DOI: 10.53992/njns.v9i2.176 NUST Journal of Natural Sciences, Vol. 9, Issue 2, 2024

Comparative analysis of antimicrobial potential of selected plant extracts against *E. coli*, *Salmonella*, and *Malassezia*

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Abstract

The present study is conducted to analyze the antifungal and antibacterial potential of some plants against three species of human pathogenic microbes i.e. *E. coli*, *Salmonella*, and *Malassezia*. The antimicrobial activity of ethanolic and methanolic extracts of *Calotropis procera*, *Prosopis juliflora*, and *Ziziphus nummularia* was assessed by employing a good diffusion method in agar. Overall results demonstrated the highly significant activity of *Ziziphus nummularia* against *E. coli*, *Ziziphus nummularia*, and *Calotropis procera* against *Salmonella* and *Calotropis procera* against *Malassezia*. Of three dilutions used, 1.0 ml, 0.8 ml, and 0.6 ml, the 1 ml dilution of methanolic extract of *Ziziphus nummularia* and *Calotropis procera* exhibited potential effectiveness against *E. coli* and *Salmonella*. While 1.0 ml dilution of ethanolic extract of *Calotropis procera* showed the best antibacterial activity. However, *Prosopis juliflora* leaf extract was found to be less effective against selected pathogens.

Keywords: Antimicrobial activities, Human pathogens, Medicinal plants, Well diffusion method.

Article History: **Received:** 22 Apr 2024, **Revised:** 8 july 2024, **Accepted**: 19 Jul 2024, **Published:** 8th Aug 2024.

Introduction

A wide variety of medicinal plants that are indigenous to Pakistan have amazing therapeutic prospects [1,2]. Medicinal plants contain many antibacterial and antifungal compounds and thus an alternate antibiotic source [3,4,5,6,7]. Plants are preferably used for such treatments because of their beneficial effects on our health [8].

There has been a rapid increase in antibiotic resistance in bacteria in the last

few decades, which poses serious problems to human health across the globe, especially for patients with weak immune systems [9]. The overuse of antibiotics and lack of advanced strategies to produce pharmaceutical products has led to the development of antibiotic resistance in bacteria [10,11,12]. This problem can be curbed efficiently with these plant-originated antibacterial compounds and in this way, various bacterial infections can be cured effectively [13,14].

Medicinal plants contain a variety of compounds with antimicrobial and antioxidant properties, which protect against pathogens and cellular oxidation in the human body. Plant therapeutics are effective for the treatment of heart diseases, cancer, and inflammatory diseases [15]. Apart from that plant medicines contain certain chemicals, which are beneficial to produce vital drugs [16,17]. Therefore, it is the need of the hour to gain knowledge about such different types of medicinal plants for their therapeutic potential [18,19].

Nowadays advancements in medicine and pharmaceutics have led microbes to change their genetic structure and metabolism and have enabled them to acquire substantial resistance against the medicines used to treat common infectious diseases [20]. Such acquired drug resistance in microbes has increased their pathogenicity making them a challenge in the field of health and medicine [21,22,23]. Bacteria have strong immunity against disease by inhibiting SOS responses and blocking various biochemical pathways to make themselves resistant to antibiotics [24].

In this context, scientists are struggling to develop alternative drugs to overcome microbial drug resistance. Antimicrobial resistance along with the lack of newly discovered drug molecules for treatment is a serious issue for the treatment of infectious diseases Kavya et al., [25], the endotoxins and increase in drug metabolism are the major cause of antimicrobial resistance [26]. Since ancient ages, plants have been used as medicine. Plants are reported to have antimicrobial, anticancer, larvicidal, hemolytic, anti-inflammatory, antioxidant, and anti-diabetic properties, etc. [27,28]. The three plants *Calotropis procera, Ziziphus nummularia,* and *Prosopis juliflora* are studied in current research. All three plants are medicinally very important and have been evaluated for their antibacterial and antifungal potential by various scientists.

