

In Vitro free Radical Quenching Effect of Essential Oil of *Citrus limetta* Risso Peel

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ABSTRACT: Antioxidants which are obtained from natural sources have been proven safe and effective for the treatment and prevention of several human diseases. This study aimed to investigate the free radical quenching activities of essential oils extracted from the peel of *Citrus limetta* Risso. The fresh peels of the fruit were subjected to hydro-distillation (modified Clevenger apparatus) to obtain essential oil. The antioxidant activities of the oil was determined by evaluating their scavenging activities against 1,1-diphenyl-2-picryl hydroxyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS⁺) cation decolorization test, hydroxyl radical, Ferric reducing antioxidant power assay (FRAP), nitric oxide scavenging assay and superoxide radical scavenging assay using different concentrations (20-100 µg/ mL). Vitamin C was taken as a positive control. The various free radical assays were concentration dependent with varying antioxidant potentials. The antioxidant activity of the essential oil extracted from the peel of *C. limetta* was similar to that of the standard Vitamin C. The present findings suggest that the essential oil of *C. limetta* having strong antioxidant potential and can be used to produce natural antioxidants.

Keywords: Essential oil, *Citrus limetta*, Clevenger Apparatus, Antioxidant, Free radicals.

1. INTRODUCTION

Antioxidants are substances which can stabilize the free radicals and prevent the cells from damage^{5,6}. Free radicals involved in the molecular alterations which are associated with various degenerative human diseases. Plants contain many chemical compounds which act as free radical scavengers, reducing agents and quenchers. Essential oils are obtained from plant material such as flowers, buds, seeds, leaves, twigs, peels, bark, herbs, wood, fruits and roots¹. They are obtained from vegetable raw material chiefly used for the fragrance and flavors and also used as pharmaceutical components, nutritious supplements and aromatherapy^{2,3,4}. Citrus fruits are one of the important horticultural crops, used as aroma flavor in many food products, including alcoholic and nonalcoholic beverages, marmalades, gelatins, sweets, soft drinks, ice creams, dairy products, candies and cakes^{5,6}. The Citrus genus is a rich source of vitamin C and contains various components such as flavonoids and terpenoids have been recognized as safe to consume by the Food and Drug Administration (FDA)⁷. The sweet lime (*Citrus limetta*) is biologically considered as a hybrid or mutation of the lemon (*Citrus limon*). The fruits of *C. limetta* has shown anti-inflammatory, anti-hyperglycemic and anti-hypertensive and anti-thrombolytic activity^{8,9,10}. The fruit peels are also having anti-inflammatory activity when compared with phenylbutazone, hence it is used for the treatment of many inflammatory diseases¹¹. The aim of the present study was to evaluate *in vitro* free radical scavenging activity of the essential oil of *C. limetta* peels.

2. MATERIALS AND METHODS

2.1 Collection and oil extraction

Fully ripened fresh fruits (*C. limetta*) were obtained from Coimbatore, India. The plant was authenticated in Botanical Survey of India (No.BSI/SRC/5/23/2017/Tech/3406), Tamil Nadu Agriculture University (TNAU), Coimbatore, Tamil Nadu, India. The fresh peels of *C. limetta* were subjected to water distillation for 3 hrs using Clevenger apparatus. The obtained essential oil was dried over anhydrous sodium sulphate and preserved in a sealed vial at 4°C for further analysis.

2.2 ANTIOXIDANT ACTIVITY

2.2.1 DPPH Radical Scavenging assay

Radical scavenging assay using DPPH is done by the method of Blois¹². A solution of DPPH in methanol (24µg/ml) was prepared and 2 ml of this solution was added to the essential oil in methanol in different concentration (20-100 µg/ml). Initially 100 µl of oil was dissolved in 1 ml of methanol. The test tubes were incubated at room temperature for 30 minutes. Then, the absorbance was measured at 517 nm. Vitamin C used as standard antioxidant. Radical scavenging activity was calculated by % inhibition.

