Volume 3 (4), 2015, Page-810-816
CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

Antioxidant and Free Radical Scavenging Activity of *Citrus Medica*

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ARTICLE INFO

Received: 18 July 2015
Accepted: 11 Aug 2015

ABSTRACT

A research was conducted for antioxidant and free radical scavenging activity of extracts of *Citrus Medica*. Total phenol content, DPPH and Nitric oxide radical scavenging effect were chosen as experimental study. The extracts were collected from root, peel and pulp. Preliminary photochemical investigation showed presence of alkaloid, carbohydrate, Glycoside, Triterpenoids, Resins and Tannins. During the study IC50 Value calculated for DPPH and Nitric Oxide radical scavenging activity. Results showed a significant value of antioxidant activity from extracts. The broad range of activity of the extracts suggests that, multiple mechanisms are responsible for the antioxidant activity

Keywords: *Citrus Medica*, Phenol Content, DPPH, Nitric Oxide, Scavenging.

1. INTRODUCTION

Plants have always played a major role in the treatment of human and animal diseases. Medicinal plants can be used as raw materials for extraction of active compounds or for extraction of abundant but inactive constituents which can be transformed by partial synthesis into active compounds; can be used as such or as extracts or as traditional preparations. World-wide interest in the use of medicinal and aromatic plants is increasing. Ethno botanical information is leading to the discovery of novel phytopharmaceutical and other. India has a very long, safe and continuous

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usage of many herbal drugs in the officially recognized alternative systems of health viz., Ayurveda, Unani, Siddha and Homoeopathy. [1, 2] These systems have rightfully existed side-by-side with Allopathy and are not 'in the domain of obscurity. Citrus fruits are among the most important horticultural products enjoying universally as articles of diet. They are one of the most important fruits in India and are in great demand as dessert fruits and also as preserves in the form of squash, cordial, marmalade or pickle. Citrus fruits can be eaten fresh like the sweet orange, mandarins, pummelo and the grapefruit or their segments may be canned. The rind of small malta is used to flavour a sweet dessert while that of citron is candied to make a sweet meat called '*turanj ka murabba*' in some parts of North India. They are rich in vitamin C, minerals and possess distinct flavours. Citrus fruits and juices have long been recognized to contain secondary metabolites including antioxidants such as ascorbic acid, flavanones, phenolics and pectin that are important to human nutrition. Limonoids are secondary metabolites present in all citrus tissues whose role in human nutrition has not been established. A recent study showed the inhibition of human cancer cell proliferation, induction of apoptosis of human breast cancer cells by citrus limonoids. In certain species of citrus, flavonoids are found as methoxylated flavones and glycoside flavones and they being used as indicators. [3]

2. MATERIALS AND METHODS

The plant sample for authentication was collected from Locally. The sample was identified by Dr. Tariq Husain, Head Biodiversity and Angiosperm Taxonomy, National Botanical Research Institute (NBRI), Lucknow, India as *Citrus medica* L. of family Rutaceae with accession no. 97840. The parts of *Citrus medica* Linn. selected for present study were root, stem bark, leaves, flowers, fruit, fruit peel and seeds. The different parts of the plant were collected when they were expected to have maximum amount of active constituents. All samples were collected locally from Mangalagiri, Guntur.

2.1 Preparation of Plant Extract

Extracts of bark and pulp were prepared by soaking 100 gm of powdered samples in 500 ml of 70% ethanol while 250 gm of root, leaf and peel samples were soaked in 500 ml of 70% ethanol for 72 h. Each mixture was stirred every 24 h using sterile glass rod. At the end of extraction each extract was passed through Whatman filter paper no. 1 (Whatman, UK). The filtrates were concentrated on a rotary evaporator under vacuum at 45ºC, dried and weighed. Percentage yield of crude extract of each sample was determined in terms of g/100 g of sample. Dried extracts were stored at 4-6ºC until used and marked as RE (root), LE (leaf), BE(bark), PE(peel) and PUE(pulp) for ethanolic extract of root, leaf, bark, peel and pulp, respectively.[4, 5]

2.2 Preliminary Phytochemical Investigation of Plant

Selected extracts were screened for the presence of various groups of compounds as mentioned below.[6-8]

