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Research Article

QUANTITATIVE PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTIVITIES OF SOME *CITRUS* FRUITS OF SOUTH INDIA

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ABSTRACT

Objective: The current investigation was contemplated to evaluate the phytochemicals and *in vitro* antioxidant activities in peel and pulp of some commercially grown citrus fruits of South India, namely, lemon (*Citrus aurantifolia*), orange (*Citrus reticulata*), sour orange (*Citrus aurantium*), pomello (*Citrus grandis*), and citron (*Citrus medica*).

Methods: The peel and pulp of the fruits were separated and subjected to cold extraction using 70% alcohol. The extracts obtained were screened for the presence of their phytoconstituents using various qualitative and further quantified for major constituents. Further, the *in vitro* antioxidant activity was assayed by different radical scavenging methods, namely, 2,2-diphenyl-1-picrylhydrazyl, superoxide anion, nitric oxide, lipid peroxidation inhibition, iron chelating activity, and reducing power assay at different concentrations.

Results: All the citrus fruits have shown significant *in vitro* antioxidant activity for the parameters assessed, wherein peel extracts recorded superior antioxidant potential than their corresponding pulps. The broad range of activity of the extracts suggests that multiple mechanisms mediated by the phytoconstituents are responsible for the antioxidant activity.

Conclusion: The study thus revealed that peel and pulp of citrus fruits are potential sources of bioactive compounds which are reflected in antioxidant activity and supports their health-promoting claims of plethora of investigations.

Keywords: Citrus fruits, Peel, Pulp, Qualitative and quantitative, *In vitro* antioxidant activity.

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INTRODUCTION

Oxygen being an obligatory element of life, living systems have evolved to survive in the presence of molecular oxygen and for most biological systems [1]. Many of the harmful effects of oxygen are ascribed to the formation and activity of reactive oxygen species (ROS) behaving as oxidants, which have a tendency to donate oxygen to other substances. Most of the ROS are free radicals and are extremely reactive and short lived [2]. Free radicals occur continuously in all cells as part of normal metabolism. At low concentration, some of the free radicals offer positive physiological effect *in vivo,* and this includes defense against infectious agents by phagocytosis, energy production, cell growth, function in different cellular signaling systems, and the induction of a mitogenic response [1]. On the other hand, these free radicals are detrimental to the integrity of biological tissue and mediate their injury. The mechanism of damage involves lipid peroxidation (LPO), which destroys cell structures, lipids, proteins, and nucleic acids [3,4]. The human body has an array of mechanisms, especially enzymatic and non-enzymatic antioxidant systems, to protect cells and their constituents against ROS and free radical-induced damage [5].

Oxidative stress is a condition which occurs due to imbalance between free radicals and antioxidant defense system. Oxidative stress plays an important contributory role in the process of aging and pathogenesis of numerous diseases such as diabetes, cancer, neurodegenerative diseases, and respiratory tract disorder [5-7]. To counteract oxidative stress, the body produces an armory of antioxidants to defend itself. It is the role of antioxidants to neutralize or clear free radicals that can affect the cells. The body's internal production of antioxidants is not enough to neutralize all the free radicals. The body can be helped to defend itself by increasing dietary intake of antioxidants [8].

Antioxidants are the substances, compounds, or nutrients in our foods which can prevent or slow oxidative damage to our bodies. These agents are able to antagonize the deleterious effects of free radicals within our body [9]. Recent investigations have shown that the antioxidants of plant origin with free-radical scavenging properties could be useful as therapeutic agents in several diseases caused due to oxidative stress [10]. Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant [1,11]. Natural products, mainly obtained from dietary sources, provide a large number of antioxidants. It is increasingly being accepted that fruits and vegetables have many health-promoting properties [12], and the consumption of fruit juices, beverages, and hot drinks has been showed to be inversely associated with morbidity and mortality from degenerative diseases [9].

