RESEARCH ARTICLE

Isolation and Identification of Phenolic Profiles in Selected Himalayan Wild Berries and Determination of their Antimicrobial Activity

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ABSTRACT

A fundamental source of food and medicine for nearby Himalayan populations is the diversity of plants. The percentage yield of extract, phytochemicals, and minimum inhibitory concentration (MIC) for the extracts *Euterpe oleraceae, Vaccinium myrtillu, Phyllanthus embilica, Rubus ellipticus,* and *Rubus niveus* were determined. The clinical and laboratory standards Institute's micro broth dilution method was used to assess these extracts' antimicrobial effects on *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, while the purification of the positive extract was done by high-pressure liquid chromatography. Phytochemical analysis was done to determine the secondary plant metabolites, including alkaloids, polyphenols, glycosides, tannins, flavonoids, carbohydrates, steroids, and saponins. All five of the studied plants' extracts showed antibacterial activity against one or more tested microorganisms. Several phenolic acids (chlorogenic acid, salicylic acid, ellagic acid, ferulic acid, gallic acid, and caffeic acid were detected in all extracts. The *R. ellipticus* extracts with petroleum ether, chloroform, methanol, and water show a maximum yield between (64–56%) except *R. ellipticus* extract with hexane (25.82%) showed a low yield. All the extracts have major quantities of carbohydrates, flavonoids, and phenols. These results suggested that produced antimicrobial activity was due to the presence of phytoconstituents in all extracts.

Keywords: Himalayan wild berries, Herbal plants, Phytochemical, Antimicrobial activity, Phenols, HPLC.

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INTRODUCTION

The treatment and prevention of human diseases has benefited greatly from the use of natural products around the globe. Medicines from natural products have been produced using a variety of materials, such as terrestrial plants and microbes, marine species, and terrestrial invertebrates and vertebrates. Its use in modern medicine is crucial.[1] Since many medicines seem to be natural compounds or their derivatives, they have had an important function in pharmaceutical research. This statistic is believed to be accurate since natural compounds or their semi-synthetic derivatives make up around 40% of all medicines.[2] Only as last-resort therapies for fatal diseases like cancer are many non-natural, synthetic medications' negative effects acceptable. Since they must accumulate within living cells, the metabolites found in therapeutic plants and other natural items may not have the side effects of synthetic and semi-synthetic drugs.[3] Natural products possess enormous potential due to their diverse chemical compositions, cost-effectiveness, intricate molecular structures, inherent biological activities, and minimal toxicity, rendering them an attractive option for developing novel therapeutic agents.[3]

Between 75–80% of the population still relies heavily on herbal treatment, and the utilization of plant extract and its active ingredients makes up most of the traditional therapy.[4] Throughout the beginning of time, higher plants have served as the foundation for the treatment of ailments in indigenous cultures. There are many different types of wild edible plants in the western Himalayas, as well as a variety of eco-geographical and ecoclimatic circumstances.[5] Hence, interest in herbal medicine has gradually increased in recent years. The therapeutic usage of medicinal plants, which are abundant in antimicrobial compounds and have fewer side effects and higher resistance, is growing in popularity.[6] Polyphenolic compounds are commonly found in both edible and inedible plants, and they have been reported to have antimicrobial activity.[7, 8] Fruits that are rich in vit C, all types of berries, and vegetables are rich sources of phenolic contents.[9] The phytochemicals in fruit, flavonols, and phenolic acids are projected to add to the health impacts of fruit-derived products and are regarded as key functional food components.[10] The studies done in recent years revealed that the role of phenolic compounds like

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phenols, flavonoids, anthocyanins, and carotenoids in berries are responsible for various pharmacological activities like anticancer, antimicrobial, antioxidants, antidiabetics, anti-inflammatory, and other bioactivities.[11]

