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Effect of Cooking Temperature on Biochemical Characteristics, Antioxidant Activity and Antimicrobial Potential of Seed Extracts of Assam, India

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The present study investigated the effects of cooking temperature on the biochemical characteristics, antioxidant and antimicrobial activity of three different seeds of *Citrus* fruits (*Citrus limon, Citrus limetta, Citrus maxima and Citrusaurantifolia*) collected from Assam, India. Total soluble sugar (72 mg/mL) were highest in *Citrus maxima*hydro 2-propanol seed extract before heating and 50 mg/mL in *Citrus limon*hydro-methanol, *Citrus limetta* hydro-methanol and *Citrus maxima*hydro-methanolseed extract after heating.Total soluble proteins before and after heating were highest 82 mg/mL and 88 mg/mL in *Citrus limon* 2 propanol seed extract. Free amino acidcontents before and after heating were highest (62 μg/mL in *Citrus limon*hydro-propanol) and (40 μg/mL in *Citrus limon*hydro-methanol) and free fatty acids were 29.2 μg/mL and 23 μg/mL in *Citrus maxima* methanol extract, respectively.H_2O_2 scavenging activity before and after heating were highest in *Citrusaurantifolia* propanol(59%) and in *Citrus limon*(67%), respectively. Total antioxidant capacity was found highest in *Citrus maxima* hydro propanol (92.5%) before heating and in *Citrusaurantifolia*2-propanol (65%) after heating.Antimicrobial activityof the seed extracts was studied on *B. subtilis,E.coli*and *P. aeruginosa*and minimum inhibitory concentration of the *four Citrus* fruitswas determined. MIC of the different seed extracts was observed for 100% (v/v), 75% (v/v), 50% (v/v) and 25% (v/v) against three test microbes viz. *Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli.* For *Bacillus subtilis*, the MIC was found to be at 100%, 100%, 75%, and 75% to the extract of *Citrus aurantifolia* hydro 2-propanol, *Citrus limon* methanol, *Citrus limetta* hydro 2 propanol and *Citrus maxima* hydro 2-propanol, respectively. For *E. coli*,the MIC was found to be at 100%, 75%, 100% and 100% for *Citrus aurantifolia* hydro 2-propanol*, Citrus limon* hydro 2-propanol, *Citrus maxima* hydro 2-propanol and *Citrus limetta* hydro 2 propanol, respectively. For *Pseudomonas aeruginosa*,the MIC was found to be at 100%, 100%, 75% and 50% for *Citrus aurantifolia* methanol, *Citrus limon* methanol, *Citrus maxima* hydro 2-propanol and *Citrus limetta* hydro 2 propanol, respectively.

Keywords: *Citrus* fruits, Seed extracts; biochemical characteristics, antioxidant activity, antimicrobial potential, hydro 2 propanol.

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Citrus fruits are biochemically important because of great medicinal properties Hammer *et al*., (1999). The genus *Citrus* belong to family Rutaceae and sub family Aurantiodeae. It is believed that most of the species under genus *Citrus* are a native to tropical and sub-tropical regions of South East Asia including India where the availability is predominant. From Indian counterpart, North-East India and almost in all the region of South-East Asia are considered to be the Centre of origin and diversity of *Citrus* species Borthakur(1992); Kumari *et al*., (2016).*Citrus* fruit isextensively used by juice processing industries while the peels are disposed as waste material. The juice yield from any*Citrus* fruit is less than half of the fruit weight and as a resultmassive amounts of wastes (peels, fibre and seeds) are generated every year around the world. In general, peels, seeds and pulps (> 50% of the fruit) are dealt with as wastes, whilepotentially; they are being used as a source of valuable daily used productsEl-Adawy *et al*.,(1999). The species of the fruit are found to be of medicinal values and are also used in confectionary, in perfume industry and toiletry. Hence, the *Citrus* waste can be very useful as they used as medicine for its pharmacological properties to counter different diseases and thereby generate revenues. There has been a recent surge of studies on the chemical composition (fatty acid content in particular) of the oil of seeds of different species of *Citrus*. El-Adawy*et al*.,(1999 revealed that the *Citrus* seeds and its flours were rich in oil and protein contents. Both *Citrus* seeds and flours proved to be a good source of minerals, e.g. K, Ca, P, Na, Fe and Mg. In many studies, the measured oil content of *Citrus* seeds: Tunisian *Citrus* seeds (26–36%) Saýdani *et al*.,(2004), Brazilian Rangpur lime seeds (32–38%) Reda *et al*.,(2005).Citrus fruits have been valued as part of a nutritious and tasty diet. The flavors provided by *Citrus* are among the most preferred food additive in the world and it is evident from the popularity of using by the people because of addinggood taste and nutritional value to the food. It is also well established that the *Citrus* and *Citrus* products are rich sources of vitamins, minerals and dietary fiber (non-starch polysaccharides) that are very essential for growth and development.Dietary guidelines and recommendations encourage the consumption of *Citrus* fruits and their products lead to widespread nutritional benefits across the population. *Citrus* is the most commonly found source of vitamin C. However, like most other whole foods, *Citrus* fruits also contain an impressive list of other essential nutrients including glycemic and non-glycemic carbohydrate (sugars and fibre), potassium, folate, calcium, thymine, niacin, vitamin B_6, phosphorous, magnesium, copper, riboflavin, panthothenic acid and a variety of phytochemicals. In addition, *Citrus* contains no fat or sodium and being a plant food, no cholesterol. The average energy value of fresh citrus is also low which can be very important for consumers concerned about putting on excess body weight.