2.2.2 ABTS⁺ Radical Scavenging assay

ABTS⁺ Radical activity was estimated by Re *et al*¹³. The stock solution contains 7 mM ABTS solution and 2.4 mM potassium persulfate solution. Prepared the working solution by mixing two stock solutions in equal quantities and kept in dark for 12 hrs at room temperature. The working solution then diluted by mixing 1 ml ABTS solution with 60 ml methanol. 1 ml of diluted ABTS added to the essential oil at different concentrations. After 7 mins incubation the absorbance was read at 734 nm. Vitamin C used as standard antioxidant. Radical scavenging activity was calculated by percentage inhibition.

2.2.3 Hydroxyl radical scavenging assay

Hydroxyl radical activity was estimated by Rajeshwar *et al*¹⁴. A reaction mixture of 3.0ml volume contained, 1.0 ml of 1.5 mM FeSO₄, 0.7 ml of 6 mM hydrogen peroxide, 0.3ml of 20 mM sodium salicylate and 1.0 ml of different concentrations (20-100µg/ml) of essential oil. After incubation for an hour at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. Vitamin C was used as a positive control. Radical Scavenging activity was calculated by percentage inhibition.

2.2.4 Nitric Oxide scavenging assay

Nitric Oxide scavenging activity was determined by Sreejayan and Rao method¹⁵. In this experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of essential oil (20-100µg/ml) and incubated at room temperature for 150 min. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H₃PO₄ and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Vitamin C was used as standard antioxidant. Radical Scavenging activity was calculated by percentage inhibition.

2.2.5 Ferric Reducing Antioxidant Power (FRAP) assay

Reducing power antioxidant activity is estimated by Benzie and Strain method¹⁶. The stock solution of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM FeCl₃, 6H₂O and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It were freshly prepared and warmed to 37°C. 900 µl FRAP reagent were mixed with 90 µl water and 30 µl test sample as well as standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 min and the amount of Fe²⁺ complex can be monitored by measuring the formation of intense blue at 700 nm. Vitamin C was used as standard antioxidant.

2.2.6 Superoxide Radical Scavenging assay

Superoxide Radical Scavenging assay was determined by Liu¹⁷. This method is based on the inhibition of production of nitroblue tetrazolium of the superoxide ion by the sample. The test tubes containing various concentration of essential oil, 0.2 ml of EDTA, 0.1 ml of nitroblue tetrazolium, 0.05 ml of riboflavin and 2.25 ml of phosphate buffer and control tubes were set up without the extract. Similarly the activity of the standard antioxidants was also carried out. After 30 mins incubation the absorbance was read at 560nm. The percentage inhibition was calculated by comparing with the optical density of the control tubes.

3. RESULTS AND DISCUSSION

3.1 DPPH radical scavenging

DPPH is a stable and organic free radical used to evaluate the scavenging activity of antioxidants from various natural products. It is more sensitive to detect active ingredients at low concentrations. The hydrogen donating capacity of the antioxidant, scavenge the DPPH radical scavenging and renovate DPPH radical into their non-radical form 1,1-diphenyl-2-picrylhydrazine¹⁸. DPPH radical scavenging activity of *C. limetta* essential and the standard antioxidant Vitamin C were shown in Figure 1. The highest scavenging activity of *C. limetta* and vitamin C was found to be 75.9% and 78.1 % respectively at the concentration 100 µg/ml with IC₅₀ value 26.32 and 59.38 µg/ml. DPPH was reduced when increasing the concentration of essential oil associated with a lower IC₅₀ value. This result indicates that the essential oil of *C. limetta* is having good quenching capacity. Our results highly correlated with previous study of Mahmud, indicated that essential peel of *Citrus acida* var. (sour lime) showed good DPPH scavenging effect in a dose dependent manner when compared with ascorbic acid as a reference standard¹⁹. Other studies of Sacchetti, also reported that essential oils of *Cananga odorata*, *Cymbopogon citratus*, *Rosmarinus officinalis* and *Curcuma longa* notably reduced the concentration of DPPH free radical in a dose dependent manner²⁰.