The genus *Citrus*, which belongs to the family of Rutaceae, is rich genera of edible fruits of various species, and they are one of the main fruit tree crops grown worldwide. *Citrus* fruits have long been valued as part of a nutritious and tasty diet. *Citrus* and their products are a rich source of vitamins, minerals, and dietary fiber that are essential for normal growth and development and overall nutritional well-being. Fruits are used raw, pickled, and are esteemed for desert, made into jams and marmalades. Many *Citrus* species are recognized for their medicinal, physiological, and pharmacological activities including antimicrobial, antioxidant, anticancer, anti-inflammatory, and hypoglycemic activities [13]. The health benefits of *Citrus* fruit

have been attributed to the presence of bioactive compounds, such as phenolics (e.g., flavanone glycosides and hydroxylcinnamic acids), Vitamin C, and carotenoids [14]. Ascorbic acid is the most important antioxidant in *Citrus* fruit juices, and it protects the organism from oxidative stress. Flavanones, flavones, and flavonols are three types of flavonoids which occur in *Citrus* fruit [9].

In view of huge importance of *Citrus* fruits as antioxidant sources, in the present research, a comparison of their antioxidant property of commonly consumed *Citrus* fruits of South India was investigated to evaluate their extent antioxidant potential. Antioxidant molecules such as phenolics, ascorbic acid, and flavonoids were also quantified to understand their contribution to the overall bioactive principles.

METHODS

Collection of plant materials

The *Citrus* fruits were procured from a local market of Shimoga, Karnataka, which were authenticated by the Taxonomist, Department of Botany, Sahyadri Science College, Shimoga. The fruits selected include lemon (*Citrus aurantifolia*), orange (*Citrus reticulata*), sour orange (*Citrus aurantium*), pomello (*Citrus grandis*), and Citron (*Citrus medica*) (Fig. 1). After selection, fruits were washed under running tap water followed by washing with distilled water to remove the surface debris. Then, the peel and pulp of the fruits were separated and were further subjected for extraction procedures.

Extraction

Exactly 1000 g of the separated peel and pulp were subjected to extraction procedure using 70% ethanol as per the method described by Jamuna *et al.* 2015 [15], and the yield of the extract was calculated.

Qualitative phytochemical analysis

The phytochemical screening was performed for testing the different chemical groups present in ethanolic extracts of both peel and pulp of all *Citrus* fruits [16-18].

Quantitative phytochemical analysis

Total phenolic content

The total phenolic content was estimated according to the method of Chandler and Dodds, 1993 [19], and the results were expressed as gallic acid equivalent in µg/mg of extract.

Total flavonoid content

A total flavonoid content of all the extracts was determined by the method of Zhishen *et al.* 1999 [20], and the values were expressed as catechol equivalent in µg/mg of extract.

Ascorbic acid content

Ascorbic acid content of peel and pulp extracts of *Citrus* was determined by 2,4-dinitrophenylhydrazine method as described by Sadasivam and

Fig. 1: *Citrus* fruits. (a) *Citrus aurantifolia*. (b) *Citrus reticulate.* (c) *Citrus grandis.* (d) *Citrus aurantium* and (e) *Citrus medica*

Manickam, 2004 [21], and the results were expressed as ascorbic acid equivalents in µg.

Total antioxidant capacity (TAC)

The TAC of all the ten extracts was performed according to the procedure of Prieto *et al.* 1999 [22]. The TAC of each extract is expressed as equivalents of ascorbic acid in µg/mg of extract.

Evaluation for *in vitro* antioxidant activities

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH free radical scavenging assay was measured using DPPH free radical test, by employing the method of Wong *et al.* 2006 [23]. DPPH radical scavenging activity of butylated hydroxytoluene (BHT) was assayed for comparison. Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula. The results are expressed as half maximal effective concentration (EC_{50}), which is the amount of antioxidants necessary to decrease the initial concentration by 50%.