Calotropis procera (Ait, AK) R. Br. (Asclepiadaceae) (Figure 1) is commonly distributed across Asia, the Middle East, and Africa [29,30]. Caloteopis procera, commonly called the apple of Sodom belongs to the Apocynaceae family and can be used as medicine, fuel derivation, and food for animals [31]. Several literature reports indicate its many therapeutic activities. Leaves of *Calotropis procera* have also been found effective in treating jaundice and in the Unani system of medicine Wadhwani et al., [32], it is also used to treat scabies, ringworm of scalps, and dropsy [33,34]. Different digestive disorders can be cured by using *Calotropis* such as constipation, stomach ulcers, and diarrhea, and for cramps, toothache, and worm's diseases Mustafa et al., [35], *Calotropis procera* is also being used for swelling, syphilis, fever, epilepsy, hysteria, leprosy, fever, gout, cancer, and snakebites [36,37].

Figure 1**.** *Calotropis procera*

Prosopis juliflora locally known as Jangli Kikar is one of the most common trees of the Pakistani provinces Sindh and Punjab, belonging to the family Mimosaceae. *Prosopis juliflora*; (Jangli kikar) an ever-green small tree or shrub with a large crown and an open canopy is also known for its medicinal value [38]. Diabetes can also be cured by using this plant [39]. *P. juliflora* (Figure 2) leaf extract is known to show antifungal, antimicrobial, anti-inflammatory, and hemolytic potential in addition to cytotoxic, antitumoral activity against human epithelial tumour cells and human hepatic tumours [40,41].

Figure 2. Prosopis juliflora

Most commonly *P. juliflora* is used as a folk remedy for Catarrh, diarrhoea, cold, excrescences, dysentery, hoarseness, sour throat, measles, and healing of wounds. The major constituents of this plant are phenolics, tannins, flavonoids, and terpenes and have a role as anthelmintic, antioxidant, antipyretic, antiulcer, cytotoxicity effect, antigiardial, and amoebicidal [42]. Decoction prepared from seed & leaf extracts is used as a disinfectant and to treat scurry. The extract of leaves exhibits very high antimicrobial activity [43].

Ziziphus nummularia, (Jangli berry) (Figure 3) belongs to the family Rhamanaceae and is found in Africa, Australia, and America and mostly in Asia [44]. This plant is used in the treatment of relief of pain, and inflammation promotes healing, and is also effective in reducing bacterial infections [45,46]. It can be used to treat fever, dysentery and mental antibacterial, and anthelmintic properties abnormalities plus has anti-inflammatory, [47]. Many diseases, like heart disease and cancer, can be treated by *Ziziphus* [48].

Figure 3. Ziziphus nummularia

Ziziphus nummularia are valued as purgative and astringent. However, its leaves are used for scabies and boils [49,50]. In the context of antibiotic resistance, medicinally important plants are essential to produce various drugs in curing diseases [51,52].

The present study was aimed to document the antibacterial and antifungal potential of methanolic and ethanolic crude extracts of *Calotropis procera*, *Ziziphus nummularia*, and *Prosopis juliflora* against isolated bacterial strains; *E. coli*, *Salmonella*, and fungi; *Malassezia.* The antibacterial and antifungal assay was performed by using the agar well diffusion method. In the diffusion essay, the wells act as reservoirs of the extract, and MIC and MFC were measured in millimetres.

Materials and Methods

Culturing of bacteria

The two bacteria (*E. coli*) and (*Salmonella*) and one fungus (*Malassezia*) were cultured in the laboratory. The sample for bacteria was sewage water and dandruff flakes for the fungi. After culturing the following identifications test i.e., (Indole Test, Citrate Test, Oxidase Test, Motility Test, Catalase Test, Urease

tests, Gram staining, Capsule staining Flagella staining, Endospore staining) were performed for bacteria and Catalase test, Esculin hydrolysis test, and Gram's staining for *Malassezia.*

Extract preparation

Collection of plant material

Leaves were preferred to check the antimicrobial activity of respective plants. The plant material (leaves) was collected from fields of Mianwali (Pakistan).

