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Research Article

IN VITRO ANTIOXIDANT ACTIVITIES OF LEAVES, FRUITS AND PEEL EXTRACTS OF *CITRUS*

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Abstract

Aim: The present study was aimed at investigating the antioxidant activities of the leaves, fruits and peel extracts of *Citrus aurantium, Citrus limetta* and *Citrus limon* belonging to the family Rutaceae.

Materials and Methods: The antioxidant activities of the hydroethanolic extracts have been evaluated by using different *in vitro* assays and the results were compared with the standard antioxidants such as butylated hydroxytoluene (BHT), ascorbic acid, curcumin, quercetin, etc. In addition, total phenolic and flavonoid contents in these extracts were determined as pyrocatechol and quercetin equivalents respectively. Among the extracts assayed, 4 extracts (leaf and peel extracts of *C.aurantium* , peel and fruit extracts of *C.limetta*) had effective H donor ability, reducing power ability, metal chelating activity, superoxide anion radical, nitric oxide radical and hydroxyl radical scavenging activities. The antioxidant activity depends upon concentration and increased with increasing amount of the extracts. The free radical scavenging and antioxidant activities may be attributed to the presence of phenolic and flavonoid compounds present in the extracts.

Result: The results obtained in the present study indicate that the leaves, fruits and peel of *Citrus aurantium, Citrus limetta* and *Citrus limon* serve as the potential source of natural antioxidants.

Keywords: Antioxidant; *Citrus aurantium; Citrus limetta; Citrus limon;* free radical; Rutaceae.

1. Introduction

The ability to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins, and carbohydrates for energy; however, it does not come without cost. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals.” Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction [1]. Overall, free radicals have been implicated in the pathogenesis of various diseases. Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. It is when the availability of antioxidants are limited that this damage can become cumulative and debilitating. Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves [2].

Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. And until subsequent free radicals are deactivated, thousands of free radical reactions can occur within seconds of the initial reaction. Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being. Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen-containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides [3]. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. Free radicals may cause reversible or

irreversible damages to biological molecules such as DNA, proteins and/or lipids [4, 5]. These damages may cause cancer, heart diseases, artherosclerosis, hypertension, arthritis, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease) and could accelerate aging of organisms[6].

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals. Many plant-derived substances, collectively termed "phytonutrients," or "phytochemicals," are becoming increasingly known for their antioxidant activity[2]. Especially Flavonoids are a group of natural products with many biological and pharmacological activities like antibacterial, antiviral, antioxidant, antiinflammatory and antimutagenic effects etc., [7,8]. However, antioxidant supplements or foods rich in antioxidants may be used to help the human body in reducing oxidative damage by free radicals and active oxygen [9]. Recently, various phytochemicals and their effects on health, especially the suppression of active oxygen species by natural antioxidants from teas, spices and herbs, have been intensively studied. Literatures suggest that the fruit of *C. aurantium* possess antianxiety activity [10] and antiobesity activity [11]. Peel of *C.limon* possess cytotoxic [12] and anti microbial activity [13], fruit of *C.limon* posses anti oxidative stress [14] and anti urinary lithogenesis [15].

Hence, the leaves, fruits and peel of *Citrus aurantium, Citrus limetta and Citrus limon* were selected for the study. These plants belonging to the family Rutaceae, is traditionally used by the local people and tribals in India to treat scurvy, rheumatism, stomachic, diarrhea, liver disorders and used as an antioxidant, anti helicobacter pylori, antiscorbutic, refrigerant, astringent, and anti lipolytic[14].

2. Materials and Methods

2.1 Plant material: The plant material consists of dried powdered leaves, peel and fresh fruit juices of *Citrus aurantium, Citrus limetta and Citrus limon* belonging to the family Rutaceae. The leaves, fruits and peel of belonging were collected from Madurai district, Tamilnadu, India during the month of June 2009. The plant was identified and authenticated by Mr. G.V.S. Murthy, Joint Director, C-I/C, Botanical survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BSI/SC/5/23/09-10/Tech-451.