Percentage effect (E%)=$(A_0-A_1)/A_0\times100$

Where A_0 is the absorbance of the control (without test samples), and A_1 is the absorbance of test samples.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat, 1964 [24]. BHT was used as positive control, and the results are expressed as EC_{50}.

Ferrous ion (Fe^{2+}) chelating activity

The chelation of ferrous ions by extracts was estimated by the method of Dinis *et al.* 1994 [25]. Ethylenediaminetetraacetic acid was used as a standard metal chelating agent, and the results are expressed as EC_{50}.

Superoxide anion radical scavenging assay

Superoxide anion radical scavenging activity of all the extracts was determined using the slight modified version of Nishimiki *et al.* 1972 [26]. BHT was used as a standard, and percentage of inhibition was calculated and expressed as EC_{50}.

Reducing power assay

Total reduction capability of extracts was estimated using the method of Oyaizu [27]. Increase in absorbance of the reaction mixture indicates increased reducing power. EC_{50} value (µg of extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid was used as a standard.

LPO inhibition assay

Thiobarbituric acid reacts with malondialdehyde to form a diadduct, a pink chromogen, which can be detected as per the method of Halliwell and Guttridge [28]. Percentage of inhibition was expressed as EC_{50}.

Statistical analysis

All the experiments were carried out in triplicates. The result of the triplicates was pooled and expressed as mean±standard error.

RESULTS

Extraction yield

Ethanolic extract of both peel and pulp of all the five *Citrus* fruits was weighed, and the yields obtained were noted. In case of peel extracts, orange showed highest yield (89.43 g), followed by pomello (86.82 g), citron (72.15 g), lemon (54.18 g), and sour orange (48.86 g), whereas in pulp extracts, pomello revealed high percentage of yield (92.27g), followed by orange (91.70 g), lemon (80.86 g), sour orange (68.36 g), and citron (58.97 g) per kg of the fruit material (Table 1).

Table 1: Yield of peel and pulp extracts of five *Citrus* fruits

Name of the fruit	Yield (g)	
	Peel extracts	Pulp extracts
Lemon	54.18	80.86
Orange	89.43	91.70
Sour orange	48.86	68.36
Pomello	86.82	92.27
Citron	72.15	58.97

Qualitative phytochemical analysis

The preliminary qualitative phytochemical investigation documented that the peel and pulp extracts of all five *Citrus* fruits showed the presence of many bioactive compounds, namely, polyphenols, flavonoids, terpenoids, steroids, glycosides, alkaloids, and carotenoids. The results also revealed that saponins were present in both peel and pulp extracts of orange and citron and in peel extract of sour orange, whereas absent in both peel and pulp extracts of lemon and pomello. The results of the analysis are shown in Table 2.

Quantitative phytochemical analysis

Total phenolic content

The amount of total phenolic contents was present in varying concentrations in the different extracts of *Citrus* fruits. The results were expressed as the number of equivalents of gallic acid (µg/mg of extract) and were found to be highest in citron peel (66.36) and pulp (51.21), followed by peel extracts of orange (48.5), lemon (46.65), sour orange (41.5), and pomello (40.14). While the pulp extracts of orange, lemon, sour orange, and pomello recorded 28.34, 34.26, 37.72, and 21.39 µg, respectively (Fig. 2a).

Total flavonoid content

The standard curve of catechol was used to express the total flavonoid content of the peel and pulp extracts of *Citrus* fruits and was expressed in terms of catechol equivalence (µg/mg of extract). The results revealed that citron peel and pulp extracts have maximum content of total flavonoids (40.17 and 37.9), followed by sour orange (39.23 and 29.83), lemon (36.49 and 15.19), pomello (28.11 and 30.22), and orange (21.61 and 20.75 µg) (Fig. 2b), respectively.