Processing of plant material

Branches were washed with running tap water and then leaves were separated from the branches with sterile forceps. Green leaves were dried in a shady place and then ground into fine powder by using a grinder.

Extraction

10.0 g dried leaves powder added in 70 % methanol and ethanol of each plant (*Calotropis procera, Ziziphus nummularia,* and *Prosopis juliflora*) was later shaken at arbitrary shaker for five days (Occasional shaking was done). The Organic extracts were filtered through filter paper to remove large plant material from extracts and then placed in a fume hood at 60 ^{o}C for fast evaporation of methanol and ethanol. The final dried crude extracts were dried and weighed and then stored in a refrigerator at 4 ^{o}C. The crude extracts of three plants were then dissolved or diluted by dimethyl sulphoxide.

Testing of antibacterial and antifungal activity

Antibacterial and antifungal activity of methanolic and ethanolic extracts of three plants i.e. *Calotropis procera, Ziziphus was* tested by the Agar well diffusion method.

Testing of antibacterial activity of methanolic extract of three plants against *Escherichia coli*

To test the activity of the methanolic extract of each plant, 25.75 g of Macconkey agar was dissolved in 500 ml of distilled water for media preparation. The media was autoclaved along with 27 petri plates to kill the germs at 121 ^{o}C temperature and 25 psi pressure. After being autoclaved, the media was poured into 27 plates and allowed to stand for a few minutes to solidify the media. 10 µl of *E. coli* strain from the nutrient broth was taken with the help of a micropipette and spread on all 27 plates (10 µl on each plate). Four wells were made with the help of tips on each plate.

After that 5 µl of each dilution of three methanolic plant extracts was poured in wells. One dilution of one plant extract was poured into three plates (triplicate). Incubation of the plates was carried out for 24 hours at 37 ^{o}C after pouring extracts into wells. On the next day, the inhibition zones were measured in mm.

Testing of antibacterial activity of ethanolic extract of three plants against *Escherichia coli*

To check the ethanolic extract's antibacterial activity of each of the three plants, the same process was repeated for the methanolic extract. Media was prepared by dissolving 25.75 g of Macconkey agar in 500 ml of distilled water and then poured into 27 plates after being autoclaved. After solidification strain was spread over plates, wells were made and after pouring of extract into wells the plates were placed in an incubator for 24 hours at 37 ^{o}C. Zones of inhibition were measured on the next day

in mm. Inhibition were measured on the next day in mm.

Testing of antibacterial activity of methanolic and ethanolic extracts of three plants against *Salmonella*

In this regard, 60 g of SS (*Salmonella*, *Shigella*) agar was added in 1000 ml of distilled water to prepare media and autoclaved along with 54 petri plates at 121 ^{o}C and 25 psi pressure to kill the germs (27 for ethanolic and 27 methanolic extracts). After being autoclaved, the media was poured into 54 plates and then on solidifying media, 10 µl of *Salmonella* strain was spread on each of 54 plates. Four wells were made with the help of tips on each plate. In 27 plates three dilutions of methanolic plant extracts were poured while ethanolic plant extracts were poured into the other 27 plates. Plates were made in triplicate for each dilution of each plant extract. All work was done carefully in the culture hood in the presence of flame to avoid any contamination. All plates were placed in the incubator for 24 hours at 37^{o}C and inhibition zones were noted on the next day.

Testing of antifungal activity of methanolic and ethanolic extracts of three plants against *Malassezia spp*

The antifungal activity of both methanolic and ethanolic extracts of plant was checked against *Malassezia*; a dandruff-causing fungi. First, media was prepared by dissolving 20 g of peptone agar, 10 g of glucose, 2 g of yeast extract, 8 g of ox bile, 10 ml of glycerol, 15 ml of tween 80, and 15 g of bacteriological agar in 1000 ml distilled water and shacked well with stirrer for complete dissolution. Media was then autoclaved along with 54 petri plates and poured into all plates. 10 ul of *Malassezia*strain was taken from nutrient broth culture with the help of a micropipette and then spread with a sterile swab. After that four wells were made with the help of tips in each plate and then three dilutions of methanolic and ethanolic extracts of *Calotropis procera*, *Ziziphus nummularia*, and *Prosopis juliflora* were poured into wells. Plates were labelled and placed in the incubator for 24 hours at 37 ^{o}C. Inhibition zones were measured after 24 hours on the next day.

