International Journal of Herbal Medicine
Available online at www.florajournal.com

IJHM
International Journal of Herbal Medicine

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2017; 5(5): 89-93
Received: 05-07-2017
Accepted: 06-08-2017

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In vitro antioxidant, antimicrobial properties and total phenolic contents of *Citrus limetta* collected from Sonapur, Assam, India

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Abstract
The objective of the present work was to evaluate the antioxidant, antimicrobial activities and phenolic contents of juice of *Citrus limetta* from Sonapur, Assam, India. Antioxidant Assay was determined by DPPH free radical scavenging activity, Super oxide dismutase (SOD) and Nitric oxide radical scavenging activities. Total phenolic content was determined by using catechol as standard. MIC for two different microbes was obtained by spectrophotometry method. The extracted juice was used for various estimations to measure total antioxidant, super oxide dismutase and nitric oxide activity *in-vitro* and determined the antimicrobial potentials. The *in vitro* antioxidant activity of the extracted juice (at concentrations 12.5-100 µl/ml of methanol) was determined by DPPH free radial scavenging activity and found in the range of 56%-95.0% respectively. Super oxide dismutase and nitric oxide scavenging activities at 100 µl of juice extract were found to be 74% and 54.8%, respectively. Total phenolic content was found 0.007 mg/ml. Minimum inhibitory concentration (MIC) was found for 75% juice for *E. coli* and at 50% juice for *P. aeruginosa*. The results obtained in this research work clearly indicated a promising potential of Citrus intake for playing a protective role against oxidative stresses and can inhibit the growth of highly resistant and opportunistic bacteria.

Keywords: Crude juice, Oxidative stress, Antioxidants, Antimicrobial activity, ROS, Hypertension.

1. Introduction
Reactive oxygen species (ROS) are very unstable and react rapidly with DNA, membrane lipids and proteins. Over production and unchecked ROS generations are associated with various health related disorders such as diabetes mellitus, hypertension, cancer, neurodegenerative, gastric ulcers, reperfusion, arthritis and inflammatory diseases [1, 2]. Antioxidants are well known scavenger of free radicals and ROS that intrude the radical chain reaction of lipid peroxidation, thus preventing oxidative damage [1]. People are now very much concern about eating different fruits including citrus fruits to reduce the deleterious effect of ROS because of its rich antioxidant contents. Citrus fruits have a small edible portion and large amounts of waste materials such as peels and seeds. The peels are used in folk medicine for the management of degenerative diseases, such as diabetes and hypertension, though there is very limited information on the mode of action of these peels in the management of diabetes and hypertension. The content of total phenols was found to be higher in peels of citrus fruits than in peeled citrus fruits [3]. Peels of citrus fruits contain significant amount of phenolic compounds, especially phenolic acids and flavonoids [4]. The interest in citrus fruits is increasing day by day for its different biological roles *viz*. anti-oxidant, antimicrobial and many more properties. Over the last decades, many other virtues and medicinal benefits of citrus fruits have been discovered besides the anti-scurvy property [5]. The consumption of fruit juices and beverages has been contrariwise associated with morbidity and mortality from degenerative diseases [6-8].
Reactive oxygen species attack and damage body cells to get the missing electron they need, but antioxidants protect the body by contributing an electron of their own, and in so doing, they neutralize free radicals and help prevent cumulative damage to body cells and tissues. Much of the total antioxidant activity of fruits and vegetables is related to their phenolic contents, not only to their vitamin C content, also a correlation exists between the polyphenol content and antioxidant activities [9]. Research suggests that many flavonoids are more potent antioxidants than vitamins C and E. Natural polyphenols exert their beneficial health effects by their antioxidant activity, these compounds are capable of removing free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals and inhibit oxidases [10, 11].
Microorganisms become resistant to most antibiotics and spread rapidly.

Therefore, antimicrobial compound study is in great demand. Microorganisms are basically used for antibiotic production but plants also represent an important source of antimicrobial compounds. Different bioactive compounds are isolated from plants which contribute for the antimicrobial activity. The effects of essential oils from *C. aurantium*, *C. limon*, *C. paradise* and many other plant oils and extracts were studied and minimum inhibitory concentrations (MIC) were found between 5-20% [12].

