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Research Article

ANTIMICROBIAL AND RADICAL SCAVENGING ACTIVITY OF *CITRUS LATIFOLIA* PEEL EXTRACT

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ABSTRACT

Citrus latifolia (*C. latifolia*) have obtained a significant role in traditional medicine system. The nutritive and medicinal values of citrus fruits with special reference to the fruit of the genus Aegle of the *Rutaceae* family, *C. latifolia* fruit and juice are excellent sources of health-promoting substances like vitamins, nutrients and factors of medicinal value cannot be under estimated. Because of its eminent medicinal property, the present study was conducted to investigate the antibacterial, antifungal, antioxidant and radical scavenging assays. Citrus peel extract were prepared in ethanol and methanol, which are taken for determining mentioned activities. Agar well diffusion assay was performed to determine antibacterial activity. Among bacteria, *Bacillus subtilis*, *Staphylococcus aureus* and *Klebsiella pneumoniae* were inhibited to high and least extent respectively. In case of antifungal activity, Poisoned food technique was performed against *Aspergillus flavus*. The colony diameter of test fungi was considerably lesser in plates poisoned with extracts when compared to control plates. Radical scavenging activity was determined by Hydrogen peroxide Scavenging assay. The extracts scavenged H_2O_2 radicals dose dependently. The scavenging effect was marked in case of ethanolic peel extract was slightly high followed by methanolic extracts. Total phenolic content of extracts was also estimated by Folin-Ciocalteu reagent (FCR) method. Where, Ethanolic extract contained high phenolic content followed by methanolic extracts. The peel of *C. latifolia* can be used to treat skin and other bacterial infections and also to control phytopathogenic fungi.

Keywords: *Citrus latifolia*, Peel extract, Agar well diffusion, Poisoned food technique

INTRODUCTION

Medicinal plants based traditional system was playing an important role in providing health care to large section of population, especially in developing and under developed countries such as India, Srilanka, Pakistan, China etc[1,2]. Traditional practices use the substances that available naturally in the surroundings. Most of such substances possess dietary values besides holding therapeutic values.

Persian lime (*Citrus latifolia*), also known as seedless lime, Tahiti lime or Bearss lime is a fruit belongs to citrus species of hybrid origin. Persian Limes are the most common type of lime grown for commercial purpose. The Persian lime is a triploid cross between key lime (*Citrus aurantiifolia*) and lemon (*Citrus limon*). They have thicker skins compare to Key limes so, they aren't easily damaged by handling and they can keep for a long duration of time. This can be used as traditional medicines because of its potent medicinal properties. Traditional systems of medicine such as Homeopathy, Ayurveda etc., are gaining interest and popularity throughout the globe due to their approaches in therapeutic and preventive measures[3].

Citrus genus (*Rutaceae*) contains about 130 genera in the seven subfamilies with many important essential oil producers. Fruits of this genus are a great source of Vitamin C, folate, flavonoids, carotenoids and coumarins[3]. Vitamin C is reported as an antiscorbutic and possesses antioxidant properties[4]. Many pharmacological studies have been done to investigate the properties of Citrus in an attempt to know its use as a medicinal agent. Few review works reported on pharmacological actions, phytochemical and health benefits of Citrus and its active components[5].

The chemical method generate toxic and harmful chemical that has an adverse effect on environment. Hence, there is a need of biosynthesis approach towards the synthesis of drugs. The fruit peels are especially available, affordable, efficient and most important eco-friendly. The citrus peel extracts has essential oils known to exhibit various biological activities such as antibacterial, antifungal and antioxidant activities. The peel of citrus fruits is a by-product of citrus processing industries. A large amount of peel is generated during the processing and it is considered as waste[6].