Total ascorbic acid content

Standard ascorbic acid was used to express the ascorbic acid content of the fruit extracts and was expressed in terms of ascorbic acid equivalence in µg/mg of extract. From the results, it is evident that orange peel extract has maximum content of ascorbic acid (52.67), followed by citron (33.77), pomello (30.81), sour orange (27.21), and lemon peel extracts (20.21), whereas pulp extracts showed orange (25.9), lemon (24.87), citron (24.69), sour orange (24.09), and pomello (23.93 µg) of ascorbic acid (Fig. 2c).

TAC

The results of TAC revealed that all *Citrus* fruit extracts under the study possess significant antioxidant potential (Fig. 2d). The results were compared with the standard curve of ascorbic acid and were expressed in terms of the equivalence of ascorbic acid in µg/mg of extract. From the results, it was found that citron peel and pulp extracts showed the maximum content of total antioxidants (140.17 and 116.11), followed by lemon (125 and 59.04), sour orange (123.77 and 53.02), orange (109.53 and 74.71), and pomello (98.91 and 87.64 µg), respectively.

Evaluation for *in vitro* antioxidant activities

DPPH radical scavenging activity

DPPH radical scavenging activity of the different extracts at varying concentrations was measured along with BHT. The EC_{50} values for fruit extracts were found to be highest in citron peel (827.26 µg/ml), followed

