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RESEARCH ARTICLE

Antioxidant and Antimicrobial Properties ofLeaves of *Lyonia ovalifolia*(Wallich)

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ABSTRACT

Lyonia ovaliafolia (Wallich) has been used in a folk medicine for the treatment of wounds, cuts, burns, scabies, etc. by different local communities of Nepal. Antimicrobial and antioxidant properties of *n*-hexane, chloroform, ethyl acetate, and ethanol extracts of leaves were evaluated. For assessing the antimicrobial property, theagar well diffusion method was used and for antioxidant property, the ferric reducing antioxidant power (FRAP) assay was used. The major classes of phytochemicals found in the chloroform fraction of leaves were alkaloids and this fraction was found to be most effective against all the tested bacteriasviz: *Staphylococcus aureus, Escherichia coli, Salmonella typhii,* and *Pseudomonas aeruginosa.* But *P. aeruginosa*is the only tested organism which is inhibited by all the fractions of plant used in both the concentrations (250 mg/ml and 500 mg/ml). Similarly, on antioxidant assayIC_{50} was found strong in ethyl acetate fraction(8.25 µg/ml) followed bychloroform (9.81 µg/ml), ethanol(15.49 µg/ml) and n-hexane(47.42 µg/ml) as compared to standard BHA with IC_{50} of 30.31 (µg/ml). Thus,this study revealed that *L. ovalifolia* (Wallich) has a potential antimicrobial and antioxidant property which scientifically justifies itsethno medicinal use.

Key words: *Lyonia ovaliafolia*, antimicrobial and antioxidant properties, chloroform fraction, ethno medicinal.

INTRODUCTION

Plants possess various secondary metabolites eliciting variety of biological activities including antimicrobial and antioxidant activity[1-3]. Ancient people used plants as a medicine to treatvarious infections, there is great interest in the plants in order to derive the new therapeutic agents[4].Among various infections, bacterial and fungal infections falls under the most common infection especially in developing countries like Nepal[5]. Increment of scope in antimicrobials from natural sources is becauseof increasing rate of multidrug resistant strains of microorganisms as well as newly developed strains with reduced susceptibility to available antimicrobial agents[6].

Reactive Oxygen Species (ROS) are highly reactive unstable ions or very small molecules formed inside living organisms as a result of oxidative stress, normal and pathological cell metabolic processes and exogenous pollutants(UV light, γ- radiation, cigarette smoke, etc.)which include hydrogen peroxide (H_2O_2), free radicals such as hydroxyl radical ($^{\bullet}OH$), superoxide anion (O^{--}), alkoxy and proxy radicals ($RO^{\bullet}$ and $ROO^{\bullet}$), etc. ROS are very harmful as they react with various cellular components including DNA, proteins, lipids, fatty acids which primarily results in lipid peroxidation. Lipid peroxidation has major role in progression of various life threatening conditions like cancer, inflammation, cardio vascular diseases, infection, etc.[7]. Though synthetic antioxidant compounds such as Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT) etc. are available, due to their carcinogenicity and other side effects, these compounds have restricted use. So, the natural plant compounds having antioxidant property which can scavenge these free radicals and thus prevent action of ROS to damage cells are found to have great potency to ameliorate the diseases[8]. Nepal has about 7,000 species of flowering plants,264 of which are endemic. The number of

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medicinal and aromatic plants used in the country has reached about 1,463 species[9]. More than 75% of the total population relies on traditional medicine and thus it has been an integral part of the national health care system[10]. In spite of tremendous medicinal use of plants bydifferent local communities of Nepal, only limited research have been carried out to prove its use in scientific basis[11-13].

Lyonia ovalifolia (Wallich) is a plant of ethno medicinal relevance used for the treatment of wounds, cuts, burns, scabies, etc. by different local communities of Nepal[16,17].Some of the preliminary studies have suggested it's in vitro antibacterial activities[18,19].However, there is lack of credible scientific data to prove its medicinal potential. Thus, this study aims to perform phytochemical screening, and antimicrobial and antioxidant activity activities of fractionated extracts of leaves of *L. ovalifolia* (Wallich).

