Section: Research Paper

PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITY STUDY OF DIFFERENT EXTRACTS OF *CITRUS MEDICA* L. LEAF AGAINST SOME BACTERIA

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ABSTRACT

The need for antibiotic-tolorated bacteria, combined with the negative effects of antibiotics on human health, necessitates the pursuit of plant-derived antimicrobial compounds. Plants with ethnomedicinal uses can be investigated to reveal their efficacy for the discovery of novel secondary metabolites and herbal drugs. *Citrus medica* L. leaf is used against loose motion and skin diseases by the tribal communities of the Balangir district of Odisha. Therefore, the study was designed to test the presence of different secondary metabolites in different leaf extracts of *Citrus medica* L., such as n-Hexane and Ethanol as solvents using the Soxhlet apparatus and to screen its antibacterial test using the agar well diffusion method as well as to determine the minimum inhibitory conc. (MIC) using the serial dilution method. The results of the phytochemical screening showed that the plant's secondary metabolites were present abundantly in the methanol extract. The leaf extracts in different test solvents were observed to have broad-spectrum activity thereby showing their efficacy against both Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Shigella flexneri, and Salmonella phimurium*). The ethanolic extract of *Citrus medica* L. showed maximum antimicrobial potential against *Salmonella typhimurium* , with a larger zone of inhibition (15.13 ± 0.14) and lower MIC value (25 mg/ml) as compared to the other two extracts against the four bacterial strains. Hence, further investigations could reveal that the leaf extracts of *Citrus medica* L. can be used as an effective natural antibacterial agent for the treatment of different bacterial diseases.

Keywords: *Citrus medica* L., Secondary metabolite, ethnomedicinal, antibacterial, MIC

1. Introduction

With the excessive use of antibiotics in the modern era, there has been an increase in the development of antibiotic-resistant bacteria, thereby decreasing their efficacy in the treatment of

bacterial diseases. In this addition, they are responsible for damaging effects on patient health, and consequently there is a worldwide increase in mortality and morbidity. It encourages the use of novel antimicrobial compounds from natural products.[1] Plant-produced antimicrobials compounds appear to be one of the most promising alternatives for dealing with antibiotic-resistant bacteria.[2] Besides that they are relatively safer and more potent than synthetic drugs. Recently the search for phytochemicals and the pharmacological investigation based upon traditional uses of medicinal plants have gained momentum for establishing their scientific authenticity and efficacy for the discovery of new drugs. Thus, several ethnomedicinal plants have been screened for their pharmacologically active secondary metabolites for the benefit of human being. Green leaves have been used as both food and medicine since ancient times, providing both nutrition and healthcare. The medicinal plants have grater antimicrobial and antioxidant properties, which can be explored for the development of therapeutically active compounds.[3] *Citrus medica* L. is an underutilised plant with various bioactive components. [4]*Citrus medica* L. commonly known as citron in English and bijapura in Ayurvedic literature, is a shrub or small tree. It has leaflets that are 3-6 inches long, elliptic-ovate or ovate-lanceolate with sort, wingless or nearly wingless petioles, flowers that are 5-10mm in a raceme, small or middle-sized petals that are generally more or less pink and fruit that is globes, ovoid, or oblong, often mamillate at the apex. It is found in all over india. [5] Various parts of *Citrus medica* L. are widely used in the Indian traditional system of medicine. Ripe fruits are antiscorbutic, stomachic, cardiac tonic, stimulant, sedative, analgesic, and used in dyspepsia, bilious vomiting, colds, fevers, palpitations, sore throats, coughs, asthma, thirst, hiccoughs, and earaches; the root is analgesic, antispasmodic, and used in diarrhea.[(6-8)]

Various phytoconstituents like alkaloids, tannins, glycoside,volatile oil and flavonoids extracted from various medicinal plants have shown their antimicrobial efficacy. [9] Till date phytochemical tests using different reagents and test their antimicrobial activity have been undertaken. Thus the present work will be aimed at determining the antibacterial activity of different extracts of *Citrus medica* L. leaves. The other goal of this study was to screen the phytochemicals found in different extracts of *Citrus medica* L. leaves for the presence of any bioactive constituents that could provide scientific support for the ethnomedicinal use of this plant against bacterial infection diseases. At the end, studies are to be conducted to determine the efficacy of the extracts of *Citrus medica* L. against selected bacterial strains.

