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EVALUATION OF PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL EFFICACY OF *MESUA FERREA* AND *PIPER CUBEBA* FRUIT EXTRACTS AGAINST MULTIDRUG-RESISTANT BACTERIA

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ABSTRACT

Objective: To investigate the *in vitro* antibacterial potential, phytochemical constituents and thin-layer chromatography (TLC) bioautography of *Mesua ferrea* and *Piper cubeba* fruit extracts. The antibacterial activities of various solvent extracts prepared were assessed against five multi-drug resistant clinical isolates from both Gram-negative and positive bacteria isolated from human infection. **Materials and Methods**: The antibacterial activity of solvent extracts from *Mesua ferrea* and *Piper cubeba* was evaluated by agar well diffusion method. The antibacterial activity was evaluated using the TLC-bioautographic method. Preliminary phytochemical analysis was conducted. **Results**: All the evaluated extracts exibited moderate to significant antibacterial activity against all clinical isolates except *Klebsiella* sp. and *Pseudomonas aeruginosa*. The maximum zone of inhibition was found to be 22.5 ± 0.90 mm in *M. ferrea* and17.6 ± 0.80 mm in *P. cubeba* against *Enterococcus* sp. The qualitative phytochemical analysis showed the presence of most of the phytochemicals such as flavonoids, steroids, reducing sugars, cardiac glycosides and triterpenoids in all the solvent extract tested. TLC and bioautography agar overlay assay of hexane extracts of *M. ferrea* indicated flavonoids as the active compounds against multidrug-resistant *Enterococcus* sp. **Conclusions**: The solvent extracts of both the plants possessing antibacterial potential against clinical isolates can be utilized in the treatment of microbial infections.

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Introduction

During the last few decades, antimicrobial resistance has been recognized as a global issue leading to ineffective treatment of infectious diseases across the world [1, 2]. The WHO in February 2017 published its first list of antibiotic-resistant pathogens threatening human health categorized as the first and second level of critical priority pathogens. [3] It includes carbapenem-resistant (*Pseudomonas aeruginosa, Acinetobacter baumannii*), methicillin-resistant (*Staphylococcus aureus),* vancomycin-resistant (*Enterococcus faecium),* fluoroquinolone-resistant (*Campylobacter* spp., Salmonellae, and cephalosporin) and many more. In the 21st century, the major challenge for human health is antibiotic resistance. The resistance to an antibiotic is resulting in inexpensive treatment, prolonged illness and high risk of death [4]. Moreover, over the last 20 years, there is a declining trend in the production of novel antibiotics for the treatment of drug-resistant pathogens. [5] Plants act as a rich source of many medicinal compounds due to their high efficacy and no side effects. Researchers are analyzing various bioactive compounds from plants that are urgently needed to combat life-threatening pathogens. In recent years, an increasing number of studies have been performed to discover and investigate new bioactive compounds of the plants for controlling antibiotic-resistant bacteria.

Mesua ferrea Linn, also known as Nagkesar is a medium-sized glabrous tree belongs to family Calophyllaceae. It originates from tropical parts of India, Srilanka, Thailand, Malaysia, and the Philippines. It can grow up to 30m tall with straight and ash colored trunk. The bark is reddish-brown to ash-grey. The leaves are simple, oblong to lanceolate and 7-15 cm long. The

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flowers are large (4-7.5 cm in diameter), white in color and fragrant with many yellow-orange stamens in the center. The fruits are oblong with one or two seeds which are smooth and chest-nut brown. [6] Traditionally, different parts of this plant are used against various ailments. [7] Flowers of *M. ferrea* have been studied for antibacterial, antineoplastic, hepatoprotective, and antioxidant activities. [8] Seeds contain essential oils, coumarins, and xanthones and are used to treat gastritis, bronchitis and to cure snake bite. [9]

Tailed pepper or *Piper cubeba*, also known as Kankol is a plant cultivated for its essential oil and berries from family Piperaceae. [10] The plant is mainly cultivated in Java, Sumatra and Southern Borneo, therefore also called as Java pepper. It is a perennial plant with round branches bearing 4-6 leaves which are half inches long. Fruits are collected before ripening and the seed is hard, oily and white. Traditionally, the plant is used to treat abdominal pain, diarrhea, dysentery, enteritis, gonorrhea, syphilis, and asthma diseases. [11] Methanolic extract of *Piper cubeba* fruit demonstrated anti-inflammatory and analgesic activity [12] and the essential oil possess antiparasitic, antimicrobial and insecticidal activities. [13] cubebol, β-elemene, and sabinene were reported to be the main components of *Piper cubeba* essential oil. [14]

The present study aimed at evaluating the *in vitro* antibacterial activity, phytochemical analysis and TLC bioautography assay of *Mesua ferrea* and *Piper cubeba* fruit extract against multi-drug resistant (MDR) Gram-negative and -positive clinical isolates.

