EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY OF *CITRUS LIMETTA* AND *CITRUS MAXIMA* ON REACTIVE OXYGEN AND NITROGEN SPECIES

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Summary

Citrus limetta and *Citrus maxima* belong to the genus *Citrus* (Rutaceae) and are rich in flavonoids, vitamin C, folate, dietary fibre, carotenoids etc. Different parts of both plants are responsible for the prevention of cancer, other degenerative diseases, anti-inflammatory activity and antidiabetic activity etc. The present study was aimed to evaluate the *in vitro* antioxidant activity of the methanol extracts of *Citrus limetta* fruit peel (MECL) and *Citrus maxima* (MECM) leaves in different models such as DPPH, superoxide radical and nitric oxide radical scavenging activity and inhibition of lipid peroxidation. The total phenolic compounds present in the extracts were estimated by Folin-Ciocalteu's reagent. Both the extracts have shown good free radical scavenging activity in a dose dependent manner. The IC_{50} values for MECM were found to be 5.5 μg/ml, 79.16 μg/ml, 174.97μg/ml and 111.5μg/ml respectively for DPPH, superoxide radical, nitric oxide radical inhibition and lipid peroxidation assays. The IC_{50} values for DPPH, superoxide radical, nitric oxide radical inhibition and lipid peroxidation assays of MECL were 7.36 μg/ml, 9.36 μg/ml, 136.45 μg/ml and 111 μg/ml respectively. Measurement of total phenolic compounds by Folin-Ciocalteu's reagent indicated that 1 mg extract contains 126.85 and 116.7 μg equivalent of pyrocatechol in MECL and MECM respectively. The present study reports significant *in vitro* antioxidant activity of the test plants.

Key words: *Citrus limetta, Citrus maxima,* free radicals, total phenolic compounds, DPPH, nitric oxide, super oxide, lipid peroxidation.

Introduction

Major diseases like cancer, atherosclerosis and inflammation are caused by generation of free radicals and lipid peroxidation inside the human body. An appropriate dietary pattern inclusive of fruit and vegetables can always decrease the risk associated with these (1,2). Presence of great amount of natural antioxidants in the plant foods aid in decreasing the occurrence of the diseases (3).

The essential oils and the extracts of different fruits have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of foods from the toxic effects of oxidating agents (4).

Citrus fruits are the largest sector for production of fruits in the world with more than 100 million tons produced per season. About 34% of them are made into juices (5). Citrus peels exhibit dominant antioxidant and anti-inflammatory effects. Peels contain more bioactive compounds such as phenolic acids, flavonoids, limonoids, and fibres than the juices (6,7). The citrus bioactive flavonoids specially the polymethoxy flavones and flavanone glycosides attract considerable attention for their significant biological activities[8].

Citrus plants (family Rutaceae) are the major source of the natural liminoids and 39 liminoid aglycones and 21 glucosides have been isolated (9). Citrus plants are also rich in naturally-occurring flavonoids, which are primarily found in peel (10). Citrus bioflavonoids like hesperidin, narirutin, naringin, neohesperidin, eriocitrin, neoeriocitrin, rutin, diosmin, neoponcirin, and nobiletin are abundant in citrus fruits and are most prominent cancer-preventing agents (11-14). Flavonoids have a wide range of biological effects, such as inhibition of key enzymes in mitochondrial respiration, protection against coronary heart disease, and anti-spasmolytic, anti-inflammatory, antioxidative, vascular, estrogenic, cytotoxic antitumor, and antimicrobial activities (15).

Citrus limetta Risso. (Rutaceae) is an Asian plant introduced by the Spaniards after the conquest, and naturalized in tropical and subtropical areas in Latin American countries and the Caribbean islands (16). *C. Limetta* fruit and leaves are used for healing of common cold, control of cholesterol and fever, regulation of inflammatory and digestive disorders and as a blood pressure modulator (17,18).

Citrus maxima Merr. (Rutaceae) is widely cultivated in warm countries. The flesh is usually separated from the skin in segments and eaten with or without sugar, and the leaves are used to flavour food. *C. maxima* Merr. fruit is used as a folk remedy for hangovers and the dried leaves are brewed in water as a drink in southern region of the Republic of Korea (19).