2. Materials and Methods

2.1 Plant Material Collection and Identification

Fresh leaves of *Citrus medica* L. were collected in March 2019 from in and around Bolangir district, Odisha, India. and definitively authenticated visually according to a taxonomic method by Dr. K.B. Satapathy of Centurion University of Technology and Management, Bhubaneswar, Odisha, India. The voucher specimen was preserved at the herbarium of TPC, Barpali, Odisha, India.

2.2 Preparation of Solvent Extracts

The fresh leaves of *Citrus medica* L. were collected in bulk and washed 2–3 times under running tap water followed by distilled water and dried at room temperature until properly dried. Then made them coarse powder by pressing the leaves of *Citrus medica* L. with hands which was then sieved and used for extraction. The dried powdered leaves (150 g) were extracted in two distinct solvent systems (400 ml each) in the order of increasing polarity (consisting of n-hexane, and ethanol) separately in a Soxhlet apparatus for seventy-four hours. The extracts collected in each solvent were filtered out one by one with the help of Whatman No. 1 filter paper then concentrated with a rotary evaporator (IKA Model RV 10D S96) and kept at 4 ^{o}C in an airtight container in the freezer until the use. All the extracts were subjected for preliminary phytochemical screening, followed by an antimicrobial assay.

Figure 1. *Citrus medica L. plant*

Figure 2. Extraction of citrus medica L. leaf

Figure 3. *Different extract of leaf of Citrus medica L.*

2.3Preliminary Phytochemical Screening

The n-hexane, and ethanol extracts of *Citrus medica* L. leaf were subjected for qualitative phytochemical screening for the presence of active chemical constituents such as alkaloids, glycoside, tannin, resin, protin, fat, flavonoid, saponin and steroids following the standard methods.

3.Antimicrobial Activity

3.1 Bacterial strains and culture media:

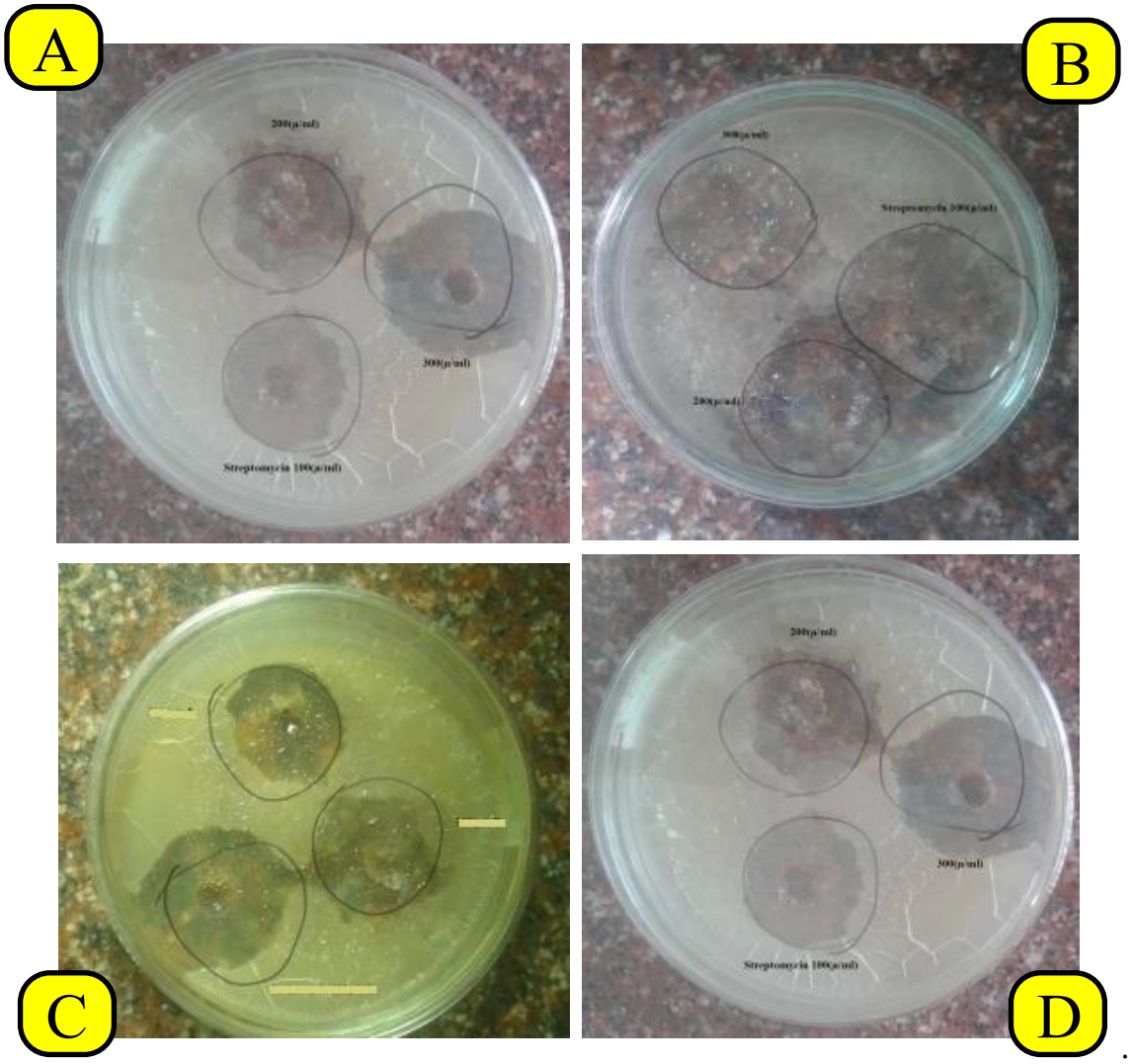
Single gram-positive human pathogenic bacteria called as *Staphylococcus aureus* (MTCC-3160) and 3 no. of gram-negative bacteria, such as *E. coli* (MTCC-119), *Shigella flexneri* (MTCC-1457), and *S. typhimurium* (MTCC 3231), were obtained from the Microbial Type Culture Collection Centre (MTCC) and collected from biotechnology department ,Sambalpur university for the study of antibacterial screening of plant extracts. All the different bacterial strains were maintained by sub-culturing them on Nutrient Agar medium (Himedia) in every fifteen days and then stored at 4°C. Ciprofloxacin was used as a standard medicine or positive control in bacterial strain testing. As a negative control, Dimethyl Sulfoxide (Himedia) was utilised.