The bacterial species *Bacillus subtilis*, initially it has been thought to be non-pathogenic but it should not be ignored as a possible human contaminant because they can cause deadly bacteraemic infections, which were identified through 16S rRNA gene in a patient[7]. Likewise, *Klebsiella pneumoniae* is the common cause of hospital-acquired Gram-negative bloodstream infection and it usually arise as a complication of gastrointestinal, focal urinary or respiratory tract infections. Although several hospital-based studies have noted a high rate of occurrence of several illnesses and a case fatality rate of 20% to 40% and the mortality rate of *K. pneumoniae* invasive pathogen seems to be increasing day by day[8]. *Staphylococcus aureus* is a gram positive bacterium found as a commensal in humans. Approximately 30% of the human population is colonized with *S. aureus*[9]. Simultaneously, it is a leading cause of Infective Endocarditis (IE) as well as osteoarticular, pleuropulmonary skin and soft tissue infections.

Among the fungi, *Aspergillus flavus*; is recognized as an important pathogen that has the ability to cause infections in humans, plants and animals. It can also survive in high temperature and widely distributed in the environment. The source of infection of this fungus is exogenous and humans as well as animals can acquire the infection by inhalation of the infectious fungal spores from the environment[10]. It is the second leading cause of invasive aspergillosis and superficial infection. Particularly common syndromes linked with *A. flavus* include keratitis, chronic granulomatous sinusitis, wound infections and cutaneous aspergillosis. *A. flavus* produces aflatoxins, which is the most harmful toxic and hepatocarcinogenic compound ever identified[11].

Here, we reported the cost effective and fragile biogenic extraction of Persian lime (*C. latifolia*) peel extract and investigation of antibacterial, antifungal and antioxidants of extracted peel extract by using Ethanol and Methanol as a solvent. It will definitely help to achieve the new era of drugs and improvement in the health condition of individuals.

MATERIALS AND METHODS

Collection of Citrus fruits

The fruit of Persian lime (*C. latifolia*), that are free from any contaminants and other kinds of damage were collected from Sagara taluk, Shivamogga District, Karnataka, India.

Extraction

The Persian limes were washed carefully under running tap water then by sterile distilled water. After that, peel of the fruits were removed and cut into small pieces. These were shade dried at room temperature (32°C) for two days, pulverized to a fine powder using a sterilized mixer grinder and stored in air-tight bottles. A known quantity of fruit peel powder (25 g) was added into separate conical flasks containing 100 ml of Ethanol (HiMedia, Mumbai, India) and 100 ml of Methanol (HiMedia, Mumbai, India) then left for 48 hours with occasional stirring. The content of flask was filtered through Whatman No. 1 filter paper[12]. Further the filtrates were dried by evaporation process. The condensed peel extract obtained after drying were used for determining antibacterial, antifungal, antioxidant and total phenolic content.

Antibacterial activity of Citrus peel extracts

Bacterial strains of gram positive *Bacillus subtilis* and gram negative *Staphylococcus aureus* and *Klebsiella pnuemoniae* were used to determine antibacterial activity of citrus peel extract by agar well diffusion method. Nutrient agar medium (Peptone - 0.5 g, Beef extract - 0.3 g, NaCl - 0.5 g, Agar – 2 g, distilled water – 100 mL) was used as culture medium. 50 µl of 24 h old mature cultures were swabbed using L-shaped rod on the medium. Wells were made by using sterile Cork borer (6 mm). 100 µl (200 mg of extract is dispersed with Dimethyl sulfoxide (20 mg/ml of 25 % Dimethyl sulfoxide [DMSO; HiMedia, Mumbai, India] of both methanolic and ethanolic extracts were filled in each wells marked as E and M. Standard (Ampicillin, 1 mg/ml) is used as a standard and DMSO (25 %, in sterile water) as a control. The plates were incubated at 37ºC for 24 hours in upright position and the zone of inhibition formed around the wells was measured by the zone measuring scale.

