Phytochemical and Antimicrobial Screening of *Pistacia Khinjuk* Plant Extract

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Abstract
To investigate medicinal properties of *Pistacia khinjuk* plant, study was conducted to determine mechanisms of antioxidant and anti-bacterial activity of crude plant extracts. Phytochemical analysis was done of four different solvent extracts (n-Hexane, methanol, ethanol and ethyl acetate) of *P. khinjuk*. Antioxidant and anti-bacterial activity were evaluated through 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay method and antibiotic susceptibility testing. Fourier Transform Infrared (FTIR) Spectroscopy was performed to study nature of chemical bonds to determine types of constituents present in the plant. Solvent extracts were used in different concentrations from 20-160µl; ethyl acetate (100-160µl) and methanol (140-160µl) extracts displayed strong scavenging effect with decrease in absorbance under Ultra-violet (UV) spectroscopy. Interferogram from FTIR spectroscopy revealed chemical bonds which confirmed presence of bioactive compounds found through phytochemical analysis. Crude plant extracts exhibited effective anti-bacterial activity, compared to positive control ceftriaxone (zone of inhibition (ZI) = ≥21mm), against urinary tract pathogens (UTPs) *Klebsiella*(*K.*) *pneumoniae* and *Pseudomonas*(*P.*) *aeruginosa* (ZIs = 24-26mm). Extracts moderately inhibited growth of *Staphylococcus*(*S.*) *aureus* (ZI = 25-27mm) and presented no effect against *Escherichia*(*E.*) *coli* (ZI = 19, 22, 24). The overall results demonstrated that crude extracts of *P. khinjuk* plant contain flavonoids, phenolics and other compounds that show significant antioxidant and anti-bacterial activity even against multidrug resistant (MDR) pathogens.
Key words: Antioxidant Activity; Antimicrobial Activity; FTIR Spectroscopy; *Pistacia khinjuk*

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INTRODUCTION

Management of wounds involves dressing, medication and traditional use of medicinal herbs is an old practice in many folk medicines [1]. *Pistacia atlantica* and *Pistacia khinjuk* are two important medicinal plant species in family Anacardaceae [2]. They are well-known therapeutic plants. The *P. khinjuk*'s fruiting body is an edible part, having long history in human therapeutics as well as flavoring agent in food industries. Seeds of *P. khinjuk* are abundant in fibers, starch, vitamins, minerals, unsaturated fats, proteins and certain elements like magnesium and potassium [3]. Its leaves, stems, flowers and roots are rich in essential oils [4] such as α-pinene, α-Terpinolene, β-caryophyllene, β-pinene, γ-terpinene myrcene, garmacrene B and spathulenol. Similarly, methanolic extracts of *P. khinjuk* contain flavonoids and phenolics [5]. Globally, most of the people rely on traditional medicine and use medicinal plants for curing diseases. In this aspect, *P. khinjuk* has anti-inflammatory, antipyretic, antioxidant, anticancer, antimutagenic and antimicrobial activity [4,6]. Its leaves' extract and endospores oils have found to have wide-spectrum antimicrobial and antimutagenic activity [6,7]. Its methanol extracts such as phenolics [8], the seeds containing Vitamin E and C [9] and sterols in cell membrane [2] are free radical scavengers (antioxidants). Likewise, methanol crude extracts of *P. khinjuk* show wound healing effect [5] and fruit oils have anti-inflammatory and antipyretic activity (10). Researchers have illustrated that *P. khinjuk* cut down blood lipid profile and its endospore oils, in specific, decrease risks associated with Coronary Heart Disease (CHD) [3]. Its bark, leaves, aerial and fruit parts have been used in traditional treatments [11]. Its role in treatment of asthma, eczema, nausea, toothaches, stomach ulcers, gastrointestinal disorders and throat infections cannot be neglected at all [12] . *P. khinjuk* plant is also useful in reducing complications associated with hyperlipidemia, cardiovascular diseases and diabetes mellitus. Its low sodium and magnesium content and excess dietary fibers and Low-Density Lipoprotein Cholesterol (LDLC) prevent obesity and hypertension and control cholesterol level in the body [13]. Antibiotic discovery has remarkable importance in history of humankind but development of antibiotic resistance in pathogens raised serious questions among scientists. For example, *Staphylococcus(S.) aureus* which was once only resistant to methicillin is now become a pan-resistant superbug [14] . The same is the case for *Streptococcus (S.) pneumoniae* and *Escherichia(E.) coli*. This resistance development in pathogens led to the discovery of other mechanisms for combating these microbes. One of the mechanisms is use of plant secondary metabolites as allopathic medicine. As previously said, *P. khinjuk* has wide-spectrum antimicrobial activity, it has activity against many pathogens including *E. coli*, *Pseudomonas(P.) aeruginosa*, *S. aureus*, *S. pneumoniae*, *Candida(C.) albicans* and many others [7]. In this respect, we will investigate, in the present study, the functional groups of *P. khinjuk* plant via FTIR spectroscopy and identify phytochemicals and check their antibacterial activity against selected pathogens.