3.2Antibacterial susceptibility assay:

The antimicrobial activity of *Citrus medica* L. leaf extracts were tested using the agar-well diffusion method using Nutrient Agar.[10] For different extracts three replicated trials were conducted against each micro organism. 30 ml of agar media (265 nm wavelength) was poured in different Petridish and allowed to coll for solidify for 15 minutes under UV light.

Subsequently, the agar plates were inoculated by streaking with a cotton swab that was dipped whole night in suspensions of test microorganisms, i.e. *S. aureus*, *E. coli*, *S. flexneri*, and *S. typhimurium* (each adjusted to turbidity of 0.5 McFarland Standard) in the agar medium. Then by using a sterile borer, wells with a diameter of 4 mm were cut in the medium. Stock solutions of different leaf extracts of *Citrus medica* L. in various solvents were diluted in DMSO solution to obtain different conc. of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, and 50 mg/ml etc.. The test samples and controls (50 µl) were loaded in the wells by using a micropipette and allowed to diffuse for 20 minutes. The plates were then incubated for one day at 37°C. The antibacterial activities of various extracts of *Citrus medica* L. leaf were assessed by using an Antibiotic Zone Scale (Hi-Media) to measure the diameter of the zone of inhibition (ZI) against the test micro organism. The recorded value so obtained by diifferent extractsof *Citrus medica* L. leaf were rounded to the nearest mm.

Fig 04: Antibacterial screening



A- Staphylococcus aureus. B- Escherichia coli c- Shigella flexneri , d-Salmonella typhimurium

3.3Minimum Inhibitory Concentration (MIC):

The broth dilution technique was used to determine the minimum inhibitory concentration (MIC) of different extract's of *Citrus medica* L. The different plant extracts of n-hexane and ethanol were taken (12.5, 25, 50,100 mg/ml) and serial dilution of the different extract with nutrient broth for different bacterial culture with necessary inoculums was used. For bacteria the tubes were incubated for one day at 37 °C. Then each test tube was checked for its turbidity. The MIC was calculated by measuring the least concentration of the extract so that no turbidity was detected.

Statistical analysis:
The average value of inhibition obtained from each experimental result were statistically analyzed and SD(standard deviation) were calculated which is expressed as a mean±standard deviation.

4 Results and Discussion

4.1Phytochemical Constituents

The extraction process is very critical for recovering and isolating phytochemicals from plant sources. The presence of more number active plant constiituent was demonstrated in n-hexane, , and ethanol extracts of *Citrus medicaL.* including alkaloids, flavonoids, tannins, saponins, steroids, glycosides, and phenols etc. (Table 1). Alkaloid is found in n-hexane extract. Flavonoid is present in n-hexane extract. Tannin is present in all two extract. Saponin is present in ethanol extract.Steroid is present in n-hexane extract .Glycoside is present in ethanol extract and Phenol is present in all two extract.