Antifungal activity of Citrus peel extracts

To investigate antifungal efficacy of citrus peel extracts against *Aspergillus flavus*, Poisoned food technique was performed. Potato dextrose agar (HiMedia, Mumbai, India) was poisoned with citrus peel extracts (1 mg extract/ml of medium). Spore suspension of *A. flavus* was prepared and spread on the poisoned plates by following spread plate technique method of fungal inoculation. Here the media with no extract is served as positive control plate. The plates were incubated at 32°C for 3-4 days in upright position. By checking the colony growth and population, we have measured the activity of different extracts by taking standard fungicide (mancozeb) as a negative control.

Hydrogen peroxide scavenging assay of *Citrus latifolia* extracts

The radical scavenging potential of Citrus extracts was tested by Hydrogen peroxide scavenging assay by the modified method of Mizel and Pick. Varied concentrations of (10, 20, 40, 60, 80 and 100 µg/mL) both ethanolic and methanolic extracts of *C. latifolia* were taken with ascorbic acid (Vit C) as a standard. Later this was mixed with 50 µL of 5 mM H_2O_2 solution and incubated for 20 minutes at room temperature. The optical density was read at 610 nm using UV-Vis spectrophotometer (eppendorf-BioSpectrometer basic/6135). The percentage of H_2O_2 scavenging was calculated using following equation.

$$\text{Hydrogen peroxide Scavenging activity (\%)} = [(A_c - A_s) / A_c] \times 100,$$

Where, A_c is absorbance of Hydrogen peroxide and A_s is absorbance of Hydrogen peroxide in presence of extract/standard[13,14]

Total phenolic content of extracts of *C. latifolia*

Folin - Ciocalteu reagent (FCR) method was used to determine the content of total phenolics in extracts[15]. In a clean test tube, 0.5 ml (10 mg/mL) of extract was mixed with 0.5 ml of FC reagent (1:1) along with 2 ml of sodium carbonate (7%) and left for 30 minutes incubation at room temperature. The optical density was read at 765 nm in UV-Vis spectrophotometer (eppendorf-BioSpectrometer basic/6135). A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml) and the total phenolic content of extracts was expressed as µg Gallic acid equivalents (GAE)[16].

RESULTS AND DISCUSSION

The result of the agar well diffusion assay and the Maximal Inhibitory Concentration (MICs) of the extracts are shown in Table 1, Figure 1 and Graph 1. The highest antibacterial activity was obtained with the Ethanol extract of *C. latifolia* against *Staphylococcus aureus* with inhibition zone diameters of 2.6 cm.

Table 1: Antibacterial activity of Citrus peel extracts

Microorganism	Ethanol (cm)	Methanol (cm)	Ampicillin (cm)	Negative control (cm)
Gram Positive Bacteria				
Bacillus subtilis	1.4	1.3	1.2	0.0
Staphylococcus aureus	2.6	2.1	2.3	0.0
Gram Negative Bacteria				
Klebsiella pneumoniae	1.3	1.2	1.4	0.0