METHODS

Study design and sampling

The study was carried out from September 2018 to May 2019 at Department of Microbiology and Biotechnology, Abasyn University Peshawar campus. Samples were collected from mountains of the Khyber, the Gilgit and the Chitral region and confirmed as *P. khinjuk* fruits from botany department university of Peshawar and Pakistan council of scientific and industrial research center (PCSIR) Peshawar. Samples were washed with distilled water, air dried (under shade), grinded, packed in sterile polythene pouches and stored at room temperature for further use.

Preparation of crude extract of *P. khinjuk* Plant

Ethyl acetate, Ethyl alcohol, Methanol, n-Hexane, and Water for injection (WFI) were used as solvents for extraction of bioactive compounds from *P. khinjuk* fruits. One kilogram (kg)/liter (L) of sample solution was made in each solvent and mixed well and kept at room temperature for several hours before use. The soluble components in a mixture were separated and filtered using Whatman filter paper and remaining solution was dried using vacuum pump with rotary evaporator under reduced pressure at different temperatures (for solvents). The resultant semi-solid crude extract was stored in sterile bottles at 4°C. For antibacterial activity, five milligram (mg) of crude extract was dissolved in 4 milliliter (ml) of Dimethyl sulfoxide (DMSO) solution.

Phytochemical analysis

Phytochemical analysis was performed for detection of bioactive compounds; Alkaloids, Tannins, Anthraquinones, Reducing Sugars, Saponins, Flavonoids, Phlobatannins, Steroids and Terpenoids [15-17]. For glycosides, Hikino and colleagues' [18] method was used (with slight modifications). Following are procedures used in present study.

For Alkaloids: 200mg of crude extract was dissolved in 2% H_2SO_4 and heated for two minutes followed by cooling at room temperature. The solution was filtered and few drops of Dragendorff's reagent was added. An orange red coloration indicated Alkaloids.

For Tannins: One gram (1g) of extract was dissolved in 50ml distilled water and heated on water bath. The solution was filtered and few drops of $FeCl_3$ were added. The dark green color indicated Tannins.

For Anthraquinones: 1.5g extract was dissolved in 10% HCL for few minutes and filtered. The solution was cool down, 10ml of chloroform was added and few drops of NH_3 were mixed followed by heating. Appearance of rose pine color showed Anthraquinones.

For Reducing Sugars: 1g of extract was dissolved in 10ml distilled water and filtered. Few drops of Fehling's solution (A and B) were added into the filtrate and kept for few minutes. An orange red precipitate showed the reducing sugars.

For Saponins: 10ml distilled water was added to 2g of plant extract and heated with vigorous shaking. Small bubbles or foam formation indicated presence of saponins.

For Flavonoids: To the 2g of extract, 1ml of 2N NaOH was added. Presence of yellow color showed flavonoids in the extract.

For Phlobatannins: Addition of 10ml distilled water in 1g of extract followed by filtration and boiling of solution with 2% HCL produces red precipitates in the presence of Phlobatannins.

For Steroids: To the 1g of extract, 2ml of acetic anhydride and 2ml of H_2SO_4 was added. The color change from violet to blue or green showed presence of steroids in the extract.

For Terpenoids: 2ml chloroform was added to 1g of extract followed by carefully adding 3ml concentrated H_2SO_4. A reddish-brown color signified terpenoids in the extract.

For Glycosides: 1g extract was hydrolyzed with HCL and neutralized with NaOH solution proceeded by addition of few drops of Fehling's solution (A and B). The brick-red precipitation suggested glycosides in the extract.