As an important food, it has antioxidant propertiesand its nutrients prevent or reduce oxidative damage to our bodies. These agents are able to remove the deleterious effects of free radicals within our body. Now a day, considerable focus is on the development and evaluation of natural antioxidants and free radical scavengers plant materials which are rich in poly-phenolic compounds. It is well known that plant derived poly-phenols have remarkable antioxidant and free radical scavenging activity adding multiple nutritional,physiological and pharmaceutical benefitto the humans. Extracts prepared from peel, flowers and leaves of bitter orange (*Citrus aurantium* L.) are popularly used in order to minimize central nervous system disorders Pultrini *et al*.,(2006). Reactive oxygen species (ROS) collectively refer to both the oxygen free radicals (the oxygen superoxide and the hydroxyl radical) and some non-radical (hydrogen peroxide) derivatives of oxygen Shoji *et al*.,(2008) are generated as products normal metabolism in the body Zhang *et al*.,(2009).

In general, bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents Gislene *et al*.,(2000). The essential extracts, oils and other plants products have evoked interest as sources of natural antioxidant and antimicrobial parameters. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases Tepe *et al*.,(2004). Essential oils are more effective in controlling biofilm cultures due to their better diffusibility and mode of contact Al-Shuneigat *etal*.,(2005). For a long period of time, plants have been a valuable source

of natural products for maintaining human health. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments Seenivasan *et al*., (2006). The antimicrobial activities of *Citrus* plants, oil and extracts were investigated from many years. The antimicrobial abilities of essential oils among which *Citrus*oils are also shown to be a particularly interesting field for applications in the food and cosmetic industries Caccioni*et al*., (1998). Hammers *et al*., (1999) studied the effect of essential oils from *C. aurantium*, *C. limon*, *C. paradisi* and many other plant oils and extracts found that the minimum inhibitory concentrations (MIC) were between 5-2% (v/v). AL- Jedah *et al*.,(2000)analyzed the action of combined spices including lemon in its mixture and found that the spices mixture was able to exert static effect on all assayed bacteria. Hayes and Markovic(2002) investigated the antimicrobial properties of lemon against *Staphylococcus aureus*, *Klebsiella*,*Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*.

This study detailed the biochemical analysis such as total soluble sugar, total soluble protein, total amino acid and free fatty acid of the seed of four different *Citrus* fruitsisolated from Sonapur and Pitonipara of Assam, India and their H_2O_2 scavenging activity and total antioxidant capacities were measured.These seeds contained antimicrobial properties and MIC of the different extracts were determined.

MATERIALS AND METHODS

Sample Collection

The fully matured and ripen fruits of *Citrus limon* and*Citrus limetta*were collected from Sonapur of Kamrup district, Assam as well as *Citrusaurantifolia* and *Citrus maxima*were collected from Pitonipara of Nalbaridistrict of Assam, India (Fig. 1). A dozen of each fruit sample was collected from the two sites and brought to the laboratory for the experimental study.

Sample preparation

Fresh fruitswere peeled off with knife to take the cotyledon. The cotyledon was rinsed with distilled water and dried with absorbent paper. Five g of the fresh seeds were grinded with the help of mortar and pestle and dissolved in water, methanol, hydro methanol, 2-propanol and hydro 2-propanol. The solution was incubated in shaker incubator at 28°C for overnight and following this centrifugation was performed at 10000 rpm for 10 min. The supernatant obtained after centrifugation was used for the further experiments.

Other set of sample was prepared by heating (boiling) at 100°C in doubled distilled water, methanol, hydro-methanol, 2-propanol and hydro 2-propanol in water bath for 2 h. All the prepared samples were stored at -20°C in the ultra-refrigeratorfor further study.

Biochemical analysis

Biochemical analysis of seeds extract was performed for macronutrients *viz*. total carbohydrate, total soluble protein, total free amino acid, total free fatty acid (acid value) and antioxidant assay.

Estimation of total soluble sugar

Estimation of total soluble sugar was made as per the standard Anthrone method Clegg(1956). To 15 µL seed extract (after centrifugation) 985µL distilled water was added. Following, 2 mL of Anthrone reagent was added. To the seed extract. The tubes were subjected to mild heating. Resultant color intensity was measured at 630 nm. Quantitative estimation was made from standard curve using glucose as standard.

Estimation of total soluble protein

Total soluble protein was estimated as per the method Lowry *et al*., (1953). To 50 µL centrifuged seeds extract 950 µL distilled water was added. To this 5 mL solution C was added. This was followed by addition of 0.5 mL Folin-Ciocaltaeu reagent. The tubes were shaken and incubated in dark for half an hour. The developed blue coloredsolution was measured in a spectrophotometer at 660 nm. Quantitative estimation was made for standard curve using BSA as standard.

Estimation of free amino acid(FAA)

Estimation of free amino acid was made using Ninhydrin reactionby the method of Jayaraman, (1981). 50 µL centrifuged seeds extract were treated with 4.5 mL mild hot 80% ethanol. To this, 3.5 mL Ninhydrin reagent was added. The tubes were warmed in a water bath for about 10 min and absorbance was taken at 570 nm. Estimation

was made from standard curve prepared with a mixture of alanine, aspartic acids, tryptophan, proline and lysine in equal proportion.