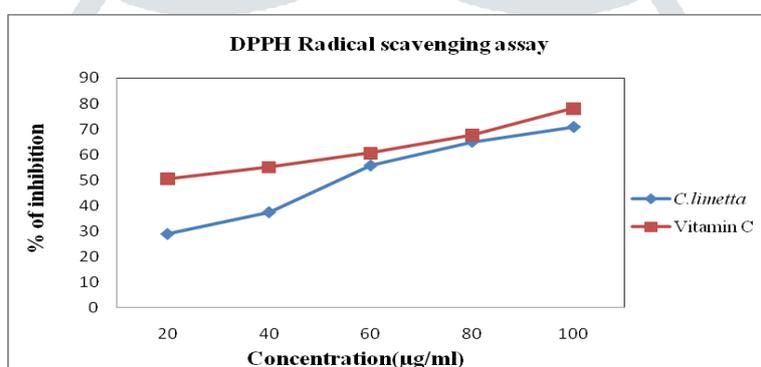


Figure 1: DPPH radical scavenging assay

3.2 ABTS⁺ Radical Scavenging assay

ABTS⁺ radical is a protonated radical has characteristic absorbance maxima at 734 nm which decreases at the scavenging of protons known as excellent substrate for peroxidases commonly used to evaluate antioxidant property of natural compounds. It is a decolonization assay in which the ABTS reacts with potassium per sulphate and generates the ABTS chromophore²¹. ABTS⁺ radical scavenging activity of *C. limetta* and standard antioxidant vitamin C were shown in figure 2. The reduction capability of the ABTS⁺ radical is determined by its decreased absorbance at 734 nm. The highest scavenging activity of *C. limetta* was found to be 55.8% and Vitamin C was found to be 63.1 % with IC₅₀ value 97.28 and 79.78 at concentration 100 µg/ml. The obtained results clearly indicated that the essential oil of *C. limetta* scavenge the radicals in a dose dependent manner. Free radical quenching ability of antioxidants towards ABTS⁺ has been reported earlier by S-Y. Li and S-P. Li²², who worked on the *in vitro* properties of essential oil extracted from *Curcuma longa* and *Curcuma wenyujin*.

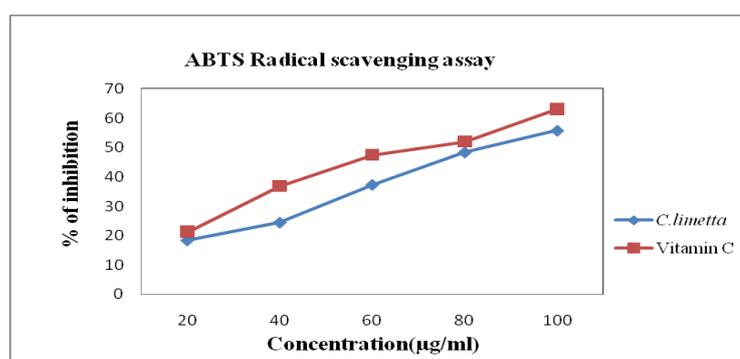


Figure 2: ABTS⁺ radical scavenging assay

3.3 Hydroxyl radical scavenging assay

Hydroxyl radical is one of the important reactive oxygen species. It can able to damage macromolecules including nucleic acids, proteins, lipids and carbohydrates resulting in genomic instability. In the presence of ferrous ions the hydroxyl radicals are produced from H_2O_2 by means of Fenton reaction. The iron homeostasis is maintained by various mechanisms in order to prevent the generation of toxic hydroxyl radicals. Hence this free radical is considered as one of the significant contributor to oxidative stress^{23, 24}. The Hydroxyl radical scavenging activity of *C. limetta* and standard antioxidant vitamin C were shown in figure 3. The essential oil exhibits hydroxyl radical scavenging capacity in a dose dependent manner. The percentage inhibition of *C. limetta* was found to be 60.3 % and for vitamin C 72.4%. The IC_{50} value of *C. limetta* was found to be 93 ± 0.42 at 100 $\mu\text{g/ml}$ concentration. This could be the presence of some phenolic compounds. The antioxidative effectiveness in natural sources was reported to be mostly due to phenolic compounds²⁵. Other studies also reported that essential oils of *Cananga odorata*, *Cymbopogon citratus*, *Rosmarinus officinalis* and *Curcuma longa* able to quench the hydroxyl radical in a concentration dependent manner indicating their strong antioxidant activities²⁶.