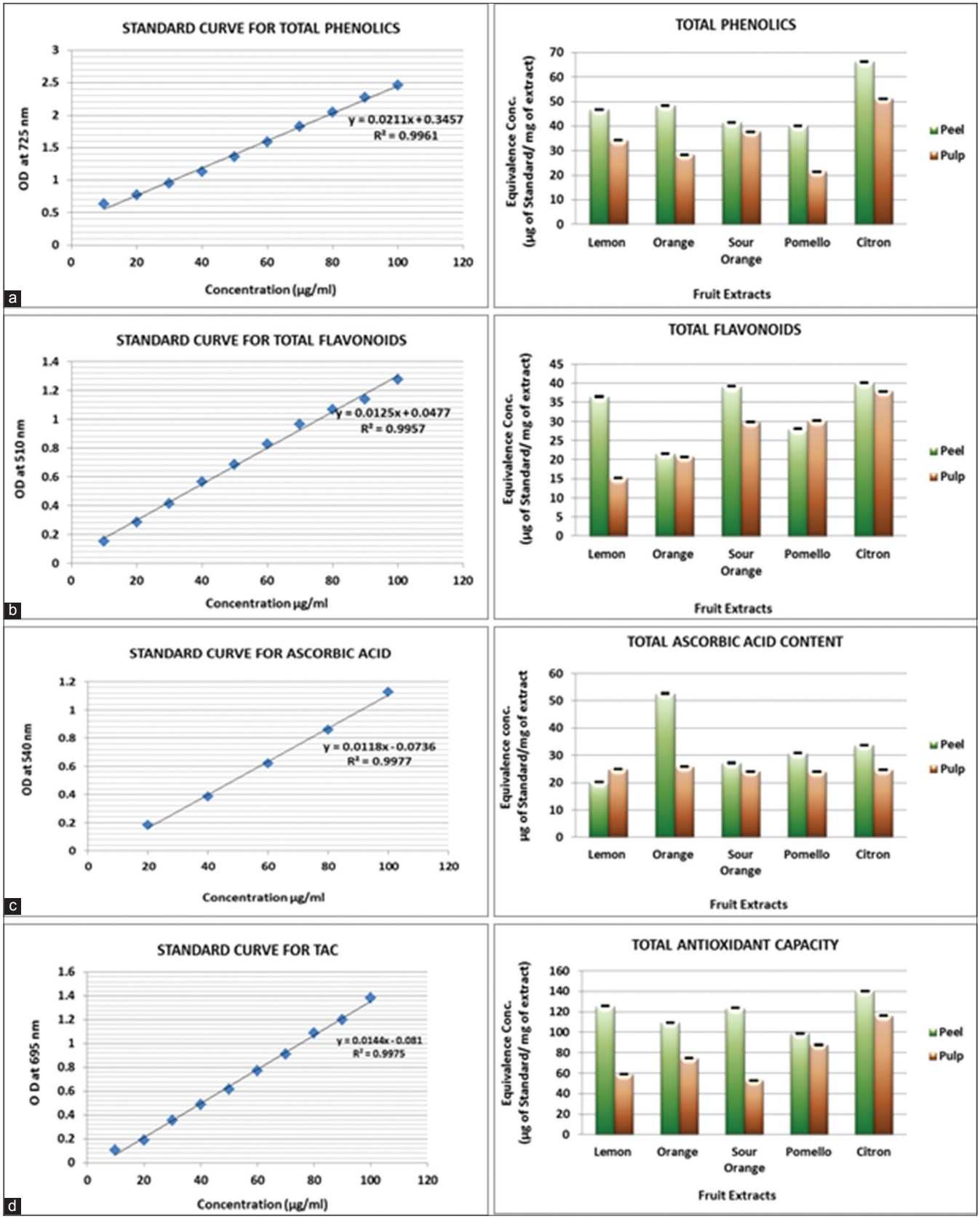

Fig. 2: Quantitative phytochemical evaluation of five *Citrus* fruits (a) standard curves and equivalence of total phenolics (b) standard curves and equivalence of total flavonoids (c) standard curve and equivalence of total ascorbic acid content (d) standard curve and equivalence of total antioxidant capacity

by peel extracts of lemon (977.89 µg/ml), orange (1097.45 µg/ml), sour orange (1213.29 µg/ml), and pomello (1485.88 µg/ml). While the pulp extracts of orange, citron, lemon, sour orange, and pomello showed EC_{50} values of 3628.44, 4089.64, 4184, 5006, and 5464.48 µg/ml,

Table 2: Qualitative phytochemical analysis of five *Citrus* fruits

Tests	Lemon		Orange		Sour orange		Pomello		Citron	
	Peel	Pulp	Peel	Pulp	Peel	Pulp	Peel	Pulp	Peel	Pulp
Steroids	+	+	+	+	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+	+	+
Saponins	-	-	+	+	+	-	-	-	+	+
Alkaloids	+	+	+	+	+	+	+	+	+	+
Carotenoids	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+
Tannins and polyphenols	+	+	+	+	+	+	+	+	+	+

respectively. Whereas BHT has registered an EC_{50} value of 65.75 µg/ml (Fig. 3a). The results demonstrated the dose-dependent DPPH radical scavenging activity in terms of EC_{50}.

Nitric oxide radical scavenging activity

All the ten extracts of *Citrus* fruits showed significant activity in competing with oxygen to react with nitric oxide and thus the inhibition of anions. The results of nitric oxide radical scavenging activity revealed that orange pulp extract possessed better scavenging effect than other peel and pulp extracts. The peel and pulp extracts of lemon, orange, sour orange, pomello, and citron recorded EC_{50} values of 761.84 and 856.16, 899.76 and 727.06, 823.85 and 792.64, 917.93 and 799.1, and 871.68 and 878.88 µg/ml, respectively (Fig. 3b).

Ferrous ion (Fe^{2+}) chelating activity

The peel extracts of sour orange and lemon possessed potent iron chelating activity with EC_{50} values of 827.95 and 871.08 µg/ml, respectively, followed by pomello (1193.03 µg/ml) and orange (1478.41 µg/ml) extracts. Among the pulp extracts, orange (1052.18 µg/ml) showed better activity than lemon (1145.73 µg/ml), sour orange (1257.54 µg/ml), and pomello (1864.97 µg/ml). While in both the peel and pulp extracts of citron, iron chelating activity was not noticeable (Fig. 3c).