Antioxidant activity of *P. khinjuk* crude extracts

Principle

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay method (19) was used with some modifications for evaluating antioxidant activity of *P. khinjuk* plant. DPPH solution, solvent extracts of plant and positive control ascorbic acid (vitamin C) were included in the requirements. A 0.1mM DPPH solution was made in ethanol. Different concentrations (20-160) in µg/ml of Plant were made in four different solvents (Ethyl alcohol, n-Hexane, Ethyl acetate, and Methanol). Two hundred microliter of each sample solution was mixed with 1800µl DPPH solution to obtain 2ml final solution. Incubation was carried out at room temperature for 20-40 minutes in dark because this method is highly sensitive to light. Antioxidant activity was measured at 517nm and detected by discoloration rate or reduction in absorption of the purple DPPH solution. Inhibition percentage (IP) or scavenging activity of antioxidant compounds (if any in the extract) was calculated using a formula given below

$$\text{Scavenging activity}(\%) = \frac{A_b - A_a}{A_b} \times 100$$

Here, A_b and A_a represents the absorbance of blank (DPPH solution without extract) and extract (DPPH solution with extract). Ascorbic acid (Vitamin C) was used as a reference (positive control) antioxidant compound and DPPH solution without any extract/Vitamin C was used as a control.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy is a technique used to determine types of chemical bonds representing functional groups present in phytochemical compound. As light travels in waves and chemical bonds can absorb light energy therefore, principle of FTIR spectroscopy is detection of wavelength of light absorbed by a chemical compound and its interpretation on FTIR graph on a computer. In present study, 10mg of all solvent extracts were encapsulated separately in 100mg KBr (potassium bromide) pellet to form a translucent sample disk. These samples were loaded onto the central region of the Shimadzu (IR Affinity 1, Japan) FTIR spectroscope and the scan range was adjusted from 400-4000cm^{-1} with resolution power of 4cm^{-1} [20].

Microbiology of the study

Sampling and study design

Urine samples (75 in number) were collected from patients at Khyber Teaching Hospital (KTH), Hayatabad Medical Complex (HMC), Lady Reading Hospital (LRH)) and Rehman Medical Institute (RMI) in the Peshawar. Samples were immediately transported to Microbiology Laboratory of the Abasyn University, Peshawar. Nutrient agar media was used for general purpose growth and Cysteine Lactose Electrolyte Deficient (CLED) media was used for selective growth as well as isolation of urinary tract pathogens (UTPs). MacConkey agar media was used for selective isolation of Gram-negative lactose fermenting bacilli and Mueller Hinton agar (MHA) media was used for cultivation of Neisseria species and determination of antibacterial susceptibility testing of isolated microorganisms.

Gram staining and biochemical analysis

Gram staining [21] and biochemical tests [22] were performed as per standards for identification of bacteria. Catalase, oxidase, urease, coagulase, indole, citrate and triple sugar iron (TSI) tests were performed in present study. Catalase and coagulase test are usually performed for differentiation of UTP staphylococci from other bacteria. Oxidase and indole test were performed for identification of cytochrome c oxidase enzyme producing bacteria and tryptophan degrading bacteria. Indole test was also used for differentiation of *Escherichia(E.) coli* from other enterobacteria. Urease and citrate tests were used, in general, for identification of enterobacteria and TSI specifically for sulphur reducing enterobacteria.

Antibacterial activity of plant extracts

Kirby-Bauer disk diffusion method was used for determining antibacterial activity of plant extracts [23]. In short, a sterile disc paper soaked in known volume of plant extract was retained on MHA

media and incubated at 37°C for 24-48hours. As volume of plant extracts diffused from higher to lower concentration, it created zone of inhibition (ZI) around each plant extract that showed antibacterial activity. The ZI was measured in millimeters (mm) with mm scale [24].

RESULTS

The present study performed phytochemical, antioxidant, FTIR spectroscopy and antibacterial analysis of the *P. khinjuk* plant. For phytochemical analysis, different tests were applied for detection of secondary metabolites in different solvent extracts of the *P. khinjuk* plant (Table 1).

Table 1. Phytochemical analysis of P. khinjuk Plant extracts.