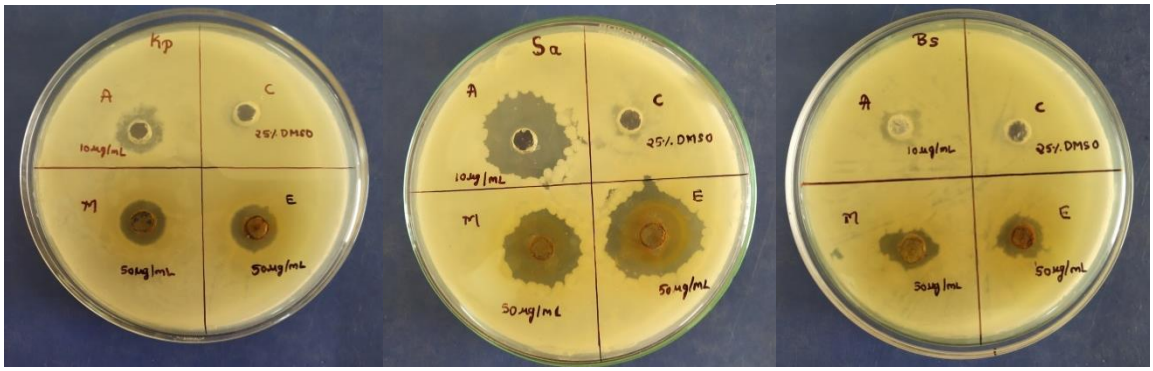
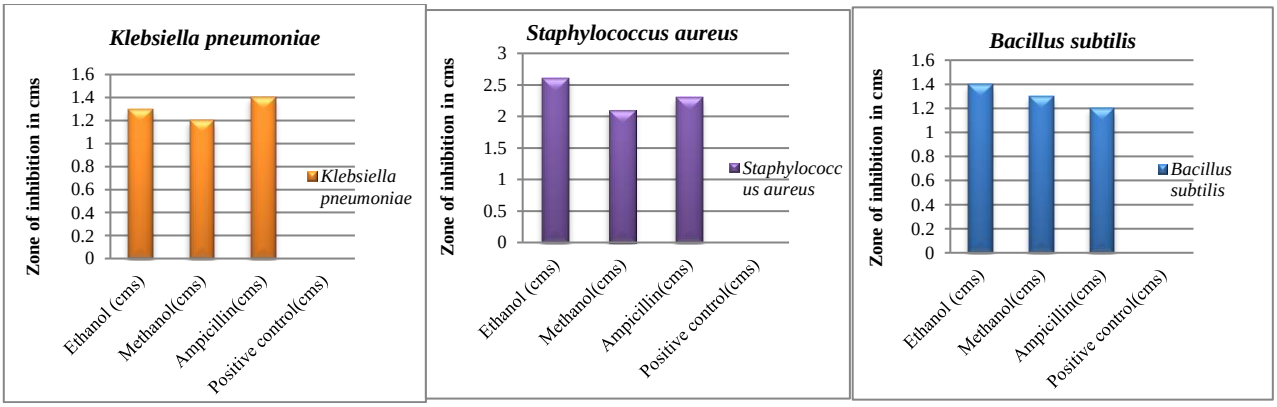


Figure 1: Plates showing zone of inhibition



(a) (b) (c)

Graph 1: (a) Zone of inhibition of *Bacillus subtilis*, (b) Zone of inhibition of *Staphylococcus aureus*, (c) Zone of inhibition of *Klebsiella pneumoniae*

The most susceptible bacteria based on the result of inhibitory zone diameters of Ethanol and Methanol extracts were the gram-positive *S. aureus* and *B. subtilis*, the gram negative *K. pneumoniae* showed lowest zone diameter of 1.2 and 1.3 cm on methanolic and ethanolic extract respectively. Both the extracts shown great result against *S. aureus* and ethanolic extract showed extraordinary result when compare to standard Ampicillin. The antibacterial activity observed in this study is very useful for developing drugs against many pathogenic bacteria. Since it suggests a different mechanism of action of *C. lantifolia* extracts than that of currently used antibiotics and further highlights its therapeutic value as an antimicrobial agent against multi-drug resistant strains. *C. latifolia* extract also contains citric acid which is very rich in vitamin C. The citric acid is a very important in treating skin diseases because it exfoliates the skin[17].

In this report, the growth of *B. subtilis*, *S. aureus* and *K. pneumoniae*, which are multi-resistant to different effective antibiotics, was strongly inhibited by both the extracts of *C. latifolia*. Whereas, the zone of inhibition shown in *K. pneumoniae* of Ampicillin is 1.4 but the zone of inhibition observed in *C. latifolia* extract with ethanol is 1.3 and methanol is 1.2 is much clearer than the standard antibiotic Ampicillin.