MATERIALS AND METHODS

Plant material

Leaves of *L. ovalifolia* were collected from Roshi (Banepa)at the height of 1439 m in 17thJuly, 2013 and a voucher specimen (Herbarium No.CISTG301/2013) was taken to National Herbarium and Tissue Culture Laboratory, Lalitpur, Nepal for authentication and kept for future reference.Collected plant material wasSoxhlet extracted using ethanol for 12 to 18 hours (≤ 50°C). The extract hence obtained was concentrated under reduce pressure using rotary evaporator (≤ 60°C) and was successively fractionated by three different solvents viz. n-hexane, chloroform and ethyl acetate.Such fractions were concentrated to near dryness under reduce pressure using rotary vacuum evaporator and further dried in petri plates using water bath (≤60ºC). Lastly, the percentage yield of extracts was calculated and these crude extracts were stored in air tight container (inside refrigerator) for further studies.

Qualitative phytochemical screening

Each fractionated extract was subjected to qualitative test for alkaloids,saponin,tannin, flavonoids,terpenoids,phenols,proteinandaminoacids,phytosterols,carbohydrate and glycosidesusing standard colour reactions to identify various phytoconstituents as described in standard texts[20,21].

Antioxidant activity

Antioxidant activity was determined as Ferric reducing antioxidant power (FRAP) assay[22]. Each ml of five different concentration (1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml and 62.5 µg/ml) of extracts were mixed with 2.5ml of phosphate buffer (adjusted to pH 6.6) and 2.5 ml of potassiumferric cyanide(30mM). The mixture was incubated at 50°C for 20 minutes. After that, 2.5ml trichloroacetic acid (600mM) was added in each test tube. Finally, the mixture was centrifuged for 10mins at 3000 rpm. After centrifugation 2.5ml of supernatant was taken in which 2.5ml distill water and 0.5ml ferric chloride (6mM) was added. Then absorbance was measured at 700nm using UV spectrophotometer (Shimazu 1700). Similarly, activity of different concentration of standard BHA was taken as positive control and DMSO as a negative control after addition of same reagents as for tests. The method was triplicated to take mean absorbance value. The percentage reduction of ferric ion was calculated as follows:

$$\%\ \text{reduction by test} = \frac{A_c}{A_t} \times 100$$

$$\%\ \text{reduction by standard} = \frac{A_c}{A_s} \times 100$$

Where; A_t, A_s and A_c are absorbance of test, standard and control respectively.

Antimicrobial activity

The agar well diffusion assay was used to access antibacterial activity[23].All the antimicrobial studies were carried out in two different concentrations of extracts(500mg/ml and 250mg/ml).Stock solutions were prepared in DMSO for antibacterial activity, however chloroform was used to prepare stock solution for antifungal activity as DMSO itself possessed some antifungal activity[24].

Four species of bacteria, one gram positive, *Staphylococcus aureus* and three gram negative, *Escherichia coli, Salmonella typhii, Pseudomonas aeruginosa* and two species of fungi, *Candida albicans* and *Asperigillus niger* were collected from Institute of Medicine (IOM),Kathmandu, Nepal. Test bacteria were maintained on Nutrient Agar (NA) at 35±2ºC while fungi were maintained on Potato Dextrose Agar (PDA) at 25±2 ºC. The concentration of bacterialand fungal cells (24 hours old) in the suspension was adjusted to 1x10^{5}CFU/ml in Muller Hinton broth solution before streaking. Gentamycin (40mg/ml) for *S.*

aureus, E. coli and *S. typhii*and Ciprofloxacin (20mg/ml) were used as positive control and DMSO as a negative control.Similarly, Fluconazole (50mg/ml) was used as positive control and chloroform as a negative control for chloroform extract for antifungal activity. 50 µl of extracts of two different concentrations were transferred in the wells by micropipette. Afterovernight incubation for 18 hours at 35±2ºC (for bacteria) and 72 hour at 28±2°C (for fungi), the diameter of each zone as judged by the unaided eye was measured and recorded in mm.

RESULTS AND DISCUSSION

The percentage yield of the extracts was found to be highest in the ethanol 23.688 %, while other fractions were 0.431% inn-hexane, 2.621% in chloroform and 0.357 % ethyl acetate.

Findings of qualitative estimation of different phytochemicals revealed that the ethanol fraction was rich in the glycosides, phenol and tannin. Other fractions of the leaves possess different phytochemicals depending on the polarity of the solvents used (**Table 1**).