The new "superfruits" *Euterpe oleraceae* (Acai), is a great source of anthocyanins, phenolic compounds belonging to the group of flavonoids, which are not only important natural pigments; but also contain proven antimicrobial and antioxidant properties.[12] Total soluble polyphenols (TSP) and total monomeric anthocyanins (TMA), two phenolic compounds that are more abundant in *Vaccinium myrtillu* (wild blue), have been shown to have some antibacterial activity towards diverse bacterial strains.[13] *Phyllanthus embilica* (gooseberry) commonly known as amla have phytochemicals such as tannins, glycosides, phenolics, flavonoids, carbohydrates, phytosterols, and alkaloids.[14] Amla fruit is used extensively in Ayurveda and is thought to boost resistance to disease.[14] The plant *Rubus ellipticus* also known as Hisalu/Hinsar,[15] and *R. niveus* Thunb a shrub known as "amora roxa"[16] both belong to the family Rosaceae; studies have demonstrated the use of *Rubus* species showing action against antimicrobial properties.[17] So, in the proposed study phenolic compounds extracted and purified from Himalayan wild berries showing anticancer, antimicrobial, and antioxidant activities can form a basis as a lead molecule for future study and evaluation.

MATERIAL AND METHOD

Plant Material

Fruits and seeds of *E. oleraceae* (Acai), *V. myrtillu* (wild blue), *P. embilica* (gooseberry), *R. ellipticus*, *R. niveus*. Berries Powder purchased from the Herbalveda

Bacterial Strains

Two bacterial strains used were *K. pneumoniae* and *P. aeruginosa* with accession numbers MCC 3094 and MCC 2265 purchased from NCMR, Pune, India.

Preparation of Plant Extracts

Soxhlet extraction of extracts

The collected fruits and seeds were allowed for surface sterilization using 0.1% mercuric chloride, followed by washing under running water, and left to air dry in the shade for 2 to 4 weeks. The dried fruits and seeds are then finely crushed and kept in an airtight container. The air-dried powder (5 g) was extracted successively by soxhlet extraction with solvents of different polarity i.e., hexane, petroleum ether, chloroform, methanol, and water. The extraction process employed 450 mLof hexane solvent and continued for 6 to 8 hours (4–6 cycles per hour i.e., 24 to 48 cycles). To monitor the progress of extraction, a sample of the extract was collected from the siphon tube of the extractor every four cycles. The extract was then spotted on a TLC plate and visualized in an iodine chamber to determine the completeness of the extraction. No spot indicates completion of extraction. The dried, sterilized, and airtight extract was collected in a hexane solvent container. A similar different extract was prepared in different solvents. All the dried extracts were weighed. All the extracts were diluted to 20 mL in their respective solvents.

Characterization of Plant Extracts

Yield

The extraction yield, defined as the ratio of the mass of extract to the mass of dry matter, served as a measure to evaluate the impact of extraction conditions.[18] The percentage of extracted yield was calculated by the formula given below.

Extraction yield/yield% = (W_O/W_t) *100

W_O = weight of initial fruit/seed sample

W_t = weight of dried extract after Soxhlet extraction

Phytochemical Analysis

Using established techniques the dried powdered sample's qualitative phytochemical properties were identified and analyzed for the presence of several secondary metabolites.[19] For determining the flavonoid and total phenolic content, the Folin-Ciocalteau reagent method was employed[20] and the aluminum chloride (AlCl3) method,[21] respectively.

Antimicrobial Activity of the Extract

All plant extracts had their antibacterial activity determined using the agar well diffusion method.[22] *K. pneumoniae* and *P. aeruginosa* were the two bacteria used to test their antibacterial activity.