Estimation of Free Fatty Acid

Free fatty acid was estimated as per the protocol ofCox and Pearson (1962). 1mL of juice (without centrifugation) was treated with 25 mL of neutral solvent (25 mL diethyl ether, 25 mL of 95% alcohol, 1 mL 1% phenolphthalein solution, the mixture was neutralized with 0.1N potassium hydroxide). Now, a few drops of phenolphthalein were added and the resultant solution was titrated against 0.001N potassium hydroxide. Free fatty acid was estimated as per the following formula:
Acid value (mg KOH/mL) = [(Titre value × Normality of KOH × 56.1)/Amount of sample in g)]

Estimation of H_2O_2 scavenging activity

H_2O_2 scavenging activity of seed extracts was determined by using the method developed by Ruch *et al*., (1989). Different extract concentrations(10 µL and 50 µL/mL) were dissolved in 3.4 mL of 0.1M phosphate buffer (pH 7.4) and mixed with 600 µL of H_2O_2 (43 mM). The absorbance of the reaction mixture was taken at 230 nm recorded after 10 min. The H_2O_2 scavenging activity was calculated by using the following formula:

$$H_2O_2 \text{ Scavenging activity (\%)} = [(A_c - A_s)/A_c] \times 100$$

Where,A_c= control absorbance
A_s= sample absorbance

Estimation of total antioxidant capacity

The total antioxidant capacity was measured by the method of Pietro *et al*., (1999). A 50 µL of seeds extracts were taken and the volume was adjusted to 500 µL with distilled water. To this, 4.5 mL phosphomolybdenum reagent (600 mM sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added and mixed properly. Blank containing 500 µL distilled water and 4.5 mL reagent and control with 5mL reagent were prepared. The tubes were capped and kept in boiling water bath at 95°C for 90 min. After incubation, optical density was taken at 695 nm and 0.25 mg/mL ascorbic acid was used as standard. Total antioxidant capacity was calculated by using the formula:

$$\text{Total antioxidant capacity (\%)} = [(A_s - A_c)/ (A_{aa} - A_c)] \times 100$$

Where, A_c = control absorbance
A_s = sample absorbance
A_{aa} = Ascorbic acid absorbance

Antimicrobial activity and determination of MIC of the extracts by Spectrophotometry method

The nutrient broth was prepared and from this broth 4.5 mL was added in each of four test tubes labelled as 100%, 75%, 50%, 25% seed extracts concentrations. 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 seeds extracts was added into the respective test tubes. After this step, 0.05 mL test pathogen suspensions were inoculated and inoculated in the respective test tubes. After inoculation, the test tubes were kept in a shaker incubator for overnight at 37°C and were observed for development of turbidity. O.D. values were recorded at 600nm. MIC for a given sample considered as the concentration which showed the minimum absorbance, Bansode and Chavan, (2013). It was determined by taking the absorbance and comparing the values at different concentration.

RESULTS

Evaluation of total soluble Sugar, total soluble protein, total free amino acid (FAA) and free fatty acid (FFA) contents

Total soluble sugar (TSS) exhibited a lot of variation among the seed extracts. In case of seeds before heating, highest total sugar content (72 mg/mL) was found in*Citrus maxima* hydro 2-propanol whereaslowest (4 mg/mL) was found in the *Citrus limetta* water. Forseeds after heating, the highest total sugar was found in *Citrus limon* hydro-methanol, *Citrus limetta* hydro-methanol, *Citrusmaxima* hydro-methanol in 50mg/mL. Lowest level of total sugar (3 mg/mL) was found in the *Citrus limetta*water.

Compared to TSS, soluble protein levels are comparatively similar. However, there was significant variation among the different species. In case of seeds before heating, the highest amount

of soluble protein (82 mg/mL) was found in the *Citrus limon* hydro-propanol and lowest level of soluble protein was found (4 mg/mL) in the *Citrus maxima*water and *Citrus maxima*methanol. In case of seed after heating, the highest amount of soluble protein (88 mg/mL) was found in the *Citrus limon*2-propanol seed extract. Lowest level of soluble protein was found in *Citrus limetta*methanol with 5 mg/mL.

Highest amount of FAA in case of seeds before heating was recorded in *Citrusaurantifolia* water with a value of 153 µg/mL and the lowest was recorded in *Citrusaurantifolia* 2-propanol and *Citrus limon*2-propanol with a value of 3 µg/mL. As in case of seeds after heating, the highest amount was recorded in *Citrus aurantifolia* water with a value of 137 µg/mL and the lowest amount was observed in *Citrus limon*methanol with a value of 2µg/mL.

Free fatty acid content is expressed as oleic acid equivalent and like acid number free fatty acid content has been found to be highly variable with similar trend to that of acid number. In case of seeds before heating, the highest free fatty acid content was recorded in *Citrus aurantifolia* water with a value of 32.5 µg/mL. Lowest free fatty acids were recorded in *Citrus limetta*water with 9.2 µg/mL; where as in case of heated sample, the highest free fatty acid content was recorded in *Citrusaurantifolia* water with a value of 24.1 µg/mL and the lowest free fatty acids were recorded in *Citrus limetta*2-propanol and *Citrus limon*2-propanolwith a value of 16.3 µg/mL.

