ANTIMICROBIAL, ANTIOXIDANT, CYTOTOXIC ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF ETHANOLIC EXTRACT OF CITRUS LIMETTA (PEEL, STEM AND LEAVES)

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ABSTRACT

Citrus limetta plant has potent activities against different infectious diseases and globally consumed citrus fruit. Studies revealed this plant has several chemical constituents that are known to have anti-microbial, anti-inflammatory, antioxidant properties. This plant is a best source to boost immunity and fight against infections and common flu and cold. The plant Citrus limetta (leaves, peel, stem) used in this research were collected from various areas of Pakpattan (Green Town) and ethanolic extract was prepared as per standard protocols. Gram-positive bacteria i.e., Staphylococcus aureus (ATCC 25923), Bacillus subtilis (ATCC 6633), Enterococcus faecalis (ATCC 29212) and Gram-negative bacteria i.e., Pseudomonas aeruginosae (ATCC 27853) and Klebsiella pneumonia (ATCC 33152) were used to check the anti-bacterial activities. Both Disc diffusion and well diffusion methods were used for the anti-bacterial pursuits. The cytotoxic pursuit of plant abstraction was set on using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] analysis. Antioxidant activities were analyzed by performing a DPHH test. Different tests have been performed including the Molisch experiment, Ninhydrin experiment, alkaline reagent experiment, Wagner’s experiment, Froth experiment, Ferric reagent experiment and Salkowski experiment for the detection of phytochemical constituents. The antimicrobial activity of ethanolic abstrac of Citrus limetta (leaves, peel and stem) tested by agar diffusion opposed to various bacterial ATCC cultures which reveal topmost zone of hindrance against Enterococcus Faecalis (24 mm) followed by Staphylococcus aureus (34 mm), whereas the minimal sector of
hindrance was shown by *Bacillus subtilis* (14 mm) after 24 and 48t hours of incubation at 37°C. The antioxidant activity results showed that it reduced the firm radical 1-diphenyl-2-picrylhydrazyl (DPPH) to yellow-tint DPPH-H outstretched 75.81% of DPPH rummage effect at its 100% attentiveness compared to vitamin c as testimonial standard being a secure antioxidant indicator. Cytotoxic activities of this plant revealed extraordinary viability of cells which is a novel property examined of this plant. Ethanolic extracts showed positive results against different test performed to check the phytochemical constituents. It was concluded that *Citrus limetta* ethanolic extract of (leaves peel and stem) possessed a potent antimicrobial, antioxidant and cytotoxic activity and the presence of phytochemical constituents of the plant make it an essential parameter in treatment of different pathological conditions.

**Keywords:** *Citrus limetta*, antioxidant activity, cytotoxic effect, ethanol extract.

**INTRODUCTION**

The Rutacea family member *Citrus limetta* Risso also known as sweet lemon in English, and Mousambi in Asian countries. This plant is native to the southern US (Damián-Reyna *et al.*, 2017). Other parts of the world and Mexico, citrus species like *C. aurantifolia*, *C. reticulata*, *C. limetta*, *C. sinensis*, *C. paradisi* are often harvested; several secondary metabolic products of these fruits have demonstrated bactericidal pursuit, serving to battle various illnesses in persons (Vikram *et al.*, 2010). They are also effective against viruses like the flu and herpes, antibacterial and antifungal (Vollumerhausen *et al.*, 2013). It has a greenish yellow peel that is tiny, oval and spherical, and rich in polyphenols, flavonoids, flavanones, and flavones (Kim *et al.*, 2009). The fruit is normally consumed by humans, but it has also been used to manage blood pressure, soothe inflammation, treat digestive issues, and lower cholesterol. Peel, which is normally a byproduct without any value and poses no environmental risk, makes up 8-10% of *Citrus limetta* (Fernández-López *et al.*, 2004). *Citrus limetta* peels are a type of agricultural waste material that are high in pectin and contain a significant amount of carboxyl corporations. They are typically produced in huge numbers by the fruit juice industr (Bulu & Saliu, 2020). Vitamins (A, C, E, and K), flavonoids, terpenoids, minerals, tannins, alkaloids, polyphenols, saponins, catalysts, pigments, and carotenoids with antibacterial cancer-preventing possessions are a few precedent of phytochemicals (Madhuri & Pandey, 2009). Antioxidants are substances that have the ability to
either prevent or delay the oxidation processes that take place when reactive or local atomic number 8 species are present. They are used to stabilize complex goods, petrochemicals, meals, cosmetics, and prescription medications. Antioxidants enact in the body's defense opposed to diseases brought on the onslaught of free radicals. DPPH, also known as two 2-diphenyl-1-picrylhydrazyls, is stable, cell-permeable leftist which is typically admittance a substance's capacity to operate as a H donor or radical scavenger as well as to assess the inhibitory effect of tissue extracts. The equivalent reductant, DPPH₂, is created when DPPH reacts with an inhibitor or reducing molecule. This reaction may result in a color different from violet to yellow (absorbance at 515-528 nm) (Badakhshan et al., 2012).