Materials and methods

Plant material: *Citrus maxima* Merr. leaves (MECM) and the *Citrus limetta* Risso. fruits were collected from Nadia region of West Bengal during the month of March-April, 2007. The plant materials were authenticated by the Botanical Survey of India, Shivpur, Howrah, West Bengal, India; and the voucher specimens (PMU-1/JU/2007 and PMU-2/JU/2007 respectively) were stored in our laboratory for further references.

Preparation of plant extracts: The leaves of *Citrus maxima* were dried under shade and powdered by mechanical grinder. About 500 g of the plant material was successively extracted with petroleum ether and methanol in a Soxhlet apparatus. The methanol extract was then evaporated under reduced pressure to get the dry extract (MECM, yield 18.1%).

Fresh fruit peels of *Citrus limetta* were taken and grounded. About 500 g of the grounded material was consecutively macerated for seven days each in petroleum ether, ethyl acetate, chloroform and methanol respectively. The methanol extract was then dried under reduced pressure to get the dry mass (MECL, yield 10.56%).

Chemicals: 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), α tocopherol, curcumin, butylated hydroxy toluene (BHT), ascorbic acid, sodium nitroprusside, sulphanilamide, naphthylethylene diamine dihydrochloride, nitroblue tetrazolium, phenazine methosulphate, nicotinamide adenine dinucleotide (NADH) were purchased from Sigma Aldrich Chemical Co., USA. Thiobarbituric acid (TBA), trichloro acetic acid (TCA), Tris HCl, sodium dodecyl sulphate (SDS), Folin-Ciocalteu's phenol reagent (FCR) and all other reagents used were of analytical grade obtained commercially.

Inhibition of DPPH radical: The free radical scavenging activity of MECM and MECL was measured by 1, 1-diphenyl-2-picrylhydrazil (DPPH) using the previously reported methods (20,21). Briefly, 200 µl of 100 µM DPPH in methanol was added to 3 ml of various concentrations (10, 25, 50, 75, 100 and 125 µg/ml) of MECM and MECL and the reference compound, α tocopherol. After incubation at 37°C for 30 min the absorbance was measured at 517 nm. . All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was calculated by the following formula.
Percentage inhibition = (Absorbance of control - Absorbance of test) × 100 /Absorbance of control.

Inhibition of nitric oxide radical: Nitric oxide radicals were generated from sodium nitroprusside solution at physiological pH which interacts with oxygen to produce nitrite ions and measured by the Griess reaction (22, 23). 1 ml of sodium nitroprusside (10 mM) in phosphate buffered saline (PBS, pH 7.4) and different concentrations of MECM, MECL and the reference compound curcumin (10, 25, 50, 75 ,100, 125, 150 and 175 µg/ml) were incubated at 25°C for 150 min. 1 ml of the incubated sample was mixed with 1 ml of the Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% Phosphoric acid), and the chromophore formed was measured at 546 nm . All the tests were performed in triplicate. The % inhibition was calculated by the above mentioned formula.

Inhibition of superoxide anion radical: Evaluation of superoxide anion scavenging activity of MECM and MECL was performed based on a modified method by Nishimiki et al (24). 1 ml of nitroblue tetrazolium (NBT) solution (156 µM NBT dissolved in 1.0 ml of 100 mM phosphate buffer , pH 7.4), 1 ml of NADH solution (468 µM of NADH dissolved in 1 ml of 100 mM phosphate buffer pH 7.4) and 0.1 ml of various concentration of MECM, MECL and the reference compound Ascorbic acid (10, 25, 50, 75 ,100 and 125 µg/ml) were thoroughly mixed in different test tubes and the reaction started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM of PMS in 100 µl of 100 mM phosphate buffer , pH 7.4). The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm. All the tests were performed in triplicate and the results averaged. The % inhibition was calculated by the formula mentioned above.