Solvent extracts	Tannins	Anthraquinones	Glycosides	Saponins	Flavonoids	Phlobatannins	Steroids	Terpenoids	Alkaloids	Reducing sugar
Ethyl alcohol	+	-	-	-	+	-	+	+	-	+
n-Hexane	-	-	-	-	+	-	+	+	-	+
Ethyl acetate	+	-	-	-	+	-	+	+	+	+
Methanol	+	-	-	-	+	-	+	+	+	+

Note: '+' for detected; '-' for not detected

The Flavonoids, Steroids, Terpenoids, Reducing sugars and Tannins (except for n-Hexane) were found in all tested extracts. Alkaloids were only present in the ethyl acetate and methanolic extracts whereas tests for other bioactive compounds (Anthraquinones, Glycosides, Saponins, and Phlobatannins) were negative for all extracts.

DPPH assay method was applied for evaluating antioxidant activity of *P. khinjuk* plant. All extracts showed similar pattern of decrease in absorbance with increase in concentration under UV spectroscopy. This decreasing pattern in absorbance of solution indicated higher antioxidant activity of solvent extracts at higher concentrations. Highest antioxidant activity (Inhibition percentage) was observed in ethyl acetate extracts followed in descending order by methanol, ethanol and n-Hexane extracts. Compared to positive control Ascorbic acid (AA), best antioxidant activity was determined in ethyl acetate extracts at 100-160µg/ml concentrations and in methanol extracts at 140-160µg/ml concentrations respectively (Table 2).

Table 2. Antioxidant activity of *P. khinjuk* plant extracts and standard antioxidant at various concentrations.

Concentration (µg/ml)	Ultraviolet absorbance in nm (Control absorbance = 0.219)				
	P. khinjuk plant extracts in different solvents				
	ME (IP%)	EE (IP%)	EAE (IP%)	n-hx (IP%)	AA (IP%)
20	0.096 (56%)	0.083 (62%)	0.090 (58%)	0.113 (48%)	0.039 (82%)
40	0.075 (65%)	0.079 (63%)	0.070 (68%)	0.090 (58%)	0.033 (84.9%)
60	0.060 (72%)	0.075 (65%)	0.056 (74%)	0.087 (60%)	0.034 (84.4%)
80	0.053 (76%)	0.072 (67%)	0.042 (80%)	0.075 (65%)	0.033 (84.9%)
100	0.040 (81%)	0.068 (68%)	0.027 (87%)	0.060 (72%)	0.031 (85.8%)
120	0.032 (85%)	0.063 (71%)	0.020 (90%)	0.055 (74%)	0.030 (86%)
140	0.024 (89%)	0.058 (73%)	0.014 (93%)	0.048 (78%)	0.029 (86.7%)
160	0.014 (93%)	0.054 (75%)	0.007 (96%)	0.036 (83%)	0.028 (87%)
Mean	0.04925 (77%)	0.069 (68%)	0.04075 (81%)	0.0705 (67%)	0.0321 (85.21%)

Note: ME = Methanol extract; EE = Ethanol extract; EAE = Ethyl acetate extract; n-hx = n-Hexane extract; AA = Ascorbic acid extract; IP = Inhibitory percentage.

FTIR spectroscopy of solvent extracts revealed important functional groups with strong bonding appearance ranged from 4000-1500 Infrared radiation (IR) spectra and confirmed by interferograms (Figure 1-4).

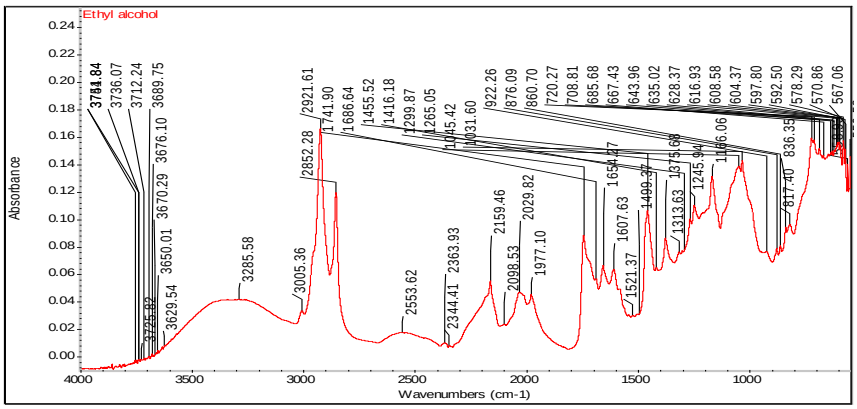

Figure 1. FTIR- Spectrum wave numbers of Ethanol extract of the *P. khinjuk* Plant.

