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Antimicrobial activity of aqueous and methanolic extracts of primula macrophylla, a medicinal herb from Ladakh region

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ABSTRACT

Primula macrophylla (Primulaceae) is a high-altitude medicinal plant often used in traditional medicine system by local practitioners. The present study investigated the antimicrobial activity of aqueous and methanolic extracts of P. macrophylla against some gram-positive and gram-negative bacteria. In the case of aqueous extracts, the maximum inhibitory effect was observed on Bacillus pumilus, a gram-positive bacterium. Here, 79.4% of growth inhibition was observed at 400 μg mL^{-1}. Whereas in the case of methanolic extracts, maximum (~70.0%) growth inhibition was observed on a gram-negative bacterium, Erwinia herbicola, at the same concentration. Based on the study, it can be concluded that P. macrophylla possesses good antimicrobial activity worth exploiting.

Keywords: Antimicrobial activity, high-altitude plants

INTRODUCTION

Plants continue to play an important role in the health care of people worldwide as 80% of the inhabitants still rely on the folk medicine system (Gurib-Fakim, 2006). The World Health Organization (WHO) has also recognized the role of traditional medicine systems, especially in developing countries, for their continued service to the people (WHO, 1978). More than 750 medicinal plants are associated with ayurveda in India and are being used to prepare herbal medicines (Nair and Mohanan, 1998). The saga of man and his search for medication to alleviate pain and cure the disease dates back to the distant past. The Indian Himalaya is considered as a remarkably diverse and important centre of biodiversity and serves as a source of major rivers and ecosystem services that has sustained huge masses (Sharma *et al.,* 2008). The cross-cultural variations and geographical divisions in the region contribute greatly to the variable applications of plants by the habitants (Gairola *et al.,* 2014). Various studies have revealed the importance of the Himalayan plants in terms of their phytochemistry and pharmacological aspects. To name a few, different species of *Aconitum*, *Taxus*, *Ephedra*, *Dactylorhiza*, *Fritillaria*, *Polygonatum*, *Podophyllum*, *Picrorrhiza*, *Nardostachys* etc are the important medicinal plants of the Himalayan region (Samant and Dhar, 1997).

The secondary metabolites found in the plants are often associated with their biological activities such as antioxidant, antimicrobial, antitumor, anti-inflammatory, antispasmodic, and antiseptic activities. These natural compounds engender biological and pharmacological properties through their interaction with the target molecules in cells such as proteins, nucleic acids or biomembranes (Wink, 2015). Plant-derived compounds such as artemisinin, atropine, cannabidiol, and capsaicin have been playing a vital role in improving human health in the last few decades (Veeresham, 2012). These natural products or compounds focus on the disintegration of the cytoplasmic membrane of the microbial cell and disturb its proton motive force, flow of electrons and coagulation of cell contents, while the target site may depend on the compound as well (Silva and Júnior, 2010). Plant-based natural products have great therapeutic potential with less side effects and lower chances of developing resistance (Lewis and Ausubel, 2006). In addition, the potency of these products in the agriculture sector in the crop management and protection against plant and soil pathogen are immense. The pesticides used against the various phytopathogens were often proven as harmful due to their residual toxicity (Campos *et al.,* 2019). The development of resistance by pathogens towards synthetic pesticides and the reduction in effectiveness demand safe and alternative agents such as

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bioherbicides which have fewer negative impacts. *Primula macrophylla* D. Don. (Primulaceae) is an important Himalayan plant which normally flourishes in cool and moist open habitats especially near wetlands including glacial forelands. The genus, *Primula* is regarded as an indicator of wetlands and glacial habitats in the mountainous regions (Khan *et al.,* 2021). *P*. *macrophylla* is extensively used by different tribes of the western Himalayan region for various purposes, such in asthma and bronchial treatments, improvement of eyesight, fever, liver inflammation and diarrhoea (Bano *et al.,* 2014; Khan *et al.,* 2022). It is also documented as a flavouring agent by different ethnic communities (Amin *et al.,* 2023). In the Indian western Himalaya, it is commonly known as 'Jalkutra' and is used to cure urinary problems (Rawat *et al.,* 2013). Different tribes in J&K, India use this plant for the treatment of cough and fever (Shah *et al.,* 2015). It is also known to possess antitumor activity and induce apoptosis in human colon cancer cells (Shou and Zheng., 2017). Some other species of *Primula* such as *P*. *denticulata* and *P*. *vulgaris* have shown to possess antioxidant, cytotoxic, antimicrobial, and wound-healing properties by inhibiting the activity of collagenase and elastase enzyme (Aslam *et al.,* 2015; Demir *et al.,* 2018; Erdem *et al.,* 2022; Kahraman *et al.,* 2022;). *P*. *macrophylla,* known by the name 'Khilchay' in Ladakh, has wide applications in the treatment of cold, cough and joint pain (Angmo *et al.,* 2012; Kala *et al.,* 2006). Considering the applications of *P*. *macrophylla* in traditional medicine, the objective of the present study was to investigate the potential antibacterial activity against different pathogens and to identify its important chemical constituents for future herbal antibacterial formulations.