Superoxide radical scavenging assay

The superoxide anion radical scavenging activity of the different extracts from peel and pulp of *Citrus* fruits assayed by the phenazine methosulfate/NADH system and their EC_{50} values were depicted in Fig. 3d. The results infer that EC_{50} values for test extracts were found to be highest in sour orange peel, followed by peel of citron (2228.165 µg/ml), pomello (2252.255 µg/ml), orange (2335.575 µg/ml), and lemon (2447.625 µg/ml). In pulp extracts, citron showed better activity with EC_{50} value of 2621.09 µg/ml, followed by orange (2629.91), lemon (2657.87), sour orange (2763.65), and pomello (2969.12 µg/ml). The standard BHT showed 50% inhibition at 49.19 µg/ml.

Reducing power assay

The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity. All the fruit extracts have shown prominent reducing power effect in dose-dependent manner and were found to be high in lemon peel extract with an effective concentration of 170.94 µg/ml, followed by peel extracts of citron (276.62), pomello (365.63), orange (497.51), and sour orange (750.46), whereas pulp extracts of pomello, orange, citron, sour orange, and lemon exhibited an EC_{50} value of 725.95, 877.19, 917.43, 1123.59, and 1412.42 µg/ml, respectively (Fig. 3e).

LPO inhibition assay

The LPO inhibition assay of extracts of five *Citrus* fruits was investigated in comparison with the known antioxidant butylated hydroxylanisole (BHA). The peel and pulp extracts of orange fruit had higher LPO inhibition in mice liver with EC_{50} values 883.7 and 946.61 µg/ml, respectively. The EC_{50} values of inhibition of LPO activity in peel and pulp extracts of lemon, citron, pomello, and sour orange was found to be 928.85 and 957.12 µg/ml, 931.27 and 951.83 µg/ml, 936.32 and 981.54 µg/ml, and 944.1 and 990.09 µg/ml, respectively. The standard BHA recorded EC_{50} values of 172.11 µg/ml (Fig. 3f).

DISCUSSION

Fruits are dietetically prominent component of foodstuffs. It has been documented that fruit and vegetables are important parts of a disease-preventing diet. The results of the recent research clearly indicate the importance of fruit and vegetables as the richest potential source of antioxidants and emphasize the need to increase the proportion of these products in the diet [29,30]. The consumption of fruits has been inversely linked with morbidity and mortality from degenerative diseases [31-33]. It is not known which dietary constituents are responsible for this association, but antioxidants seem to play the crucial function in the protective functions of plant foods [32-35].

In the present study, peel and pulp of five different *Citrus* fruits, namely, lemon, orange, sour orange, pomello, and citron commonly cultivated in South India were tested for different phytoconstituents. The result of qualitative phytochemical analysis documented that all the ten extracts of *Citrus* fruits are bestowed with the presence of several bioactive compounds, namely, polyphenols, flavonoids, terpenoids, steroids, glycosides, alkaloids, and carotenoids. In the recent years, there is an increasing interest in finding antioxidants of natural origin. The most effective antioxidants are flavonoids, and other phenolic compounds present in plants particularly in herbs, seeds, and fruits [36,37]. Okwu and Emenike 2007 screened phytochemicals of five *Citrus* species and revealed the presence of saponins, tannins, flavonoids, alkaloids, and phenols. Further, the results of quantitative estimation revealed the presence of higher concentration of several potential antioxidant components such as total phenols, flavonoids, ascorbic acid, and total antioxidants in *Citrus* peel extracts than the pulp extracts [38]. Phenolics in fruits and vegetables, as well as Vitamin C, are said to be effective antioxidants. Both juice and peel contain nutraceuticals; nevertheless, it has been demonstrated that they are more abundant in *Citrus* peel [39-41]. The results of the present investigation, therefore, are in accordance with others who indicated that peels are an important source of phenolics [42,43].