2.2.1 Detection of alkaloids

Small portion of the solvent free extract was stirred with few drops of dilute HCl and filtered. The filtrate was then tested for following tests:

a) Mayer's test: Few drops of Mayer's reagent were added with 2-3 ml of filtrate. It gives a cream coloured precipitate.

b) Dragendorff's test: Few drops of Dragendorff's reagent were added with 2-3 ml of filtrate. It gives an orange brown precipitate.

c) Hager's Test: Few drops of Hager's reagent were added with 2-3 ml of filtrate. It gives yellow precipitate.

d) Wagner's test: Few drops of Wagner's reagent were added with 2-3 ml of extract. It gives a reddish brown precipitate.

2.2.2 Detection of carbohydrates

Small quantity of the extract was dissolved in distilled water and filtered. The filtrate was then subjected to Molisch's test (general test); Benedict's and Fehling's test for reducing sugars.

a) Molisch's test: 2-3 ml of aqueous extract was mixed with few drops of α- naphthol solution in alcohol. It was shaken and concentrate H2SO4 was added from the side of the test tube. It shows a violet ring at the junction of two liquids.

b) Benedict's test: 2 ml of Benedict's reagent was mixed with 2 ml of test solution in a test tube. The mixture was heated in boiling water bath for 5 min. The solution appeared green, yellow or red depending on the amount of reducing sugar present in test solution.

c) Fehling's test: 1 ml of Fehling's A and 1 ml of Fehling's B solution were mixed, then boiled for 1 min. Equal volume of test solution was added and heated in boiling water bath for 5-10 min. Appearance of first yellow and then brick red precipitate indicates the presence of reducing sugars.

2.2.3 Detection of glycosides

Small portion of the extract was hydrolysed with dilute HCl for few hours on water bath and was subjected to Legal's test and Keller-Kiliani test for cardiac glycosides; and Borntrager's test for anthraquinone glycosides.

a) Legal's test (for Cardenolides): 1 ml pyridine and 1 ml sodium nitroprusside was mixed with 2-3 ml of the extract. A pink colour appears.

b) Keller-Kiliani test (for deoxysugars): Glacial acetic acid, one drop of 5% FeCl3 solution and concentrate H2SO4 were added to 2-3 ml of the extract. Reddish brown color appears at the junction of two layers and upper layer turns bluish green.

c) Borntrager's test: Dilute H2SO4 was added to 3 ml of extract, boiled and filtered. To the cold filtrate, equal volume of chloroform was added, shaked well and organic layer was separated. On addition of ammonia, ammoniacal layer turns pink.

d) Modified Borntrager's test for C-glycosides: To 5 ml extract, 5 ml of FeCl3 (5%) and 5 ml dilute HCl were added and heated in boiling water bath for 5 minutes. Benzene was added in cooled solution and organic layer was separated after shaking. Equal volume of dilute ammonia was added in organic layer. Ammoniacal layer shows pinkish red colour.

e) Test for coumarin glycosides: Alcoholic extract when made alkaline, shows blue or green fluorescence.

2.2.4 Detection of triterpenoids and sterols

Triterpenes and sterols were detected by performing following tests:

a) Liebermann-Burchard test: 2 ml of the extract was added with chloroform and mixed, to this mixture 1-2 ml acetic anhydride was added and 2 drops of concentrate H2SO4 was added from the side of test tube. First red, then blue and finally green color appears.

b) To the chloroform extract acetic anhydride was added. Then concentrate H2SO4 was added from the side wall of test tube, upper layer turns green and indicates presence of steroids.