H_2O_2 scavenging activityandevaluation of total antioxidant capacity

H_2O_2 was determined to evaluate the total scavenging activity of the different *Citrus* seed extracts. Among the different seed extracts in case of seeds before heating, the highest activity content was recorded in *Citrus aurantifolia*propanol with a value 59%. Lowest activity was recorded in *Citrus limetta*methanol with a value of 12%; whereas in case of heated sample, the highest activity content was recorded in *Citrus limon*methanol with a value of 67%.

TAC was determined to evaluate the total antioxidant activity of the different *Citrus* seed extracts. Among the different seed extracts in case

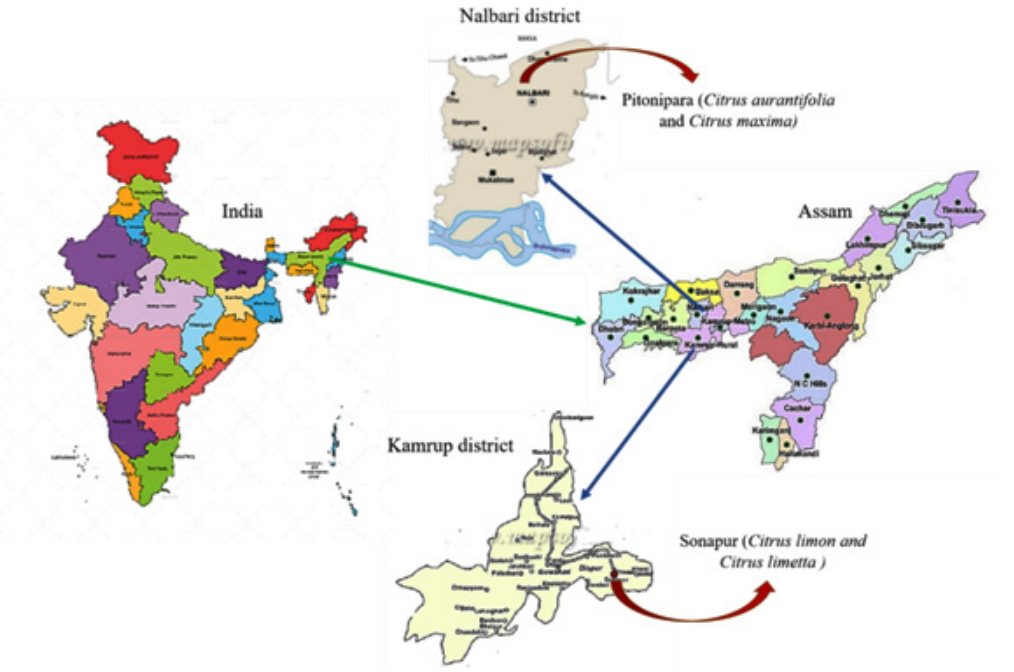

Fig. 1. Collection sites of the *Citrus* fruits are presented in the above figure -*Citrus limon*(lemon), *Citrus limetta*(mosumi), *Citrusaurantifolia*(lime) and *Citrus maxima*(robab). *Citrus limon and Citrus limetta* were collected from Sonapur of Kamrup district and *Citrusaurantifolia* and *Citrus maxima* were collected from Pitonipara of Nalbari district of Assam, India

of seeds before heating, the highest activity content before heating was recorded in *Citrusmaxima*hydro propanol with a value of 92.5%. Lowest activity was recorded in *Citrus limon*water 5%; whereas in case of heated sample, the highest activity content after heating was recorded in *Citrusaurantifolia*

