Journal of Global Biosciences
ISSN 2320-1355
Volume 8, Number 8, 2019, pp. 6382-6396
Website: www.mutagens.co.in

Research Paper

THE PHYTOCHEMICAL AND PHARMACOLOGICAL ACTIVITY OF *Citrus limetta* PEEL EXTRACTS

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Abstract

Phytochemical screening of citrus peel extracts was performed for the presence of various phytochemicals. The phytochemical screening results indicated the presence of different phytochemical and bioactive compounds such as flavonoids, steroids, alkaloids, terpenoids, tannins, phenols carotenoids, minerals and vitamins. Citrus plants are used for the different purposes such in the food and beverage industries, cosmetic, and medicinal applications. The Citrus plants are not only providing nutritional supplements but also help for the reducing risk of several illnesses. Some research articles suggested that C*itrus limetta* is a potential source of antibacterial, antioxidant, anti-inflammatory and antitumour activities. We focused on *Citrus limetta* peels that are the important constituents of phenolic compounds and it's free radical scavenging ability facilitated by their hydroxyl groups. The result indicates a good radical scavenging ability of C*itrus limetta* peel that will be useful in medicine, pharmaceutical, and cosmetic industries. It can be also used as a preservative in food industries due to its antioxidant properties. This study highlighted the industrial, pharmaceutical and medical potentials of the *Citrus limetta* peel.

Key words: World health organization, Phytochemicals, Bioactive compound, Medicinal, Antioxidant, Antimicrobial.

INTRODUCTION

The World Health Organization report indicating that there are 21,000 plant species used for medicinal purposes. In India around 2500 plant species are used which belongs to more than 1000 genera are to cure several diseases [1, 2]. The medicinal plants are the best source to obtain a variety of drugs. The World Health Organization 2008 report indicating that about 80% of individuals from developed countries are depending on traditional medicine for the primary health care [3]. Especially for the phenolic compounds that vary from simple phenolic acids to highly polymerized compounds such as tannins and phenolic compounds [4]. The largest category is the flavonoid group, which comprises 13 classes with over 5,000 compounds [3]

Phytochemicals have several activities but it's mainly known for the antioxidant activity [5]. The plant family *Rutaceae* is also known as 'Citrus Fruit Family' which is the rich source of antioxidant. Citrus fruits belong to six genera that are Fortunella, Eremocitrus, Clymendia, Poncirus, Microcitrus, and Citrus. The family *Rutaceae* is a family of trees, shrubs, and herbs [6]. These are the native plant of tropical and subtropical regions of Asia, but the genus citrus is the major commercial fruits [7]. The most important products of citrus fruits are the essential oil that is obtained from the citrus peels [8]. The peels of *Citrus limetta* and orange usually discarded as a byproduct which represents around 50 to 65% of the weight [9]. These residues can be used as livestock feed, although it has very low nutritional value. The peels possess quite good radical scavenging and antimicrobial abilities [10]. The citrus peels contain high quantity of phenolic compounds which includes several flavonoid compounds. The citrus peel extracts just like essential oils are known to exhibit various biological activities such as antimicrobial, antioxidant activities and the potential sources for the screening of anticancer [7]. Citrus essential oils are a mixture of volatile compounds and mainly contain monoterpene hydrocarbons [11]. Citrus flavonoids have a broad spectrum for the biological activity such as antibacterial, antifungal, antidiabetic, antiviral activities [5]. There are many studies which are suggesting that the endogenous antioxidants, or exogenous antioxidants which are supplied by the diet, can function as free radical scavengers and will help to improve human health [4].

Whereas the reactive oxygen species (ROS), are formed under normal physiological conditions which are becoming deleterious if it's not eliminated by the endogenous systems. In the body, free radicals originate from two sources: endogenous

sources, such as nutrient metabolism, ageing process etc. and the exogenous sources such as tobacco smoke, ionizing radiation, air pollution, and organic solvent [12, 13]. The imbalance between the generation of reactive oxygen species and the endogenous antioxidant systems creates oxidative stress that is causing numerous diseases and disorders [14] such as cancer [15] Alzheimer's diseases [16] ageing [17]. The secondary metabolites especially phenolic and flavonoids are potent free radical scavengers play a defensive role and are able to modulate enzymatic activities and inhibit cell proliferation [18, 19].