The objectives of this work are to look into the different antioxidant and antimicrobial properties of *Citrus limetta in-vitro*. In this study, we have seen the antioxidant potential of *Citrus limetta* by determining super oxide dismutase and nitric oxide activities and total phenol contents. We have also determined antimicrobial potential against *E. coli* and *P. aeruginosa* in presence of different concentrations of juice extracts.

2. Materials and methods

2.1. Collection of Samples

Fresh ripen samples of *Citrus limetta* were collected from Sonapur, Kamrup, Assam, India (fig. 1). A dozen of fruits was collected and brought to the laboratory for further processing and investigations (fig. 2).

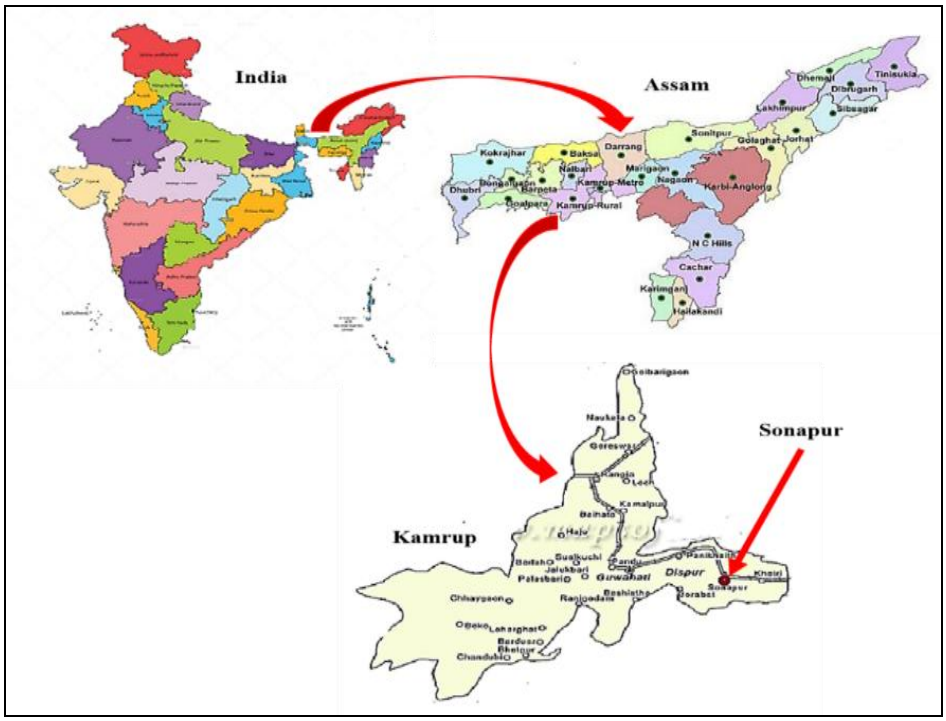

Fig 1: Collection site of *Citrus limetta* from Sonapur of Kamrup district of Assam, India**.**

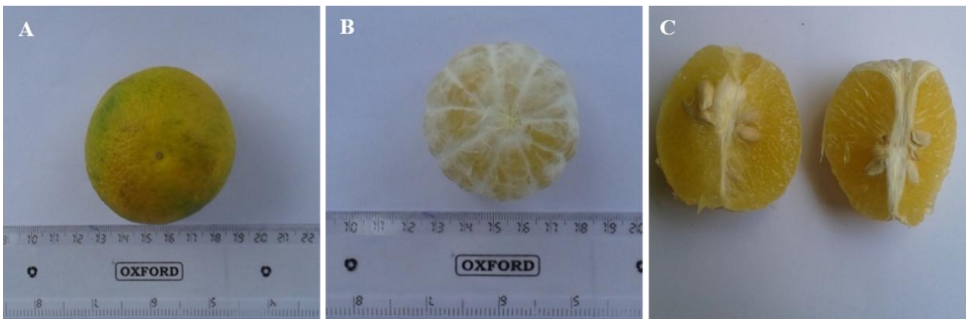

Fig 2: *Citrus limetta* fruit collected from Sonapur of Kamrup district of Assam, India and measured the size when brought into the laboratory. Pictures are- A: whole fruit; B: peeled fruit and C: slices of the fruit.