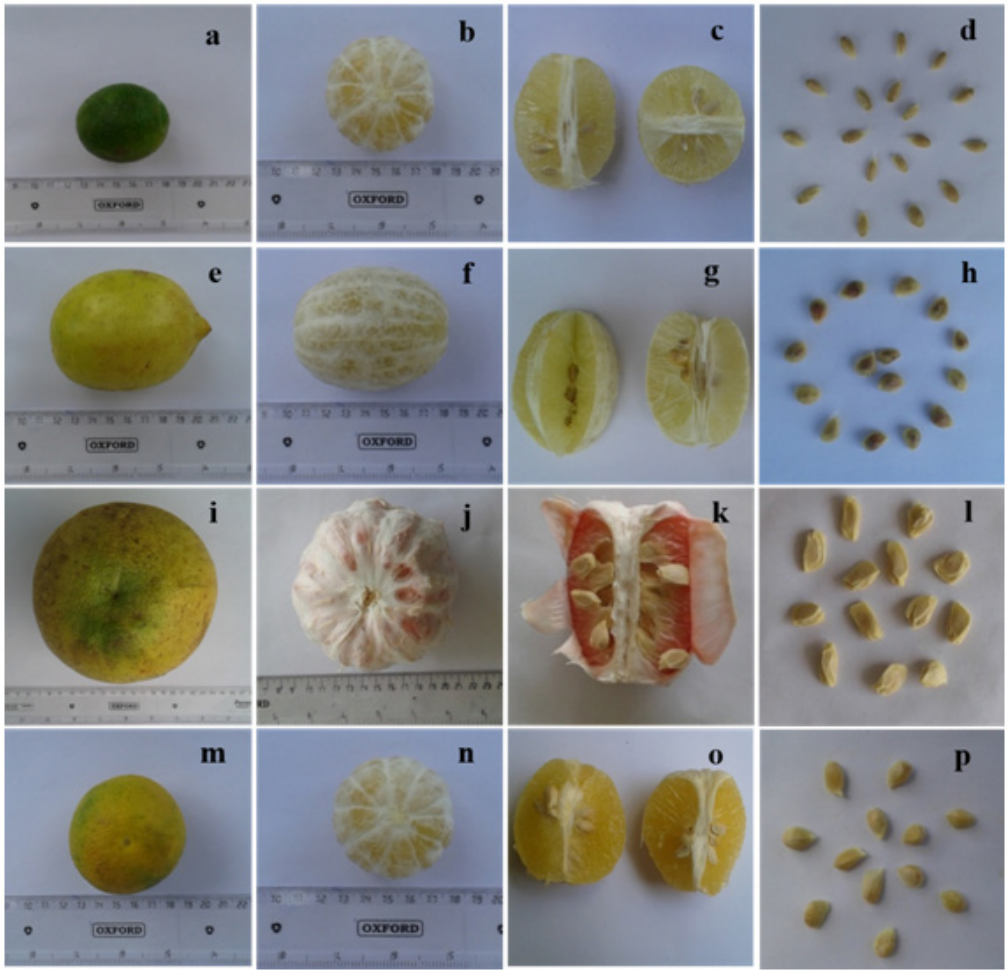

Fig. 2. Citrus fruits, their size and seeds are presented in the above figure.a.*Citrus limon* (lemon), b. peeled*Citrus limon*, c. sliced*Citrus* limon, d.*Citrus limon* seeds, e.*Citrus limetta* (mosumi),f.peeled *Citrus limetta*, g. sliced *Citrus limetta*, h. seeds of *Citrus limetta*,i.*Citrusaurantifolia,* j. peeled *Citrusaurantifolia*, k. sliced *Citrusaurantifolia*, l. seeds of *Citrusaurantifolia*, m.*Citrus maxima* (robab), n. peeled *Citrus maxima*, o. sliced *Citrus maxima* and p. seeds of *Citrus maxima*

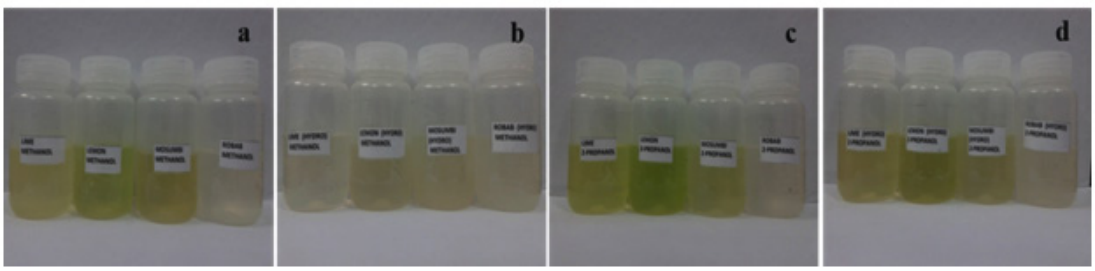

Fig. 3. Samplesprepared in water, methanol, hydro-methanol, 2-propanol and hydro 2-propanol from the four different Citrus seeds.a.*Citrus limon*, b. *Citrus limetta*, c.*Citrusaurantifolia* and d.*Citrus maxima*

Table 1. TSP, TSS, FAA and FFA of different citrus seed extracts before heating and after heating at 50 µL/mL concentration. All values are expressed in mean ± SEM. Samples taken in triplicate (n=3).

Sample	TSS (mg/mL)		TSP (mg/mL)		FAA (µg/mL)		FFA (µg/mL)	
	Before heating	After heating	Before heating	After heating	Before heating	After heating	Before heating	After heating
Citrusaurantifolia methanol	42.0±0.25	34.0±0.26	6.0±0.24	8.0±0.05	16.0±0.11	4.0±0.10	21.9±0.10	19.6±0.6
Citrusaurantifolia hydro-methanol	52.0±0.26	46.0±0.33	6.0±0.08	10.0±0.31	30.0 ±0.06	5.0±0.11	14.6±0.03	20.2±0.5
Citrusaurantifolia 2-propanol	16.0±0.26	17.0±0.12	68.0±0.3	74.0±0.11	3.0±0.09	21.0±0.11	14.6±0.17	16.8±0.40
Citrusaurantifolia hydro-propanol	55.0±0.06	48.0±0.25	12.0±0.3	14.0±0.26	40.0±0.08	13.0±0.07	14.6±0.03	16.8±0.17
Citrus limon methanol	33.0±0.05	20.0±0.32	6.0±0.15	6.0±0.08	45.0± 0.09	2.0±0.10	14.0±0.10	19.1±0.30
Citrus limon hydro-methanol	56.0±0.11	50.0±0.30	10.0±0.33	10.0±0.44	23.0± 0.11	40.0±0.15	14.0±0.03	17.4±0.28
Citrus limon 2-propanol	14.0±0.11	10.0±0.05	82.0±0.31	88.0±0.29	3.0±0.15	4.0±0.15	19.6±0.09	16.3±0.19
Citrus limon hydro propanol	44.0±0.31	44.0±0.06	8.0±0.33	12.0±0.44	62.0±0.18	24.0±0.10	22.4±0.10	17.4±0.16
*Citrus limetta*methanol	38.0±0.05	41.0±0.15	7.0±0.30	5.0±0.46	33.0±0.12	17.0±0.14	19.6±0.14	18.0±0.30
Citrus limetta hydro-methanol	60.0±0.08	50.0±0.15	10.0±0.05	6.0±0.12	25.0±0.17	28.0±0.09	22.4±0.12	17.4±0.19
Citrus limetta 2-propanol	18.0±0.30	17.0±0.30	64.0±0.37	70.0±0.18	8.0±0.09	21.0±0.10	19.0±0.02	16.3±0.18
Citrus limetta hydro-propanol	46.0±0.15	38.0±0.14	24.0±0.46	18.0±0.08	53.0±0.07	20.0±0.09	15.1±0.05	17.4±0.24
*Citrus maxima*methanol	28.0±0.08	24.0±0.30	4.0±0.31	6.0±0.39	12.0±0.14	10.0±0.09	29.2±0.08	23.0±0.01
*Citrus maxima*hydro-methanol	56.0±0.26	50.0±0.28	12.0±0.23	12.0±0.54	47.0±0.15	24.0±0.18	23.0±0.02	20.2±0.08
*Citrus maxima*2-propanol	20.0±0.12	15.0±0.08	76.0±0.28	82.0±0.33	4.0±0.24	21.0±0.12	17.4±0.11	16.8±0.33
*Citrus maxima*hydro-propanol	72.0±0.26	36.0±0.27	12.0±0.31	10.0±0.31	50.0±0.14	20.0±0.11	14.1±0.09	18.0±0.32

