EUROPEAN ACADEMIC RESEARCH
Vol. IX, Issue 7/ October 2021

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Characterization and Antimicrobial activity of *Pinus halepensis Mill* tree needles of Quetta valley, Balochistan

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Abstract

The study of the biological properties of various plants for their medicinal uses has grown remarkably. Literature shows that different species of Pines are rich in several bioactive molecules. Physicochemical examination showed that pine needles are of green color with piney and citrus smell. They are 12.3cm long, slender, needle-like leaves that are produced in pairs and have a smooth texture. The pH of pine needles in 70% methanol and pure methanol was 5.5 and 5.3 respectively. The percentage of moisture content was 58.15. The findings revealed the presence of carbohydrates, amino acids, alkaloids, flavonoids, glycosides, phytosterols, steroids, terpenoids, phenols, and saponins. The antibacterial activity was examined via agar well diffusion method on Gram positive and Gram negative bacteria that is Staphylococcus aureus and Escherichia coli respectively. The study confirmed that Pinus halepensis Mill. needles exhibit antibacterial activity against Staph aureus and E.coli. In case

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of Pine needles in pure methanol showed higher antibacterial activity against Staph aureus with IZ of 14mm and E. coli with IZ of 10mm than pine needles in 70% methanol with IZ of 8mm and 7mm respectively.

Keywords: Pinus halepensis Mill., needles, phytochemicals, qualitative screening, antibacterial activity, physicochemical properties.

INTRODUCTION

The use of natural compounds from plant source for treating various diseases was primordial and is the basis for recent therapies to maintain a healthier life. For thousands of years, plants are known for their ethnopharmacological properties and more than 50% of therapeutic agents are derived from plants and other natural sources. The extracts from different parts of plants possess curative properties but they are also used as preservatives, sweeteners, and coloring agents in many medicinal formulations (Heinrich, 2000; Gurib, 2006). Extracts of medicinal plants consist of bioactive chemical compounds for which they are of extreme importance to the pharmaceutical industry. Over the past two decades, harmful side effects and resistance towards certain antibiotics were observed. To overcome these disadvantages different plant extracts were examined for new antimicrobial agents (Joshi and Joshi, 2006; Alexa et al., 2018).

Phytochemicals are biologically active chemical compounds present in plants, accountable for several biochemical characteristics of plants. Plants are the rich source of bioactive molecules, over 0.25 million phytochemicals have been identified so far. They are also part of our diet mainly in fruits, vegetables, grains, cereals, and pulses. Phytonutrients have potential health-beneficial properties. Plants consist of several phytochemical molecules like tannins, vitamins, amines, phenolic, quinones, terpenoids, flavonoids, alkaloids, and other metabolites (Cai et al., 2003; Ashokkumar et al., 2008; Gracelin et al., 2013; Roghini and Vijayalakshmi, 2018).

Phytochemicals produced by plants help during plant growth because of metabolic reactions. Environmental factors like rainfall,

altitude, climate, and others affect plant growth as a result the quality of herbal ingredients is also affected in particular species produced in the same country. These conditions also cause variations in the bioactive compounds of plants (Okwu, 2004; Ezeonu and Ejikeme, 2016).

The genus Pinus belongs to the family Pinaceae which consists of around 250 species that are rich in secondary metabolites. They are naturally found in temperate regions of the earth's Northern hemisphere particularly in the Caribbean area, Central and North America, Europe, Asia, and the Mediterranean region. In Asia, it's commonly found in Pakistan, China, Thailand, Myanmar, the Philippines, and India. They have also been planted in temperate regions of the southern hemisphere. They are resinous and evergreen trees that grow 3-80m tall and have gray-green needle-like leaves that grow in pairs. (Fekih et al., 2014; Mohareb et al., 2017; Ustun et al., 2012).