Table 1: Preliminary phytochemical analysis of fractionated extracts of leaves

S. No	Phytochemical	n-Hexane	Chloroform	Ethyl acetate	Ethanol
1	Alkaloids	-	++	-	+
2	Saponins	-	-	++	-
3	Tannins	-	-	-	++
4	Flavonoids	-	-	+	+
5	Terpenoids	++	-	-	-
6	Phenols	-	-	-	++
7	Protein and amino acids	-	-	-	-
8	Phytosterols	++	+	+	-
9	Carbohydrates	-	-	-	++
10	Glycosides	-	-	+	++

Note:"++": appreciable amount, "+": trace amount, and "-": completely absent

Result of assessment of antibacterial activity revealed variability in activity of different fractions (**Table 2**). Present finding shows that only the chloroform fraction is able to inhibit all the test bacteria in both the tested concentrations. However, a study done by Negi *et. al.* reported that chloroform fraction of leaves is ineffective to *E. coli*[18]. Negi *et. al.* used 100(mg/ml)of extract concentration which is not comparable with our concentrationsviz: 500 (mg/ml) and 250(mg/ml). Therefore, it is confirmed from our study that chloroform fraction of leaves this plant is effective on higher concentrations against *E.coli.* It gave maximum ZOI of 13.00±0.50 (mm) against *S. aureus*followed by ZOI of 10.00±0.00 (mm) with *P. aeruginosa*at 500(mg/ml). However, *P. aeruginosa* is only the test bacterial which was inhibited by all four tested fractions. Similar findings were also reported by Panthi *et. al.* showed that methanolic extract of young leaves and apical buds inhibited the growth of *P. aeruginosa*[19]. *n*-hexane fraction of leaves gave maximum ZOI of 10.50±0.86(mm) with *P. aureginosa* at 500 (mg/ml). Terpenoids are generally recognized as safe and have been found to inhibit the growth of microorganisms[25].As the n-hexane fraction of leaves were rich in terpenoids, the additive and synergistic effects of phytochemicals in extract might be responsible for their potent antimicrobial action against *P. aureginosa*.

Table 2: Antibacterial activity of fractionated extracts of Leaves as ZOI [mm]

Plant extracts		Bacteria			
Fractions	**Concentration**	***S. aureus***	***E. coli***	***S. typhi***	***P.aeruginosa***
n-hexane	E1	-	-	-	10.50±0.86
	E2	-	-	-	7.17±0.28
	Std.	13.83±1.25	14.00±0.50	17.33±0.28	21.50±0.50
Chloroform	E1	13.00±0.50	6.50±0.25	8.92±1.50	10.00±0.00
	E2	12.67±1.25	4.75±0.05	8.33±2.52	7.50±0.50
	Std.	16.42±0.95	13.83±0.28	16.83±1.75	21.50±0.50
Ethyl acetate	E1	-	-	-	5.33±0.57
	E2	-	-	-	2.67±0.28
	Std.	15.17±2.40	15.00±0.50	16.17±1.26	21.17±1.04
Ethanol	E1	-	-	-	5.00±0.00
	E2	-	-	-	2.50±0.00

	Std.	17.25±1.50	15.00±0.00	17.50±0.50	21.33±0.57

Results expressed as Mean ± SD of triplicated experiment and there was confluent growth in DMSO (negative control).

Note: E1: fractionated extract of 500 (mg/ml), E2: fractionated extract of 250 (mg/ml), Std: Gentamycin (40mg/ml) for *S.aureus, E.coli and S.typhii* and Ciprofloxacin (20mg/ml) for *P. aureginosa*, "-": no zone of inhibition, Bacteria, *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, S. typhi: Salmonellatyphi, P.aeruginosa: Pseudomonas aeruginosa*

Antifungal activity was tested for only the chloroform fraction which possessed promising antibacterial effects against two species of fungi, *Candida albicans and Aspergillus niger*. No measurable ZOI was observed with *A. niger* (which is not shown here). However, fractionated chloroform extract showeda ZOI of 6.06±0.05 (mm) at 500(mg/ml) and 3.00±0.57 (mm) at 250(mg/ml) against *C. albicans*(**Table 3**). This is the first study to elucidate the antifungal activity of leaves of *L. ovalifolia*(Wallich).