2.2 Preparation of the extract: Fresh leaves and peel of the above plants were collected, dried in shade under room temperature, powdered mechanically and sieved through No.20 mesh sieve. The finely powdered leaves were kept in an airtight container until the time of use. About 120g of the dried powder was soaked with 1200 ml of ethanol: water (6: 4) for 12 h and then macerated at room temperature using a mechanical shaker for 4 h. The extract was filtered off and the marc was again soaked with the same volume of ethanol: water for 12 hour and then further extracted for 4 hour and filtered. The filtrates were then combined, concentrated under reduced pressure and evaporated at 40ºC. About 50g of peel powder was taken in soxhlet apparatus, and extracted with 450 ml of ethanol: water (6:4). After extraction, filtered and the filtrate was concentrated and evaporated at 40ºC. The fresh juice was collected from the fruit and dried at 40ºC.

Table1. Percentage of yield of extracts

Extract	Leaf	Peel	Fruit
C.Aurantium	17.5 %	22.12%	10%
C.Limetta	19.38 %	24%	11.32%
C.Limon	22.67%	20.52%	10.37

2.3 Drugs and chemicals: Pyrocatechol, DPPH, 2-deoxy-2-ribose, quercetin, ascorbic acid, nitro blue tetrazolium , butylated hydroxyl toluene, xanthine oxidase were obtained from Himedia labs ltd., Mumbai and trichloroacetic acid, Folin ciocalteau reagent, were purchased from SD Fine Ltd., Mumbai. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

2.4 Phytochemical screening: Preliminary phytochemical screening of the plant belongings were performed for the presence of alkaloids, phenolics, tannins, flavonoids, terpenoids and glycosides [17].

2.5 Superoxide anion scavenging activity: A reaction mixture with a final volume of 3 ml per tube was prepared with 1.4 ml of 50 mM KH_2PO_4-KOH, pH 7.4 containing 1 mM EDTA, 0.5 ml of 100

μm hypoxanthine, 0.5 ml of 100 μM NBT. The reaction was started by adding 0.066 units per tube of xanthine oxidase freshly diluted in 100 μl of phosphate buffer and 0.5 ml of test extract (50-800μg/ml) in saline. And absorbance measured at 560 nm. Ascorbic acid was used as the standard [18].

2.6 Hydroxyl radical scavenging activity: Hydroxyl radical scavenging activity was measured by the ability of the extract to scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) [25]. The reaction mixture contained 100 μl of 2-deoxy2-ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 500 μl of the extracts at various concentrations (50-800 μg/ml) in buffer, 200 μl of 1.04 mM EDTA and 200 μM $FeCl_3$ (1:1v/v), 100 μl of 1.0 mM hydrogen peroxide (H_2O_2) and 100 μl of 1.0 mM ascorbic acid. Test samples were kept at 37^0C for 1 h. The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid test. One ml of 1% thiobarbituric acid and 1.0 ml 2.8% trichloroacetic acid were added to the test tubes and were incubated at 100^0C for 20 min. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Quercetin was used as the standard [3].

2.7 Reducing power ability: It was measured by mixing 1.0 ml extract of various concentration (50-800μg/ml) prepared with distilled water to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min at 3000 g, 2.5 ml from the upper part were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm. Butylated hydroxyltoluene (BHT) was used as the standard [33].

2.8 DPPH radical scavenging activity: The hydrogen donating ability of the extract was examined in the presence of DPPH stable radical. One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentrations (10-160 μg) of extract and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm. Ascorbic acid was used as the standard [18].

2.9 Nitric oxide radical scavenging assay: Various concentrations of the extract and sodium nitroprusside (5mM) in phosphate buffer saline (0.025 M, pH 7.4) in a final volume of 3 ml are incubated at 25° C for 150 min. Control experiments without the test compounds but with equivalent amount of buffer is prepared in the same manner as done for the test. There after, 0.5 ml of incubation solution is removed and diluted with 0.5 ml Griess' reagent (1% sulphanilamide, 2% *O*-Phosphoric acid and 0.1% naphthyethylene diamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthyethylene diamine dihydrochloride is read at 546 nm. The percentage inhibition is calculated. The experiment is done in triplicate using curcumin (50-800 μg/ml) as positive control [19].