Several natural compounds obtained from various parts of Pinus trees were used in traditional medicines as ointments, decoctions, bathing oils and as drugs that could be inhaled to cure many health-related problems especially respiratory diseases such as pulmonary ailments, bronchitis, and cough, others include muscle disorders and wound healing of infectious, cardiac diseases, rheumatic or neuralgic disorders and hypertension (Eddouks et al., 2002; Grassmann et al., 2003; Mustafa et al., 2012; Süntar et al., 2012

People not only in Pakistan but across the globe have been reported to develop resistance towards several medicines. They are also prone to multiple harmful side effects of these medicines. This is threatening our capability to cure common infections that result in prolonged health issues. In Pakistan, 80% of the total population is a rural community that relies on available conventional medicines for remedial curative therapies. These natural drugs are more beneficial than synthetic drugs because of their innocuous nature (Kayani et al., 2014; Bibi et al., 2015). The present study's motive is to identify the phytochemical, antimicrobial and physicochemical properties of Pinus halepensis Mill. so that it can potentially be used as a source of new molecule.

MATERIAL AND METHODS

Collection of Plant Material

Pinus halepensis Mill. needles were collected in August from different areas of Quetta, Balochistan. The plant sample was identified in the Department of Botany, University of Balochistan. To eliminate dust particles, plant needles were washed with tap water and then air-dried under shade at room temperature for 10 days. Needles were then stored in the dark.

Sample Extraction

The dried samples were ground to a fine powder in an electrical grinder at 8800rpm and accurately weighed in a digital balance and deposited in labeled polythene bags, before further analysis. The dry extracts of each sample were extracted into pure and 70% ethanol by immersing the powder in the solvent for three days at room temperature and was shaken thoroughly during different time intervals. This procedure was repeated thrice. The methanolic fractions were filtered on muslin. The resulting filtrates were then combined and concentrated by evaporating methanol under vacuum with a rotary evaporator at 60ºC to give crude extracts. The extracts were stored in dark bottles.

Physicochemical Analysis

The physicochemical properties were determined by using Londonkar and Jayashree, 2014 methods with slight modification.

i) Organoleptic studies

The organoleptic properties of Pinus halepensis Mill. needles such as colour, odour, size, shape, and texture were assessed.

ii) Determination of pH

The pH of 70% and pure methanolic extracts of Pinus halepensis Mill. needles were evaluated with the use of a pH meter.

iii) Determination of Moisture content

To assess the moisture content of the sample, needles of Pinus halepensis Mill. were weighed and then taken in a flat-bottomed dish and kept in a hot air oven at 100-110ºC overnight. The loss in weight was considered as the amount of moisture content in the sample. The percentage moisture was calculated by using the given formula.

$$\% \text{ Moisture} = \frac{\text{wt of wet sample} - \text{wt of dry sample}}{\text{wt of wet sample}} \times 100$$

Qualitative screening of phytochemicals

a) Detection of Carbohydrates

The presence of carbohydrates in 70% and pure methanol extracts of Pinus halepensis Mill. needles were determined by Molisch's test, Fehling's test and Benedict's test (De Silva et al., 2017 and Shaikh and Patil 2020).

b) Detection of Proteins and amino acids

The standard procedures of Biuret and Xanthoproteic tests were followed to determine the presence of proteins (Shaikh and Patil 2020) while presence of amino acids was determined by ninhydrin test given (Roghini and Vijayalakshmi 2018).

c) Detection of Alkaloids

To evaluate the existence of alkaloids by Mayer, Wagner, Dragendroff and Hager's test were performed (Shaikh and Patil 2020).

d) Detection of Flavonoids

The presence of flavonoids was examined Alkaline reagent test, Shinoda test and Lead acetate test (Shaikh and Patil 2020).

e) Detection of Glycosides

Salkowski's test, Keller-Kiliani test and Liebermann's test were performed were performed to investigate the presence of glycosides (Gul et al., 2017).

f) Detection of Phytosterols

The determination of phytosterols was carryied out Libermann-Burchard's and Salkowski's method (De Silva et al., 2017).

g) Detection of Steroids

The presence of steroids was examined by the test stated by (Roghini and Vijayalakshmi 2018).

h) Detection of Terpenoids

The detection of terpenoids was evaluated by performing Salkowski test (Roy et al., 2018).

i) Detection of Phenols

The ferric chloride method was carried out to monitor phenols (Shaikh and Patil 2020).

j) Saponins

The presence of saponins in the extracts were investigated by Shaikh and Patil., 2020 with slight modification.