Inhibition of lipid peroxidation: Lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate was estimated by the method of Ohkawa et al (25). The reaction mixture (0.5 ml) containing rat liver Homogenate [25 % w/v in Tris-HCl buffer (20 mM, pH 7.0)] 0.1 ml; 0.1 ml of KCl (30 mM); 0.1ml of $FeSO_4.6H_2O$ (0.16 mM); 0.1 ml of ascorbic acid (0.06 mM) and 0.1 ml of various concentrations of MECL, MECM and reference compound BHT (10, 25, 50, 75, 100 and 125 µg/ml) were incubated at 37°C for one hour. After the incubation

period, reaction mixture was treated with 0.2 ml sodium dodecyl sulphate (SDS) (8.1%); 1.5 ml TBA (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by distilled water and then kept in a water bath at 95-100°C for 30 minutes. After cooling, 1.0 ml of distilled water and 5.0 ml of *n*-butanol and pyridine mixture (15:1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance was measured at 532 nm. The IC_{50} of the MECL, MECM and BHT was calculated from the % inhibition curve.

Amount of total phenolic compounds: Total soluble phenolics present in the MECM and MECL were determined by the method reported earlier (26). 1 ml of suspension containing 100µg of MECM and MECL in water was transferred into 100 ml Erlenmeyer flask. The final volume was adjusted to 46 ml with distilled water and then 1 ml of Folin-Ciocalteu's reagent (FCR) was added to this mixture. After 3 min, 3 ml of Na_2CO_3 (2%) was added, and was shaken for 2 h at room temperature and absorbance measured at 760 nm. All the tests were performed in triplicate and the results averaged. The concentration of total phenolic compounds in MECM and MECL was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from the standard pyrocatechol graph. The equation is given below.

$$Absorbance = 0.001 \times Pyrocatechol\ (\mu g) + 0.0033$$

Statistical analysis: Experimental results were mean ± standard error of mean (SEM) of three parallel measurements. IC_{50} values were calculated by Graph Pad Prism ver. 5.0.

Results and discussion

DPPH radical is a stable free radical in methanol solution. Because of the odd electron of DPPH, it gives a strong absorption maximum at 517 nm by visible spectroscopy. The odd electron of the radical becomes paired off in the presence of extract (hydrogen donor). When it becomes paired off, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured (27).

Nitric oxide (NO), a short lived free radical generated endogenously, exerts influence on a number of functions including vasodilation, neurotransmission, synaptic plasticity and memory in the central nervous system (28).

Superoxide anion radical is formed by activated phagocytes such as monocytes, macrophages, eosinophills and neutrophills. The production of superoxide anion is an important factor in phagocytes for killing bacteria. In the PMS-NADH-NBT system superoxide anion is derived from the dissolved oxygen released from the coupling reaction of PMS-NADH. This superoxide radical reduces NBT (27).

Both ferrous and ferric ions induce lipid peroxidation through various mechanisms involving reactive species. Most of the antioxidants inhibit iron-induced lipid peroxidation (28).

The test extracts showed a dose dependant free radical scavenging activity in all the *in vitro* models. The % inhibitions in the various models are shown in figures 1, 2, 3 and 4 for DPPH, nitric oxide radical, superoxide radical scavenging activities and lipid peroxidation respectively. The IC_{50} values for MECM and MECL for the above mentioned assays are stated in Table 1. Determination of the total phenolic contents showed that 1 mg of MECL contained 126.85 µg equivalent of pyrocatechol whereas 1mg of MECM contains 116.7 µg equivalent of pyrocatechol (Table 1).

Table 1: IC_{50} values of MECM and MECL for DPPH, nitric oxide, super oxide radical scavenging and inhibition of lipid peroxidation.

Extracts	IC_{50} value for DPPH (μg/ml)	IC_{50} value for superoxide radical (μg/ml)	IC_{50} value for nitric oxide radical (μg/ml)	IC_{50} value for lipid peroxidation (μg/ml)	Total Phenolic content (μg/mg)
MECL	7.36	9.36	136.45	111.0	126.85
MECM	5.57	79.16	174.97	111.50	116.70

Preliminary phytochemical screening of the methanol extract of MECL showed the presence of alkaloids, flavonoids, saponins and tannins. The phytochemical evaluation of MECM and MECL showed the presense of flavonoids mainly in both the extracts.

In the process of utilization of oxygen in physiological and metabolic processes, about 5% of oxygen gets reduced to oxygen derived free radicals which are capable of attacking lipids, proteins and damage of DNA (21).

High content of phenolic compounds are known to have direct antioxidant property due to presence of hydroxyl groups which can function as hydrogen donar (27). High phenolic component in an extract is a valid reason to expect potentially high radical scavenging activity.