Minimum Inhibitory Concentration (MIC)

The microdilution method was utilized to determine the MIC of the extract against the bacteria *K. pneumoniae* and *P. aeruginosa*. MIC refers to the minimum concentration of the sample at which visible microbial growth is inhibited.[23]

Isolation and Purification of Phenolic Compound

HPLC analysis of column fractions

EOH10, EOM10, EOW10, VMW10, and REP10 were selected for HPLC analysis. The phenolic chemicals were examined using a UV-vis DAD HPLC system from the Agilent 1100 series. A reverse-phase chromatography column made of zorbax C18 (5 µm, 4.6 × 250 mm) was used. A 50 µg/mL of all the standard phenolic compounds (Gallic acid, Caffeic acid, vanillic acid, coumaric acid, ellagic acid, benzoic acid, phenylacetic acid, quercetin) were prepared and run separately. A combination of acetonitrile (HPLC grade, 99.9%) and phosphoric acid (mobile phase B) was prepared to create the mobile phase for HPLC. This was accomplished by gradually adding 85% orthophosphoric acid to HPLC grade water until the pH reached 2. The temperature was maintained at 5°C, and the flow rate was set at 0.5 mL/min.

RESULTS AND DISCUSSION

Extraction Yield

Biologically active substances are typically found in small amounts in plants. An extraction technique is one that can produce extracts with a high yield while requiring little modification to the extract's necessary functional qualities. One indicator of the impacts of the extraction conditions was

Table 1: Inferences of Phytochemical analysis of Plant samples

Sample	*Alkaloid*			*Flavo-noid*	*Phenol*	*Glycos-ides*	*Tannins*	*Carbohydrate*			*Saponins*	*Steroids*
	Mayer's test	*Drage-ndorff's test*	*Wagner test*					*Molisch*	*Fehling's*	*Benedicts*		
EOH	+	+	+	+	+++	-	-	+	-	+	-	-
EOP	+	-	+	+	+	-	-	+	-	+	-	-
EOC	-	-	-	-	-	-	-	+	-	+	-	-
EOM	-	-	-	+++	+++	-	+	++	++	++	-	-
EOW	-	-	-	+++	+++	-	+	++	++	++	-	-
VMH	-	-	-	++	+	-	-	++	++	++	-	-
VMP	-	-	-	+	+	-	-	++	++	++	-	-
VMC	-	-	-	+	+	-	-	++	++	++	+	+
VMM	+	+	+	+++	+++	+	+	+++	+++	+++	+	+
VMW	+	+	+	+++	+++	+	+	+++	+++	+++	+	+
PEH	-	-	-	+	+	++	++	+	-	-	+	-
PEP	-	-	-	+	-	+	+	-	-	-	+	+
PEC	+	-	-	+	-	+	-	-	-	-	+	+
PEM	++	+	+	++	+++	-	++	+++	+++	++	-	-
PEW	-	-	-	-	++	+	++	+++	+++	++	-	-
REH	+	+	-	+	-	++	-	-	-	-	-	-
REP	++	++	-	++	+	++	-	-	-	-	-	-
REC	+	++	+	++	++	++	-	-	-	-	-	-
REM	++	+++	-	+++	++	++	+++	+	+	++	+	-
REW	++	+	+	++	++	++	+	++	++	++	+	-
RNH	-	-	-	+	+++	-	-	+	+	+	-	-
RNP	-	-	-	+	++	-	-	+		+	-	-
RNC	-	-	-	+	+++	-	+	+	++	+++	-	-
RNM	+	-	-	++	+++	-	+	++	++	++	+	+
RNW	+	-	-	++	+++	-	+	++	++	++	+	+

(+): Present, (-): Absent

EOH: *E. oleraceae* in hexane, EOP: *E. oleraceae* in petroleum ether, EOC: *E. oleraceae* in chloroform, EOM: *E. oleraceae* in methanol, EOW: *E. oleraceae* in water; *VMH: V. myrtillu* in hexane, VMP: *V. myrtillu* in petroleum ether, VMC: *V. myrtillu* in chloroform, VMM: *V. myrtillu* in methanol, VMW: *V. myrtillu* in water; PEH: *P. embilica* in hexane, PEP: *P. embilica* in petroleum ether, PEC: *P. embilica* in chloroform, PEM: *P. embilica* in methanol, PEW: *P. embilica* in water; REH: *R. ellipticus* in hexane, REP: *R. ellipticus* in petroleum ether, REC: *R. ellipticus* in chloroform, REM: *R. ellipticus* in methanol, REW: *R. ellipticus* in water; RNH: *R. niveus* in hexane, RNP: *R. niveus* in petroleum ether, RNC: *R. niveus* in chloroform, RNM: *R. niveus* in methanol, RNW: *R. niveus* in water.