Antibacterial Assay

Agar well diffusion method

The antibacterial activity against Gram positive, Staphylococcus aureus, and Gram negative, Escherichia coli was determined by the agar-well diffusion method performed with few modifications. In 1000ml of distilled water, 28g of nutrient agar was dissolved to prepare bacterial culture. The resulting media, petri dishes, borer, and yellow and blue tips were autoclaved for 20 minutes at 37°C. After autoclave, the media was allowed to cool and bacterial strains were evenly poured on the petri plates. Wells were punched at equal distance and 100µL of samples were introduced into the wells. For 24 hours at 37°C agar plates were incubated. The antibacterial effect of each sample was evaluated by measuring the diameter of inhibition zones produced around the wells as the antibacterial agent present in plant extract diffuses in agar medium and inhibits the growth of tested bacterial strain. Controls were maintained for both bacterial strains for Gram positive (Staphylococcus aureus) streptomycin and for Gram negative (E.coli) ampicillin were used as positive control while for negative control NaCl was used (Stephen et al., 2005).

RESULTS AND DISCUSSION

Physicochemical Analysis

The physicochemical properties of Pinus halepensis Mill. needles are summarized in Table 1. The needles were green in color with piney and citrus odour. They were long, slender, and needle-shaped that were produced in pairs with smooth texture. Their size was 12.3cm, which was taken as the average of three different needle pairs. The pH of pine needles was 5.5 and 5.3 in 70% and pure methanol extracts respectively. This shows that pine needles are slightly acidic in nature. These results are in agreement with the study of Voulgaridis et al., 1985. The moisture content was measured by drying 10.06g of pine needles in the oven. After drying the needles lost 5.85g mass. The percentage moisture calculated shows that 10.06g of pine needles

consist of 58.15% moisture. The result is in correlation with Kadri et al., 2015.

Sample	Property	Observation
Needles	Color	Green
	Odor	Piney and Citrus Smell
	Length	12.3cm
	Shape	Long, slender, needle like leaves, produced in pairs.
	Texture	Smooth
	Moisture Content	58.15%
Needles (Methanol 70%)	pH	5.5
Needles (Methanol 100%)	pH	5.3

Table 1. Physicochemical characteristics of Pinus halepensis Mill. needles.

Phytochemical Screening

The qualitative screening of pure and 70% methanolic extracts of Pinus halepensis Mill. is presented in Table 2. The analysis showed that several phytochemicals that include carbohydrates, amino acids, alkaloids, flavonoids, glycosides, phytosterols, steroids, terpenoids, phenols, and saponins were present. Surprisingly, proteins were not observed in both extracts.

Carbohydrates

The carbohydrates content in the methanolic extract was determined by applying Molisch, Fehling, and Benedict's test. In Molisch's test, a violet ring was formed after the addition of concentrated sulphuric acid while in case of Fehling and Benedict's test red and reddish-green precipitates appeared (Table 2). These results confirmed the presence of carbohydrates in Pinus halepensis Mill. needles. These results are in accordance with the results reported by Mohareb et. al., 2017 also showed the presence of carbohydrates in leaf and bark extract of Pinus halepensis.

Proteins and Amino Acids

The presence of protein and amino acids were observed by performing Biuret and Xanthoproteic tests. Both Biuret and Xanthoproteic tests gave negative results for protein which might be due to its extremely low concentration whereas in the Ninhydrin test deep blue color

appeared that indicated the presence of amino acids (Table 2). The results are in correlation with Tukan et al., 2013, their results revealed the presence of proteins in plant extract. Aditi Sharma et.al (2016) also reported the presence of amino acids and protein but our results are are not in accordance with Atul Kabra et.al (2013), reported the absence of protein in plant extract.

Alkaloids

The alkaloids content was monitored by performing Mayer, Wagner, Dragendroff, and Hager's test. In case of Mayer's test white creamy precipitates appeared while Wagner and Dragendroff's tests reddish-brown precipitates were appeared indicate the presence of alkaloids. Yellow precipitates appear in Hagers test that was an indication of the presence of alkaloids (Table 2). These results are in accordance with Zerroug et al., (2021), reported the presence of alkaloids in Pinus halepensis Mill.