Results

Comparison of different plants, extracts, and dilutions for inhibitory zones against *E. coli*

When all three dilutions i.e., 1.0 ml (50 mg/ml)**,** 0.8 ml (40 mg/ml), and 0.6 ml (30 mg/ml) were compared with different plant extracts for inhibitory zones against *E. coli*, the 1.0 ml dilution of methanolic *Ziziphus nummularia* extract showed the highest value. Whereas the 0.8 ml dilution and the 0.6 ml showed less activity. Overall results indicate the best activity of methanolic extract than the ethanolic extract. A comparison of different plants, extracts, and dilutions for inhibitory zones against *E. coli* is given in Table 1.

Comparison of inhibitory zones of selected plant extracts and their dilutions against *Salmonella*

Table 1 presents the comparison of some selected plant extracts and their dilutions for the inhibitory zones against *E. Coli*, *Salmonella*, and *Malassezia*. Table 1 shows that 1 ml dilution (50 mg/ml) of each plant extract exhibited a prominent value for the zone of inhibition. However, the 1 ml dilution (50 mg/ml) of *Z. nummularia* and *C. procera* methanolic extract had the highest zones of inhibition against *Salmonella*. While 0.8 ml (40 mg/ml) and 0.6 ml (30 mg/ml) concentrations were not found so effective.

Table 1. Comparison of antimicrobial activity of different plant species against *E. coli*, *Salmonella*, and *Malssezia.*

Plant	Extract	Concentration (mg/ml)	Inhibition zones (mm)		
			E. coli	***Salmonella***	***Malassezia***
Ziziphus nummularia	Methanolic	50	14.9	14.4	13.8
		40	14.4	14.3	13.5
		30	13.5	13.8	13.1
	Ethanolic	50	14.5	14.8	13.6
		40	13.8	13.3	13.4
		30	13.2	12.8	12.6
Prosopis juliflora	Methanolic	50	14.4	13.4	13.6
		40	13.6	13.2	13
		30	13.3	13.1	12.3
	Ethanolic	50	13.5	15.3	14.5
		40	13.4	13.1	13.3
		30	12.5	12.3	13.2
Calotropis procera	Methanolic	50	13.8	14.4	13.1
		40	13	14.3	12.3
		30	13	13.8	12
	Ethanolic	50	14.2	13.1	15.3
		40	13.2	12.9	14.3
		30	12.2	12.8	13.7

Comparison of inhibitory zones of selected plant extracts and their dilutions against *Malssezia*

A comparison of different plant extracts and dilutions for the inhibitory zones against *Malassezia* (Table 1) exhibits that 1.0 ml dilution of methanolic extract had a pronounced effect on the *Malassezia* spp, but 1.0 ml dilution of ethanolic *C. procera* leaf extract showed highest zones of inhibition.

Comparison of antimicrobial activity of different plant species against *Salmonella, E. coli* and *Malassezia*

Table 2 shows that *Ziziphus nummularia* and *Calotropis procera* presented the highest antibacterial activity against *Salmonella* and *E. coli* species with the highest mean value of zones of inhibition of 13.6 mm i.e., for both *Z. nummularia* and *C. procera*. and 14.2 mm for *Z. nummularia* respectively. Whereas *Prosopis juliflora* showed the smallest zone of inhibition i.e., 13.3 mm for *Salmonella* and 12.9 mm for *E. coli*. *Calotropis procera* was found effective against *Malassezia* species on comparison of antifungal activity of selected plants. However, *Ziziphus nummularia* and *Prosopis juliflora* both possessed the second-highest antifungal activity with a similar zone of inhibition of 13.3 mm (Table 2).

