

PHYTOCHEMICAL COMPOSITION AND IN- VITRO ANTI-MICROBIAL STUDIES OF WILD CINCHONA (*NEOLAMARCKIACADAMBA*) FRUIT EXTRACTS

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Abstract:

Kadamb (Neolamarckiacadamba Roxb.) tree has wide spectrum of bioactivities such as analgesic, antipyretic, anti-inflammatory hypolipidemic and antidiabetic. However, very little is known about anti-microbial properties of its fruits. Therefore, the present study was undertaken to investigate Phytochemical constitution, antibacterial and antifungal properties of N. cadambafruits. Methanol and ethylacetate Extracts of the fruits are prepared and were used to investigate Phytochemical constituents and antimicrobial properties. Out of the selected solvent extracts, the methanolic extract(1000µg/ml) showed maximum zone of inhibition (19.3 mm) against Bacillus subtilis and minimum (14.9 mm) against Enterobacter aerogenes. Ethylacetate extract showed maximum zone of inhibition against Staphylococcus aureus(16.7mm) and minimum against Pseudomonas aeruginosa(11mm). The preliminary phytochemical analysis showed the presence of phytosterols, tannin, phenol, saponins and flavonoids in the methanolic extract. The antimicrobial effects of Neolamarckiacadamba fruit extracts indicate that this wild cinchona contains substantial number of bioactive agents are responsible for antimicrobial efficacy. The study also concludes that methanolic extract of N. cadambafruit can be used as a potential antimicrobial source for various infections.

Introduction

Plants as sources of bioactive compounds continue to play a dominant role in the maintenance of human health. Plants are rich sources of secondary metabolites with interesting biological activities. They are the best sources for obtaining natural antimicrobial drugs for various medicinal uses and disease related mechanism. The recent investigations are concentrating on the exploring of antiviral, antimicrobial and ant insecticidal activities of different plants extracts [1]. *Neolamarckiacadamba* is one of the medicinal plants traditionally used by the Indian. *Neolamarckiacadamba*(Roxb.) (English name: Wild cinchona; Hindi name: Kadamb, Kadam; Telugu name: Rudraskamba, Kadamba) belongs to the family Rubiaceae. The other names of the plant are *Naucleacadamba* (Roxb.), *Anthocephaluscadamba* (Roxb.)

Miq., *Samamacadamba* (Roxb.)

Kuntze, *Anthocephalusmorindifolius* Korth., *Naucleamegaphylla* S.

Moore, *Neonaucleamegaphylla* (S. Moore) S. Moore, etc. It is an evergreen, tropical tree native of South and Southeast Asia with scented orange flowers in dense globe-shaped clusters. The flowers are used in perfumes [2]. Bark of the plant is used in fever, inflammation,

cough, vomiting, diarrhea, diabetes, burning sensation, wounds, ulcers and snakebite. The bark is also used for its significant diuretic and laxative property [3]. Since ancient times different parts of this plant are used as anti-diuretic, anti-pyretic, in the treatment of anemia, tumor and for the improvement of semen quality [4, 5]. Previous investigations of *N. cadamba* reported isolation of cinchotannic acid, quixotic, cadambagenic acids, quinovic acid, saponins, steroids, alkaloids, one new secoiridoid, 3'-Ocaffeoyl sweroside and two new phenolic apio-glucosides, kelampayoside A and kelampayoside B [6]. The fruit extract exhibited good membrane stabilizing activity inhibiting both hypotonic solutions and heat induced haemolysis in comparison to inhibition by standard acetyl salicylic acid. As the fruit is edible, its juice is given to children for the remedy of gastric irritability [7]. Timber of cadamba tree is used for making pulp and paper, boxes and furniture and its wood is used as fuel [8]. Aqueous flower extract of *Neolamarckia cadamba* have been used to synthesize silver nanoparticles at room temperature [9].