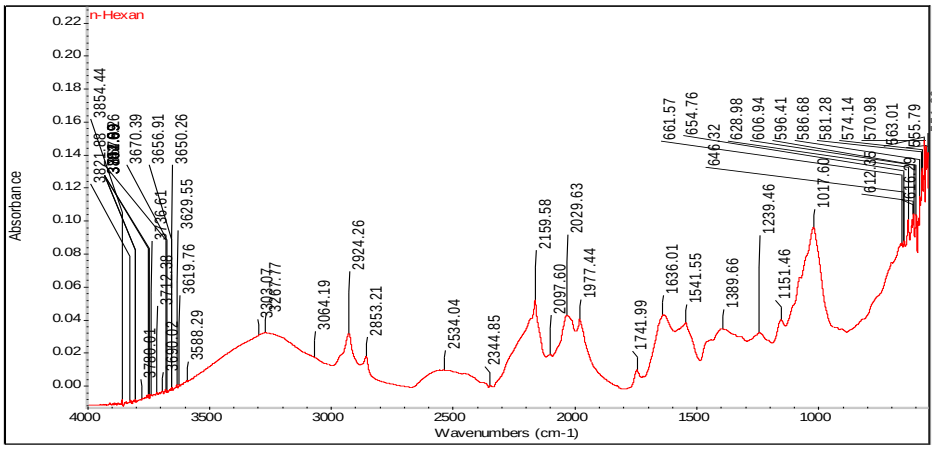

Figure 2. FTIR- Spectrum wave numbers of n-Hexane of the *P. khinjuk* Plant.

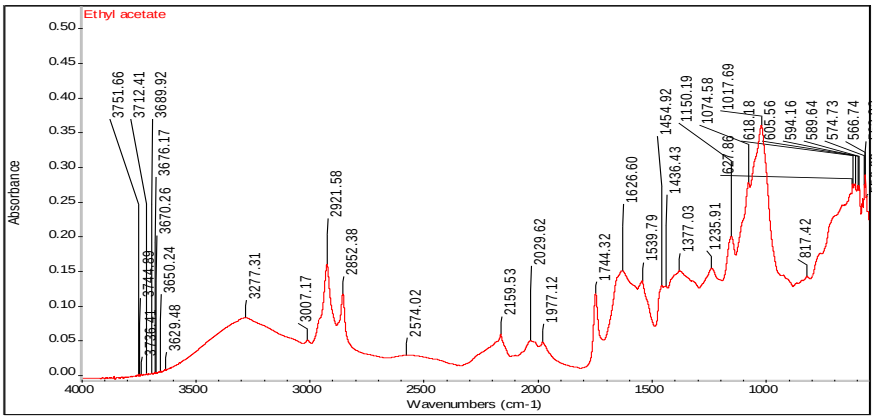

Figure 3. FTIR- Spectrum wave numbers of Ethyl acetate extract of the *P. khinjuk* Plant.