Table 2. Comparison of antimicrobial activity of different plant species and extracts against *Salmonella*, *Ecoli*, and *Malssezia*

Plant/Extracts	N	*Salmonella*	*E. coli*	*Malssezia*
		Mean values (mm)		
Ziziphus nummularia	144	13.6	14.2	13.6
Calotropis procera		13.6	13.6	13.3
Prosopis juliflora		13.3	12.9	13.3
Methanolic		13.7	13.8	13.8
Ethanolic		13.3	13.4	13.1

Comparison of different plant extracts against *Salmonella, E. Coli,* and *Malassezia*.

The methanolic extract of *Salmonella* and *E. coli* had better extraction power than the ethanolic extract when both extracts were Figure 4). In the comparison of ethanolic and methanolic extracts, for *Malassezia* species, the ethanolic extract of *C. procera* exhibited better antifungal activity than the methanolic extract (Table 2 and Figure 4.

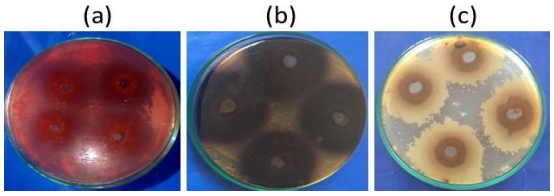

Figure 4. The highest inhibition zones of methanolic leave's extract (1 ml dilution) against **(a)** *E. coli* on Macconkey agar after 24 hrs of incubation, **(b)** *Salmonella* on SS agar with a mean diameter of 13.6 mm after 24 hrs of incubation and **(c)** *Malassezia spp* after the incubation of 48 hrs.

Discussion

Different concentrations of ethanolic and methanolic extracts (50 mg/ml, 40 mg/ml, and 30 mg/ml) employed, were screened for their antibacterial activity. However, the concentration 50 mg/ml (1.0 ml) of methanolic extract was found to have the best antibacterial properties than ethanolic extract (Table 1). The methanolic extract of *Ziziphus nummularia* was found to be more effective against *E. coli* (Table 2) significantly with $p > 0.05$ and inhibition zones of 14.1 mm followed by *Prosopis juliflora*; Keeka (13.4 mm) (Figure 4; Table 2) and *Calotropis procera*; Aak (13.2 mm); (Table 2).

The results of the present study match with the study of Beg et al., [53]. They also used the agar plate well diffusion method for the determination of the antibacterial activity of methanolic, hexane, and aqueous extract of three varieties of *Ziziphus* including *Z. jujube, Z. mauritiana,* and *Z. nummularia,* against two humans pathogenic; *E. coli* and *S. aureus*. The methanolic extract of all three varieties of *Ziziphus* showed good antimicrobial activity as compared to the aqueous extract, which failed to show a significant effect. Hexane extracts of fruit were also found to be effective against tested organisms.

According to Beg et al., [53], *Ziziphus* and its species have tremendous medicinal properties, and some other studies also reported their medicinal potential as antibacterial, neuro-protective antifungal, anti-inflammatory, anti-ulcer, anti-allergic, and anti-cancer. The results of the present work also collaborate with the study of Abdulla et al., [54], who carried out the study of ethanolic and aqueous extract of *Ziziphus* leaves against various food-borne bacterial strains. Both aqueous and ethanolic extracts showed considerable antibacterial activity at a dose of 100

mg/ml against all strains including *E. coli*, *P. aeruginosa, Neisseria gonorrhoea, S. aureus, B. subtilis,* and *S. faecalis*, with 17.6-21.7 mm of the inhibition zones.

The antimicrobial potential of *Ziziphus* may be due to the oxygenated mono and sesquiterpene hydrocarbons such as caryophyllene [55,56]. The concentration of phenolic compounds or glycosidic contents, phenol, tannins, and saponins compounds have a powerful antibacterial effect [57]. That's why Ziziphus possesses good antibacterial activity against human pathogenic bacteria, especially *E. coli*.