The leaf extracts of *N. cadamba* revealed the presence of various secondary metabolites and these include glycosides, alkaloids, tannins, phenolic, steroids, and flavonoids [10, 11]. The bark contains alkaloids like cadambine and its derivatives, saponins, glycosides, triterpenoids, cadambagic acid, quinovic acid and β -sitosterol [12, 13]. Those alkaloids, steroids and flavonoids have potent antiepileptic effect in various seizure models [14]. In addition to this, saponins have also been able to modulate the neurotransmitter levels in the brain and to possess potent anti-convulsant activity [15]. The qualitative chemical tests revealed the presence of saponins, proteins, terpenes, carbohydrates and alkaloids in the bark powder of *N. cadamba* [16]. Phytochemical evaluation of methanolic extract of *N. cadamba* showed the presence of flavonoids, alkaloids, carbohydrate, proteins and glycoside compounds [17]. The flowers of *N. cadamba* yield essential oil and the main constituents of the essential oils were linalool, geraniol, geranylacetate, linalyl acetate, α -selinene, 2-nonanol, β -phellandrene, α -bergamottin, p-cymol, curcumen, terpinolene, camphene and myrcene [18]. The seeds of *N. cadamba* are composed of water-soluble polysaccharides D-xylose, D-mannose and D-glucose in the molar ratio 1:3:5 [19]. The literature review informs that the plant contains saponins, indole and quinoline alkaloids, secoiridoids and triterpenes [20]. In folk medicine it is used in the treatment of fever, uterine complaints, blood diseases, skin diseases, eye inflammation, diarrhoea, anaemia, leprosy, dysentery and stomatitis [21]. The pharmacological studies have revealed that the extract of the plant have antimicrobial, antioxidant, and wound healing as well as anti-diarrheal properties [22].

As part of our investigation the crude methanol and ethylacetate extract of fruits of *N. cadamba* were studied for antimicrobial potential in terms of zone of inhibition by using agar well diffusion method for the first time and we, here in, report the results of our preliminary investigations.

Experimental

Reagents

Solvents used for extraction (AR grade) were purchased from Merck India (Mumbai, India). Peptone and Beef extract of microbiology grade was purchased from Merck chemicals

Mumbai. Agar of bacteriological grade, sodium chloride (laboratory reagent), distilled water (Emplura double distilled water) were obtained from Merck chemicals, Mumbai.

Collection of plant material

Fruits of *N. cadamba* were collected from K L University campus, Vaddeswaram, Guntur, AP, India. These fruits were classified into immature fruits (small green fruits) and ripe fruits (yellow to slight orange) based on their maturity stages. The fruits were identified by Dr. G. Vijayalakshmi, Principal, Southern International Institute of Hotel Management, Visakhapatnam. Ripped fruits were used to carry out present study.



Neolamarckia Cadamba Fruit

Preparation of fruit extract

The fruits were washed, cut into pieces and air dried and ground to a coarse powder. The powder of fruits of *N. cadamba* was extracted using Methanol as solvent (1:10 w/v) at 60° C for 48 hours. Extract thus obtained were filtered through Whatman filter paper No. 1 and filtrate was concentrated using rotavapor at 40° C for 2 h. The residue was again extracted with ethylacetate as a solvent at 60° C for 48 hours. Extract thus obtained were filtered through Whatman filter paper No. 1 and filtrate was concentrated using rotavapor. These crude extracts were dried under vacuum (25 mm Hg at 30°C) for complete removal of solvent and stored in a desiccator. The dried extracts were dissolved in known volume of solvent (25 mg/mL) for analysis.

Screening of phytochemicals

The freshly prepared fruit extracts of *Neolamarckia cadamba* were qualitatively tested for the presence of phytochemicals. Phytochemical screening of the extracts was performed using the following reagents and chemicals: alkaloids with Mayer's, Wagner's, Hager's and Dragendorff's reagent; carbohydrates with Molish's, Fehling's, Barfoed's and Benedict's reagents; glycosides with modified Borntrager's test and Legal's test; saponins were tested with

froth and foam test; phytosterols with salkowski's test and Liebermann burchard's test; fixed oils and fats with stain test and acetone- water test; phenols with ferric chloride test; Tannins with gelatin test and lead-acetate test; Flavonoids with lead acetate test, alkaline reagent test, Shinoda test and Zinc hydrochloric acid reduction test; Proteins and amino acids with Xanthoproteic test, Biuret test and Ninhydrin test; Diterpenes and Triterpenoids with copper acetate test, Noller's test and Tshugajen test. They were identified by characteristic color changes and precipitation reactions using standard procedures [23, 24].