Nowadays, there are many synthetic antioxidants which are frequently used to cure the disease. They cause several side effects, on the different organ such as kidney and liver etc. Some study also suggesting carcinogenesis effect in laboratory animals [20, 21 & 22] also another side. Now microorganisms are getting resistance against the antimicrobial drugs, which is becoming a big challenge.

The aim of this study is to evaluate the phytochemical composition, antioxidant, antimicrobial, anti-inflammatory and cytotoxicity activities of citrus limetta peels. Citrus limetta peels are getting more attention to the scientific community due to its connection between flavonoids intake and disease prevention [23]. Analysis of phenolic and flavonoid compounds in citrus limetta peels will help to get most effective, less toxic and cost-effective antioxidants.

MATERIALS AND METHODS

Plant Material and preparation of the Extracts

The peels of *Citrus limetta* were collected from the market surrounding region of Gulbarga, Karnataka, and authenticated at the Department of Botany, Gulbarga University, Gulbarga. The peels of *Citrus limetta* were dried under the shade, chopped into small pieces, and coarsely powdered by using a chopper. Then these coarse powders were subjected for the successive extraction by using different solvents ranging from non-polar to polar i.e. Chloroform, methanol, and hexane by Soxhlet method. The extracts were then collected and dried inside the hot air oven then citrus peel extracts were collected and stored at 4˚C.

Phytochemical Screening

Phytochemical screening of citrus peel extracts was performed by using the method described by Kokate [24] for the presence of various phytochemicals. The extracts of

citrus limetta peel were used in different concentrations for the bioassay.

Test Organisms

All the microbial strains of human pathogens were used for antimicrobial bioassay were procured from Department of Biotechnology, Gulbarga University Gulbarga, Karnataka, India. These microbes are the Gram-negative bacteria such as Salmonella typhimurium (MTCC 98), Klebsiella pneumonia (MTCC 109), *Escherichia coli* (MTCC 724), and Pseudomonas aeruginosa (MTCC 424).

Bioassay for antimicrobial activity

The antimicrobial activities were determined by using Agar well-diffusion method by Perez [25]. Petri plates were prepared with specific inoculums. Four wells with 10mm diameter were made in each of the plates using sterile tips. We used 0.5 ml of different concentrations of solvent extracts were added by using sterilized pipettes into the wells and in the control experiments without plant extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens. The diameter of the inhibition zones was measured. The experiment was repeated thrice and the average values were recorded.

Antioxidant activity:

ABTS radical scavenging activity

ABTS radical scavenging activity was estimated by the method of Thoo [26] and Surveswaran [27] with slight modification. ABTS 7mM concentration was dissolved in water, ABTS radical cation was produced by reacting stock solution with 2.45mM potassium persulfate solution. The two stock solution was mixed in equal quantities (1:1) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The solution was diluted by mixing 100 µl of ABTS solution with 3.9 ml of ethanol to obtain an absorbance of 0.700 ± 0.02 units at 734nm. Fresh ABTS solution was prepared for each assay. Different concentrations of methanolic extracts (1 mL) were allowed to react with 1 mL of the ABTS solution and the absorbance was measured at 734 nm after 6 min using a UV-Visible Spectrophotometer. ABTS radical scavenging activity was calculated according to the following equation:

ABTS radical scavenging activity % = (Ac - As) /Ac * 100

Where Ac is the absorbance without samples and As the absorbance in the presence of the samples

Reducing power

The reducing power of methanolic extracts was determined according to the method of Oyaizu [28] with slight modification. The different concentration of methanolic extract 0.25ml was mixed with 0.25 ml phosphate buffer (0.2 M, pH 6.6) and 0.25 mL, of 1% potassium ferric cyanide [K3Fe (CN) 6]. The mixture was incubated at 50°C for 20 min. the reaction was terminated by adding 0.25 ml of trichloroacetic acid solution (10%, w/v). The mixtures were centrifuged at 5000 rpm for 5 min. The supernatant (0.5 ml) was mixed with an equal volume of distilled water and 0.1 ml of ferric chloride solution (0.1%, w/v). The intensity of the Prussian blue color was measured at 700 nm using a spectrophotometer. Results are expressed as the mean absorbance value.