2.10 Ferrous chelating ability: The ferrous level is monitored by measuring the formation of the ferrous ion-ferrozine complex. The reaction mixture containing different concentrations of extracts (50-800μg/ml) were added to 2 mM ferrous chloride (0.1 ml) and 5 mM ferrozine (0.2 ml) to initiate the reaction and the mixture is shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution is measured at 562 nm. The positive control are those using ascorbic acid and all tests and analysis are run in triplicate. The percentage chelating effect of Ferrozine-Fe^{2+} complex formation is calculated [20].

2.11 Total phenolic content: Total soluble phenolics of the extracts were determined with Folin-Ciocalteu reagent using pyrocatechol as the standard [21]. An aliquot of 0.1 ml suspension of 1 mg of the extracts in water was totally transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by the addition of distilled water. Folin-Ciocalteu reagent (1 ml) was added to this mixture, followed by 3 ml of 2% sodium carbonate 3 min later. Subsequently, the mixture was shaken for 2 h at room temperature and the absorbance was measured at 760 nm. The concentration of total phenolic compounds in the fractions was determined as μg pyrocatechol equivalent by using the standard pyrocatechol graph [22].

2.12 Total flavonoid content : Total soluble flavonoid content of the extract was determined with aluminium nitrate using quercetin as the standard [35]. One mg of the extract was added to 1ml of 80 % ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10 % aluminium nitrate, 0.1 ml of 1 M potassium acetate and 4.3 ml of 80 % ethanol. The absorbance of the supernatant was

measured at 415 nm after incubation at room temperature for 40 min. The total flavonoid content in the fractions was determined as µg quercetin equivalent by using the standard quercetin graph [22].

2.13 Statistical analysis: All data were expressed as mean ± standard error of mean (S.E.M.) and statistical analysis was performed using one way analysis of variance (ANOVA) followed by Turkey's test $P < 0.05$ was considered significant.

3. Results

3.1 Superoxide anion scavenging activity: Among the extracts assayed, 7 extracts (Leaf, peel and fruit of *C.aurantium*, peel and fruit of *C.limetta* and *C.limon*) exhibited better superoxide anion scavenging activity in a concentration dependent manner in the xanthine oxidase-NBT system. The scavenging activity of *C.limetta* peel extract (IC_{50} 114.83±1.48 µg/ml) was higher than that of other extracts compared with standard ascorbic acid (IC_{50} 39.3±2.92 µg/ml) (Table 1). The scavenging effect of extracts on superoxide radical increased in order with IC_{50}: *C.limetta* peel (114.83±1.48 µg/ml) > *C.aurantium* peel (137.08±1.47 µg/ml) > *C.limon* peel (162.5±1.44 µg/ml) > *C.limetta* fruit (178.50±0.76 µg/ml) > *C.aurantium* fruit (223.16±0.44 µg/ml) > *C.limon* fruit (258.83±1.09 µg/ml) > *C.aurantium* leaf (332.83±1.04 µg/ml).

3.2 Hydroxyl radical scavenging ability: Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of deoxyribose by the free radicals generated by the Fenton reaction. Among the extracts assayed, 7 extracts (Leaf, peel and fruit of *C.aurantium*, peel and fruit of *C.limetta* and *C.limon*) and the standard (quercetin) inhibited the production of hydroxyl radicals. The scavenging activity of the *C.limetta* peel (IC_{50} 25.50±1.60 µg/ml) was higher than that of other extracts (Table 1) compared with the standard quercetin (IC_{50} 12.8±3.45 µg/ml) (Table 1). The scavenging effect of extracts on the hydroxyl radical increased in order with IC_{50}: *C.limetta* peel (25.50±1.60 µg/ml) > *C.aurantium* peel (31.50±1.04 µg/ml) > *C.limon* peel (45.56±1.22 µg/ml) > *C.limetta* fruit (52.50±1.75 µg/ml) > *C.aurantium* fruit (64.80±0.75 µg/ml) > *C.limon* fruit (72.16±1.74 µg/ml) > *C.aurantium* leaf (96.76±0.95 µg/ml).