Materials and Methods

Plant Materials and Extracts Preparation

Fruits of *Mesua ferrea* and *Piper cubeba* were purchased from the local herbal market, Noida, Uttar Pradesh, India. The specimens were authenticated and identified taxonomically. The fruit samples were washed thoroughly with tap water followed by washing with sterile water, air-dried powdered and used for extraction. Fifty grams of air-dried and powdered sample was successively extracted using a soxhlet evaporator with 200ml of each acetone, hexane, methanol, and ethanol in the increasing order of their polarity for 48h. The extracts after solvent evaporation was subsequently dissolved in 10% dimethyl sulphoxide (DMSO) to a final concentration of 50 mg/ml and stored at 4°C till use.

Test Cultures and Growth Conditions

Pure cultures of bacteria used include clinical isolates of *Klebsiella sp., Staphylococcus aureus, Escherichia coli, Enterobacter* sp*.,* and *Pseudomonas aeruginosa*. The cultures and their antibiotic resistance profiles (Table 1) were obtained from the Department of Microbiology, Rajiv Gandhi Cancer Research Institute, Delhi, India. The bacteria cultures were maintained in their proper agar slants at 4°C during the study and used as stock cultures. They were sub-cultured on to nutrient broth for 24 h before testing. These bacteria were as test pathogens to assess their antibacterial activities.

Phytochemical Screening

Hexane, acetone, methanol, and ethanol extracts obtained were assessed for the presence of various phytochemicals to determine the presence of metabolites such as tannins, steroids, reducing sugars, alkaloids, anthraquinones, glycosides, flavonoids, saponins, triterpenoids, and phlorotannins. [15]

Antibacterial Activity Assay

In order to determine the antibacterial effect of various extracts, the agar well diffusion method [16] was employed with minor modification. Inoculum containing 10^8 cfu/ml of each of the bacterial cultures were spread on nutrient agar plates using a sterile swab moistened with the bacterial suspension. Then, 100 µl (25mg/ml) of plant extract was added to the 8-mm wells punched into the agar medium and allowed to diffuse at room temperature for 2h. Subsequently, the plates were incubated at 37°C in the upright position for 24h. Wells containing the same volume of ethanol methanol, acetone, hexane, and DMSO (10%) were considered as negative controls while standard antibiotic discs of imipenem (10 µg) and vancomycin (30 µg) were used as the positive controls. All tests were donr in triplicate and the antimicrobial activity was expressed as the mean of inhibition with their standard deviation.

Thin-layer Chromatography Bioautography Assay

As determined by agar well diffusion method, Hexane extracts of *Mesua ferrea* fruit extract exhibiting significant antimicrobial potential against Enterococcus sp. were analyzed using TLC bioautography assay. [16]

Results and Discussion

Medicinal plant extracts and plant essential oils have attracted enormous attention recently because of increasing concern of MDR clinical isolates. The scientific community is looking for some natural alternates to cater to the ever-increasing cases of infectious diseases and resistant microorganisms present in the community. Therefore, the present study emphasizes on solvent extracts from two selected medicinal plants and its screening to analyze the phytocompounds, antimicrobial potential against selected MDR clinical isolates and identification and separation of a bioactive component by TLC bioautography.

Antibacterial Activity Assay

The antibacterial potential of *Mesua ferrea* and *Piper cubeba* fruit extracts was determined against five bacterial isolates by agar well diffusion technique whose antibiotic resistance profiles are as given in Table 1. The *in vitro* antibacterial activity evaluated by agar well diffusion confirmed the potential antibacterial activity of acetone and methanol extracts as compared to hexane and ethanol extracts against most of the clinical isolates. The *Messua ferrea* extracts showed significant to moderate antimicrobial activity toward tested strains except for *Klebsiella* sp. and *Pseudomonas aeruginosa*. *Messua ferrea* hexane and acetone extracts showed significant inhibitory effects against the tested cultures like *E. coli, Staphylococcus aureus*, and *Enterococcus* sp. (Table 2). Maximum inhibition was observed in the case of *Enterococcus* sp with the zone of inhibition (22.5 ± 0.90 mm) followed by *S. aureus* (20.2 ± 0.34 mm) in the hexane extract of *Mesua ferrea.* Least inhibition was observed in the case of ethanol extract. None of the *Messua ferrea* solvent extracts showed inhibition against *P. aeruginosa.* The antibacterial effect of *M. ferrea* tested against various human pathogens such as *Bacillus* sp., *Proteus* sp., *S. aureus,* and *Enterococcus* sp. has been reported by various researchers. [9, 17, 18] The results are consistent with the reports of previous investigators. However, Chandra *et al.* [9] reported antibacterial potential against various human pathogenic bacteria except for *E. coli* and *Enterococcus* sp. which was contrary to the present investigation. It is also observed that the antibacterial activity of nonpolar solvent extracts is higher as compared to solvents in increasing polarity. Similar outcomes have been demonstrated in *M. ferrea* seed extracts by Chandra *et al.* [9].