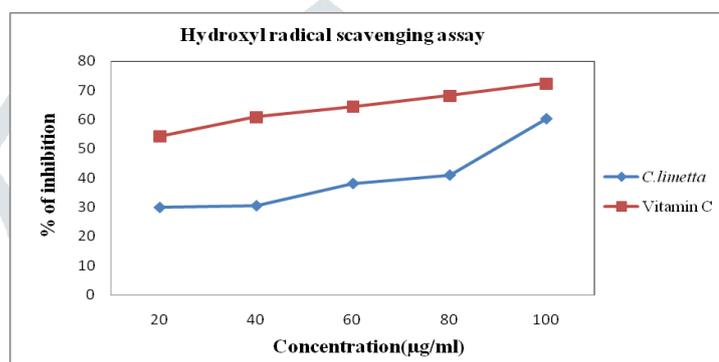


Figure 3: Hydroxyl radical scavenging assay

3.4 Nitric oxide radical scavenging assay

Nitric oxide is a short lived free radical that plays an important role in a variety of physiological processes. It is synthesized by nitric oxide synthase (NOS) enzymes. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside on buffered saline which reacts with oxygen to produce nitrite molecules which is measured by Griess reagent²⁷. Under normal physiological conditions, nitric oxide generates in the cells which contribute to regulation of anti-inflammatory and antioxidant properties. During inflammatory processes, the immune system produces both the superoxide anion and radical and nitric oxide²⁸. Nitric oxide radical scavenging activity of *C. limetta* and standard antioxidant vitamin C were shown in figure 4. The percentage inhibition of *C. limetta* found to be 68.9% with IC_{50} value 63 ± 0.14 at 100 $\mu\text{g/ml}$ concentration when compared with the standard antioxidant vitamin C (71%) with the IC_{50} value 35 ± 0.54 $\mu\text{g/ml}$. Similar results obtained in the studies of Sacchetti, who reported that the essential oil from *Cananga odorata*, *Cymbopogon citratus* showed good quenching ability on nitric oxide when compared to the standard ascorbic acid in a concentration dependent manner²⁹.

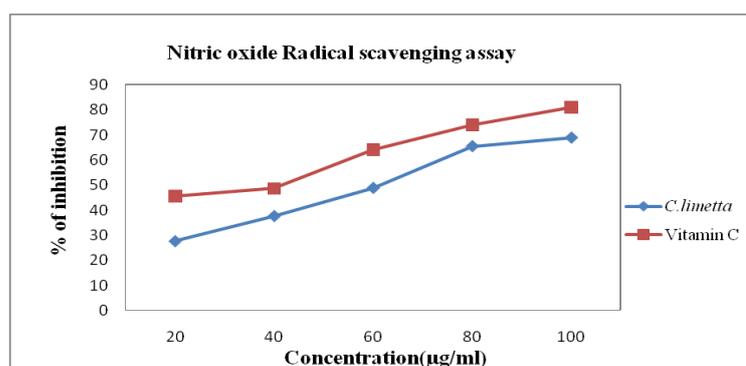


Figure 4: Nitric oxide radical scavenging assay

3.5 Ferric Reducing Antioxidant Power (FRAP) assay

In ferric reducing antioxidant power non- enzymatic antioxidant reacts with prooxidants and inactive them. In this assay the oxidant Fe (III) TPTZ complex converted into Fe (II) TPTZ³⁰. In the FRAP assay the hydrogen donating capacity of the essential oil *C. limetta* and standard antioxidant vitamin C were shown in figure 5. The hydrogen donating ability was found to be 42.57 µg/ml for *C. limetta* and 69.32 µg/ml for standard antioxidant vitamin C. The reducing power indicates that the antioxidant compounds are electron donors and reduce the oxidized intermediate of lipid peroxidation processes hence they can also act as primary and secondary antioxidants. In the previous studies Ganiyu, reported that the essential oil of lemon peel had a dose dependent ferric reducing ability with IC₅₀ value of 133.5 µl/L showed similar result with our present study³¹.