The present study reveals that methanolic extract of *Ziziphus nummularia* and *Calotropis procera* exhibited remarkable inhibitory effects for *Salmonella* (Table 1). This study somehow correlates with the findings of Chanda et al., [58], who evaluated the antibacterial activity of five medicinally important plants including *Ziziphus nummularia* against *Salmonella* and some other bacteria through the agar well diffusion method. They used petroleum ether, acetone, and methanol as a solvent for extraction and found that methanolic extract showed moderate to good antibacterial activity. They also found that among Gram-negative bacteria, *Salmonella* is the most resistant bacteria. *Ziziphus nummularia* is rich in alkaloids and, due to these compounds *Z. nummularia* has the best antimicrobial activity against *Salmonella* [59]. On comparison of *Calotropis procera*'s antibacterial activity against *Salmonella*, it is found that this plant also showed the highest activity against *Salmonella* (Table 1) similar to *Ziziphus nummularia* (inhibition zones of 13.6 mm for both plants). On comparison of these results with the study of Rahman et al., [60], the present result has an advantage in the sense that *C. procera* showed remarkable inhibitory effects on *Salmonella.* However, according to Rahman et al., [61], *C. procera* had the least activity against *Salmonella* (10 mm) but the highest activity against *E. coli* (21 mm).

The present research also correlates with the study of Sanafi, [62], in which the antibacterial potential of the methanolic and aqueous extract of *Calotropis procera's* leaves was examined. Like the present study, Al Sanafi also concluded the methanolic extract of leaves is active against Gram-negative bacteria including *Salmonella* at low concentrations, while this study predicts the highest antibacterial action of both *Z. nummularia* and *C. procera* against *E. coli* and *Salmonella. Prosopis juliflora* exhibited the least antibacterial activity against *Salmonella* (Table 2). It might be due to environmental or any other factor.

The present study depicts the highest antifungal activity of ethanolic extracts of *Calotropis procera* leaves against *Malassezia* spp (dandruff-causing fungi) with the inhibitory zones of 13.6 mm followed by kikar (13.3 mm) and Berri (13.3 mm), (Table 2). The antifungal activity of *Calotropis procera* extract has been demonstrated by a few other scientists for other fungal strains but for *Malassezia*, its antifungal activity has not been well reported yet. So, in this regard, the present study has the advantage that this plant can also be used to treat fungal infections.

The present study also correlates with the study of Manoorkar V B, et al., [63], that ethanolic extract of leaf and latex of *Calotropis procera* has the best fungicidal properties. They also used agar well diffusion method to determine the Antifungal effect of *C. procera* against some selected fungi including, *Alternaria alternata, Cuvularialunata, Rhizoctonia-solani, Penicillium chrysogenum, Fusarium solani, Aspergillus niger, A. flavus, A. terrus A. fumigatus,* and *Rhizopus sp.*,

realized that ethanolic extract of both leaves and latex of this plant have inhibitory effect on the growth of tested fungi with the concentration of 100 mg/ml applied. The ethanolic leaves extract was proven significantly better with the inhibition zone of 15.0 mm but aqueous extract was comparatively less effective. Similarly, the latex ethanolic extract showed the highest zone of inhibition with a diameter of 20.0 mm.

Recent research indicates that the chemical compounds present in plants are responsible for their anti-inflammatory and antibacterial properties (64,65]. After *Calotropis procera*, both *P. juliflora* and *Z. nummularia* exhibited the second-highest antifungal activity against *Malassezia spp.* The results demonstrate that *P. juliflora* also has the potential to restrict the growth of fungi. This statement is supported by the research work of Satish (2007) who investigated the antifungal activity of *Prosopis juliflora* along with some other plants against *Aspergillus spp* using the agar well diffusion method. *Ziziphus muritiana*, a plant of *Ziziphus* has antifungal activity [66,67].

Conclusion

The present research analyzed the potential use of plants as natural resources to cure bacterial and fungal diseases. This research can provide a bedrock trend to decrease the use of synthetic drugs. Due to the presence of numerous active antimicrobial natural compounds in plants used in the present work, their capacity to restrict bacterial and fungal growth is well-proven. In present days, bacteria and fungi have developed resistance against applied drugs so extracts of these plants can be used as efficient tools to combat the resistance. So, the current research provides a way to use the plants at a larger scale for the welfare of mankind.

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