MATERIALS AND METHODS

*Citrus limetta* (leaves, peel and stem) used in this research were collected from various areas of Pakpattan (Green Town). *Citrus limetta* was dried, dipped in ethanol and shifted to shade and then rotary evaporator was utilized for extract preparation. The dried concentrate was then freezeed for further examination. Two Gram negative and three Gram positive bacterial ATCC cultures were used in this study i.e., *Pseudomonas aeruginosae* (ATCC 27853), *Klebsiella pneumonia* (ATCC 33152), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 25923) (Siddiqa et al., 2022). Mueller-Hinton agar, nutrient agar, nutrient Broth, normal saline were used for antimicrobial activity. Positive (Ciprofloxacin) and negative (DMSO) controls were used an inoculation of bacteria was done in normal saline solution. 5 ml solution was filled in each test tubes (6 test tubes). Afterwards each sample of bacteria dipped in each labled tube. Filter paper sterilized discs were placed on MH agar plates which was already swabbed bacterial ATCC cultures and plant extract was loaded along with negative and positive control (Rajiv et al., 2013). In well diffusion method, a hole was made with the help of a sterilized tip and then the plant extract with the given designated concentration was loaded in the well along with positive and negative controls. The dishes were then nurture at 37°C for twenty-four hours (Smahane et al., 2016). For phytochemical analysis, Muller Hinton Agar (MHA) medium was poured on petri dishes for testing of microorganism. The channel paper hovers (5 mm in width) was determined to the agar plates and that were stacked with 20 µl of plant separate. The plates were along these lines put at 37°C for 24 hours. In the wake of putting the advancement limitation zone were estimated
in mm (Kumar et al., 2009). For testing of carbohydrates, Molisch’s test was used. In this test a solution in a test tube was mixed with 2 dribble of Alpha-Naphtholbenzene mixture. Then 2 ml sulphuric acid was added along the side walls of the vacutainer. A purple violet loop was observed which designate the existance of carbohydrates (Pathare et al., 2014). Plant extract was mixed with Ninhydrin reagent and heated till boiling temperature reached. Upon boiling, the color change was detected as blue-violet which interpreted as amino acids or proteins. Alkaloids were examined using Wagner’s assay. In a test tube, Wagner's reagent (iodine in potassium iodide) was combined with this filtrate solution. Alkaloids were present as indicated by a brown or reddish precipitate. Alkaline reagent testing was done to determine the presence of flavonoids. A few dribbles of a NAOH suspension were added to the plant abstraction during this experiment. When diluted acid was added, an intense yellow colour development in the test tube, which indicated the presence of flavonoids. Froth testing was done to identify saponins. For this test, 2 ml of distilled water and dry powder extract were dissolved, mixed, and left to stand for 10 minutes. Saponins were present because of the frothy look. Using Keller-technique, Kiliani's cardiac glycoside was found to be present. In a test tube, dried extract and 1 ml of FeCl$_3$ reagent were combined for the experiment. In the vacutainer, a few dribbles of concentrated H$_2$SO$_4$ were computed. After a few minutes, a test tube's greenish blue tint designate the involvement of cardiac glycosides. The unfold loop-closed loop reaction test was conducted using Coumarins experiment. For this test, a test tube was filled with two drops of a 1% NaOH solution and warmed up over a flame to a short period of time. In the test tube, 4 dribble of 2% HCl were computed. Presence of coumarins is indicated by cloudy ring development. Salkowski testing was done to check for steroids. In this test, the extract was combined with chloroform, little quantity of concentrated H$_2$SO$_4$ was appended. Presence of steroids indicated by the colours cherry red, bluish red, and purple. Braymer's test was conducted as part of Tannin's exam. For this test, 10% alcoholic FeCl$_3$ was combined with crude dry powder. The existance of tannins is suggested by the green colour or blue-black. For antioxidant experiment, a cylinder held 50 ml of concentrates with concentrations ranging from 1 to 5 mg/ml and 5 ml of a 0.1 mM DPPH tincture (4 mg/100 ml alky). The obtained mixture vortexed, nurtured for 30 minutes at 25ºC in an usually faint area, then examined with a spectrophotometer at 517 nm. Ethanol made up 80% (v/v) of the clear. For correlation, ascorbic acid corrosive (10 mg/ml DMSO) was used. The accompanying condition was used to test the
influence of DPPH searching. DPPH searching impact (%) = \([(AB - AA)/AB]\) × 100 (Badakhshan et al., 2012). Through the use MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium sedative] test, cytotoxic activity of plant extracts was assessed (Applichem, USA). 19 exponentially developing cells were painted in threefold into 96-well Greiner dishes (Germany) in 200 l of development media, the cells were nurtured for twenty-four hours before abstractions were computed.