In the recent years, potent free radical scavengers have attracted a huge interest as possible therapeutics against free radical-mediated diseases [44]. Natural compounds show stronger antioxidant activity which is likely to quench free radicals. The antioxidant activity may act in various ways by scavenging the radicals, decomposing peroxides, and chelating metal ions [45]. Frankel and Kolevaet suggested that the use of different methods is necessary in antioxidant activity assessment to understand the various antioxidation mechanisms which are operating in the cellular system [46-48]. In the present study, the antioxidant potentials of five *Citrus* fruits peel and pulp extracts were assessed using various *in vitro* assays at different concentrations. From the results, it was observed that all the *Citrus* fruit extracts manifested potent antioxidant activities. For all *Citrus* fruits, peels gave pronounced

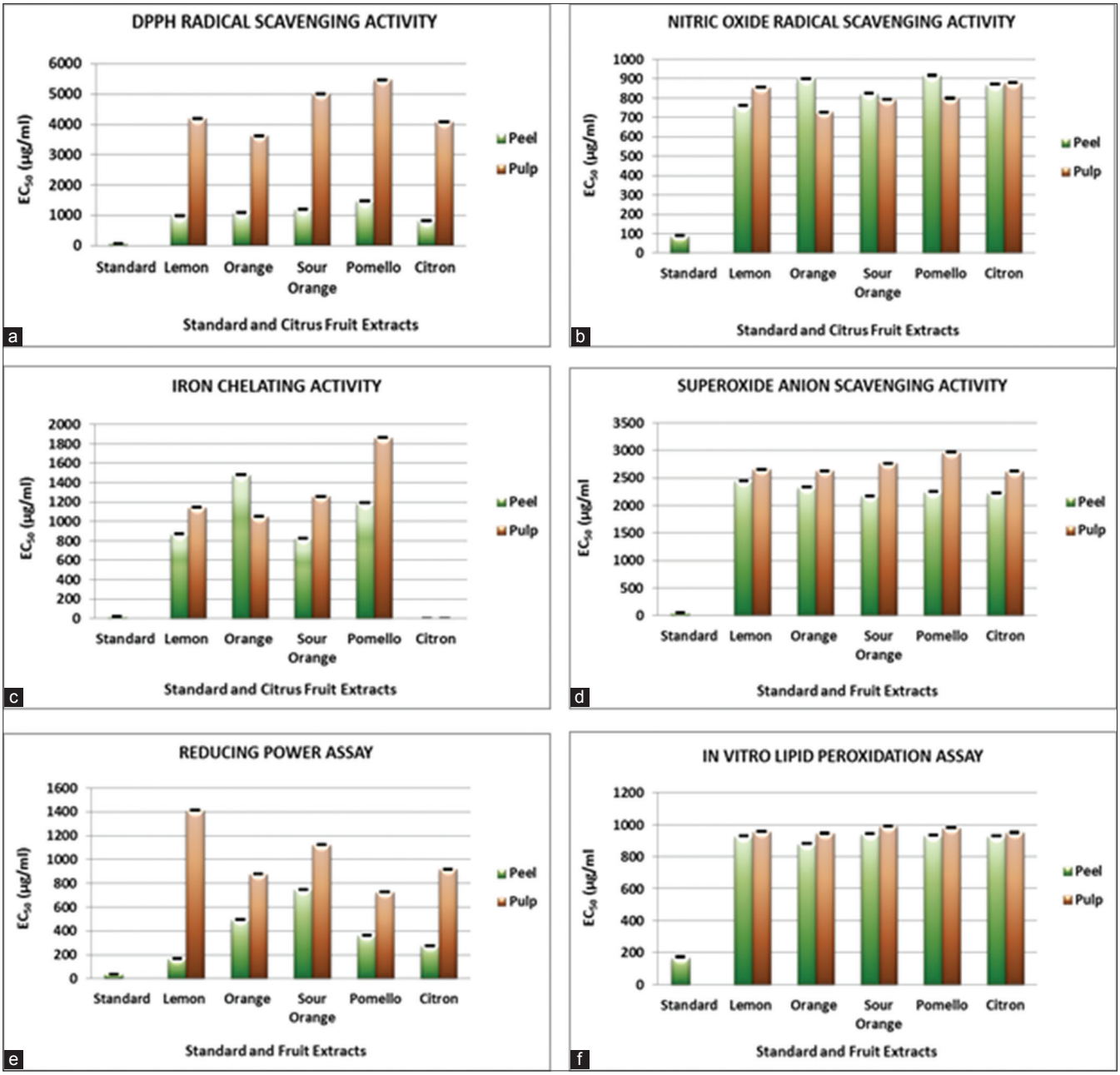

Fig. 3: *In vitro* antioxidant activities (half maximal effective concentration) of five *Citrus* fruit extracts (a) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, (b) nitric oxide radical scavenging activity, (c) Fe^{2+} chelating activity, (d) superoxide radical scavenging assay, (e) reducing power assay, (f) lipid peroxidation inhibition assay

results than their corresponding pulps which are in agreement with reports available in the literature [9]. Peel extracts manifested differential expression of antioxidant capacity in different assays due to their phytoconstituents operational under different mechanism. Lemon peel showed highest activity in nitric oxide scavenging and reducing power assay while sour orange peel demonstrated maximum action against superoxide anion and ion chelating activity. Whereas in DPPH and LPO inhibition assay, peels of citron and orange showed highest activity. On the other hand, orange pulp exhibited superior antioxidant potential in all the assays studied with the exception of reducing power and superoxide anion scavenging, in which pomello and citron pulp gave the best results, respectively. Further, the results are corroborative to the quantitative assays, namely, total phenolic, flavonoids, ascorbic acid, and total antioxidants. The data therefore suggest that the extracts of *Citrus* are a potential source of natural antioxidants.