MATERIALS AND METHODS

The fresh plants of *P*. *macrophylla* were collected from Leh district of Ladakh (UT) at 5119 m amsl (34°01'48.27"N 077°43'02.90" E) (Fig.1) and identified from Botanical Survey of India, Dehradun (voucher code 453). The plants were shade-dried and powdered, their water and methanolic extracts were prepared as per the method of Truong *et al.* 2019. Different concentrations of the extract (ranging from 25μg mL^{-1} to 400 μg mL^{-1}) were prepared using dilution method. To determine their antibacterial activity, different gram-positive [*Bacilllus cereus* (MTCC 480), *Streptomyces scabiei* (MTCC 3966), *Bacillus pumilus* (MTCC 2296) and *Rhodococcus fasciens* (MTCC 8495)] and gram–negative [*Pseudomonas syringae* (MTCC 1604) and *Erwinia herbicola* (MTCC 6720)] bacterial strains were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh (IMTECH). *B*. *cereus* is the food and soil borne bacteria, while the other five bacteria are plant-borne pathogens.

The effect of aqueous and methanolic extracts of *P*. *macrophylla* was investigated using disc diffusion assay as suggested by Gedikoğlu *et al*. (2019). Briefly, the bacterial strains were grown on culture media and maintained on Agar media slants. The Petri plates were also prepared using nutrient agar and agar powder and kept overnight to solidify. The bacterial culture was prepared in a culture tube containing bacterial broth. After solidification of media on plates, 100 μl of bacterial suspension from the bacterial culture was spread evenly on the media plates using the spreader and allowed to settle. The sterile discs were then placed equidistantly on the medium with bacterial culture. The 20 μl of extracts of different concentrations prepared in different solvents were impregnated on each sterile disc (Himedia 6 mm in diameter). The plates were then allowed to stand in an incubator at 35 ºC for overnight to allow the growth of bacteria and to observe the inhibition of bacterial growth by each extract. The solvent used for the preparation of each extract was taken as a negative control while rifampicin (25 μg $disc^{-1}$) was used as positive control in the assay. After 24 hours of incubation, the zone of inhibition (ZOI) of bacterial growth was measured with the help of a measuring scale. Further, the analysis of the chemical constituents of *P*. *macrophylla* was done using LC-MS and total phenolic and flavonoid contents were estimated. The phenolic content was determined in terms of ferulic acid as standard and results were expressed as mg of ferulic acid equivalent (FAE) per g of the dry plant material while the flavonoid content was estimated with respect to quercetin as standard and results were expressed as mg quercetin equivalent (QE) per g of the dry plant material (Swain and Hillis, 1959; Meda *et al.,* 2005).

All the assays were performed in replicates and results for each assay were expressed as the mean ± SE. One-way ANOVA was used for the data analysis and *post hoc* Tukey's test was applied for differentiating the mean values at $p \leq 0.05$. SPSS Inc., Chicago, IL, 16.0 Version was used for performing all the statistical analyses.