Hydrogen peroxide-scavenging activity

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Cetinkaya [29]. Hydrogen peroxide solution (1 mM/L) was prepared with 50 mM phosphate buffer (pH 7.4). Different concentrations of methanolic extracts (1 mL) were allowed to react with 0.6 mL of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Hydrogen peroxide scavenging activity was calculated according to the following equation:

$$\text{Hydrogen peroxide scavenging activity\%} = ((Ac - As) / Ac) * 100$$

Where Ac is the absorbance without samples and As is the absorbance in the presence of the samples.

Cytotoxic study

Determination of cell viability by MTT Assay

The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue colored product (formazen) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazen production by the cells used [30].

The monolayer MCF-7 cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well

microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 ml of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 ml of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 ml of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

% Growth inhibition = 100 - (Mean OD of individual test group / Mean OD of control group * 100).

RESULTS

Phytochemical screening of *Citrus limetta* peel

The extractive yield of *Citrus limetta* peel extract were 15% for hexane extract, 4% for chloroform extract and 6% for methanol extract. Phytochemical screening of the hexane and chloroform extract revealed the presence of Alkaloids, terpenoids, steroids, tannins, and phenols, whereas methanolic extract showed the presence of Alkaloids, terpenoids, flavonoids, steroids, tannins and phenols as shown in Table 1.

Antimicrobial Activity of *Citrus limetta* peel extract

In the present investigation, *Citrus limetta* peel extract was screened for its antimicrobial activity. The result of antibacterial activity obtained for the different concentration of three crude extracts from *Citrus limetta* peel extract by the agar well diffusion method was shown in Table 2. Bacterial strains i.e. *Salmonella typhimurium* (MTCC 98), *Klebsiella pneumonia* (MTCC 109), *Pseudomonas aeruginosa* (MTCC 424) and *Escherichia coli* (MTCC 724) were used. The methanolic extract of Citrus limetta peel showed the highest antibacterial activity that could be considered as one of the

sources of natural antibiotics for medicinal use against an opportunistic pathogen result shown in fig.1.

Antioxidant assay

ABTS radical scavenging activity

The methanolic extract was more potent than reference standards used (98.81%, 68.83%, and 55.05%) in scavenging $ABTS^{+}$ radicals. Fig.2 demonstrated a steady increase in the percentage of inhibition of the $ABTS^{+}$ radicals by the methanol extract and maximum inhibition was achieved above 1000 µg/mL. IC50 values for methanolic extract, Ascorbic acid and BHT were 280, 500 and 450µg/mL respectively. In contrast, the Ascorbic acid and BHT did not show a leveling off at the highest concentration, however, its radical scavenging effects were much less than the methanol extract.

Ferrous Reducing Antioxidant Power (FRAP)

The antioxidant activity was investigated by methanolic extract along with Ascorbic acid and BHT as a standard reference. The reducing power of Citrus limetta peel extract along with Ascorbic acid and BHT is shown in Fig.3 BHT showed high reducing power than ascorbic acid and methanolic extract. The reducing power increased with increase in concentration. The reducing power is due to the presence of phenolic content which acted as a free electron donor and converted free radicals into a more stable product and terminating the free radical chain reaction [32]. In contrast, when compared to methanolic extract and ascorbic acid, both showed similar reducing power and less effective compared to BHT.

Hydrogen peroxide-scavenging activity

Hydroxyl radical inhibition from methanolic extracts of *Citrus limetta* peel was investigated and the results obtained were concentration gradient Fig.4 BHT showed the highest inhibition along with ascorbic acid and methanolic extract (96.57%, 94.05%, and 84.32%) respectively. The methanolic extract was similarly effective in comparison with Ascorbic acid and BHT. The IC50 values were 80, 70 and 60 µg/mL respectively.