Flavonoids

The flavonoids content was monitored by applying alkaline reagent, magnesium ribbon and lead acetate test. In alkaline reagent test, the yellow reaction mixture changed to a colorless solution indicated the presence of flavonoid. In case of Shinoda the crimson color appeared while in lead acetate test shows yellow precipitation (Table 2). These results showed the presence of flavonoids in Pinus halepensis Mill. needles and are in accordance with the results reported by Meziti et al., 2019.

Glycosides

The presence of glycosides was observed by performing Salkowski's , Keller-Kiliani and Liebermann's test. In Salkowski's test reddish-brown color appeared while in case of Keller-Kiliani test brown ring was formed after the addition of concentrated sulphuric acid whereas in Liebermann's test green color appeared (Table 2). The color formation indicates the pressence of glycoside in the sample. The results are in correlation with the work reported by Al-Bazaz et al., 2018.

Phytosterols

The phytosterols content were monitored by Libermann-Buchard's and Salkowski's test. In Libermann-Buchard's test, an array of color changes was observed while in Salkowski's test a brown ring formed at the junction of two liquid indicated the presence of phytosterols (Table 2). The results are in agreement with the study of Cheikh-Rouhou et al., 2008. They stated the existence of phytosterols in seed oil of Pinus halepensis Mill. in Tunisia.

Steroids

The test for steroids was performed to determine the presence of steroids in needles of Pinus halepensis Mill. The formation of a bluish brown ring after the addition of concentrated sulphuric acid that indicated the presence of steroids (Table 2). The result is in correlation with the results of Mohareb et al., 2017. Their study also revealed the presence of steroids in leaf and bark extracts of Pinus halepensis of Libya.

Terpenoids

The presence of terpenoids was determined by performing Salkowski's test. The appearance of reddish-brown color at the interface after addition of few drops of concentrated sulphuric acid showed the presence of terpenoids (Table 2). The result is in accordance with the work of Mohareb et al., 2017, they also reported the presence of terpenoids in leaf and bark extract.

Phenols

Ferric chloride test was performed to monitor the presence of phenols in Pinus halepensis Mill. needles. In the ferric chloride test bluish-black color was observed (Table 2). This result confirmed the presence of phenols and are in agreement with the results of Pasqualini et al., 2003. They also revealed the presence of phenols in needles of Pinus halepensis Mill. sample collected from different locations.

Saponins

To monitor the presence of saponins foam test was performed. A persistent foam appeared that indicated the presence of saponins in Pinus halepensis Mill. (Table 2). The result is not in accordance with

the studies conducted by Atul Kabra et.al., (2013) reports the absence of saponins while our results are in agreement with Mohareb et al., 2017 that also reported the presence of saponins in leaf extract.

S. No.	Phytochemical Constituent	Observation	Inference	
			Pine Needles in 70% Methanol	Pine Needles in Pure Methanol
1	**Carbohydrate**			
	Molisch	Ring formation	++	++
	Fehling	Red ppts	++	++
	Benedict	Reddish green ppts	++	++
2	**Proteins and amino acids**			
	Biuret	No color change	--	--
	Xanthoproteic	Brick red color	--	--
	Ninhydrin	Deep blue color	++	++
3	**Alkaloids**			
	Mayer	White creamy ppts	++	++
	Wagner	Reddish brown ppts	++	++
	Dragendroff	Reddish brown ppts	++	++
	Hager	Yellow ppts	++	++
4	**Flavonoids**			
	Alkaline reagent	Yellow to colorless	++	++
	Shinoda	Crimson color	++	++
	Lead acetate	Yellow ppts	++	++
5	**Glycosides**			
	Salkowski	Reddish brown color	++	++
	Keller-Kiliani	Brown ring	++	++
	Liebermann	Green colour	++	++
6	**Phytosterols**			
	Libermann-Buchard	Color change	++	++
	Salkowski	Brown ring	++	++
7	**Steroids**	Bluish brown ring	++	++
8	**Terpenoids**			
	Salkowski	Reddish brown color	**++**	**++**
9	**Phenols**			
	Ferric chloride	Bluish black color	**++**	**++**
10	**Saponins**			
	Foam test	Persistent foam	++	++

Table 2. Qualitative phytochemical screening of Pinus halepensis Mill. needles.