Nowadays, there is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress, and there is incessant interest in the protective biochemical functions of natural antioxidants contained in spices, herbs, and medicinal plants [44]. Natural antioxidants, particularly in fruits, can be phenolic compounds (tannins, flavonoids, phenolic acids, and tocopherols), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid [2,49]. The antioxidant activity of phenolics is mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators [30,50]. In the present study, antioxidant activity mainly attributed to the presence of Vitamin C and phenolics. Flavonoids are most important natural phenolics. These possess broad-spectrum chemical and biological properties including radical scavenging properties [51]. Most

polyphenols, especially flavonoids, are very effective scavengers of hydroxyl and peroxyl radical [52]. The correlation between total phenol contents and antioxidant activity has been widely studied in different foodstuffs such as fruit and vegetables [53-56]. The study of Ghafar *et al.* has shown a direct relation between antioxidant activity of *Citrus* species and phenolic contents. Ascorbic acid is highly bioavailable and is the most important water-soluble antioxidant vitamin in cells, effectively scavenging ROS. When relating the antioxidant activities of fruit juices to health and disease risk, it is important to consider the contribution of ascorbic acid in addition to that of phenolic compounds with antioxidant activity [57].

In addition to the prominent role of phenolics, the presence of other phytoconstituents, namely, alkaloids, sterols, glycosides, R-tocopherol, β-carotene, and reduced glutathione may also play a crucial role [58]. The presence of these phytochemicals has been recently considered of crucial nutritional importance in the prevention of chronic diseases such as cancer, cardiovascular disease, and diabetes [59].

Thus, the present study documents that all the five extracts of *Citrus* fruits commonly used in South India for various purpose either in pulp or peel form possess powerful antioxidant capacity, wherein the latter was found to be distinctly superior for the parameters assessed and are therefore potential sources of bioactive compounds and can be considered as antioxidant constituents for developing functional foods and for allied benefits.

CONCLUSION

In the present study, the results proved that the highest activity was shown by the *Citrus* peel extracts in most of the assays performed than that of the pulp extracts. The broad range of activity of the extracts suggests that multiple mechanisms mediated by the phytoconstituents are responsible for the antioxidant activity. The study, therefore, suggests that the *Citrus* fruits possess potent antioxidant activity, which might be helpful in preventing or slowing the progress of various oxidative stress-related diseases.

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