Cytotoxicity Assay

The effect of the methanolic extracts of the *Citrus limetta* peel was investigated by the MTT assay on the MCF-7 cell lines for the cytotoxicity against MCF-7 cell lines at the different concentrations to determine the IC50 value (concentration mg/ml) that causes a 50% cell death). The USNCI plant screening program has mentioned that the crude extract considered having in vitro cytotoxic effect if the IC50 value of 48-72h pretreated

carcinoma cells is less than 20 mg/ml and 4 mg/ml for pure compounds. Fig.5 shows that only methanolic extract of *Citrus limetta* peels exhibited high in vitro cytotoxic activity against MCF-7 cell lines. Results show that the 79 % growth inhibition was increasing steadily with increasing concentration up to 1000 µg/ml on MCF-7 cell lines. IC50 value of this assay for MCF-7 cell lines pretreated with methanolic peels extract was 153.33±1.6 µg/ml.

Table 1. Screening of *Citrus limetta* peel extracts

Phytochemical Screening	He	Ch	Me
Alkaloids	+	+	+
Terpenoids	+	+	+
Flavonoids	-	-	+
Steroids	+	+	+
Tannins	+	+	+
Phenols	+	+	+

The tested samples were He: Hexane extract, Ch: Chloroform extract, Me: Methanol extract.

+ indicates the presence and – indicates absence of the compound.

Table 2.Antibacterial activity of crude extract obtained from *Citrus limetta* peel extract.

Test Extract Concentration		Zone of Inhibition (mm)			
		E.coli	*S.typhimurium*	*K.pneumonia*	*P.aeruginosa*
Met	75	18	16	17	15
Chl	75	14	14	14	15
Hex	75	11	9	11	8
DMSO	75	--	--	--	--
Chp	Disc	30	28	26	29

(–) no growth inhibition zone observed. The tested samples were: Met- Methanol extract, Chl- Chloroform extract, Hex- Hexane extract, DMSO- Dimethyl Sulfoxide, Chp- Chloramphenicol disc.

Figure 1: Antibacterial activity of crude extract obtained from *Citrus limetta* peel extract.

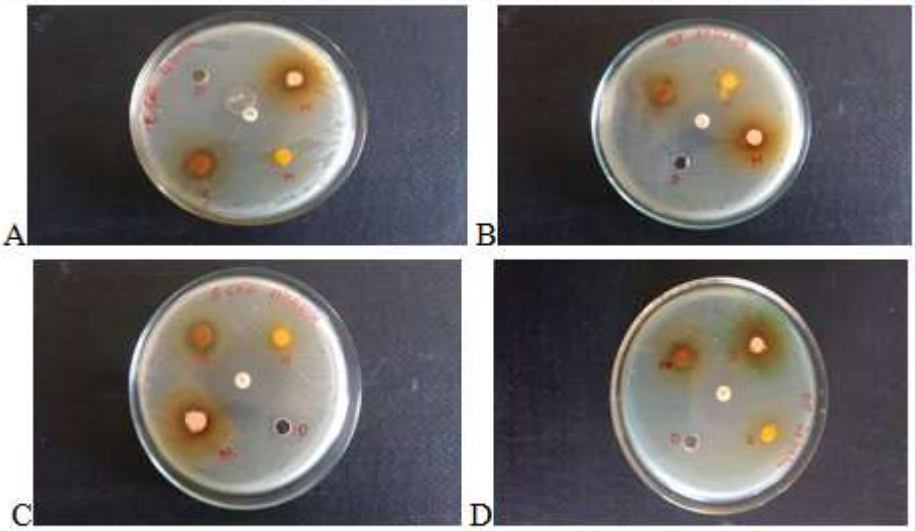


Fig1. Antibacterial activity of crude extract against pathogenic Gram- negative bacteria: A - *E coli*, B - *S.typhimurium*, C - *K.pneumonia*, D - *P.aeruginosa*