Test Organisms

The test pathogens used for screening the efficacy of *N. cadamba* fruit extracts were *Bacillus cereus*(MTCC – 1307), *Bacillus subtilis*(MTCC – 1427), *Escherichia coli* (MTCC – 294), *Pseudomonas aeruginosa* (MTCC – 1748), *Candida albicans* (MTCC – 3017) and *Aspergillus niger* (MTCC –616).

Determination of antimicrobial activity of fruit extracts

The antimicrobial activity of the plant extracts was evaluated by agar well diffusion method [25]. Bacteria were grown in Nutrient Agar media to match the turbidity of 0.5 McFarland standards to be inoculated on Nutrient Agar media agar. After inoculation, plates were dried for 15 min, and the wells were punched using sterile cork borers. Once wells were formed, they were filled with 50µL of isolates and blank (water). Commercially available Gentamycin (10 µg) discs were used as a positive control in this study. Plates were incubated for 24 h at 37 °C to allow leaf extracts to diffuse through the agar media to form zones of inhibition. The diameters of the zone of inhibition for different isolates against different bacteria were measured in millimetre for further analysis. An agar well (6 mm) showing no zone of inhibition was considered as no antimicrobial activity.

Results and Discussion

Phytochemical screening:

Phytochemical screening of the various extracts of *N. cadamba* fruit were done for the presence of some secondary metabolites such as alkaloids, terpenoids, flavonoids, saponins, carbohydrates, glycosides, phytosterols, oils & fats, tannins etc., and the results are presented in table 1. Phytochemical compounds such as alkaloids, saponins, tannins, flavonoids and steroids have been known to be biologically active and thus partially responsible for the antimicrobial activities of plants, hence their use in traditional medicine [26].

Table 1

Chemical Constituent	Ethylacetate extract	Methanol extract
Alkaloids	-	-
Carbohydrates	+	+
Glycosides	+	+
Saponins	+	+

Phytosterols	-	+
Fat & oils	-	-
Resins	-	-
Phenols	-	+
Tannins	+	+
Flavonoids	-	+
Proteins & amino acids	-	-
Terpenoids	-	+

Antimicrobial activity:

The antimicrobial activity was examined by agar well diffusion method against all the selected human pathogens at concentration levels of 1µg/ ml, 10µg/ ml, 100µg/ ml, 250µg/ ml, 500µg/ ml and 1000µg/ ml. Gentamycin is used as the standard drug. The results indicate that the samples have the potential to use as an inhibitor agent for the growth of human pathogens. The inhibitory effect of the crude extracts against bacteria and fungi compared to gentamycin as control is given in table 2, 3 & 4. As shown in table 2 & 3, the methanol extract of cadamba fruit exhibited a promising inhibitory power against tested organisms. The inhibitory potential of the methanol extract was high at a concentration of 1000µg/ ml against the bacteria *Bacillus subtilis* followed by *staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes*. Ethylacetate extract was less potent than methanol extract, ethylacetate extract didn't show any inhibitory zone at concentration of 1µg/ ml and 10 µg/ ml. *Staphylococcus aureus* was more susceptible with ethyl acetate extract exhibiting maximum inhibitory zone (16.7mm) at 1000µg/ ml followed by *Bacillus subtilis* (15.8mm), *Enterobacter aerogenes* (11.4mm) and *Pseudomonas aeruginosa* (11mm). Antifungal activity was studied against *Candida albicans* and *Aspergillus niger* and the zones of inhibition values are given in table 5. Methanolic extract of cadamba fruit was more potent against *Candida albicans* and *Aspergillus niger* at various concentration levels (1µg/ml to 1000µg/ ml). *Candida albicans* was more sensitive to methanolic extract with inhibitory zones of 3.3mm at 100 µg/ ml, 4.9mm at 250 µg/ml, 5.8mm at 500µg/ml and 9.6mm at 1000µg/ ml. *Aspergillus niger* was more sensitive to methanolic extract with inhibitory zones of 3.1mm at 1 µg/ ml, 3.3mm at 10 µg/ ml, 3.9mm at 100 µg/ ml, 5.2mm at 250 µg/ ml, 6.9mm at 500 µg/ ml and 11.8mm at 1000 µg/ ml. *Candida albicans* was susceptible with ethyl acetate extract with maximum inhibitory zone of 6.4mm at 1000 µg/ ml *Aspergillus niger* with maximum inhibitory zone of 9.5mm at 1000 µg/ ml.