3.3 DPPH radical scavenging activity: DPPH can be used to determine the free radical scavenging activity as it forms a stable molecule on accepting an electron or hydrogen atom [36]. There was a reduction in the concentration of DPPH due to the scavenging effect of extracts. Among the extracts assayed, 8 extracts (Leaf, peel and fruit of *C.aurantium* and *C.limetta,* peel and fruit of *C.limon*) and standard demonstrated H-donor activity. The extracts and standard reduced DPPH to yellow coloured product in concentration dependent manner. The DPPH scavenging activity of *C.limetta* peel extract was higher than that of other extracts and its IC_{50} was 70.08±0.93µg/ml compared with the IC_{50} of standard ascorbic acid (26.5±0.71µg/ml) (Table 1). The scavenging effect of extracts on the DPPH radical increased in order with IC_{50}: *C.limetta* peel (70.08±0.93 µg/ml) > *C.aurantium* peel (86.83±1.09 µg/ml) > *C.limon* peel (104.33±1.45 µg/ml) > *C.limetta* fruit (119.41±0.87 µg/ml) > *C.aurantium* fruit (130.06±0.96 µg/ml) > *C.aurantium* leaf (142.25±0.86 µg/ml) > *C.limon* fruit (143.33±0.88 µg/ml) > *C.limetta* leaf (148.16±0.44 µg/ml).

3.4 Nitric oxide radical scavenging assay: The scavenging of nitric oxide by the extracts were concentration dependent. Incubation of solutions of sodium nitroprusside in phosphate buffered saline at 25˚C for 150 min resulted in the generation of nitric oxide. Among the extracts assayed, 5 extracts (Leaf and peel of *C.aurantium*, peel and fruit of *C.limetta*, peel of *C.limon*) effectively reduced the generation of nitric oxide radicals. *C.limetta* peel showed highest nitric oxide scavenging activity (IC_{50} 290.33±0.44µg/ml) compared with standard curcumin (IC_{50} 78±0.83µg/ml) (Table 1). The scavenging effect of extracts on nitric oxide radical increased in order with IC_{50}: *C.limetta* peel (290.33±0.44 µg/ml) > *C.aurantium* peel (459.75±0.44 µg/ml) > *C.limon* peel (565.25±1.09 µg/ml) > *C.limetta* fruit (705±0.56 µg/ml) > *C.aurantium* leaf (765.41±0.82 µg/ml).

3.5 Ferrous chelating ability: The formation of the Fe^{2+} - ferrozine complex was not completed in the presence of extracts, indicating that the extracts chelate the iron. The absorbance of the extracts decreased with increasing concentration (from 50 to 800µg). *C.limetta* leaf showed highest chelating activity (IC_{50} 286.16±0.60µg/ml) compared with standard ascorbic acid (IC_{50} 93.24±0.35µg/ml) (Table 1). The chelating effect of extracts on iron increased in order with IC_{50}: *C.limetta* leaf (286.16±0.60 µg/ml) > *C.aurantium* leaf (362±1.73 µg/ml) > *C.limetta* peel (445.08±1.08 µg/ml) > *C.aurantium* peel (586.66±1.20 µg/ml) > *C.limon* leaf (707.16±0.44 µg/ml) > C.limetta fruit (795.50±0.86 µg/ml).

Table 2: Antioxidant activities of the leaves, fruits and peel extract of *Citrus aurantium, Citrus limetta* and *Citrus limon*