RESULTS and DISCUSSION

The proposed study was comprised of Citrus limetta also known as sweet lime. Ethanolic extract of the stem, leaves and peels of the plant collected from Pakpattan was prepared by following standard protocols. ATCC cultures of Gram negative and Gram-positive bacteria were applied. Nutrient agar was used to grow the bacteria in controlled conditions. Muller Hinton Agar was used to perform the antibacterial activity. Disc diffusion and well diffusion techniques were applied to assess anti-bacterial proceedings of the manufactured extracts against different bacterial cultures along with positive (Ciprofloxacin) and negative controls (DMSO) (Figure 1 and 2).

For antioxidant activity DPPH assay (Figure 4) was used and for cytotoxic activity MTT assay was performed (Figure 3). Different tests have been performed including Molisch experiment, Ninhydrin experiment, alkaline reagent experiment, and Wagner’s experiment, Froth experiment, Ferric reagent experiment and Salkowski experiment for the detection of phytochemical constituents (Table 1). Ethanolic extract of Citrus limetta capsule, leaf, stem abstractions, Pseudomonas aeruginosa (ATCC 27853), Bacillus subtilis (ATCC 6633), and Enterococcus faecalis (ATCC 29212), Staphylococcus aureus (ATCC 25923), Klebsiella pneumonia (ATCC 33152) exhibited maximum zones of inhibition against bacterial growth. Researchers found that after 48 hours of maturation at 37°C, peel extract showed greatest segment of hindrance against B. subtilis ATCC 6633 (26 mm), B. cereus ATCC 14579 (28 mm) and stalked S. aureus ATCC 25923 (21 mm), whereas F. oxysporum ATCC 48122 showed the least amount of inhibition after forty-eight hours at 25°C in differentiation to streptomycin/fluconazole at 20µ platter (Javed et al., 2013). Another group of studies presented by Mahmud and his colleagues in 2009 reported the maximum segment of hindrance against B. subtilis was demonstrated from C. acida peel oil.
Figure 1: Disc diffusion method ethanolic extract of *Citrus limetta* plant (75mg and 100mg/1ml DMSO).