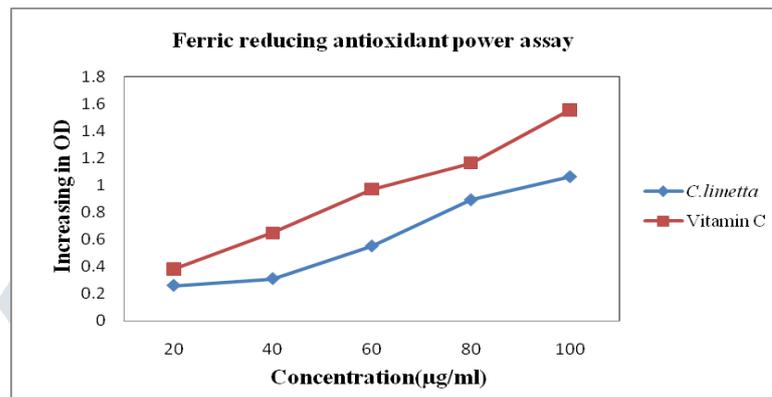


Figure 5: Ferric reducing antioxidant power assay

3.6 Superoxide Radical Scavenging assay

Superoxide is one of the proximal reactive species that is produced by the mitochondrial respiratory chain. It is a weak oxidant but it can generate more dangerous species such as singlet oxygen which could damage the tissues³². In this method, superoxide anion is derived from dissolved oxygen by riboflavin-methionine illuminate reaction and reduces the yellow dye to blue formazan which is measured at 560nm³³. Superoxide radical scavenging activity of *C. limetta* and standard antioxidant vitamin C were shown in figure 6. The percentage inhibition of *C. limetta* was found to be 75.9% with IC₅₀ value 60 at 100 µg/ml concentrations when compared with the standard antioxidant vitamin C (81.8%) with IC₅₀ value 37±0.19 µg/ml. this results shows that the essential oil of *C. limetta* posses antioxidant activity. Our findings similar to the previous studies of Kim, who reported that the essential oil extracted from *Citrus obovoides* and *Citrus natsudaidai* posses strong antioxidant capacity against superoxide radicals³⁴.

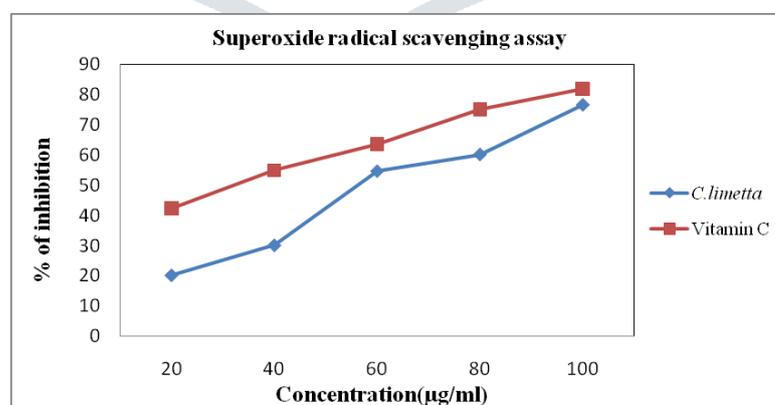


Figure 6: Superoxide radical scavenging assay

4. CONCLUSION

Plants contain natural antioxidants which are the important source for the drug development. *C. limetta* is a well known citrus species commonly called as Mosambi. All parts of the plant such as peel, flower, and fruit juice are used as the traditional medicine. Literature available does not give the sufficient information about the toxicity and adverse effects of any part of the plant. Ascorbic acid is a natural antioxidant which may inhibit the development of major clinical conditions including heart diseases and certain cancers. From the above results we have concluded that the essential oil of *C. limetta* peel contribute to the prevention of oxidation as antioxidants and free radical scavengers. This might be due to the presence of secondary metabolites presented in the essential oil of *C. limetta* peel.

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