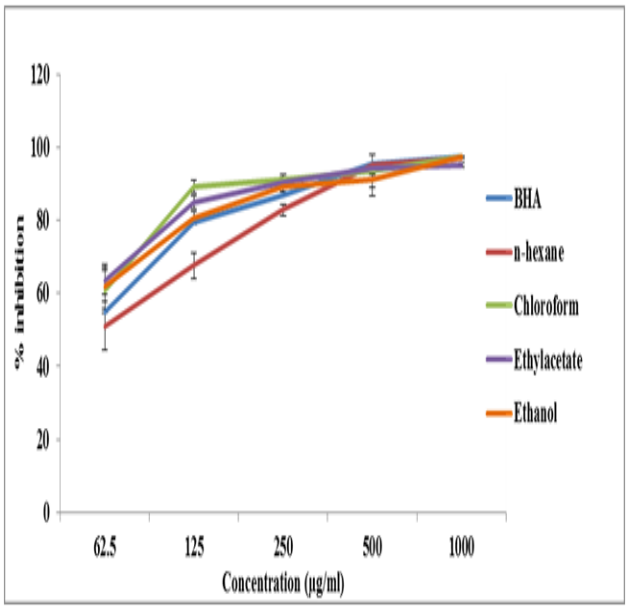
Table 3: Antifungal activity of fractionated chloroform extract of leaves against *Candida albicans*

Concentration	ZOI [mm]
E1	6.06±0.05
E2	3.00±0.57
Std.	11.33±0.60

Results expressed as Mean ± SD of triplicated experiment and there was confluent growth in chloroform (negative control)

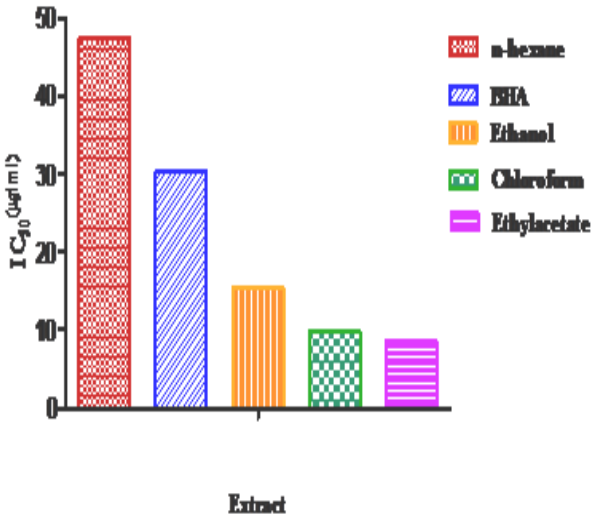
Note: E1: fractionated extract of 500 (mg/ml), E2: fractionated extract of 250(mg/ml), Std: Fluconazole (50mg/ml).

Figure 1: Percentage reduction of ferric ion of fractionated extract of leaves



Above line graph (**Figure 1**) represents the dose dependent reduction of ferric ion to ferrous ion by fractionated extracts and standard at various concentrations while bar graph (**Figure 2**) represents anti-oxidant activity as IC_{50}(µg/ml). This anti-oxidant activity was determined by calculating the ferric reduction ability power where higher absorbance of Prussian blue color indicates a higher ferric reducing power. Comparing IC_{50} of all fractionated leaves extracts, n-hexane being non polar solvent, its extracts was found to be least potent as it is expected to take steroidal compounds. IC_{50} was in the order of ethyl acetate(8.25 µg/ml), chloroform(9.81 µg/ml), ethanol(15.49 µg/ml) and n-hexane (47.42 µg/ml) as compared to Standard BHA with IC_{50} of 30.31 (µg/ml). Out of these, n-hexane fraction of leaf extract showed least reduction at 47.42(µg/ml) which was 64% effective as compared to standard. Plants containing polyphenolic compounds like flavonoids, saponins, terpenoids, tannins are antioxidant in nature[26]. Thus, the strong antioxidant activity shown by ethylacetate fraction may be due to presence of flavonoides and saponins(Table 1). Further more Lyoniside is a lignan glycoside which is found in leaves of Lyonia ovalifolia[27]. A study by Anna S*et al*. showed Lyoniside isolated from ethanolic extracts of rhizome and stem of bilberry (*Vaccinum myritillus* L.) have significant radical scavenging activity in DPPH assay with IC_{50} of 23 µg/ml[28]. Thus we can assume that the lyoniside present in the ethyl acetate fraction of leaves is responsible for the shown antioxidant activity.

Figure 2:IC_{50} value of fractionated extracts of leaves



This study concluded that the leaves extracts of *L. ovalifolia* (Wallich) possess antioxidant and antimicrobial properties and could serve as free radicals scavenging as well as natural antibiotic agent.Thus, our findings support the traditional

use of *L. ovalifolia* (Wallich) as natural remedy for wounds, cuts and burns.

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