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Comparative Evaluation of Extracts of *Citrus limon* Burm Peel for Antioxidant Activity

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ABSTRACT

Cellular damage arising from free radicals is one of the fundamental mechanisms underlying a number of human neurodegenerative disorders like diabetes, inflammation, Alzheimer's disease, autoimmune pathologies, and digestive system disorders. Thus, antioxidants play an important role in the treatment of such diseases. Natural sources of antioxidants, free of carcinogenicity unlike synthetic ones, are being tapped for antioxidant formulations. The present study aims at a comparative evaluation of hexane, alcoholic, and aqueous extracts of fresh lemon peel for antioxidant activity. All extracts were subjected to phenolic content estimation by the Folin-Ciocalteau method and flavonoid content estimation by the aluminium chloride colorimetric method. Results revealed that the alcoholic extract had the maximaml content of both phenolics and flavanoids. Antioxidant activity was evaluated by using the beta-carotene bleaching method, the nitric oxide radical scavenging and the hydrogen peroxide scavenging assays. The alcoholic extract was found to have good free radical inhibitory property as well as nitric oxide radical scavenging activity. The hexane extract, however, showed only good hydrogen peroxide scavenging activity. Thus, the higher antioxidant activity of the alcoholic and hexane extracts of *Citrus limon* peel could have wide therapeutic utility against various diseases.

Key words: Antioxidant activity, *Citrus limon*, flavonoids, phenolics

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INTRODUCTION

Antioxidants are molecules that can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition of these free radical species. They act in low concentrations relative to the oxidizable substrate and protect other chemicals of the body from damaging oxidation reactions by reacting with free radicals, mainly reactive oxygen species (ROS), the most dangerous byproduct of the aerobic environment. During these reactions, as the beneficial antioxidant molecule sacrifices itself by becoming oxidized, there is a constant need to replenish antioxidant sources, either endogenously or through supplementation. There are two basic categories of antioxidants: Synthetic and natural. The use of synthetic antioxidants is being restricted because of their carcinogenicity. Hence, herbs which have been reported to possess antioxidant properties, are being used for antioxidant formulations.[1]

Phenols and polyphenols including flavanoids in wine, fruits, and vegetables have been reported to exhibit a wide range of biological activities and their effects are mainly attributed to antioxidant properties that prevent free radical-mediated cytotoxicity, lipid peroxidation, and oxidation of low-density lipoproteins. The ability of these compounds to scavenge ROS is well documented.

In addition, they are reported to be nontoxic and nonmutagenic. Among the fruits, citrus fruits are the most common dietary sources of phenolics, flavanones, and flavones. Thus, extracts from these fruits could prove to be beneficial for the prevention and treatment of many neurodegenerative disorders.[2] In the present work, we intend to evaluate various extracts of fresh peels of *Citrus limon* for their antioxidant property.

MATERIALS AND METHODS

Plant source

Fresh lemon fruits used in the present study were collected from the local market at Ettumanoor, Kottayam (dist) and authentified by the botanist, Mr. Joby Paul, Department of Environmental sciences, M.G University as *Citrus limon* Burm. The voucher specimens are deposited in the herbarium of the Dept. of Pharmaceutical Sciences, M.G. University, Kottayam. Lemon fruits were peeled off and the fresh peels were subjected to extraction for further studies.

Apparatus and chemicals

Our study involved the use of a UV-VIS spectrophotometer (Shimadzu UV-1601 UV-VIS spectrophotometer). All reagents used were of analytical grade. Gallic acid (Nice Chemical Private Ltd, New Delhi), rutin and ascorbic acid (Loba Chemie Private Ltd, Mumbai), propyl gallate (Merck Speciality Private Ltd, Mumbai), beta-carotene and linoleic acid (Research Lab Fine Chem. Industries, Mumbai) and quercetin (Chemika-Biochemika-reagents, Mumbai) were procured and used for the studies.

Extraction

Extraction of lemon peel was carried out by using solvents of increasing polarity: *n*-hexane, alcohol, and water. Extraction of lemon peel with *n*-hexane was carried out by using a simple maceration technique: 500 g of *C. limon* peel (80 fruits) was taken, size-reduced, and extracted with 2 L of *n*-hexane in an amber-colored bottle with intermittent shaking for five days. After five days, the extract was decanted off and allowed to evaporate at room temperature in an evaporating dish. The residue obtained was weighed and kept closed in a desiccator for further studies. Extraction of 125 g of lemon peel marc (left after *n*-hexane extraction) using alcohol was carried out by a hot continuous extraction method in a Soxhlet apparatus. Extraction was carried out for nine hours with the lemon peel marc packed in the extractor and 300 mL of alcohol in a round bottom flask. The extract obtained was collected and concentrated by gentle heating. The concentrated extract was then weighed and stored in the desiccator for further studies. Aqueous extraction was carried out by the reflux method with the marc left over after Soxhlet extraction using 50 g of the marc packed in a round bottom flask and refluxed for two hours. The extract obtained was then concentrated to a dry residue by heating and the dry residue obtained was weighed and used for further studies.[3,4]