2-propanol with a value of 65%. Lowest activity was recorded in *Citrus limetta*water with a value of 16.6%.

Selection of microbial cultures

Microbial culture including *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* were collected from the microbiology laboratory of University of Science and Technology Meghalaya, Baridua - 793101, Meghalaya, India. Microbes were sub-cultured in nutrient broth (pH 7.4) and stored in refrigerator 4 °C for further study.

Antimicrobial activity and MIC determination of the extracts

MIC was determined in the form of turbidity and taking optical density (O.D.) at 600 nm in an UV-Vis spectrophotometer.MIC of the different seed extracts was observed for 100% (v/v), 75% (v/v), 50% (v/v) and 25% (v/v) against three test microbes viz. *Bacillus subtilis, Pseudomonas aeruginosa* and*Escherichia coli.* For *Bacillus subtilis*, the MIC was found to be at 100%, 100%, 75%, and 75% to the extract of *Citrus aurantifolia*hydro 2-propanol, *Citrus limon*methanol, *Citrus limetta* hydro 2 propanol and *Citrus maxima* hydro 2-propanol, respectively. For *E.coli*,the MIC was found to be at 100%, 75%, 100% and 100% for *Citrus aurantifolia* hydro 2-propanol*, Citrus limon*hydro 2-propanol, *Citrus maxima* hydro 2-propanol and *Citrus limetta* hydro 2 propanol, respectively. For *Pseudomonas aeruginosa*,the MIC was found to beat 100%, 100%, 75% and 50% for *Citrus aurantifolia*methanol, *Citrus limon*methanol, *Citrus maxima* hydro 2-propanol and *Citrus limetta* hydro 2 propanol, respectively.

DISCUSSION

A very least work has been done on nutritional, antioxidant and antimicrobial property of *Citrus* seed extracts. However, work has been found on the antioxidant and antimicrobial property of *Citrus* juice Kumari and Handique(2013). They found that the total soluble sugar, total soluble protein, free amino acid and free fatty acid in *Citrus reticulata* are 102.6 mg/mL, 8.9 mg/mL, 0.50µg/mL and 0.07µg/mL. Similarly, the present study revealed that the total soluble sugar (TSS),