Antibacterial Activity

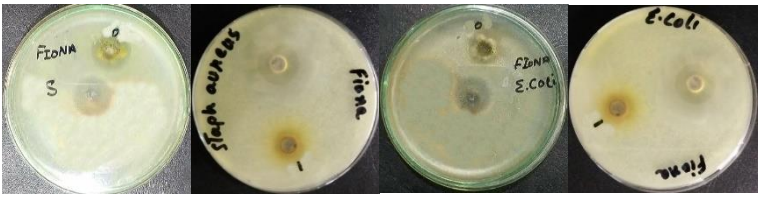
The antibacterial activity of pure and 70% methanol fraction against Gram-positive and Gram-negative bacterial strains that are Staphylococcus aureus and Escherichia coli respectively are presented in Table 3.

Bacteria Type	Pathogenic Strains	Diameter of inhibition zones (mm)	
		Extract	
		Pine needles in Pure Methanol	Pine needles in 70% Methanol
Gram positive	Staph aureus	14mm	8mm
Gram negative	E.coli	10mm	7mm

Table 3. Antibacterial activity of methanolic extracts

The inhibition zone observed against Staphylococcus aureus was 14mm for the extract of pine needles in pure methanol while 70% methanol showed value of 8mm. Similarly, needles of Pinus halepensis Mill. also showed antibacterial activity against Escherichia coli. The inhibition zone observed for the pure methanolic extract of pine needles was 10mm and for 70% methanolic extract was 7mm. The results attained are in correlation with the study of Salim et al., 2019. The positive control used against E.coli was ampicillin with an inhibition value of 24mm and against Staph aureus, the positive control used was streptomycin with an inhibition value of 20mm. In all experiments performed the negative control used was NaCl with no inhibition zone. Hence, it is concluded that the activity of both Gram positive, Staph aureus, and Gram negative, E.coli can be inhibited by the leaves of Pinus halepensis Mill.

In recent years, the remarkable interest of researchers has been seen in developing novel antimicrobial agents to combat microbial resistance. Several studies showed that extracts of various parts of Pinus halepensis Mill. bear antibacterial activity. Fekih et al., 2014 reported that essential oil from the aerial parts such as needles, buds, and twigs of Pinus halepensis Mill. has antimicrobial properties against different bacteria. Hence, can be utilized in the food and pharmaceutical industries. Aloui et al., 2021 recently proved that essential oil from needles of Pinus halepensis Mill. exhibit antibacterial activity against various Gram positive and Gram negative bacteria. This is due to the presence of phenolic compounds like terpenoids, flavonoids, and alkaloids. The antibacterial agents are present in Pinus halepensis Mill. needles might play important role in wound healing as they protect wound from infections and microbial attacks (Süntar et al., 2012). The methanolic extracts of Pinus halepensis Mill needles showed good inhibitory effects towards Staph aureus and E.coli.



a) Pure methanol b) 70% Methanol c) Pure methanol d) 70% methanol

Figure 1 shows antibacterial activity against staph aureus (a, b) and E.coli (c, d).

CONCLUSION

Pinus halepensis Mill. is an extensively spread species of pine present in Quetta Valley . The present study shows that methanolic extracts of Pinus halepensis Mill needles consist of various phytochemical compounds like carbohydrates, alkaloids, flavonoids, glycosides, phytosterols, steroids, terpenoids, phenols, and saponins. These phytochemical compounds bear several properties for which they can be used to treat diseases.

These phytochemicals are accountable for the antibacterial characteristics of the plant. The needles showed antibacterial activity against Staph aureus and E.coli. Staph aureus is known to cause food poisoning, boils, and impetigo whereas E.coli is known to cause pneumonia, traveler's diarrhea, urinary tract infection, bacteremia, and many other clinical infections. The antibacterial agents present in Pinus halepensis Mill needles can be used to treat such diseases. As far as we know, we report the foremost study on the characterization, antimicrobial properties of leaves extract of Pinus halepensis Mill. of Quetta, Balochistan.

FUTURE RECOMMENDATIONS

Regardless of exploring many chemical compounds, the complete complexity of phytochemicals remains to be identified. Further investigations are necessary to understand the correlation between the biological properties and chemical composition and to recognize the medicinal properties of the molecular entities present.

ACKNOWLEDGEMENT

We express our sincere thanks and gratitude to the entire faculty members of Institute of Biochemistry, University of Balochistan for supporting this research.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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