Although the inhibitory potential of the crude extracts was distinct against the selected organisms the results indicated that they could be used as a source of antimicrobial drugs. It is because of the use of herbal medicines can reduce side effect and combinations of herbal plants and drugs in the treatment of infectious diseases can prominent effect.

Table 2: Anti-bacterial activity of methanolic extract of *Neolamarckiacadamba* fruit (zones of inhibition in mm)

S No	Name of the organism	Zone observed in mm for studied concentration					
		1µg/ml	10µg/ml	100µg/ml	250µg/ml	500µg/ml	1000µg/ml
1	<i>Pseudomonas aeruginosa</i>	---	3.2	6.9	7.4	12.9	17.1
2	<i>Bacillus subtilis</i>	---	3.6	6.8	7.1	11.7	19.3
3	<i>Staphylococcus aureus</i>	---	3.3	6.5	7.9	13.5	18.7
4	<i>Enterobacter aerogenes</i>	---	---	4.6	7.0	11.2	14.9

Table 3: Anti-bacterial activity of Ethyl acetate extract of *Neolamarckiacadamba* fruit (zones of inhibition in mm)

S No	Name of the organism	Zone observed in mm for studied concentration					
		1µg/ml	10µg/ml	100µg/ml	250µg/ml	500µg/ml	1000µg/ml
1	<i>Pseudomonas aeruginosa</i>	---	---	---	6.1	8.7	11.0
2	<i>Bacillus subtilis</i>	---	---	4.3	5.9	9.6	15.8
3	<i>Staphylococcus aureus</i>	---	---	6.9	7.8	12.3	16.7
4	<i>Enterobacter aerogenes</i>	---	---	5.7	6.2	9.8	11.4

Table 4: Anti-bacterial activity of standard (Gentamycin) (zones of inhibition in mm)

S No	Name of the organism	Zone observed in mm for studied concentration		
		100µg/ml	250µg/ml	500µg/ml
1	<i>Pseudomonas aeruginosa</i>	9.1	14.0	19.8
2	<i>Bacillus subtilis</i>	14.8	16.4	21.7
3	<i>Staphylococcus aureus</i>	8.7	15.7	18.2
4	<i>Enterobacter aerogenes</i>	9.5	13.7.	18.9

Fig.1: Anti-bacterial activity of *N. cadamba* fruit extracts (concentration-500µg ml; zones of inhibition in mm)

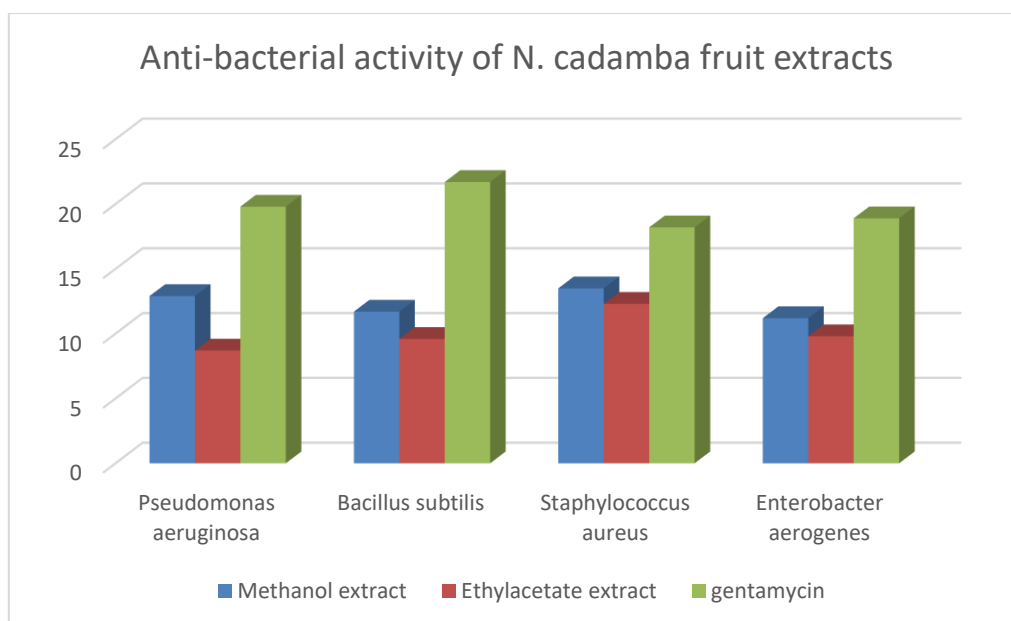


Table 5: Anti-fungal activity of methanol and ethyl acetate extract of *Neolamarckiacadamba* fruit (zones of inhibition in mm)