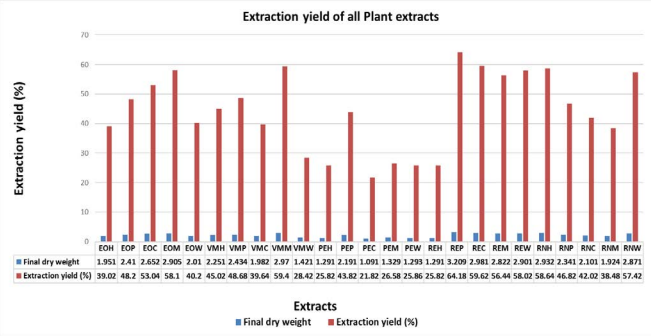

Figure 1: Extraction yield (%) of all extract samples

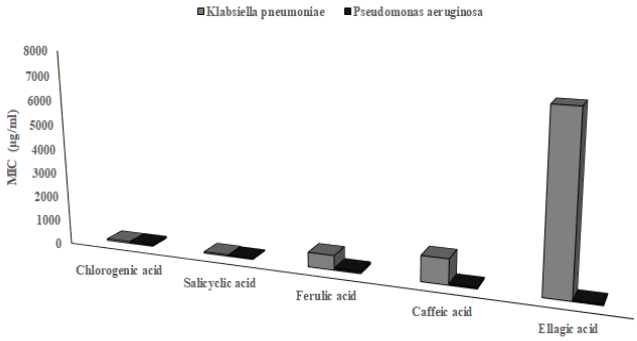

Figure 2: Minimum inhibitory concentration (MIC) of compounds

extraction yield (mass of extract/mass of dry matter).[24] Extract yield of all the plant extracts was prepared by refluxing method using hexane, petroleum ether, methanol, chloroform, and water, summarized in Figure 1. In reflux, REP yield (64.18%) obtained with petroleum ether was the maximum yield followed by REC (59.62%), VMM (59.04%), RNH (58.64%), REW (58.02%), EOM (58.01%), RNW (57.42%), REM (56.44%) AND EOC (53.04%) all having the highest extraction yield. The extract yield PEC (21.82%) was found to be the lowest extract yield obtained with chloroform, followed by PEW (25.86%), PEH (25.82%), REH (25.82%), PEM (26.58%), and VMW (28.42%) all having the lowest extraction yield. The *R. ellipticus* extracts with petroleum ether, chloroform, methanol, and water show a maximum yield between (64–56%) except *R. ellipticus* extract with hexane (25.82%) showed a low yield. Whereas the *P. embilica* extracts with chloroform, methanol, hexane, and water showed the lowest yield in the range of (21–26%) except for *P. embilica* extract with petroleum ether (43.82%) showed high yield.

Phytochemical Analysis

According to a preliminary investigation, all the extracts of Himalayan wild berries contained alkaloids, phenolics, flavonoids, glycosides, carbohydrates, tannins, saponins, and steroids. Table 1 displays a comparative summary of the phytochemical analysis. Alkaloids are a unique class of nitrogenous substances that have been utilized for centuries to treat a variety of human and animal diseases. High molecular weight polyphenols known as tannins defend plants from microbes. They result in animal protein indigestion, which slows growth.[25,26] Important dietary components for humans including flavonoids and polyphenols may be used to treat cancer, cardiovascular and inflammatory illnesses, as well as protect radiation damage.[27] All the extracts have major quantities of carbohydrates, flavonoids, and phenols. These results suggested produced antimicrobial activity due to the presence of almost similar phytochemicals in all extracts.