Table 1: Antibiotic resistance profile of Gram negative and positive isolates

Antibiotics	Test Bacteria				
	Ec	*E* sp	*K* sp	*Pa*	*Sa*
AK	S	R	S	S	S
AC	R	R	R	R	S
CFX	R	R	R	R	R
CS	S	R	R	S	S
CE	R	R	R	R	R
CI	R	R	R	R	R
CF	R	R	R	S	S
GF	S	R	S	S	S
G	R	R	S	S	S
I	S	S	S	S	S
LE	R	R	S	S	S
MR	S	S	S	S	S
OF	R	R	R	S	S
PT	S	S	S	S	S
VA	-	-	-	-	S
LZ	-	-	-	-	S

AK=Amikacin, AC=Amoxycillin/Clavulanic acid, CFX=Cefixime, CS= Cefoperazone+ Sulbactam, CE=Cefotaxime, CI=Ceftriaxone, CF=Ciprofloxacin, GF=Gatifloxacin, G=Gentamicin, I=Imipenem, LE=Levofloxacin, MR=Meropenem, OF=Ofloxacin, PT=Piperacillin/tazobactam, VA=Vancomycin, LZ=Linezolid, R=Resistant, S=Sensitive, *Ksp= Klebsiella species, Ec=Escherichia coli*, *Sa=Staphylococcus aureus, Pa=Pseudomonas aeruginosa,*

In the case of *Piper cubeba*, all extracts showed significant to moderate antimicrobial activity towards various cultures tested except hexane extract which showed no inhibition (Table 2). Highest inhibition was observed in the case of *Enterococcus* sp with wide inhibition zone (17.6 ± 0.80 mm) followed by *E. coli* (16.3 ± 0.75 mm) and *Pseudomonas aeruginosa* (15.3 ± 0.62 mm). Similar results were reported by Khan and Siddiqui. [19] No inhibition was observed in hexane extract when tested against different cultures. Both *Mesua ferrea* and *Piper cubeba* fruit extracts were found more effective on Gram-positive bacteria as compared to Gram-negative isolates. The results obtained confirm the observations of previous researchers. [9, 17] This differences in the activity is due to the cell wall in Gram-positive bacteria, which is single-layer, while the Gram-negative cell wall is multilayered comprising of phospholipids and lipopolysaccharides which are acting as a barrier for the entry and reaction of most antibiotics or antimicrobial agents through the cell envelope as observed by Wang *et al.* [20].

Table 2: Antibacterial activity of *Mesua ferrea* and *Piper cubeba* fruit extracts determined by agar well diffusion method

Plant	Ex	Test Bacteria (Zone of inhibition in mm)				
		Sa	***K* sp**	***E* sp**	***Pa***	***Ec***
Mesua ferrea	H	20.2 ± 0.34	-	22.5 ± 0.90	-	17.0 ± 0.11
	A	18.0 ± 0.80	9.3 ± 0.23	19.3 ± 0.10	-	19.2 ± 0.52
	M	13.2 ± 0.16	-	12.0 ± 0.11	-	15.5 ± 0.37
	E	9.7 ± 0.75	-	-	-	11.3 ± 0.44
Piper cubeba	H	-	-	-	-	-

	A	14.0 ± 0.70	14.3 ± 0.18	15.2 ± 0.52	15.3 ± 0.62	16.3 ± 0.75
	M	11.5 ± 0.30	-	17.6 ± 0.80	13.2 ± 0.06	15.0 ± 0.30
	E	9.0 ± 0.05	8.5 ± 0.10	11.3 ± 0.16	9.6 ± 0.34	8.5 ± 0.17

Sa: Staphylococcus aureus, K sp: Klebsiella sp, E sp: Enterococcus sp, Pa: Pseudomonas aeruginosa, Ec: Escherichia coli, Ex: Extract, H: Hexane extract, A: Acetone extract, M: Methanol extract, E: Ethanol Extract, Zone of inhibition is expressed as mean ± standard deviation, -: no inhibition