Fig-2 illustrating ABTS radical scavenging activity

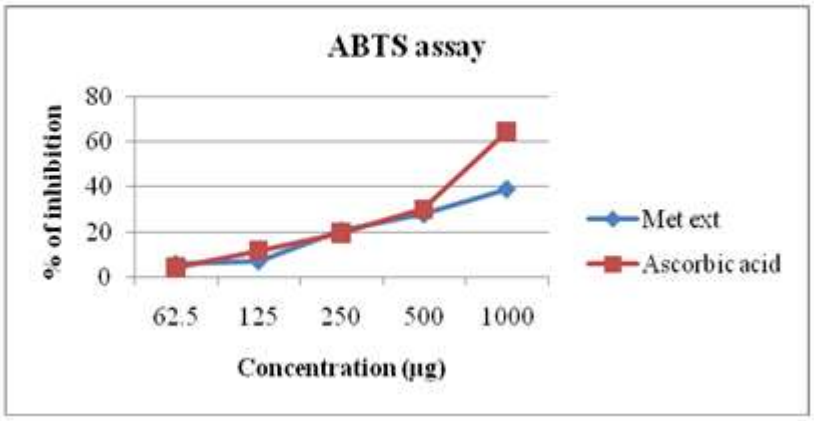


Figure-3 illustrating Ferrous Reducing Antioxidant Power assay

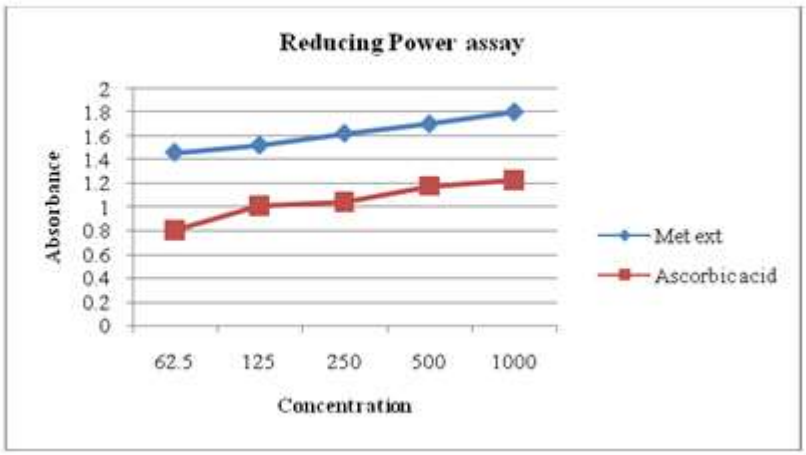


Figure-4 illustrating Hydrogen peroxide-scavenging assay

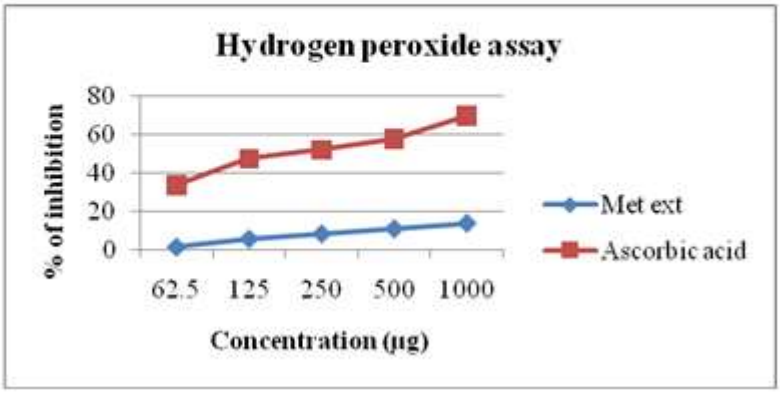
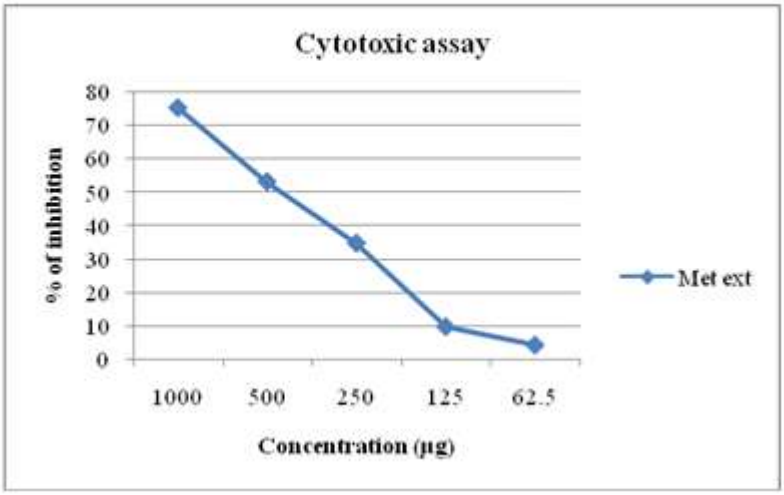