RESULTS AND DISCUSSION

The results of the study revealed that with increasing concentration of the water and methanol extracts of *P. macrophylla*, more detrimental effect was observed on the growth, both gram-positive and gram-negative bacteria. The inhibitory effect of water extracts was observed to be more on gram-positive bacteria, with the exception of *R*. *fasciens*. In case of *B*. *pumilus*, the largest diameter of zone of inhibition by water extracts was recorded to be 79.4% over that of rifampicin used as positive control. At 400 μg mL^{-1}, whereas growth inhibition upon the treatment of methanolic extract at the same concentration was only ~56% (Table 1 and 2). The efficacy of aqueous extract to inhibit bacterial growth was reported against *B*. *subtilis* and *Staphylococcus aureus* by *Moringa oleifera* seed extracts (Saadabi and Zaid, 2011). Similar reports are available for the other species of genus *Bacillus*, where aqueous extracts of garlic effectively inhibit the bacterial growth (Mukhtar and Ghori, 2012). The inhibition zones varied depending on the type of extract and bacterial species. In case of methanolic extracts, maximum effect (~70%) was observed on *E*. *herbicola* – gram-negative bacterium. This bacterium causes browning disease at different growth stages in a wide range of crop plants including rice (Lorenzi *et al.,* 2022; Sandhu *et al.,* 2023). Methanolic extracts of *Avicennia alba* was found to be effective against *E*. *herbicola* at 400 μg mL^{-1} (Das, 2020) and methanolic leaf extracts of *Lantana camara* showed potential antibacterial activity against *E*. *herbicola* (Kumar and Prabha, 2019), which supports out results. Methanol was reported to be considered as an effective solvent in plant extraction than other solvents like ethanol, n-hexane, and water (Eloff, 1998; Durmaz *et al.,* 2006). Organic solvents are found to be efficient against many bacterial species, which indicate that compound responsible for antibacterial activity can be readily extracted by these solvents (Karaman *et al.,* 2003; Kaushik and Goyal, 2008). Methanolic extracts of *P*. *macrophylla* produced clear zone of inhibition against gram-negative bacteria, *E*. *herbicola* and *P*. *syringae* even at lower concentrations while aqueous extracts of the studied plant failed to produce clear zone against these pathogens depicting the efficacy of methanolic extracts against gram-negative bacteria. The inhibitory effect of water and methanolic extracts against food borne pathogen, *B*. *cereus* was found to be 55.4% and 50.0%, respectively at highest concentration of 400 μg mL^{-1}. *B*. *cereus* is often associated with food poisoning cases where heat-stable toxins are produced which cause diarrhoea and emesis (Bottone, 2010; Ehling-Schulz *et al.,* 2019).

Table 1: Antibacterial activity of aqueous and methanolic extract of *P*. *macrophylla* against some gram-positive pathogenic bacteria

Concentration (μg mL^{-1})	Gram-positive							
	Bacillus pumilus		*Streptomyces scabiei*		*Rhodococcus fasciens*		*Bacillus cereus*	
	Water	Methanol	Water	Methanol	Water	Methanol	Water	Methanol
25	9.5±0.29c (55.9)	12.3±0.25d (30.9)	8.8±0.25d (30.4)	ND	ND	9.3±0.25d (47.4)	8.8±0.25d (33.9)	9.0±0.41d (38.7)
50	12.3±0.25b (72.0)	14.5±0.29c (46.0)	9.3±0.25cd (32.2)	ND	ND	9.5±0.25cd (48.7)	11.3±0.48c (43.7)	9.8±0.25cd (41.9)
100	12.8±0.25b (75.0)	16.5±0.29b (52.4)	10.3±0.25c (35.6)	9.3±0.25b (37.0)	ND	10.3±0.25bcd (52.6)	13.3±0.25b (51.5)	10.3±0.25cd (44.1)
200	13.3±0.25b (77.9)	16.8±0.25b (53.2)	11.5±0.29b (40.0)	9.8±0.25b (39.0)	9.5±0.29b (46.3)	10.8±0.25bc (55.1)	13.5±0.29b (52.5)	10.8±0.25bc (46.2)
400	13.5±0.29b (79.4)	17.5±0.29b (55.6)	12.3±0.25b (42.6)	10.3±0.25b (41.0)	10.3±0.25b (50.0)	11.5±0.50b (58.9)	14.3±0.25b (55.4)	11.8±0.25b (50.53)
Positive control/ Rifampicin	17±0.41a	31.5±0.64a	28.75±0.25a	25±0.41a	20.5±0.64a	19.5±0.29a	25.75±0.25a	23.25±0.25a

ND- Not Detected; Values are mean (diameter of zone of inhibition) ± standard error. Different alphabets within a column represent significant differences at p ≤ 0.05 applying post hoc Tukey's test