Table 2: Minimum Inhibitory Concentration (MIC)

Bacterial Strains	*Compounds*	*(MIC) µg/mL*
K. pneumoniae	Chlorogenic acid	68.95837
	Salicylic acid	53.36428
	Ferulic acid	590.9484
	Caffeic acid	1031.675
	Ellagic acid	7080.266
	Ceftriaxone	2.536150293
P. aeruginosa	Chlorogenic acid	72.3210001
	Salicylic acid	31.79972958
	Ferulic acid	94.93205791
	Caffeic acid	54.89317672
	Ellagic acid	40.03856163
	Cefepime	68.64248771

Antimicrobial Activity

The antimicrobial activity (expressed as µg/ mL) of the given compounds against two strains of bacteria *K. pneumoniae* and *P. aeruginosa* are summarized in Table 2 and Figure 2. The minimum inhibitory concentration was used to assess the extracts' efficacy against the tested bacteria (MIC). The test was performed for all the extracts which showed high antimicrobial activity. The organism *K. pneumoniae* was found to be more susceptible to ceftriaxone with a MIC value of 2.536150293 µg/mL. *K. pneumoniae* was less susceptible to salicylic acid with MIC value of 53.36428 µg/mL. *K. pneumoniae* was less susceptible to chlorogenic acid, ferulic acid, caffeic acid, and ellagic acid with higher MIC50 values (MIC ≥ 64 µg/mL). The organism *P. aeruginosa* was found

Table 3: Retention times, areas, and response factors of identified compounds

Secondary Metabolites	*R.T. (min)*	*Area (mAU)*	*Response factor*
Chlorogenic	7.124	156.00	7.80
Ellagic	18.656	62.20	3.11
Caffeic acid	14.852	1112.01	55.60
Gallic acid	16.092	1654.02	82.70
Syringic	7.092	204.01	10.20
Salicylic	12.588	79.02	3.95
Ferulic	13.728	1920.02	96.00
Vanillic acid	9.968	659.02	32.95

RT=Retention Time, Initial Concentration of Std =20 µg/mL

to be more susceptible to salicylic acid with a MIC value of 31.79972958 µg/mL. *P. aeruginosa* was less susceptible to ellagic acid and caffeic acid with MIC50 values of 40.03856163 and 54.89317672 µg/mL, respectively. *P. aeruginosa* was less susceptible to cefepime, chlorogenic acid, and ferulic acid with higher MIC50 values (MIC ≥ 68 µg/mL).

Purification of the Positive Extract by High-Pressure Liquid Chromatography

Table 3 provides an overview of compounds found in all plant extracts' retention factor, area, and response factor. All plant extracts contained one or more of the following phenolic acids: Chlorogenic acid, ellagic acid, caffeic acid, gallic acid, salicylic acid ferulic acid syringic acid, and vanillic acid. The initial concentration of the standard was taken as 20 µg/mL.

CONCLUSION

According to this study, all plant extracts contain interesting antibacterial characteristics that can be related to their abundance in phytochemicals, including phenolics and flavonoids. All five of the studied plants' extracts showed antimicrobial activity against one of the two tested microorganisms. All extracts showed several phenolic acids, including chlorogenic acid, salicylic acid, ellagic acid, ferulic acid, caffeic acid, and gallic acid. Except for the *R. ellipticus* extract in hexane (25.82%), all the *R. ellipticus* extracts in petroleum ether, chloroform, methanol, and water exhibit a maximum yield between (64–56%). All the plant extracts contain a significant number of phenols, flavonoids, and carbohydrates. These findings suggested produced antimicrobial activity because all extracts included almost identical phytochemicals. Based on the results, plants are considered to be an exceptional reservoir of secondary metabolites, which can be isolated into pure compounds possessing in vivo biological activities. These compounds exhibit mechanisms of action that can potentially be utilized for developing herbal remedies against infectious diseases

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