Fungal Strains

Aspergillus flavus has the ability to cause harmful effect in human, animal, plant and even birds. *C. latifolia* has shown great inhibitory minimal concentration towards it. The *C. latifolia* extract mixed with ethanol has shown more significant result compare to the *C. latifolia* extract mixed with methanol, which are shown in Figure 2. Numerous studies have described the inhibitory activities of *Citrus* against human pathogens, fungi, yeasts, and food pathogens[18].

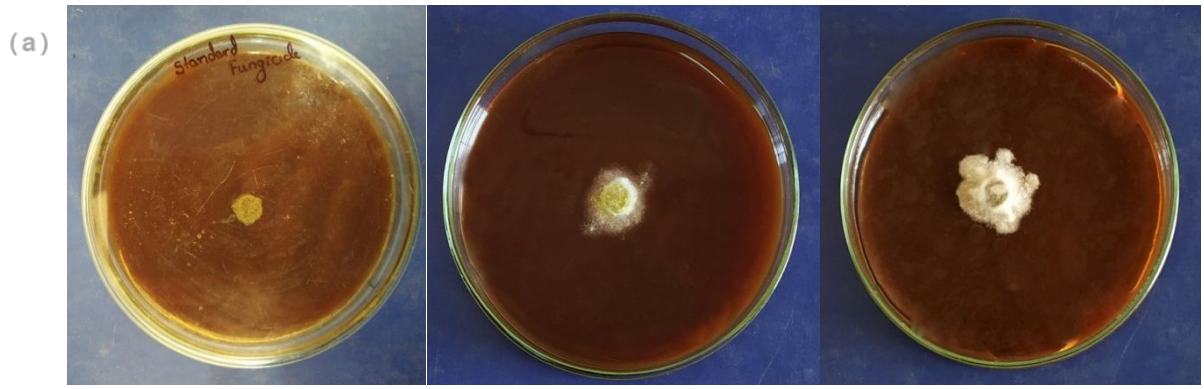
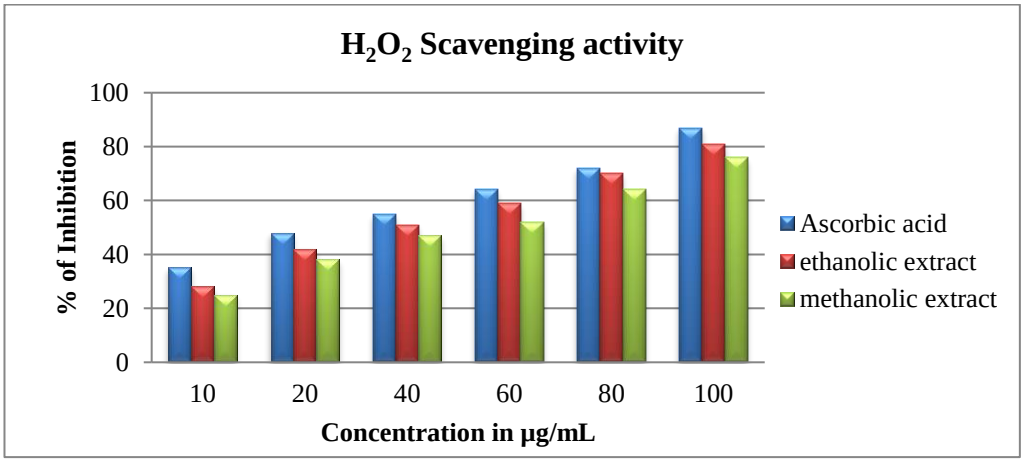


Figure 2: Plates showing growth inhibition of fungal colony. (a) Standard fungicide, (b) Ethanolic extract and (c) Methanolic extract

While, *A. flavus* the fungus were inhibited by the *C. latifolia* extract with Methanol and Ethanol extract shown a great result. Fungicides such as benlatecaptan, thiram, mancozeb, bavistin, cosan and copper oxychloride are routinely used for disease control. The resistance against these fungicides has been noticed in most fungal strains including *A. flavus*[19]. Hence, search for alternative disease control strategies has been triggered which gives more effective result against any fungi[20]. In the present study, we have determined the efficacy of *C. latifolia* extract and the extracts were found effective in inhibiting *A. flavus*. Peel extract had high inhibitory potential shown in the result.