S No	Sample	Organism	Inhibition zone in mm observed for the sample concentration in $\mu\text{g/ml}$					
			1	10	100	250	500	1000
1	NC EA	<i>C albicans</i>	-	-	3.4	5.1	6.2	6.4
2		<i>A niger</i>	-	3.1	3.6	6.7	8.3	9.1
3	NC ME	<i>C albicans</i>	-	-	3.3	4.9	5.8	9.6
4		<i>A niger</i>	3.1	3.3	3.9	5.2	6.9	11.8
5	Standard	<i>C albicans</i>	4.1	6.4	9.8	NT	NT	NT
6		<i>A niger</i>	3.9	7.2	12.4	NT	NT	NT

NC EA: Ethyl acetate extract of *Neolamarckiacadamba* fruit

NC ME: Methanol extract of *Neolamarckiacadamba* fruit

- : No inhibition zone observed

NT: Not Tested

Standard: Fluconazole

Fig.2: Anti-fungal activity of *N. cadamba* fruit extracts (concentration-100µg ml; zones of inhibition in mm)

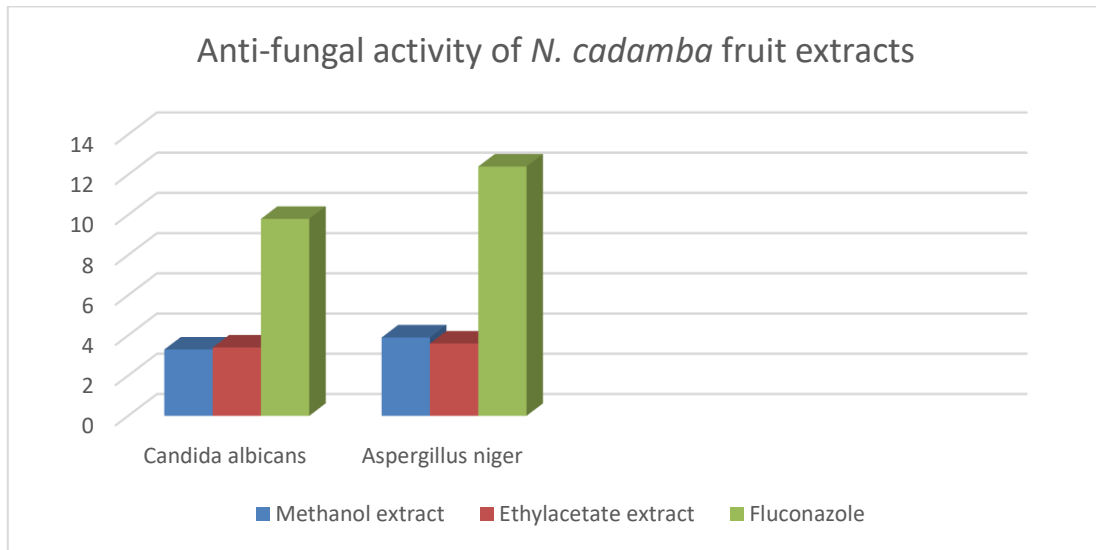


Fig. 3: Antifungal activity of *N. cadamba* fruit extract against *Aspergillus niger* and *Candida albicans*