Extracts		IC_{50} µg/ml				
		$O_2\cdot^-$	OH·	DPPH	NO·	Fe^{2+}
C.aurantium	**Leaf**	332.83±1.04	96.76±0.95	142.25±0.86	765.41±0.82	362±1.73
	Peel	137.08±1.47	31.50±1.04	86.83±1.09	459.75±0.44	586.66±1.20
	Fruit	223.16±0.44	64.80±0.75	130.06±0.96	-	-
C. limetta	**Leaf**	-	-	148.16±0.44	-	286.16±0.60
	Peel	114.83±1.48	25.50±1.60	70.08±0.93	290.33±0.44	445.08±1.08
	Fruit	178.50±0.76	52.50±1.75	119.41±0.87	705.16±0.56	779.5±0.86
C.limon	**Leaf**	-	-	-	-	707.16±0.44
	Peel	162.5±1.44	45.56±1.22	104.33±1.45	565.25±1.09	-
	Fruit	258.83±1.09	72.16±1.74	143.33±0.88	-	-
Standard	**Ascorbic acid**	39.3±2.92	-	26.50±0.71	-	93.24 ± 0.35
	Quercetin	-	12.8±3.45	-	-	-
	Cucumin	-	-	-	148.36±0.83	-

Results are expressed as mean± SEM of three parallel measurements, P<0.01 when compared with control**.**

3.6 Reducing power ability : Leaves, fruits and peel extract of *Citrus aurantium, Citrus limetta and Citrus limon* have reductive capability when compared to the standard, BHT, the activity of the extracts was less than the standard (Table 2).

Table 3. Reducing power ability of the leaves, fruits and peel extract of *Citrus aurantium, Citrus limetta* and *Citrus limon*

Plant	Part	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml
Citrus aurantium	**Leaf**	0.034±0.004	0.101±0.004	0.147±0.003	0.205±0.004	0.262±0.002
	Peel	0.101±0.004	0.187±0.003	0.256±0.003	0.371±0.003	0.536±0.003
	Fruit	0.048±0.003	0.107±0.003	0.170±0.003	0.231±0.004	0.314±0.003
Citrus limetta	**Leaf**	0.023±0.002	0.067±0.003	0.137±0.002	0.177±0.003	0.242±0.003
	Peel	0.137±0.003	0.240±0.002	0.3140.002	0.412±0.003	0.560±0.002
	Fruit	0.060±0.002	0.124±0.003	0.188±0.004	0.239±0.004	0.332±0.002
Citrus limon	**Leaf**	0.016±0.003	0.027±0.002	0.043±0.003	0.084±0.002	0.159±0.004
	Peel	0.083±0.003	0.128±0.004	0.197±0.004	0.290±0.003	0.391±0.004
	Fruit	0.025±0.002	0.087±0.002	0.132±0.002	0.177±0.003	0.252±0.003
BHT		0.601±0.003	0.713±0.005	0.839±0.003	0.911±0.008	1.190±0.110

Values are expressed as mean± SEM of three parallel measurements, P<0.01 when compared with the standard, BHT

3.7 Total phenolic content: The content of total phenolics in the extracts was determined using the standard pyrocatechol graph and it was expressed as µg pyrocatechol equivalents (PCE). Total phenolic content of the extracts varied from 7.39 to 33.05 µg PCE/mg. Total phenols of extracts were found to be in the following order: *C.limetta* peel > *C.aurantium* peel > *C.limon* peel > *C.aurantium* leaf > *C.limetta* leaf > *C.limon* leaf > *C.limetta* fruit > *C.aurantium* fruit > *C.limon* fruit.

3.8 Total flavonoid content: The total flavonoid content in the extracts was determined using the standard quercetin graph, and it was expressed as µg quercetin equivalent (QE). Total flavonoids of extracts varied from 0.51 to 21.62 µg QCE/mg. Total flavonoids of extracts were found to be in the following order: *C.limetta* peel > *C.aurantium* peel > *C.limon* peel > *C.aurantium* leaf > *C.limetta* leaf > *C.limon* leaf > *C.limetta* fruit > *C.aurantium* fruit > *C.limon* fruit

4. Discussion and Conclusion

Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms.

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts [23] DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow coloured product, diphenylpicryl hydrazine, with the addition of the fractions in a concentration-dependent manner. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. The extracts showed significantly higher inhibition percentage (stronger hydrogen-donating ability) and positively correlated with total phenolic content.

The transformation of Fe^{3+} into Fe^{2+} in the presence of various fractions was measured to determine the reducing power ability. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom [24]. The antioxidant principles present in the extracts caused the reduction of Fe^{3+}/ ferricyanide complex to the ferrous form, and thus proved the reducing power ability.