2.2. Extraction of Juice

The fruits were washed thoroughly in water and dried. The juices were extracted by manual squeezing. The collected juice was centrifuged at 10000 rpm for 10 minutes, supernatant was used for the present study and pH of the juice was recorded.

2.3. Antioxidant Assay

2.3.1. Antioxidant activity by DPPH free radical scavenging assay

Different concentration of juices was prepared (from 12.5 µl to 100.0 µl) in methanol. 2ml of DPPH was added and incubated. After incubation at room temperature for 30 min, optical density was measured at 517 nm [13]. The scavenging activity was calculated by the formula-

$$\text{Scavenging activity (\%)} = \frac{(A-B)}{A} \times 100$$

Where, A = absorbance of DPPH and B = absorbance of fruit juice and DPPH combination.

2.3.2. Super oxide dismutase (SOD) activity

All the solutions were prepared in phosphate buffer (pH 7.4). 1 ml of NBT (156 µM), 1 ml NADH (468 µM) and 3 ml of sample were mixed. The reaction was started by adding 100 µM phenazine methosulphate (60 µM). It was incubated at 25 °C for 5 min. Absorbance was read at 560 nm [14]. The percentage activity was calculated by the formula-

Scavenging activity (%) = $\frac{(A-B)}{A} \times 100$

Where, A = absorbance of control and B = absorbance of sample.

2.3.3. Nitric oxides (NO_x)

Nitric oxide radical scavenging activity was determined. 1 ml of sodium nitroprusside (10 mM) was mixed with 1 ml of sample in phosphate buffer (pH 7.4). It was incubated at 25 °C for 150 min. To the mixture 1 ml griess reagent (1% sulphanilamide, 2% ortho-phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride was added. Absorbance was read at 546 nm [15]. The percent activity was determined by the formula-

Scavenging activity (%) = $\frac{(A-B)}{A} \times 100$

Where, A = absorbance of DPPH and B = absorbance of sample.

2.3.4. Total phenolic content

Total phenolic content was determined [16]. 1 ml of juice was mixed with 2 ml water followed by addition of 0.5 ml Folin reagent. It was then incubated for 3 min at room temperature. 2 ml of 20% sodium carbonate was added and incubated for 1 min in water bath. Absorbance was read at 650 nm. The phenolic content was determined by using catechol as standard.

2.4. Antimicrobial Assay

2.4.1. Evaluation of antimicrobial property and MIC determination

The nutrient broth was prepared. From this broth 4.5 ml broth was added in each of four test tubes labelled as 100%, 75%, 50%, 25% concentrations. Sets of four test tubes containing 4.5 ml nutrient broth were prepared for two test microorganisms. Then 0.5 ml of each concentration of sample was added into the respective test tube. After this step, 0.05 ml test pathogen suspensions were inoculated were inoculated respective labelled test tube. After inoculation, the test tubes were kept in a shaker incubator for overnight at 37 °C and were observed for development of turbidity and O.D. values were recorded at 600 nm. MIC for a given sample considered as the concentration which showed the minimum absorbance [17]. It was determined by taking the absorbance and comparing the values at different concentration.

3. Results & Discussion

The experimental findings are calculated and recorded.

3.1. pH and total antioxidant activity

pH of *Citrus limetta* juice extract was recorded using pH meter and found to be ~ 3.46. The pH of the juice extract is highly acidic.Total antioxidant activity of *Citrus limetta* was evaluated in 12.5, 25.0, 50.0 and 100 µl concentration of juice extract. The maximum total antioxidant activity was found at 100 µl with a scavenging activity of 97%. At 12.5 µl concentration of juice extract, scavenging activity was found 55% whereas at concentrations 25.0 and 50.0 µl, the scavenging activity was increased by 80% and 91.1%, respectively. These results implied that with increase in the juice extract concentrations of the *Citrus limetta*, percent scavenging activity increased. The percent scavenging activity was increased by 31.2% when the juice concentration increased to 25.0 µl from 12.5 µl and 11.1% activity was increased when the juice extract concentration was increased from 25.0 µl to 50.0 µl. However, a poor augmentation of scavenging activity (6.1%) was observed when juice extract concentration increased from 50.0 µl to 100.0 µl (Table 1).