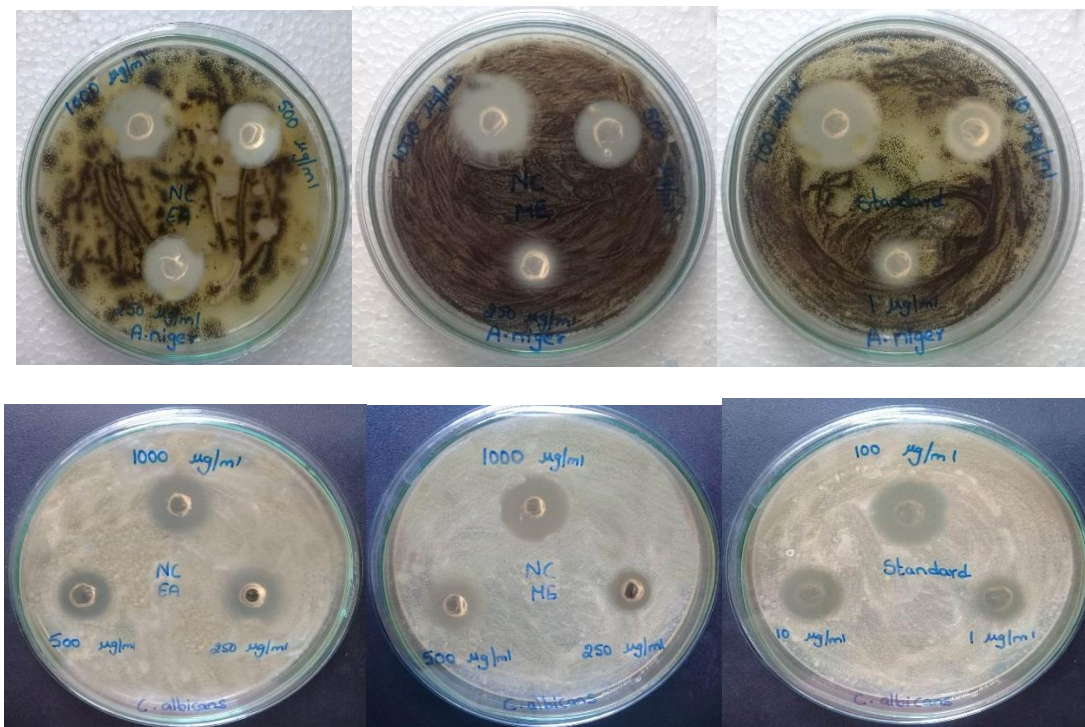
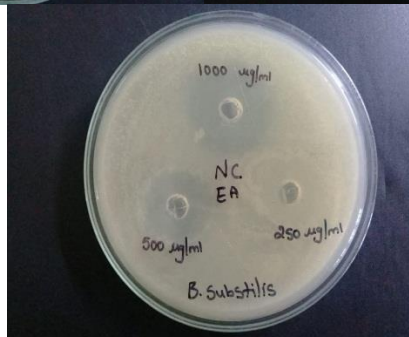
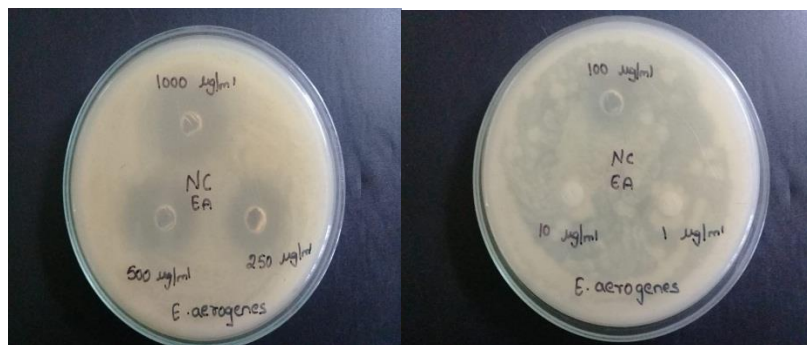
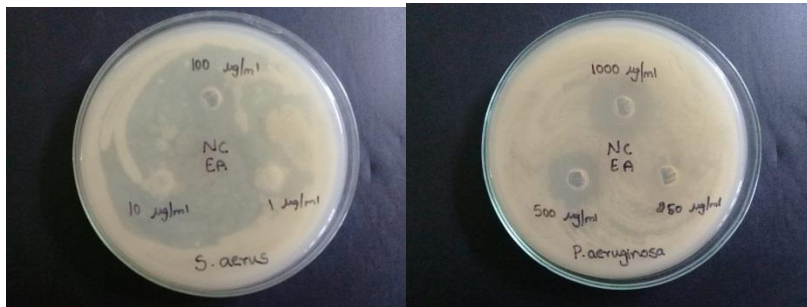
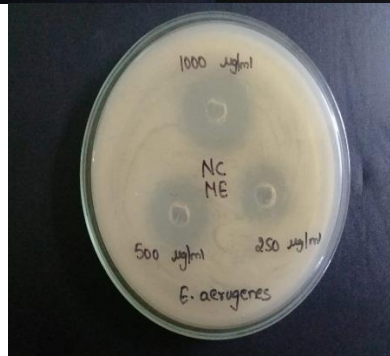
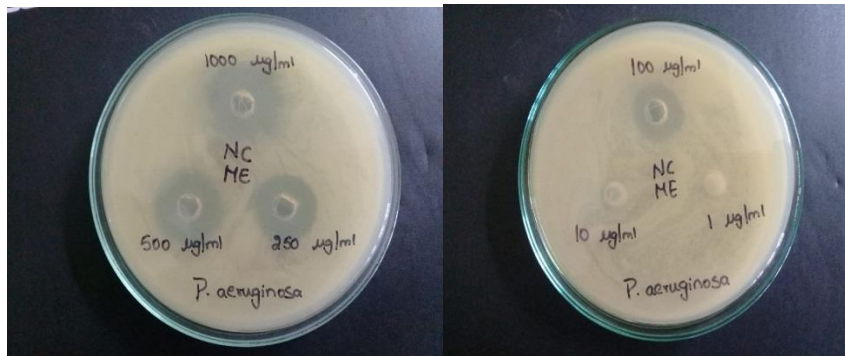
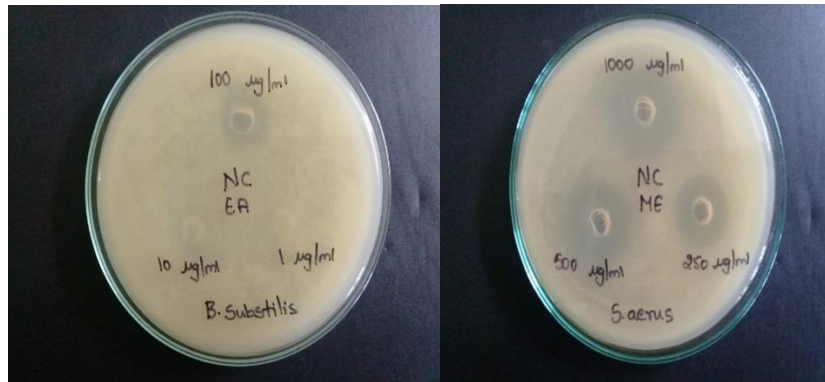
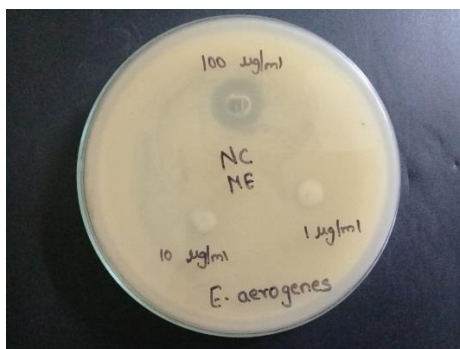