Hydrogen peroxide scavenging assay of *Citrus latifolia* extracts

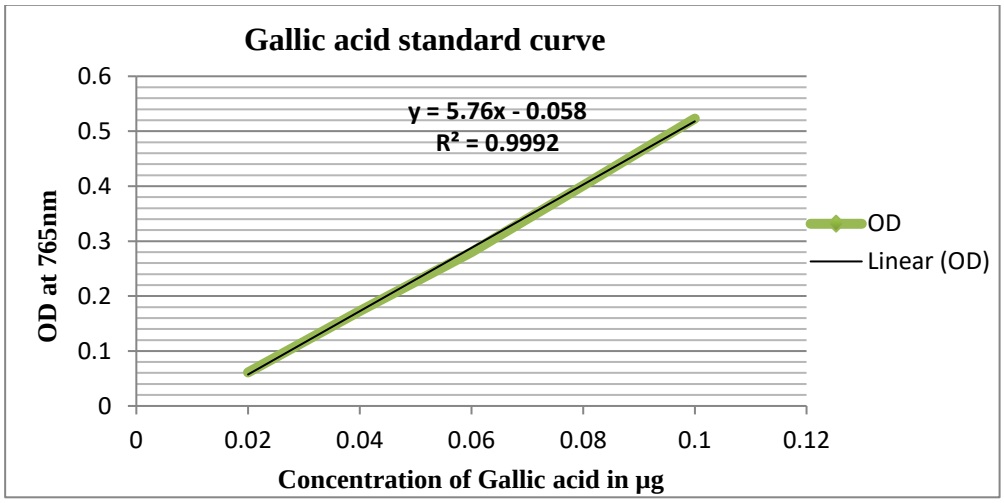
There are about 170 antioxidants from citrus fruit that have been reported in the literature, including vitamins, mineral elements, phenolic compounds, terpenoids and pectin[21]. Here we have reported the result of antioxidant activity in terms of Hydrogen peroxide radical scavenging effect of extracts of *C. latifolia* is shown in Graph 2. The extracts exhibited nearly equal scavenging of Hydrogen peroxide radicals when compared to Standard Vitamin C.



Graph 2: H_2O_2 Scavenging activity

Total phenolic content of extracts of *C. latifolia*

The Citrus fruits contains abundant amount of phenolic compounds and it show strong antioxidant properties through the dehydrogenation of hydroxyl groups. Table 2 shows the content of total phenolics in *C. latifolia* peel extracts (as estimated by FCR method). Total phenolic content, as estimated in terms of µg GAE/mg extract, was high in ethanolic extract of *C. latifolia* peel followed by methanolic extract.



Graph 3: Gallic acid standard curve

Table 2: Total phenolic content (µg GAE/mg) of extracts of *C. latifolia* peel

Extract	Total phenolic content (µg GAE/mg)
Ethanolic	0.297
Methanolic	0.259

CONCLUSION

The peels of citrus fruits are considered to be waste product of citrus processing industries. In the present report, the peel extract of *C. latifolia* exhibited tremendous inhibitory effect on microorganisms. So many countries have a high rate of skin infections but are not regarded as a significant health problem because of low level of lethality and severity. However, skin ailments present a major health concern because they occur worldwide. They are often persistent, long lasting and difficult to treat. The fact that extracts of *C. latifolia* exhibited a broad spectrum of antibacterial, antifungal activity and comparable efficacy to the synthetic antioxidants. The therapeutic value of *C. latifolia* as a potential source for drug development amidst the obvious dearth of effective and safe antibacterial drugs and also validates the claim of the peel extract. Since *C. latifolia* have shown considerable antibacterial, antifungal and antioxidant properties, it could be used as a preventive drug for many health problems that too without any side effects.

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