Hydroxyl radical is the most deleterious and reactive among the ROS and it bears the shortest half-life compared with other free radicals. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid [25]. The extracts when added to the reaction mixture, scavenged the hydroxyl radicals and prevented the degradation of deoxyribose.

The most commonly occurring cellular free radical is superoxide anion radical. When an oxygen molecule gains one electron from another substance, the formation of superoxide radical takes place spontaneously. Superoxide is also produced endogenously by flavonoenzymes like XO activated in ischemia-reperfusion, lipoxygenase and cycloxygenase. A variety of enzyme system catalyzes the univalent reduction of molecular oxygen to superoxide. Two molecules of superoxide rapidly dismutase to hydrogen peroxides and molecular oxygen and this reaction is further accelerated by superoxide dismutase (SOD) [22]. The extracts exhibited better superoxide anion scavenging activity in a concentration dependent manner in the xanthine oxidase-NBT system.

In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Nitric oxide is a free radical which plays an important role in the pathogenesis of pain, inflammation, etc. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent [26]. The absorbance of the chromophore is measured at 546 nm in the presence of the fractions. The extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. This may be due to the antioxidant principles in the extracts which compete with oxygen to react with NO· thereby inhibiting the generation of nitrite.

The metal chelating ability of the leaves, fruits and peel extract of *Citrus aurantium, Citrus limetta* and *Citrus limon* was measured by the formation of ferrous ionferrozine complex. Ferrozine combines with ferrous ions forming a red coloured complex which absorbs at 562 nm [34]. It was reported that the chelating agents which form σ bond with a metal, are effective as secondary antioxidants, because they reduce the redox potential thereby stabilising the oxidised form of the metal ion [27]. The results of our study demonstrate that the extracts have an effective capacity for iron binding, suggesting its antioxidant potential. In addition, the metal chelating ability of the extracts demonstrated that they reduce the concentration of the catalyzing transition metal involved in the peroxidation of lipids.

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and also decrease cardiovascular complications [28]. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Total phenolic assay by using Folin-Ciocalteu reagent is a simple, convenient and reproducible method. It is employed routinely in studying phenolic antioxidants [29].

Flavonoids are a group of polyphenolic compounds, which exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral, anticancer activities. They also inhibit enzymes such as aldose reducatse and xanthine oxidase. They are capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidants [30]. In view of their wide pharmacological and biological actions, they have a greater

therapeutic potential. The presence of high phenolic and flavonoid content in the extracts has contributed directly to the antioxidant activity by neutralising the free radicals.

On the basis of the results of this study, it is clearly indicated that the leaves, fruits and peel extract of *Citrus aurantium, Citrus limetta* and *Citrus limon* has a powerful antioxidant activity against various oxidative systems *in vitro*. Recent years have witness increased interest in the role of free radical oxidative damage in human diseases and aging. Free radical oxidative stress has a probable role in the pathogenesis of variety of human diseases which has lead to the use of agents that can supplement the natural antioxidant defences [2]. Plant based medicines contain rich antioxidants and are traditionally used in different parts of the world [31], this lead to high antioxidant status at low metabolic cost. The hypothesis of obtaining plant based medicine is beneficial to human health because this increases antioxidant defence against oxidative damage [32]. Based on the active profile exposed through various *in vitro* assays, it can be concluded that the leaves, fruits and peel extract of *Citrus aurantium, Citrus limetta* and *Citrus limon* showed significant antioxidant activities.

The various antioxidant mechanisms of these extracts may be attributed to strong hydrogen donating ability, a metal chelating ability, reducing power ability and their effectiveness as scavengers of hydroxyl, superoxide, and nitric oxide. The above results indicate that the peel extract of *C.limetta* showed a stronger antioxidant activity than the other extracts and it was followed by the peel extract of *C.aurantium* and *C.limon*. Further investigations on the isolation of active compounds present in the extracts are necessary to identify a potential chemical entity for clinical use in the prevention and treatment of free radical related disorders.

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