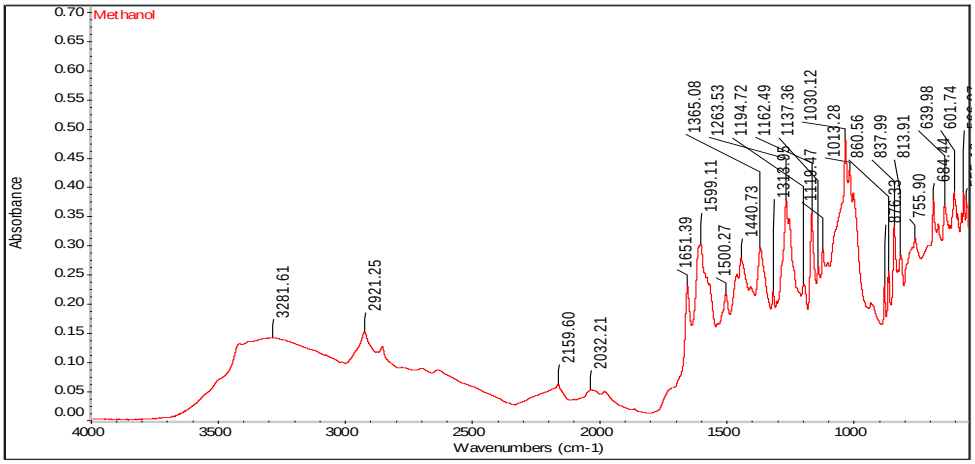

Figure 4. FTIR- Spectrum wave numbers of Methanol extract of the *P. khinjuk* Plant.

These functional groups included Alcohols (3689-3285), Aliphatic primary amines (3303), Carboxylic acids (3267), Alkynes (3218), C-H alkenes (3064), Alkanes (2921), Thiocyanates and Azides (2159), Isothiocyanates (2032), Allenes (1977), Aromatic compounds (1741), C=C Alkenes (1651), Cyclic alkenes (1599) and Nitro compounds (1541). Only alcohols, carboxylic acids and

allenes were primary functional groups found in all solvent extracts except for absence of alcohols in Methanol extract and Carboxylic acids in Ethyl acetate extract. Other functional groups were either present only in single extract or in two of all solvent extracts.

Fifty (50 in number) out of 75 urine samples were positive for bacterial growth on culture media. Presumptive analysis revealed presence of four bacterial species (*K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *E. coli*) through biochemical identification and Gram staining (Table 3).

Table 3. Gram staining and biochemical identification of bacteria.

Identified Microorganisms	Catalase	Urease	Coagulase	Oxidase	Indole	Citrate	TSI	Gram staining
Escherichia(E.) coli	+	-	-	-	+	-	A/A	-
Klebsiella(K.) pneumoniae	+	+	-	-	+	+	A/A	-
Pseudomonas(P.) aeruginosa	+	-	-	+	-	-	N/C	-
Staphylococcus(S.) aureus	+	+	+	-	-	+	N/C	+

Note: TSI = Triple sugar iron; '+' = positive; '-' = negative; N/C = No Change; A/A = Acid production

Antibacterial activity of solvent extracts of *P. khinjuk* plant was checked against identified bacterial species using MHA media. Ceftriaxone was used as positive control antibiotic and DMSO solution as negative control. All extracts, ceftriaxone and DMSO solution were used at 100µl concentration. All extracts effectively inhibited all bacterial isolates of present study except *E. coli* as compared to published ZI ranges: 16-19mm (*K. pneumoniae*), 18-22mm (*P. aeruginosa*), 25-31mm (*S. aureus*) and 29-35mm (*E. coli*) (Figure 5). Effective ZIs (24-26mm) were appeared against *K. pneumoniae* and *P. aeruginosa*, lower to middle ZI ranges (25-27mm) against *S. aureus* and no effective ZI (19, 22, 24mm) against *E. coli*. The published ZI range for positive control ceftriaxone is ≥21mm and all extracts displayed good antibacterial activity against three of four UTPs isolated in present study. The blank solution DMSO did not show any antibacterial activity against all bacteria and is therefore not depicted in Figure 5.