Therefore, it can be concluded from the entire study that the plants *C. Limetta and C. maxima* exhibited marked *in vitro* antioxidant effects and appear to be very suitable candidates for further investigations in other fields of its reported medicinal usage.
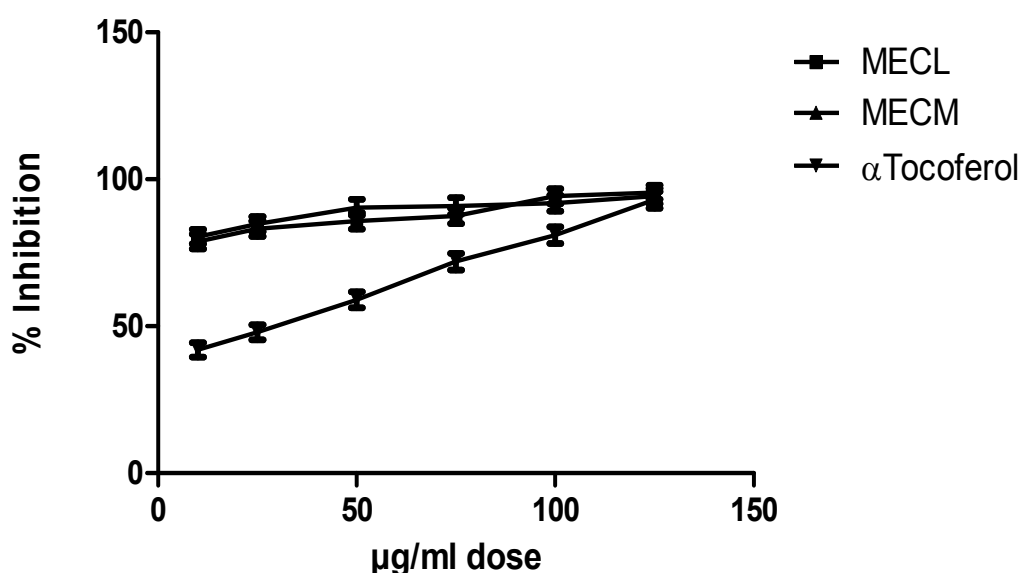
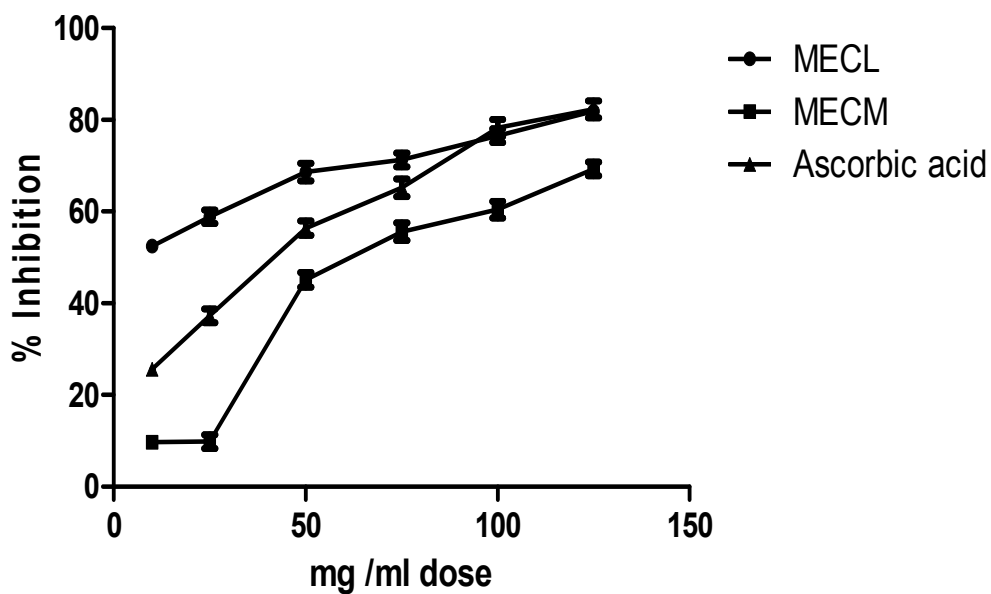**Fig. 1.** DPPH radical scavenging activity of MECL, MECM and α-tocoferol.