Fig. 4: Antibacterial activity of *N. cadamba* fruit extract against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterobacteraerogenes*.







The study offers a source for the discovery of alternative antimicrobial drugs. The study also concludes that cadamba fruit contains potent medicinal properties.

Conclusion

From the present investigation it is clearly indicated that the extracts of *N. cadamba* fruit have moderate to potential antimicrobial properties and can be used in the treatment of infectious diseases caused by enteric and other pathogenic microorganism to human beings. Of the two extracts tested (methanol and ethyl acetate), the methanolic extract exhibited antimicrobial potential indicating that the solvents used in extraction procedure have prominent effect on solubility of the antimicrobial compounds that are present in the plant material [27].

The presence of a moderate content of flavonoids, tannins and phenol in this study may have contributed to the observed pharmacological activity of this plant in their therapeutic potential.

Most plant extracts are believed to be more active against Gram positive bacteria because the cell wall is easier to penetrate than Gram negative ones, which contain outer membrane with a lipopolysaccharide layer that is impermeable to certain antibiotics and antibacterial compounds [28, 29].

These results provide evince that crude fruit extracts of *Neolamarckia cadamba* might be potential sources of new antimicrobial agents. The reason could be that the extracts were able to penetrate the lipopolysaccharide layer more readily thereby making them susceptible.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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