Table 1: DPPH free radical scavenging activity (%) of *Citrus limetta* at different concentrations**.**

Sl. No.	Concentration (µl/ml)	Scavenging activity (%)
1	12.5	56.0
2	25.0	81.0
3	50.0	92.1
4	100.0	95.0

Note: 100 µl concentration of juice was taken for measuring the antioxidant activity i.e. super oxide dismutase (SOD) and nitric oxide activity. All the readings of the total antioxidant activity were taken in triplicates.

3.2. Super oxide dismutase activity

Superoxide dismutases (SOD) are metallo-enzymes that scavenges the superoxide anion into oxygen and hydrogen peroxide. These enzymes are very important in regulating the super oxide anions in body, therefore, SOD activity of *Citrus limetta* juice extract per µl concentration is important to evaluate for determining the medicinal and pharmacological potentials. The percent scavenging activity of SOD was revealed 74% per 100 µl of juice extract as represented in table 2.

3.3. Nitric oxide scavenging activity

Nitric oxide is a well-known anti-inflammatory mediator as well as important regulatory molecules in various physiological functions. Role of nitric oxide in human physiology is in neurotransmission, vasodilator and in host defence. The toxicity of nitric oxide enhances greatly when it reacts with super oxide radicals by forming highly reactive peroxynitrite anion ($ONOO^-$) [17-19]. Peroxynitrite is very stable and can damage membranes, proteins, DNA and tissues. Nitric oxide radical scavenging activity was found 52.8% per 100 µl of juice extract as summarized in table 2. Scavenging activity of *Citrus limetta* juice extract inferred their biomedical importance as a potent anti-oxidising agent.

Table 2: Percent scavenging activity of SOD and nitric oxide per 100 µl *Citrus limetta* juice extracts.

	Volume of juice (µl)	Percent activity
SOD activity	100	74.0
Nitric oxide activity	100	52.8

Note: All the readings of the total antioxidant activity were taken in triplicates.

3.4. Total phenolic content

Phenols are secondary metabolites found largely in plants. They are commonly found in fruits, vegetables and beverage. They have certain clinical importance in preventing free radical related anti-inflammatory diseases. The prevention of the chain initiation step by scavenging various reactive species such as free radicals is considered to be an important antioxidant mode of action [20]. Total phenolic content of juice extract originated was 0.007 mg/ml. Presence of phenolic contents in juice is responsible for its significant free radical scavenging activity.

The importance of eating citrus fruits have been widely known. Scientifically it was proven that the eating of any types of citrus fruits is very useful for health. Citrus fruits are

the important source of antioxidants, phenolic and antimicrobial compounds. They contain different antioxidant vitamins such as Vitamin A, C and E and bioactive compounds [21, 22]. Antioxidants and antimicrobial activities of *Citrus limetta* juice and crude extract were seen and found very potential in regulating oxidative stress and inhibiting two different bacterial growths. Many papers have reported antioxidants in juice and edible parts of Citrus of different origin and from different varieties [23]. Antioxidant activities at different concentrations were measured against DPPH and for 100 µl of juice was found to be highest (95%) whereas at 12.5 µl, the percent scavenging activity was lowest (56%) implied that the percent activity is depend on concentrations. Adequate concentration is very important to perform total antioxidant activity at maximum level. The percent activity of super oxide dismutase and nitric oxide at 100 µl of juice concentration was 74% and 52.8%, respectively. All these results revealed that *Citrus limetta* juice is biochemically important regulating oxidative stresses and thus prevent from oxidative damages [24]. The content of phenols present in *Citrus limetta* was 0.007 mg/ml which probably helped in activating antioxidant enzymes thereby regulating various oxidative stress.

