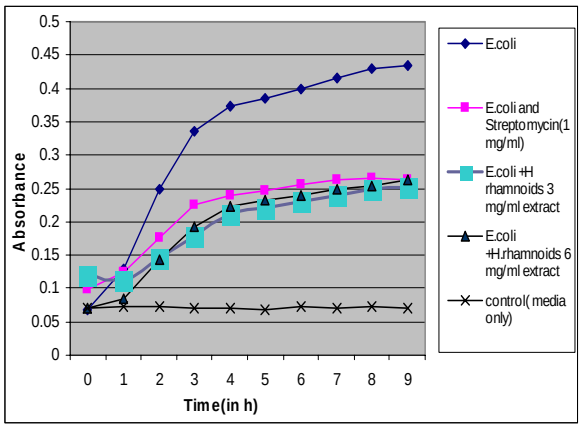

Fig. 1b. Effect of *H. rhamnoides* methanol extract on *E. coli*

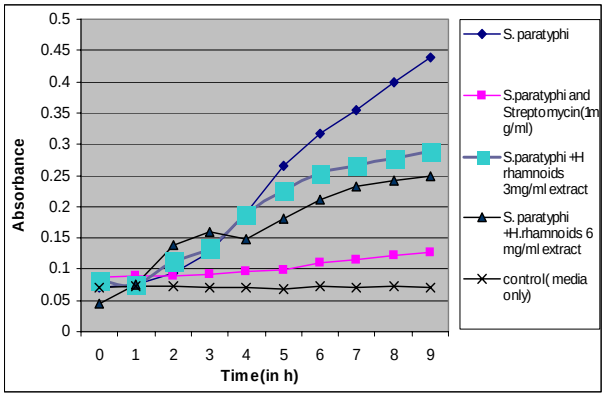

Fig. 1c. Effect of

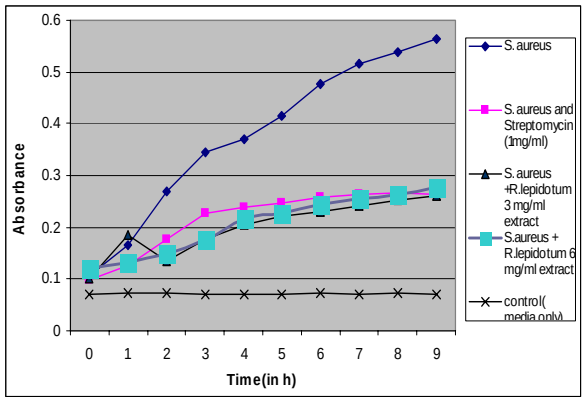

Fig.1d. Effectt of *R. lepidotum* methanol extract on *S. aureus*

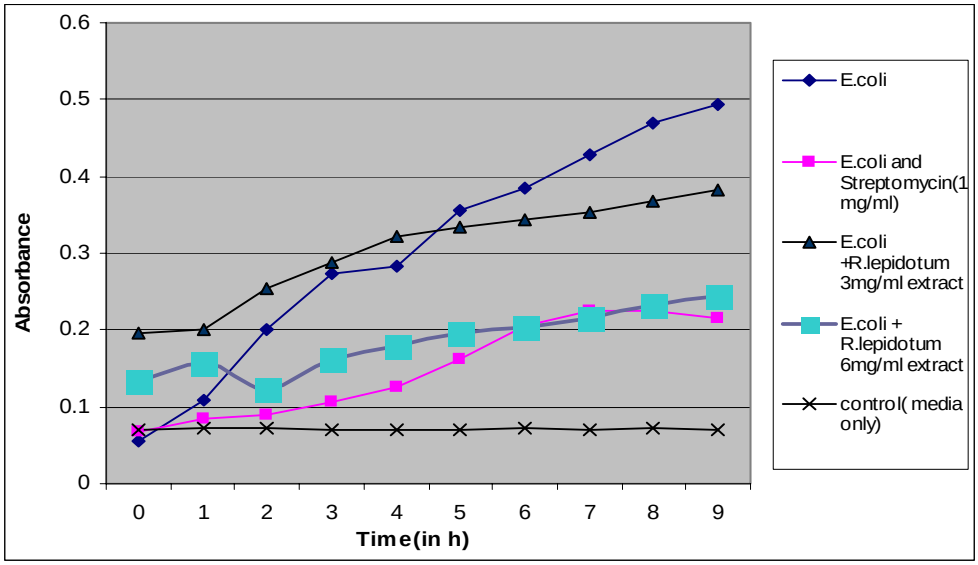

Fig. 1e. Effect of *R. lepidotum* methanol extract on *E. coli*

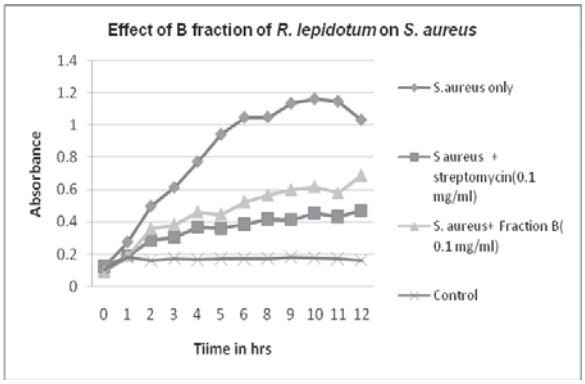

Fig. 2a. Effect of B fraction of *R. lepidotum* on *S. aureus*

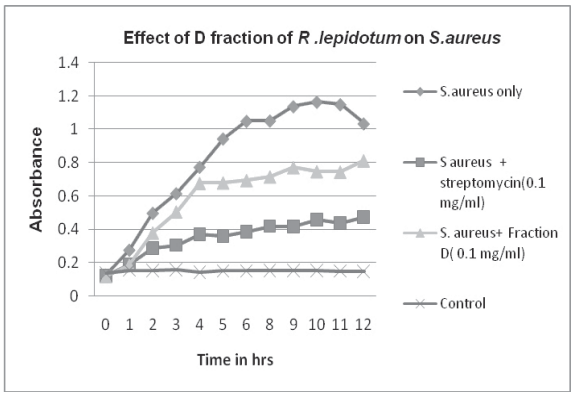

Fig. 2b. Effect of D fraction of *R. lepidorum* on *S.aureus*

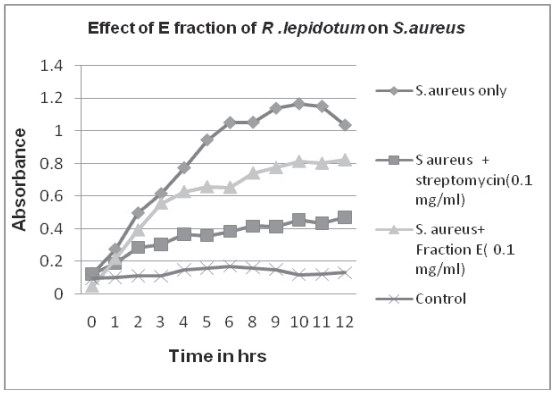

Fig. 2c. Effect of E fraction of *R. lepidotum* on *S.aureus*

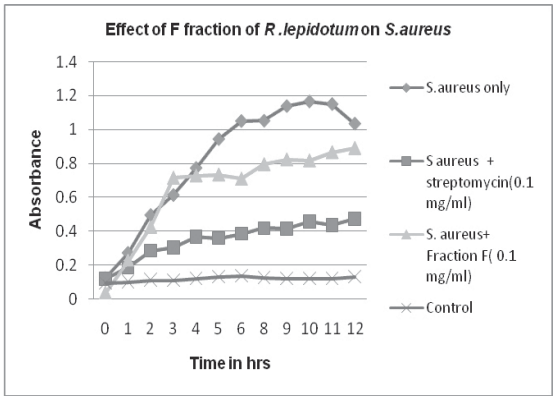

Fig. 2d. Effect of F fraction of *R. lepidotum* on *S. aureus*

In vitro growth assay on HeLa cell line