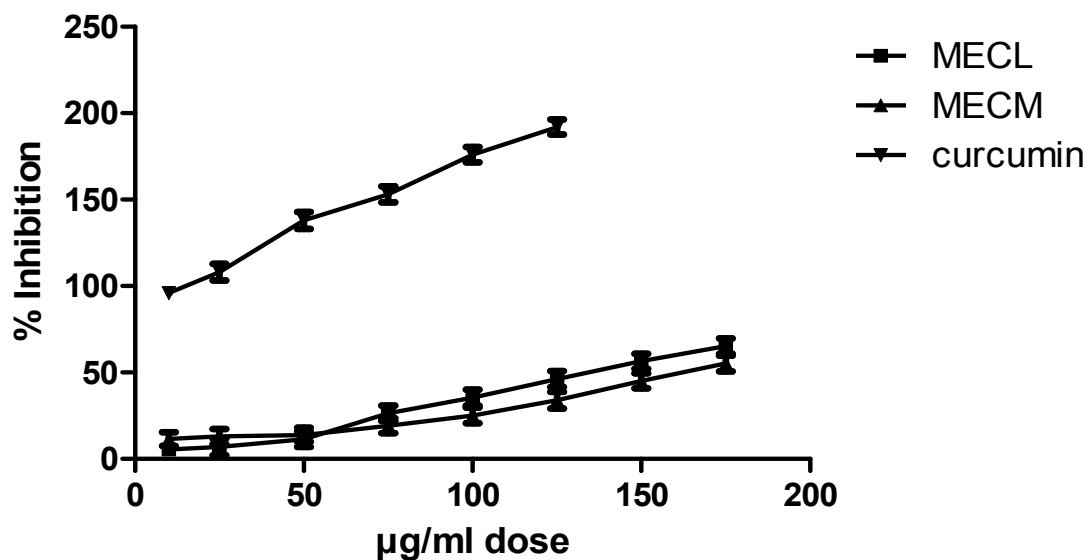**Fig. 2.** Superoxide radical scavenging activity of MECM, MECL and ascorbic acid.

Fig. 3. Nitric oxide scavenging activity of MECL, MECM and curcumin.
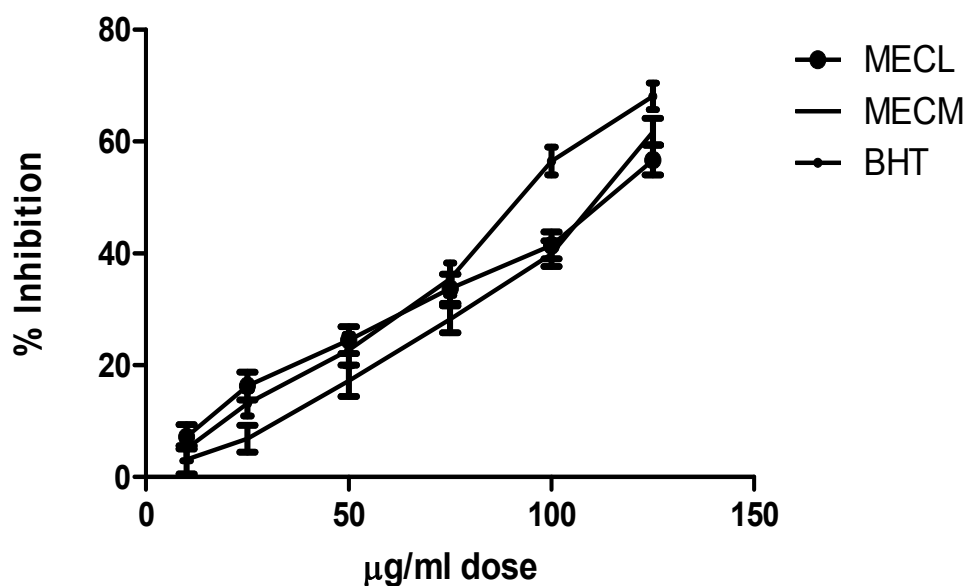
Fig. 4. Inhibition of lipid peroxidation by MECL, MECM and BHT.

Acknowledgements

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