3.5. Antimicrobial activity and MIC determination of the extracts by spectrophotometry

The nutrient broth was prepared. From this 4.5 mL of broth was added in each of four test tubes labelled as 100%, 75%, 50%, 25% concentrations (fig. 3). Five sets of four test tubes containing 4.5 ml nutrient broth were prepared for three test microorganisms. Then 0.5 ml of each concentration of sample was added into the respective test tube. After this step, 0.05 ml test pathogen suspensions were inoculated were inoculated respective labelled test tube. After inoculation, the test tubes were kept in a shaker incubator for overnight at 37 °C and were observed for development of turbidity and O.D. values were recorded at 600 nm. The antimicrobial potential of different parts of *Citrus maxima* like leaves, fruit wastes and essential oil against was proved against microbes like *Bacillus sbtilis*, *Pseudomonas* aeruginosa, and *Staphylococcus aureus* [25].

Antimicrobial activities of *Citrus limetta* were also found as like anti-oxidative capacities. In terms of O.D. The MIC was determined for *E. coli* and *P. aeruginosa* MIC was recorded at the concentrations of 75 µl/ml and 50 µl/ml, respectively

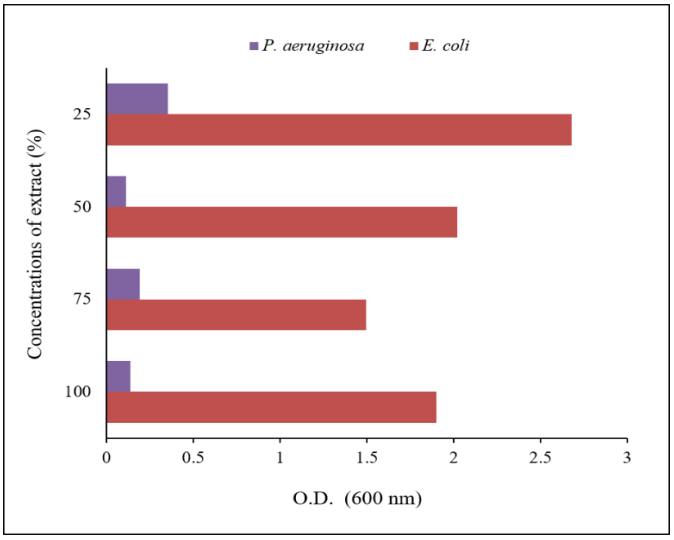

Fig 3: Antimicrobial activity shown by different concentrations of juice extract of *Citrus limetta* in *E. coli* and *P. aeruginosa*.

MIC is the concentration which showed the minimum absorbance. It was determined by taking the absorbance and comparing the values at different concentration. MIC against *E. coli* was found at a juice extract concentration of 75% whereas MIC against *P. aeruginosa* was found at 50% juice extract concentration. *E. Coli* is more resistant to juice extracts of *Citrus limetta* than *P. aeruginosa*.

Table 3: Antimicrobial activity (MIC) of *Citrus limetta* of different juice percent.

Microbes	Juice extracts (%)	O.D. (600 nm)
E. coli	100	1.901
	75	1.495
	50	2.021
	25	2.678
P. aeruginosa	100	0.137
	75	0.193
	50	0.113
	25	0.354

4. Conclusions

Citrus limetta is an ideal source of antioxidants and antibiotics. More of clinical and biological benefits from a single source are highly valuable. Antioxidant activities (total, SOD and nitric oxide activities) are concentrations dependent. Intake of *Citrus limetta* juice in adequate amount could be highly beneficial to achieve antioxidant and antimicrobial activities. A very less concentration (100 µl) of juice extract revealed ~ 95% of antioxidant activities. Antimicrobial activity of *Citrus limetta* crude juice could replace commercial antibiotic ampicillin sources. Crude juice extract is beneficial to kill/ inhibit the growth of highly resistant and opportunistic bacteria *P. aeruginosa*. Crude extract could be used externally where *P. aeruginosa* infected such as surgical wounds, burns, ear infections, etc. Similarly, *Citrus limetta* juice intake in sufficient amount could help in recovery of diarrhea in diarrhea patients. The source of *Citrus limetta* juice also could be very much effective to microbes which are easily get resistant to different types of drugs.

Acknowledgments

The authors thank to the University of Science and Technology Meghalaya, Baridua - 793101, Meghalaya, India for research support in terms of financial assistance and providing laboratory facilities.

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