Phytochemical Screening

Phytochemical analysis of *Mesua ferrea* and *Piper cubeba* fruit extracts showed the presence of most of the phytochemicals such as flavonoids, steroids, reducing sugars, cardiac glycosides, and triterpenoids in all the studied extracts (Table 3). It was observed that saponins were present only in methanol and ethanol extracts of *Mesua ferrea* plant and reported present only in methanolic extract of *Piper cubeba* plant. Flavonoids were reported in all the extracts of *Mesua ferrea* plant except in ethanolic extract while present in all the extracts of *Piper cubeba* fruit extracts. None of the extracts showed the presence of anthraquinones and phlobatanins. Our results were in agreement with Barbade and Datar [21] who observed that steroids and terpenoids were found present in n-hexane *M. ferrea* stamen extract and total absence of saponins and tannins. However, Khandia *et al.* [22] reported the presence of saponins and tannins in *M. ferrea* stamen extract. Similar results for *P. cubeba* were reported by Nahak and Sahu [10] who reported the presence of flavonoids, tannins, terpenoids, and steroids in the solvent extracts.

Table 3: Phytochemical screening of *Mesua ferrea* and *Piper cubeba* fruit extracts

Phytoconstituents	**Plant extracts**							
	Mesua ferrea				***Piper cubeba***			
	H	**A**	**M**	**E**	**H**	**A**	**M**	**E**
Saponins	-	-	++	+	-	-	+	-
Flavonoids	++	+	+	-	+	+	+	+
Steroids	++	++	++	++	++	++	++	+
Tannins	-	+	++	+	-	+	++	+
Reducing Sugars	++	+++	++	+	+	+	+	++
Cardiac Glycosides	++	+++	++	+	+++	-	++	+
Phlobatanins	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-
Triterpenoids	+++	++	++	+	+++	++	+++	+

H: Hexane extract, A: Acetone extract, M: Methanol extract, E: Ethanol Extract, +: Positive, -: Negative

TLC Bioautography Assay

In TLC bioautography analysis, different compounds available in the extracts were separated using chromatographic plates. These plates are further overlaid by a molten agar medium to screen the antibacterial potential of the compounds separated. TLC analysis confirmed the presence of flavonoids, steroids, reducing sugars and triterpenoids in all the solvent extracts tested (data not shown). In the bioautography assay, hexane extract of *the Messua ferrea* plant which exhibited maximum zone of inhibition against tested isolates *Enterococcus* sp. was separated by solvent system toluene and ethyl acetate (93:7 v/v) on TLC. Inhibition zones against the growth of tested bacteria *Enterococcus* sp. was observed on the TLC plates of *Messua ferrea* fruit extract as clear spots on the pink background when sprayed with the aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride. The hexane extracts from *Messua ferrea* extract showed two inhibition zones with R_f value ranging from 0.10- 0.36 and 0.52-0.63. The big spot at R_f values of 0.10- 0.36 was found to exhibit antibacterial activity against tested *Enterococcus* sp. Whereas, the small spot with R_f value 0.52- 0.63 showed no visible inhibition against the tested strain respectively on plate B. This could be due to the evaporation of the active compounds or photo-oxidation. Sometimes the limited or insufficient quantity of the active component may also result in no inhibition. [23] Spots with R_f value ranging from 0.10 - 0.36 corresponds to the spots representing flavonoids when sprayed with 1% ethanolic aluminum chloride spray reagent (data not shown). The present findings are in line with the results obtained by Sharif *et al.* [24] and Dahiya and Purkayastha [25] who reported the antibacterial potential of flavonoids. This result suggests that the antimicrobial activity present in *Messua ferrea* hexane extract may be due to the presence of flavonoids. Inhibition zones obtained were may be due to more than one active component present in the extract which is overlapping due to the choice of solvent system utilized for screening purposes.

Conclusion

The present study revealed that both the selected plants have immense potential to be utilized as antibacterial agents against selected clinical isolates. The medicinal properties of fruit extracts of both plants evaluated in the current study can be attributed to the presence of various phytocompounds including steroids, flavonoids, triterpenoids, reducing sugars and

cardiac glycosides. The results further indicate that flavonoids present in the hexane extract of *M. ferrea* fruit has significant antibacterial properties. Hence the present finding supports the use of the hexane extract of the plant in folk medicine for the formulation of antimicrobial agents. Further studies related to Bioactivity-guided fractionation and identification of pharmacologically active components need to be done.

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Conflict-of-Interest Notification Page:
The authors declare that we have no conflict of interest.

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