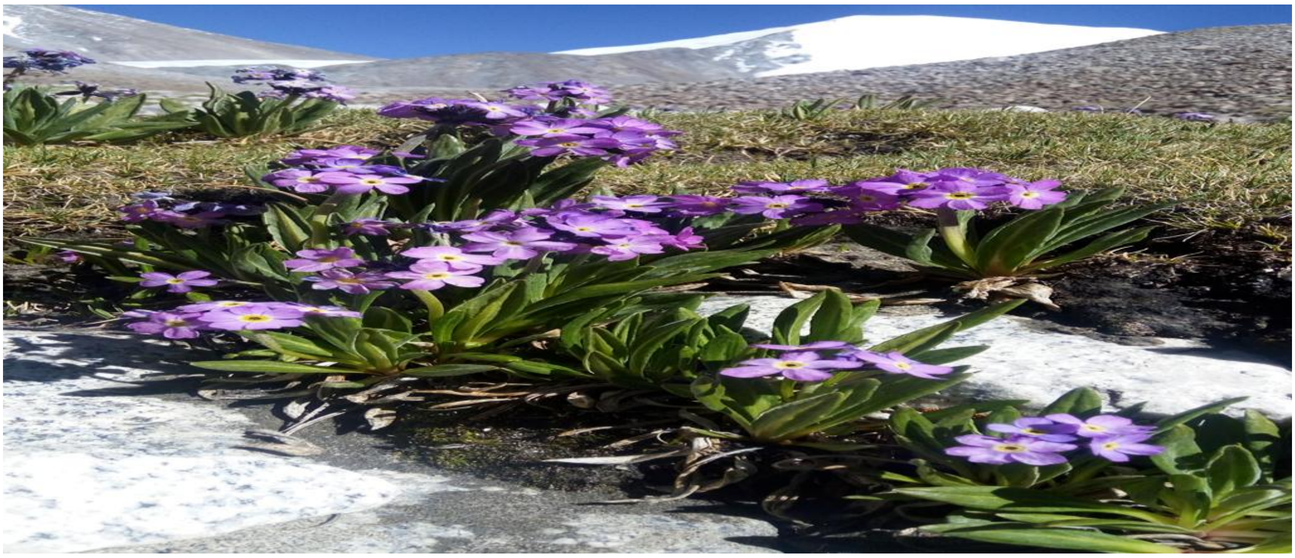

Figure 1: Photograph showing *P*. *macrophylla* in natural habitat

The antibacterial activity of methanolic extracts was found to be significantly better against gram-negative bacteria (*E*. *herbicola* and *P*. *syringae*) as well as a gram-positive bacterium *R*. *fasciens* since it exhibited inhibitory effect even at lower concentrations, while in the case of aqueous extracts, no activity was detected at lower concentrations (25, 50 and 100 µg mL^{-1}) against these bacteria (Table 1 and 2). Based on the study, it can be said that both aqueous and methanol extracts of *P. macrophylla* possess good antimicrobial potential and thus could be used for the development of plant-based antimicrobial drugs against the tested microbes. Methanolic extracts was found to be effective against the gram-negative bacteria while aqueous extract was effective against gram-positive bacteria except for *R*. *fasciens* in present study. In addition, *P. macrophylla* has also been reported to possess effective antifungal, antileishmanial, cytotoxic and phytotoxic properties in different formulations /extracts (Najmus-Saqib *et al.,* 2009). Başbülbül *et al.* (2008) have reported the antibacterial activity of alcoholic formulation of *P*. *veris*. The use of plant-based antimicrobial agent is safe and alternate approach to use as antimicrobials for pathogen reduction (Gyawali and Ibrahim, 2014).

Table 2: Antibacterial activity of aqueous and methanolic extract of *P*. *macrophylla* against some gram-negative pathogenic bacteria

Concentration (µg mL^{-1})	Gram-negative			
	Erwinia herbicola		*Pseudomonas syringae*	
	Water	Methanol	Water	Methanol
25	ND	9.5±0.29d (55.1)	ND	11.0±0.41c (31.9)
50	ND	9.8±0.25cd (56.5)	ND	12.5±0.29c (36.2)
100	ND	10.8±0.25cd (62.3)	ND	12.8±0.25bc (36.9)
200	ND	11.8±0.25bc (68.1)	ND	13.3±0.25b (38.4)
400	10.8±0.25b (34.9)	12.0±0.41b (70.0)	9.5±0.64b (33.04)	13.8±0.25b (39.8)
Positive control/ Rifampicin	30.75±0.25a	17.25±0.48a	28.75±0.25a	34.5±0.64a

ND- Not Detected; Values are mean (diameter of zone of inhibition) ± standard error. Different alphabets within a column represent significant differences at p ≤ 0.05 applying post hoc Tukey's test