The polarity of the solute determines the solvents used for extraction. Ethanol is very effective in the extraction of lower molecular weight phenols.[11] According to previous preliminary phytochemical study , high molecular weight chemicals were extracted with a highly polar solvent such as water, whereas small and medium molecular weight compounds were extracted with less polar solvents such as ethanol. Hence, the result of the present study revealed that the majority of the phytochemicals were obtained from the ethanolic extract of *Citrus medica L.*.

Table 1. Qualitative preliminary phytochemical analysis of leaf extracts of *Citrus medica L.*

Phytoconstituents	**Tests**	**n-h**	**et**
Alkaloids	Mayers test	+	--
Flavonoids	Alkaline Reagent Test	+	+
Tannins	Lead acetate test	+	+

Saponins	Foam test	--	--
Steroids	Salkowski's test	+	--
Glycosides	Born trager test	--	+
Phenols	Ferric chloride test	+	+

+ Present, -- Absent.n-h- n-hexane, , et-- ethanol

4.2Antimicrobial Activity

The antibacterial activity of *Citrus medica L.* was studied in terms of the inhibition zone (ZI) formed by the agar well diffusion test .The results of the antibacterial assay showed different antibacterial activity of different extract of leaf against the tested bacteria. All two extracts of *Citrus medica L*. showed antibacterial effect in the following inhibition sequence: *Escherichia coli >Salmonella flexneri>Salmonella typhimurium>Staphylococcus aureus*. The minimum antibacterial effect of the leaf extract was against the bacterial strain *Staphylococcus aureus*. The antibacterial competency of the plant extract was in the order of ethanol extract > n-hexane extract. The antibacterial effect of the leaf extract of *Citrus medica L.* was compared with the antibacterial effect of the standard drug ciprofloxacin (0.1 miligram/ml). At 0.1 miligram/ml, the antibiotic ciprofloxacin inhibited the growth of microbes in the following order: *Staphylococcus aureus* (26.3±0.63mm) >*Shigella flexneri*(25.8±0.75mm) >*Salmonella typhimurium*(24.5±0.86mm) >*Escherichia coli* (22.1±0.89mm). *Citrus medica L.* antibacterial effect was examined in terms of minimum inhibitory conc. as shown in Table 3. MIC is the minimum conc. of an antibacterial agent that retards the visible growth of pathogen. The MIC conc. range of the various leaf extracts varied from 12.5 to 100 mg/mL. The ethanol extract showed the minimum inhibitory concentration of *Salmonella typhimurium* (50 mg/ml). This study shows that ethanol extracts had lower MIC values for the tested microorganism than n-hexane extract In this study, gram-negative bacteria, such as *Escherichia coli* showed high sensitivity towards *Citrus medica L.* extract.

Table 2. Antibacterial activity (zone of inhibition ZI) of different leaf extracts of *Citrus medica(Linn.)*,

		Concentration of plant extract					
Bacterial strains	**Solvent**	**6.25 mg/ml**	**12.5 mg/ml**	**25 mg/ml**	**50 mg/ml**	**Negative control [DMSO]**	**Positive Control Ciprofloxacin [0.1mg/ml]**
Staphylococcus aureus	n-hexane	5.44±0.36	5.89±0.15	6.11±0.05	6.13±0.16	00±0.00	26.3±0.63
	ethanol	5.99±0.5	9.83 ± 0.43	10.26±0.15	10.78 ± 0.14	00±0.00	
Escherichia	n-hexane	10.29±0.23	10.04±0.13	11.18±0.25	12.37±0.16	00±0.00	22.1±0.89
	ethanol	10.52±0.21	10.19±0.17	12.84±0.13	13.43±0.12	00±0.00	

coli							
Shigella flexneri	n-hexane	10.32±0.54	11.22±0.12	12.19±0.38	14.24±0.12	00±0.00	25.8±0.75
	ethanol	10.32±0.14	10.17±0.16	10.28±0.13	11.13±0.19	00±0.00	
Salmonella typhimurium	n-hexane	9.26±0.53	10.16±0.02	10.18±0.7	10.25±0.12	00±0.00	24.5±0.86
	ethanol	9.18 ± 0.26	13.13 ± 0.14	14.23 ± 0.14	15.13 ± 0.14	00±0.00	