2.3 Antioxidant Activity

a) Chemicals

1, 1-Diphenyl-2-picryl hydrazyl (DPPH) (HiMedia Pvt. Ltd., Mumbai), butylatedhydroxytoluene (BHT) (SRL, Mumbai), Folin-Ciocaleteau reagent (CDH, New Delhi), ascorbic acid (SRL, Mumbai), potassium ferricyanide (Loba Chemie, India), sodium nitroprusside (CDH, New Delhi), pyrocatechol (Loba Chemie, India), methanol (Loba Chemie, India),

trichloroacetic acid (Loba Chemie, India), sulphanilamide (Loba Chemie, India), naphthylethylenediamine dihydrochloride (CDH, New Delhi) were used in this study. Spectral measurements were done by using UV-visible spectrophotometer (UV- 601, Shimadzu Scientific Instruments).

b) Plant material and preparation of extract

The powdered root, peel, pulp and juice were used to evaluate their antioxidant potential. Ethanolic extracts of root, peel and pulp were used in this.

c) Determination of total phenolics

The concentration of total phenolic compounds in the extracts was determined as modified by Negi and Jayaprakasha (2003) and the results were expressed as Nainital pyrocatechol equivalents. The dried extracts of root, peel and pulp were dissolved in a mixture of methanol and water (6:4 v/v). Different concentrations (10, 25, 50, 75, and 100 µg) of standard pyrocatechol and one concentration (100 µg) of extract/ juice were taken in separate test tubes and the volume was adjusted to 0.2 ml by addition of distilled water. One milliliter of 10-fold diluted Folin-Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate solution were added to all the tubes. After 30 min incubation at room temperature the absorbance was measured at 765 nm using a UV-visible spectrophotometer. All experiments were performed in triplicate and the results were averaged. Total phenolic compounds in the extracts and juice was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph. [9] The equation is given below;

Absorbance = 0.253 × pyrocatechol (µg) + 1.835

2.3.1 Radical scavenging activity using DPPH method

The free radical scavenging activity of extracts was measured by 1, 1-diphenyl-2- picryl hydrazyl (DPPH) method. Used as a reagent, DPPH evidently offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants. Various concentrations (50, 75, 100, 200, and 300 µg/ml) of juice and ascorbic acid and dried extracts of root, peel and pulp were prepared in ethanol (70%) in different test tubes. Volume of the samples and ascorbic acid were adjusted to 3 ml by adding ethanol (70%). Methanolic solution of DPPH (100 µM or 0.1 mM) was prepared and 1 ml of this solution was added to each test tube, shaken vigorously and were allowed to stand at 27ºC for 30 min. A control was prepared as described above without samples or standard. A mixture of 3 ml ethanol (70%) and 1 ml methanol was used for the baseline correction. Absorbance was measured at 517 nm. All the tests were performed in triplicate and the results averaged. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity

Radical scavenging activity was expressed as the inhibition percentage and was calculated by using the formula. [10]

% Radical scavenging activity = [(A *control* – A *sample* / A *control*)] × 100

Where, A*control* is the absorbance of the control and A*sample* is the absorbance of the test compound. The antioxidant activity was also expressed as IC50. The IC50 value was defined as the concentration (µg/ml) of extract that inhibits the formation of DPPH radicals by 50%.

2.3.2 Nitric oxide radical scavenging effect

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by the Griess Reagent. The reaction mixture (3 ml) containing 2 ml of sodium nitroprusside (5 mM) in phosphate buffered saline and 1 ml of different concentrations (50, 75, 100, 200 and 300 µg/ml) of extract in DMSO (Dimethyl Sulphoxide). The reaction mixture was incubated at 25ºC for 30 min. After 30

min, 1.5 ml of incubated sample was taken and 1.5 ml of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylene diamine was measured at 546 nm. A control was taken without test compound or standard. Ascorbic acid was taken as a reference antioxidant. All the tests were performed in triplicate and the results averaged. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test samples using following formula. [11, 12]

% Radical scavenging activity = [(A *control* – A *sample* / A *control*)] × 100

Where, A *control* is the absorbance of the control, and A *sample* is the absorbance of the test compound. The IC50 values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression equation, prepared from the concentration of the samples and percentage inhibition of free radicals.