Figure 2: Well diffusion method ethanolic extract of *Citrus limetta* plant (75mg and 100mg/1ml DMSO).
Table 1: Phytochemical Analysis of *Citrus limetta* plant

<table>
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<tr>
<th>Phytochemicals</th>
<th>CL-01(P)</th>
<th>CL-02(S)</th>
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<tr>
<td>Corbohydrates</td>
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<td>Proteins</td>
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<td>Phenols</td>
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Numerous more investigations also demonstrated the potent antibacterial properties of limonene, and linalool (Mahmud *et al.*, 2009). The capacity of important extracts to act as a donor for electrons or hydrogen atom in alteration of DPPH leftist into its reduced form DPPH-H (which is measured spectrophotometrically) gives them tocopherol pursuit attributes. The results of DPPH scavenging mechanism of *C. limetta* peel, stem and leaves extracts contrast with vitamin c as a testimonial standard are designating that it has lower antioxidant pursuits relative to testimonial standard, vitamin c, being a strong antioxidant indicator. The peel extract of *C. limetta* was able to reduce the stable radical DPPH to yellow-colored DPPH-H reaching 75.81%, for leaves 59.56% and stem 75.37% of DPPH scavenging effect at their 100% concentration whereas the reference standard, ascorbic acid, gave a 92.56% DPPH scavenging effect at its 100% concentration. Mahmud *et al* studies also indicated that essential peel of *C. acida* var. sour lime showed 91.7% of DPPH scavenging effect at its 100% concentration compared with ascorbic acid as a reference standard. Sacchetti *et al* studies also showed that essential oils of *Cananga odorata*, *Cymbopogon citratus*, *Rosmarinus officinalis* and *Curcuma longa* notably reduced the concentration of DPPH free radical indicating their strong antioxidant activities.
Cytotoxic activity of *Citrus limetta* Peel

Cytotoxic activity of *Citrus limetta* Stem

Cytotoxic activity of *Citrus limetta* leaves

**Figure 3:** MTT assay of Leaves, Peels and Stem of *Citrus limetta*. 
Figure 4: DPPH assay of ethanolic extract of leaves, peels and stem of *Citrus limetta*.
Other studies also showed that radical-scavenging activities using DPPH of 31 kinds of citrus essential oils were comparable with or stronger than that of Trolox (standard antioxidant) (Choi et al., 2000). *Citrus limetta* peel, stem and leaves ethanolic extracts have very essential compounds those are effective against many food borne bacteria and pathogenic bacteria. Peels extract is the most efficient in antibacterial, anti-oxidants and anti-tumor activity. This fruit is normally consumed by humans, but it has also been used to reduce blood pressure, treat irritability, treat digestive issues, and lower cholesterol. Ascorbic acid usually used in comparison of DPPH for antioxidant activity, and these *Citrus limetta* stem, leaves and stem extracts showed greatest comparison in DPPH assay and have strong antioxidant effect. In a recent study in Mexico showed that its culmination is used for his or her antihyperglycemic and antihypertensive activity (Damián-Reyna et al., 2017). Limonoids and flavonoids found in them are responsible for their use in traditional antitumor and anti-inflammatory purposes. In current study, effective cytotoxic activity has found in the ethanolic extract of *Citrus limetta* (Simonne et al., 2011).

**CONCLUSION**

This study indicates the ethanolic extract of *Citrus limetta* (leaves, peel and stem) possess a potent antioxidant, anti bacterial and cytotoxic activity and phytochemical constituents indicates the biochemical potential of the plant which may help in treating many diseases and cope up the conditions like common flu and cold by boosting immune responses. Peel extract is a promising candidate for use as natural products based antioxidants for the health of human being. In the present study, different tests has been performed to identify and characterize the specific phytochemicals. It is proposed that there are many emerging opportunities and strategies present which can enable the successful development of *Citrus limetta* extracts from the peel, stem, and leaves which can be used as a natural component in food preservation, cosmetics, and medications because to their potent antioxidant, antibacterial, and phytochemical properties.

**Conflict of interest**

The authors declare no conflict of interest.
REFERENCES


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