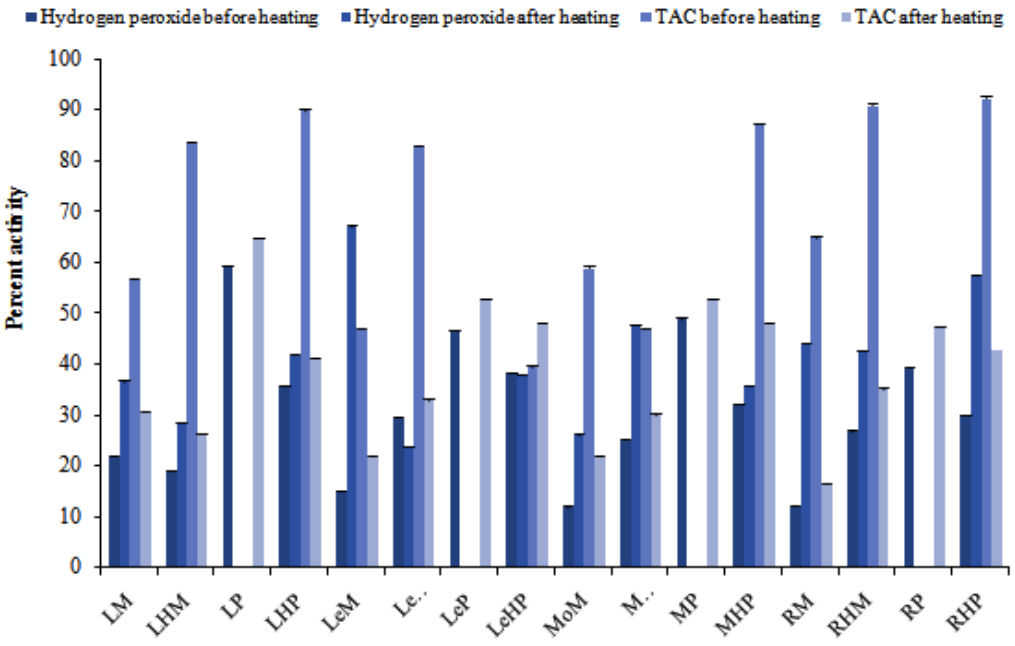

Fig. 4. Percent H_2O_2 scavenging activity and total antioxidant capacity (TAC) of *Citrus limon, Citrus limetta, Citrusaurantifolia* and *Citrus maxima* seeds. All values are expressed in mean ± SEM and samples were taken in triplicate (n=3). L-*Citrus aurantifolia*, M-methanol, HM-hydro methanol, P-propanol, HP-hydropropanol, Le- *Citrus limon*, Mo- *Citrus limetta*, R- *Citrus maxima*

Table 2. Evaluation of antimicrobial activity of different juice extracts on *E.coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* determination of MIC with respect to extract solution

Microbe	Sample	Solvents	Concentration (%)	O.D. (600 nm)
Bacillus subtilis	*Citrusaurantifolia*	Water		
		Methanol	100	0.005
		Hydro methanol	75	0.375
		2-propanol	100	0.620
		Hydro 2-propanol	100	0.002
	Citrus limon	Water		
		Methanol	100	0.009
		Hydro methanol	75	0.340
		2-propanol	75	0.359
		Hydro 2-propanol	100	0.023
	Citrus limetta	Water		
		Methanol	100	0.022
		Hydro methanol	100	0.279
		2-propanol	100	0.437
		Hydro 2-propanol	75	0.016
	Citrus maxima	Water	100	0.287
		Methanol	100	0.138
		Hydro methanol	100	0.429
		2-propanol	100	0.274
		Hydro 2-propanol	75	0.020
Escherichia coli	*Citrus aurantifolia*	Water	25	0.933
		Methanol	25	0.046
		Hydro methanol	100	0.298
		2-propanol	75	0.633
		Hydro 2-propanol	100	0.024
	Citrus limon	Water	75	1.495
		Methanol	50	0.029
		Hydro methanol	100	0.337
		2-propanol	75	0.650
		Hydro 2-propanol	75	0.010
	Citrus maxima	Water	100	1.408
		Methanol	100	0.041
		Hydro methanol	100	0.531
		2-propanol	100	0.594
		Hydro 2-propanol	100	0.030
	Citrus limetta	Water	100	0.364
		Methanol	100	0.106
		Hydro methanol	100	0.337
		2-propanol	75	0.503
		Hydro 2-propanol	100	0.034
Pseudomonas aeroginosa	*Citrusaurantifolia*	Water	100	0.037
		Methanol	100	0.015
		Hydro methanol	100	0.219
		2-propanol	75	0.214
		Hydro 2-propanol	50	0.620
	Citrus limon	Water	25	0.343
		Methanol	100	0.021
		Hydro methanol	100	0.224
		2-propanol	100	0.442
		Hydro 2-propanol	50	0.032
	Citrus maxima	Water	100	0.824
		Methanol	100	0.011
		Hydro methanol	25	0.232
		2-propanol	100	0.361
		Hydro 2-propanol	75	0.010
	Citrus limetta	Water	75	0.371
		Methanol	100	0.120
		Hydro methanol	100	0.323
		2-propanol	75	0.608
		Hydro 2-propanol	50	0.011

total soluble protein (TSP), free amino acid (FAA) and free fatty acid (FFA) was for the *Citrus* seeds extracts are explained in the following. The highest total sugar was observed in*Citrus maxima*hydro 2-propanol with 72 mg/mL and lowest level of total sugar was found in the *Citrus limetta*waterwith 4 mg/mL before heating, after heating the seed extract, the highest total sugar (50 mg/mL) was observed in *Citrus limon*hydro-methanol, *Citrus limetta*hydro-methanol,*Citrus maxima*hydro-methanol and lowest level of total sugar was found in the *Citrus limetta*water (3 mg/mL). The highest amount of soluble protein was found in the *Citrus limon*hydro-propanol with 82 mg/mL and lowest level of soluble protein was found the *Citrus maxima*water and *Citrus maxima*methanol with 4 mg/mL in the seed extract before heating. In case of heated seed extracts, the highest amount of soluble protein (88 mg/mL) was found in the *Citrus limon*2-propanol and lowest level of soluble protein (5 mg/mL) was found in *Citrus limetta*methanol. For FAA, highest amount of FAA before heating was recorded in *Citrusaurantifolia* water with a value of 153 mg/mL and the lowest was recorded in *Citrusaurantifolia* 2-propanol and *Citrus limon*2-propanol with a value of 3 mg/mL in case of cool sample. As in case of heated seed extracts, the highest amount was recorded in *Citrusaurantifolia* water with a value of 137 mg/mL and the lowest amount was observed in *Citrus limon*methanol with a value of 2 mg/mL. For FFA, the highest free fatty acid content was recorded in *Citrusaurantifolia* water with a value of 32.5 mg/g and lowest free fatty acids were recorded in *Citrus limetta*water with 9.2 mg/g in case of cool; whereas in case of heated sample, the highest free fatty acid content was recorded in *Citrusaurantifolia* water with a value of 24.1 mg/g and the lowest free fatty acids were recorded in *Citrus limetta*2-propanol and *Citrus limon*2-propanolwith a value of 16.3 mg/g.