It has previously been established that flavonoids, alkaloids, terpenes, and phenolic compounds are largely responsible for the antimicrobial activity in plants (Rios and Recio, 2005). The quantitative estimation of total phenolic and flavonoid confirmed the presence of phenolic and flavonoid compounds. The amount of total phenolics was estimated to be 18.59 ± 0.99 mg FAE g^{-1} and that of total flavonoids was found to be 7.70 ± 0.04 mg QE g^{-1} (data not presented). The present study also indicates the presence of 25 compounds in *P. macrophylla* via LC-MS which include important compounds like quercetin, rhamnetin and anethole and also the derivatives of catechin and gallic acid (Table 3). The polyphenolic compounds like quercetin and gallic acid are well known for their activities like antimicrobial, antidiabetic, anticancer, antiulcer, and antimalarial (Ahmad *et al.,* 2018). Rhamnetin is known to possess several pharmacological properties including antioxidant, anticancer, anti-inflammatory, antiviral and antibacterial (Medeiros *et al.,* 2022). Anethole, a type of polyphenol, has multiple beneficial effects on human health, as it is known to possess anti-inflammatory, antidiabetic, chemopreventive, neuroprotective, and also immuno-modulatory properties (Aprotosoaie *et al.,* 2016). Plant-derived polyphenols acts as natural microbial growth inhibitors against human pathogens, food-borne pathogens, and post-harvest control of pathogens in crops which minimise the use of synthetic and chemically derived antimicrobial agents (Daglia, 2012; Gutiérrez-del-Río *et al.,* 2018; Aguilar-Veloz *et al.,* 2020). The presence of polyphenolic compounds in *P. macrophylla* justifies the antimicrobial activity of aqueous and methanolic extract against both gram-positive and negative bacteria.

Table 3: Chemical constituents of *P. macrophylla* identified by LC-M

Name of the compound	Formula	Molecular Weight	Class of compound
Dihydrocaffeic acid	$C_9H_{10}O_4$	182.17	Polyphenols
Trans-methyl geranoate	$C_{11}H_{18}O_2$	182.26	Terpenoids
Geranyl acetone	$C_{13}H_{22}O$	194.31	Terpenoids
Hydroxy caffeic acid	$C_9H_8O_5$	196.15	Polyphenols
Caryophyllene-5,6-Oxide-2,12-glycol	$C_{15}H_{26}O_3$	254.37	Terpenoids
6-hydroxyluteolin	$C_{15}H_{10}O_7$	302.23	Polyphenols
Fernesyl monophosphate	$C_{15}H_{27}O_4P$	302.35	Metabolite or derivatives
Quercetin	$C_{15}H_{10}O_7$	302.23	Flavonoid
Cyanidin-3-O-galactoside	$C_{21}H_{21}O_{11}$	449.38	Polyphenols
Iso-quercetin	$C_{21}H_{20}O_{12}$	464.37	Polyphenols
Myricetin-3-O-galactoside	$C_{21}H_{20}O_{13}$	480.37	Polyphenols
Myricetin-3-O-glucoside	$C_{21}H_{20}O_{13}$	480.37	Polyphenols
Carotene-5,6-epoxide (beta-)	$C_{40}H_{56}O$	552.88	Terpenoids
Cyanidin-3-O-rutinoside	$C_{27}H_{31}O_{15}$	595.52	Polyphenols
Quercetin-3-O-glucosyl-xyloside	$C_{26}H_{28}O_{16}$	596.49	Polyphenols
Anethole	$C_{10}H_{12}O$	148.20	Polyphenols
Caffeic acid	$C_9H_8O_4$	180.16	Polyphenols
5,5-Dicaffeic acid	$C_{18}H_{14}O_8$	358.30	Polyphenols
Gallic acid 4-O-glucoside	$C_{13}H_{16}O_{10}$	332.26	Polyphenols
5-O-Caffeoylshikimic acid	$C_{16}H_{16}O_8$	336.29	Polyphenols
β-Sitosterol	$C_{29}H_{50}O$	414.71	Terpenoids
(+)-Catechin-3-O-glucose	$C_{21}H_{24}O_{11}$	452.41	Polyphenols
Isorhamnetin	$C_{16}H_{12}O_7$	316.26	Polyphenols
Rhamnetin	$C_{16}H_{12}O_7$	316.26	Polyphenols
Apigenin-7’4-dimethylether	$C_{17}H_{14}O_5$	298.29	Polyphenols

CONCLUSION

Based on the observations, it can be concluded that *P. macrophylla* has a potent antimicrobial activity against both gram-positive and gram-negative bacteria known to be harmful to crops or food. The methanolic extracts of *P. macrophylla* were found to be more successful towards supressing microbial growth in gram-negative bacteria than aqueous extracts while both aqueous and methanolic extracts inhibited the growth of gram-positive bacteria significantly. Phenolic compounds identified through LC-MS may be responsible for the observed antibacterial activity and thereby suggesting more eco-friendly and cost-benefit alternative as natural antimicrobial agents. However, future investigations are encouraged to study the toxicity in order to determine the actual effectiveness and relevance for the treatment against pathogens.

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