3. RESULTS AND DISCUSSION

3.1 Various Phytochemical constituent as mentioned in table 1.

Table 1: Phytochemical Constituent

S.No	Chemical compounds	ChE (F)	EE (F)	EtOH (root)	EE (peel)	EE (pulp)	Fruit deco
1.	Alkaloids	+	+	+	+	+	+
2.	Carbohydrate	+	+	+	+	+	+
3.	Glycosides (G)	+	+	+	-	-	-
4.	Triterpenoids &steroidsSteroids	+	+	+	-	-	-
5	Resins	+	+	+	NA	NA	NA
6	Tannins	+	+	+	+	+	+
7	Protien & amino acid	-	-	-	+	+	-

3.2 Total Phenolic Content

Generally, hexane is used for the extraction of non-polar compounds like fatty material or some carotenoids, while ethyl acetate for carotenoids and some phenolics. The other solvents are used for the extraction of polar compounds like aglycones and glucosides of flavonoids and limonoids depending upon their polarity. Ethanol is reported to extract tannins, polyphenols, polyacetylenes, flavonol, terpenoids, sterols and alkaloids; so used in this study for extraction of phytoconstituents because polyphenols and flavonoids are having potent antioxidant activity. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. Some of the studies reported correlations between total phenolics and radical scavenging activity of citron fruit extracts. Table 2 shows the yield and phenolic content of each extract.

Table 2: Percentage yield and phenolic content of *C. medica* L. extracts.

Extracts	Yield (g/100 g of Crude drug)	Phenolics as pyrocatechol equivalents (µg/mg of extract)
Root	6.25	725.37
Peel	1.28	98.81
Pulp	29.55	390.74
Juice	24.72	428.26

3.3 DPPH radical scavenging activity

The free radical scavenging potentials of extracts from citron and ascorbic acid at different concentrations were tested by DPPH method and the results are depicted in Figures 5.2-5.6. Antioxidants react with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm and convert to 1, 1-diphenyl-2- picryl hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extracts. The results showed that all the extracts of *C. medica* L. have reduced the DPPH radical in a concentration.

At 300 µg/ml conc, extracts and ascorbic acid exhibited 87.10% (root), 88.28% (juice), 20.55% (pulp), 88.28% (peel) and 98.05% (ascorbic acid) free radical scavenging activity. IC50 values were found to be 87.10 µg/ml (root), 88.28 µg/ml (juice), 20.55 µg/ml (pulp) and 88.28 µg/ml (peel) for different extracts (Table 3). Results indicated that among all tested extracts pulp extract was found highly effective having IC50 value 18.25 µg/ml.

3.4 Nitric oxide radical scavenging activity

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess NO to generate nitrite and peroxynitrite anions, which act as free radicals. In the present study, possibly the extracts compete with oxygen to react with NO and thus inhibits generation of the anions. Figures illustrate the percentage inhibition of NO generation by *C. medica* L. extracts. At 300 µg/ml concentration percentage inhibition were found to be 78.93% (root), 76.28% (juice), 78.48% (pulp), 79.42% (peel) for extracts and 95.38% for ascorbic acid. The IC50 values of extracts were found to be 155.37, 134.89, 178.87 and 146.84

Table 3: IC50 Value (µg/ml) for DPPH and NO Methods

Extracts	IC50 Value (µg/ml)	
	DPPH Methods	NO Methods
Root	87.10	155.37
Peel	88.28	134.89
Pulp	20.55	178.87
Juice	27.65	146.84

4. CONCLUSION

It has been shown that some antioxidant assay methods give different antioxidant activity trends. Therefore, the present antioxidant activity was determined by three different methods. Based on our results, the highest activity was shown by the pulp extract by DPPH method, fruit juice followed by the pulp extract by nitric oxide method and the peel extract by reductive ability method. Phenolic compounds and ascorbic acid were identified as possible antioxidants in orange juice, a citrus fruit. Phenolic compounds were able to scavenge radicals and to chelate metals while ascorbic acid can play a pro-oxidant role in the presence of transition metals. The results of present study demonstrate that *C. medica* L. extracts can effectively scavenge various free radicals under *in vitro* conditions. The antioxidant activity may be due to the presence of phenolic compounds and/or vitamin C present in the samples. The broad range of activity of the extracts suggests that multiple mechanisms are responsible for the antioxidant activity.

5. ACKNOWLEDGEMENT

Authors thanks to the faculty and Principal of Nirmala College of Pharmacy, Mangalagiri for encouraging and providing chemicals to carryout research work.

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Conflict of Interest: None

Source of Funding Nil