Figure 5- illustrating Cytotoxicity Assay



DISCUSSION

The aim of our present study was to utilize the waste that is generated from the *Citrus limetta* fruit. Usually, these samples are regarded as waste materials after extracting juice. The phytochemical screening of the *Citrus limetta* peel extract indicated the presence of different phytochemical compounds; flavonoids, steroids, alkaloids, terpenoids, tannins, and phenols. The rapid emergence of multiple drug resistance strains of pathogens to current antimicrobial agents has generated an urgent intensive for new antibiotics from medicinal plants. Many medicinal plants have been screened extensively for their antimicrobial potential worldwide. The most important of these bioactive constituents which are mainly secondary metabolites are alkaloids, flavonoids, tannins and phenolic compounds. These phytochemicals are toxic to microbial cells. Phenolic compounds are a class of chemical constituents containing one or more acidic

hydroxyl residues attached to an aromatic arene (phenyl) ring. They are one of the most effective antioxidative constituents that contribute to the antioxidant activity of plants. Antioxidant activity is manifested in a wide variety of actions, such as inhibition of oxidising enzymes, chelation of transition metals, transfer of hydrogen or a single electron to radicals, singlet oxygen deactivation, or enzymatic detoxification of reactive oxygen species. The overall antioxidant activities should be evaluated by different methods in order to extensively characterise the antioxidant potential of pure compounds or extracts. Therefore, the extract of *C. limetta*, as well as standard antioxidant ascorbic acid and BHT, were examined with regard to scavenging capacity towards ABTS, hydrogen peroide and reducing power. Antioxidant activity of *C. limetta* extract was concentration-dependent in all the applied tests. Overall, our results demonstrate that *C. limetta* showed a level of antioxidant activity that was better than synthetic antioxidant ascorbic acid and BHT in scavenging capacity towards ABTS, hydrogen peroide and reducing power, which is in accordance with previous reports. However, the presence of the aforementioned phenolics could be at least partially responsible for the antioxidant activity of *C. limetta*, since antioxidant effects have often been regarded as dependent on the presence of this class of compounds. Furthermore, a dietary intake of phenolics has been associated with reduced risk of different diseases, such as cancer, cardiovascular disease, diabetes, or atherosclerosis, probably due to their potent antioxidant properties. Thus, the estimated antioxidant activity of *C. limetta* can contribute to the benefits of this species as a food or food supplement. Whereas in our cytotoxicity assay we found that the methanolic extract of *Citrus limetta* peels exhibited a high in-vitro cytotoxic activity against MCF-7 cell lines. Based on our results citrus limetta peel will be useful in medicine, pharmaceutical, cosmetic industries. However, a further phytochemical analysis is needed for the isolation of pure bioactive molecules from the *Citrus limetta* peel that may show a broad spectrum of pharmacological activities.

CONCLUSION

In conclusion, the results obtained in this study are clearly indicating a broad spectrum antibacterial activity of *Citrus limetta* peel against pathogenic bacteria. The comparative analysis of commercially available standard antibiotics and *Citrus limetta* peel extracts on tested bacteria were better than available antibiotics. The methanolic extract of

Citrus limetta also showed a good radical scavenging, antimicrobial, antioxidant, anti-inflammatory and cytotoxic activities. This study highlights the industrial, pharmaceutical and medical potentials of the samples studied.

ACKNOWLEDGEMENT

The author thanks to Department of Biotechnology, Ministry of Science and Technology for the financial support.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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