The antioxidant property of *Citrus maxima* and the study material used in their work was mainly leaves Kundusen *et al*., (2011) or juice Oyedepo, (2012). In the present study, for in *vitro* antioxidant analysis non-enzymatic assay was followed. In non-enzymatic assay in vitro antioxidant activity of the extracts was evaluated by total Antioxidant Capacity (TAC) and H_2O_2 scavenging activity. For TAC, the highest activity content was recorded in *Citrusaurantifolia* 2-propanol with a value of 59.2% and lowest activity was recorded in *Citrus limon*water 5% in case of sample before heating; whereas in heated sample, the highest activity content was recorded in *Citrusaurantifolia* 2-propanol with a value of 64.8% and lowest activity was recorded in *Citrus limetta*water with a value of 8%. For H_2O_2 scavenging activity, the highest activity content was recorded in *Citrus maxima*hydro-propanol with a value 92.5% and lowest activity was recorded in *Citrus limon*hydro-propanol with a value of 39.6% in case of cooled sample; whereas in case of heated sample, the highest activity content was recorded in *Citrus limon*methanol with a value of 67.2 % and lowest activity was recorded in *Citrus limon*hydro-methanol with a value of 23.9% (Table 1).

Works of; Tao *et al*.,(2008)and Mathur *et al*.,(2011) proved the antimicrobial potential of different parts of *Citrus maxima* like leaves, fruit wastes and essential oil against microbes like *Bacillus subtilis,Pseudomonas aeruginosa,* and *Staphylococcus aureus*,etc. In our present study, antimicrobial activity of the *Citrus* seed extracts was studied against *Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa* by spectrophotometry method. MIC was observed in the form of turbidity and taking the optical density (OD) at 600 nm. In this method, MIC of seed extracts against *Bacillus subtilis* was found in *Citrusaurantifolia* hydro 2-propanol 100%, *Citrus limon* methanol seed extract (100%),*Citrus limetta* hydro 2-propanol (75%)and *Citrus maxima* hydro 2-propanol (75%). The MIC towards*E. coli* was found in*Citrusaurantifolia* hydro 2-propanol (75%), *Citruslimon* hydro 2-propanol (75%), *Citrus maxima* hydro 2-propanol (100%) and *Citrus limetta* hydro 2-propanol (100%).The MIC of *Citrusaurantifolia* methanol, *Citrus limon* methanol, *Citrus maxima* hydro 2-propanol and *Citrus limetta* hydro 2-propanol to*Pseudomonas aeroginosa* was 100%, 100%, 75% and 50%, respectively (Table 2).

The concept of eating *Citrus* fruits in the form of raw, juice and powder has been tremendously increasing in the community as because people believe that the *Citrus* fruits contain antioxidative compounds, antimicrobial properties, etcGillman *et al*.,(1995); Rimm *et al*., (1996); Kumari and Ahad(2017). Eating *Citrus* fruits as a medicine to recover scurvy is an old

practice done by the common people from few decades, Rapisarada *et al*., (1999). The waste seeds of the *Citrus* fruits carried various antioxidative, nutritive value and antimicrobial property that was proved in this study. The seed extracts of the *Citrus limon, Citrus maxima, Citrus aurantifolia* and *Citruslimetta* contained tremendous amount of soluble free sugars, soluble proteins, free amino acids and free fatty acids. They also possessed high H_2O_2 scavenging activity, antioxidative capacities as well as antimicrobial properties. ROS after crossing the threshold limit in any organism is harmful and damage lipid membranes and degrade proteins and amino acids, Tchimene *et al*.,(2016). Eating *Citrus* fruits in raw form, juices can reduce the oxidative stress as the juice contains non enzymatic antioxidants such as phenolics, flavonoidsand ascorbic acid, tocopherol, vitamiins, etc. Kumari *et al*.,(2016). We also looked into the effects of cooking temperature on the biochemical parameters (free sugars, amino acids, fatty acids, etc.), antioxidative capacities as well as antimicrobial properties. The results determined that heat also slightly reduce these properties. Therefore, eating raw seed extracts, or powdered form of seeds is more effective against oxidative stress and microbial infections.

CONCLUSION

The Citrus species namely *Citrus limon, Citrus limetta, Citrus maxima and Citrusaurantifolia* are predominantly found in the North-Eastern region of India. These fruits are rich in biomolecules (free sugars, amino acids, fatty acids) and carryantioxidant and antimicrobial properties. In general, the seeds of these fruits are thrown/ disposed after eating the fruits. In this study, we have looked into the free sugars, amino acids and fatty acids contents of *Citrus limon, Citrus limetta, Citrus maxima and Citrusaurantifolia* seeds and their antioxidative capacities and antimicrobial properties before and after heating. Among the seeds of the *Citrus*, *Citrus limon*seeds carried the highest antioxidative capacities and antimicrobial properties before and after heating. All the *Citrus* seeds carried antioxidative capacities and antimicrobial properties and hence these seeds can be processed into pharmaceutical products to treat against ROS stresses and different antimicrobial diseases. The suggestion is to eat the seed as raw or in the powdered form to achieve the maximum response against oxidative stresses and microbial infections. The product will be a cheaper one and can attain higher market value and popularity as this product do not carry any side effects. The source (seed) is considered as waste. So, the waste can be converted into useful pharmaceutical product. This will increase the economic status of N. E. India.

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