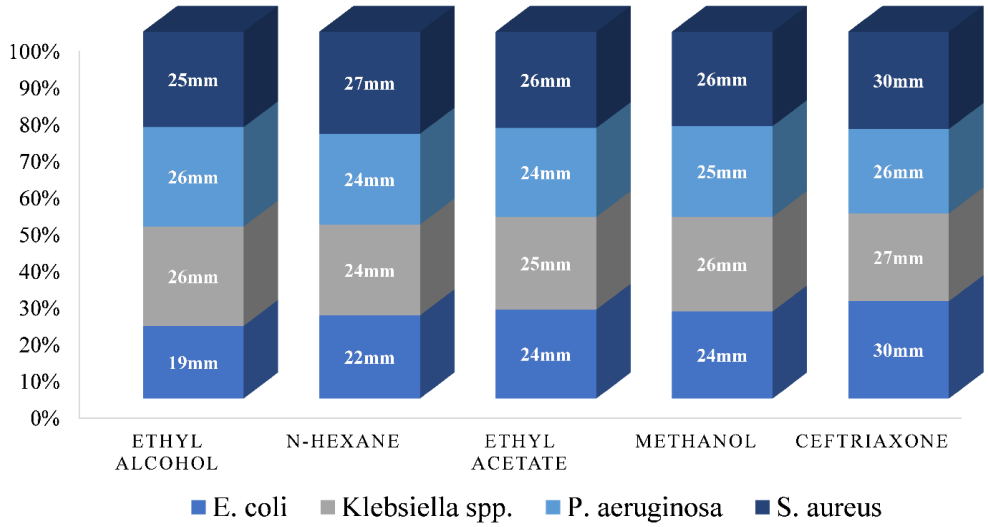

Figure 5. Antibacterial activity of solvent extracts of *P. khinjuk* plant.

DISCUSSION

Medicinal plants have a prolonged history in human life. Traditional healers and tribal people have been using medicinal plants since ancient times. Some species of Pistacia including *P. khinjuk* plant are medicinal in nature and contain important secondary metabolites that show life-extending properties. In present study, phytochemical analysis of different solvent extracts of *P. khinjuk* plant revealed presence of flavonoids, steroids, terpenoids, reducing sugars, tannins and alkaloids. The presence of these secondary metabolites in *P. khinjuk* plant proved its medicinal importance in traditional systems. This is confirmed by previous studies which have reported anticancer, antimutagenic, antioxidant, antimicrobial, anti-inflammatory, anti-pyretic, anti-diabetic, antihyperlipidemic, antidiarrheal and wound healing activity of the above-stated secondary metabolites [4–8,12-13,32] .

Antioxidant activity of *P. khinjuk* plant extracts was evaluated through DPPH assay method and UV spectroscopy. DPPH is a free radical compound having strong scavenging effect on antioxidant compounds and hence, is a best choice for analysis of antioxidant activity of plant extracts. The present study found good antioxidant activity of *P. khinjuk* plant in all solvent extracts. However, ethyl acetate (Mean value = 0.04075, 81%) and methanol extracts (0.04925, 77%) confirmed to have robust antioxidant activity. The results of present study coincide with the published studies probably because methanol and ethyl acetate extracts of *P. khinjuk* plant contain higher percentage of flavonoids and phenolics which both are potent antioxidants in nature [8,25].

FTIR technique is a useful spectroscopy which identifies type of organic/inorganic compound or functional group present in a plant. Commonly found functional groups of *P. khinjuk* plant revealed in present study were carboxylic acids, alcohols, and allenes. Besides were the aliphatic primary amines, alkanes, alkenes, alkynes, cyclic alkenes, thiocyanates, isothiocyanates, azides, aromatics and nitro compounds. FTIR analysis in present study is in accordance with the previous studies that conducted research on *Ichnocarpus frutescens* [26], *Sageretia thea* [27] ,*Solanum torvumto*

[28] and *Gmelina asiatica* [29] plants with slight inconsistency for aldehydes and alkyl halides in *Gmelina asiatica* and chlorides in *Sageretia thea*.

With emergence of antibiotic resistance in bacteria, anti-bacterial plant species are of major focus in recent years. Good antibacterial activity was observed in different solvent extracts of the *P. khinjuk* plant in present study, making the plant promising alternative to commercially available antibiotics against UTPs. In overall comparison, ZIs (24-26mm) against UTPs *K. pneumoniae* and *P. aeruginosa* were more than their published ZI ranges (16-19mm and 18-22mm). Against *S. aureus,* lower to middle ZI ranges (25mm, 26mm, 27mm) were detected compared to published ZI ranges (25-31mm). Our results indicated significant antibacterial activity of *P. khinjuk* fruit extracts compared to previous study which conducted research on antibacterial activity of *P. khinjuk* [19] and other plants [30,31] against *S. aureus*, *P. aeruginosa* and *E. coli*. ZIs showed against UTPs in previous studies were too low to compare with present study and published ZI ranges.

CONCLUSION

In situation of antibiotic resistance and limited therapeutic options to treat multidrug resistant bacteria, present study demonstrates strong antioxidant and anti-bacterial activity of *P. khinjuk* fruit extracts.

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Ethical Issue
None to declare

Conflict of interest
Authors declare no conflict of interest

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