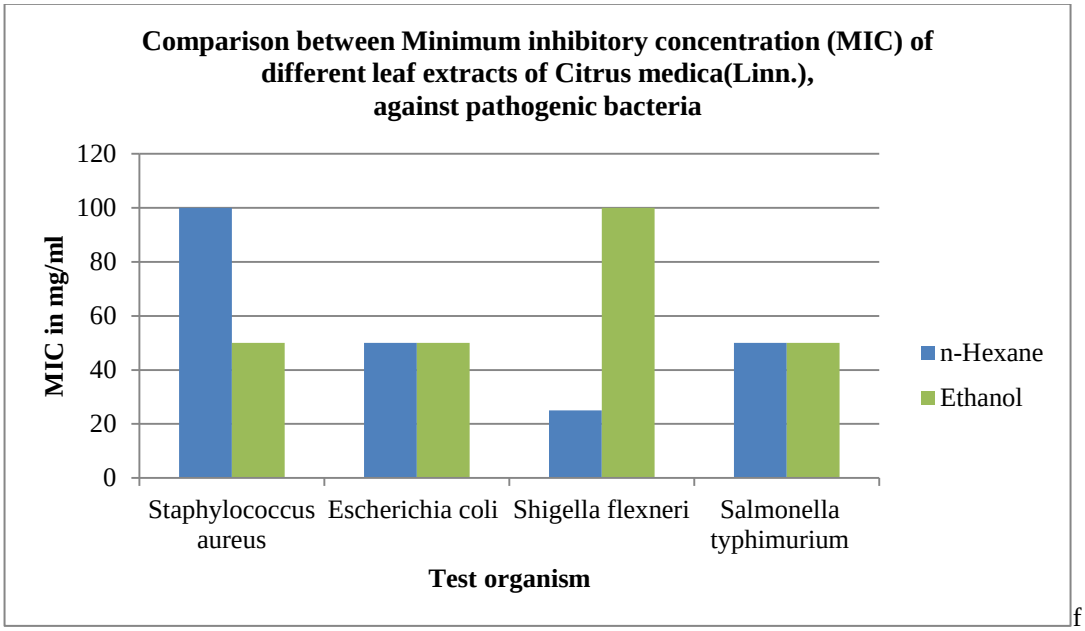
*Values in the table are mean ± SD of 3 replicates.

From this study, it seems that microorganism can be inhibited by various extracts of the leaves of *Citrus medica L.* The leaf extract had shown its efficacy by inhibiting almost all bacterial strains . The potency of leaf extracts may be attributed to the presence of certain secondary metabolite present in them, which play a major role in retarding bacterial pathogens in the human body. The antibiotic ciprofloxacin, on the other hand, had shown inhibiton activity against the tested micro organism. But long-term usage of synthetic antibiotics results in bacterial resistance, endangering the efficacy of antibiotics against those bacteria. Therefore, herbal drugs, being inexpensive with no detrimental effects, can be used as natural antibiotics for treatingvarious bacterial deases .

Table 3. Minimum inhibitory concentration (MIC) of different leaf extracts of *Citrus medica L.* against pathogenic bacteria.

Microorganisms	**n-hexane**	**ethanol**
Staphylococcus aureus	100 mg/ml	50mg/ml
Escherichia coli	50 mg/ml	50 mg/ml
Shigella flexneri	25mg/ml	100 mg/ml
Salmonella typhimurium	50 mg/ml	50 mg/ml

Figure 2. Comparison between Minimum inhibitory concentration (MIC) of different leaf extracts of *Citrus medica L.* against pathogenic bacteria.



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5.Conclusion

The present study authenticates that the antimicrobial aspect of *Citrus medica L.* which is an traditional medicine. In this addition, the existence of several phytoconstituent in plant extracts may be the reason for their effectiveness. All the extracts were found to be more or less effective against all test bacteria, revealing their broad-spectral activity. Further, the methanolic extract of *Citrus medica L.* showed the strongest antimicrobial activity in terms of larger zones of inhibition and lower MIC values among the three extracts against the *E. coli* bacterial strains. Hence, the leaf extracts of *Citrus medica L.* can be used as alternative herbal treatment for treating various bacteria. Additionally, it has the capacity for the development of pharmaceuticals and new drugs in the future.

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Conflicts of interest

The authors do not have any conflict of interests to declare.

Ethical approvals

This study does not involve experiments on animals or human subjects.

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