In-vitro antiproliferative activity of methanol extract of *R. lepidatum* is high in HeLa cell line. *R. lepidotum* methanol extract showed relatively high killing potential compared to *H. rhamnoids* (Fig 3). *H. rhamnoids* showed minimal activity at low concetration but on increasing concentration, killing rate sharply increased. Similar pattern was observed in BSLA test (Table 3) for *H. rhamnoids*, indicating direct correlation between BSLA and antiproliferative activity of methanol extract against HeLa cell line. The result indicated that *R. lepidotum* has high potency of antibacterial and antiproliferative activity whereas *H. rhamnoids*, the fruits of which is edible, has nothing or very less antibacterial and hence the antiproliferative activity.

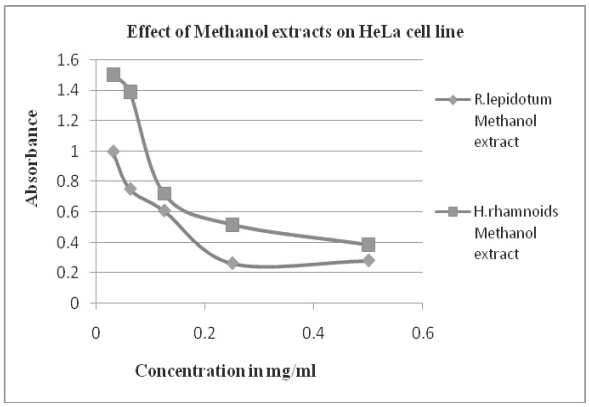

Fig. 3. Effect of methanol extracts on HeLa cell line

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