Total phenolic estimation

Total phenolic estimation was carried out by using the standard Folin-Ciocalteau method.[5] The extracts and the standard, gallic acid, were dissolved in methanol separately for the total phenolic estimation. One milliliter of each extract was mixed with 5 mL of Folin-Ciocalteau reagent, and 4 mL of sodium carbonate solution added after 5 min and kept at room temperature for two hours. Absorbance values were measured at 750 nm and a standard curve was prepared using gallic acid. The absorbance values obtained for the extracts were interpreted from the standard curve to get the total phenolic content expressed as milligram equivalents of gallic acid.

Total flavonoid content

Estimation of flavonoids was carried out by the aluminium chloride colorimetric method.[6] The extracts and the standard, rutin, were dissolved in methanol separately for the total flavonoid estimation. To each extract (1 mL), 4 mL of water followed by 0.3 mL of sodium nitrate were added. After 5 min, 0.3 mL of 10% aluminium chloride solution was added and at the 6th minute, 2 mL of 1 M sodium hydroxide were added. After proper mixing, absorbance was measured at 510 nm and a standard curve was prepared using rutin. The absorbance values obtained for the extracts were interpreted from the standard curve to get the total flavonoid content expressed as milligram equivalents of rutin.

In vitro antioxidant activity

Beta-carotene bleaching method

The mechanism of bleaching of β-carotene is a free radical-mediated phenomenon, resulting from the hydroperoxides formed from linoleic acid. In this reaction, β-carotene undergoes rapid discoloration due to the attack of free radicals formed upon abstraction of a hydrogen atom from the diallylic methylene group of linoleic acid. Absorption at 470 nm is consequently decreased. The presence of an antioxidant in the reaction mixture hinders the rate of bleaching by neutralizing free radicals formed in the system during incubation at 50ºC.[3]

The antioxidant activity of extracts and the standard were assessed on the basis of free radical scavenging using the beta-carotene bleaching model.[3] Both the sample solutions (extracts) and the standard solution (propylgallate) were prepared in methanol and 0.2 mL of each solution was placed in different test tubes. To each test tube, 5 mL of the β-carotene emulsion was transferred and gently shaken before being placed at 45°C in a water bath for 60 min. The absorbance of the samples, the standard, and the control were measured at 470 nm against a blank consisting of an emulsion without β-carotene. The measurements were carried out initially (t = 0) and at 30 and 60 min. All samples were assayed in triplicate and averaged; the antioxidant activity was measured using the formulae:

$$AA = 1 - \frac{A_o - A_t}{A^{\circ}_o - A^{\circ}_o} \times 100$$

Where, A_o and A°_t: Are the absorbance values measured at the initial incubation time for sample/standard and control respectively

At and A°_t: Are the absorbance values at time t (t = 30 min and t = 60 min) for the sample/standard and control respectively.

Nitric oxide radical scavenging assay

NO is an important bioregulatory molecule that has a number of physiological effects, including control of blood pressure, neural signal transduction, platelet function, as well as antimicrobial and antitumor activities. Low concentrations of NO are sufficient, in most cases, to effect these beneficial functions. However, during infections and inflammations, the formation of NO is elevated and may bring about some undesired deleterious effects. NO does not interact with bioorganic macromolecules such as DNA or proteins directly. However, the NO molecule is very unstable in aerobic conditions and reacts with oxygen to produce intermediates such as NO_2, N_2O_4 and N_3O_4. The stable products of these reactions, nitrate, nitrite, and peroxynitrite, react with superoxide and these products are highly genotoxic. The deamination of guanine, cytosine, and adenine is mediated primarily by N_2O_3. In addition to the formation of nitrosoamines and deamination of the DNA bases, NO may also act by affecting the enzymatic activities of several thiol-rich DNA repair proteins like DNA alkyl transferase, formamopyrimidine-DNA glycosalase, and also carcinogenesis in general. As excess generation of NO is deleterious to human health, extracts should be screened for their capacity to scavenge nitric oxide *in vitro*.[7]

The antioxidant activity of extracts and the standard were assessed on the basis of their nitric oxide scavenging ability.[8] The standard, quercetin, and the extracts were prepared in phosphate-buffered saline at 50, 100, 150 and 200 μg/mL concentrations. The assay reaction mixture (3 mL) was prepared by mixing 2 mL of 10 mM sodium nitroprusside solution, 0.5 mL of phosphate-buffered saline, and 0.5 mL of the sample or standard solution. These were then incubated at 25°C for 2.5 hours. After incubation, 0.5 mal of the reaction mixture was pipetted out and mixed with 1 mL of sulphanilic acid reagent (final concentration of 0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for complete diazotization. Then, 1 mL of 1-naphthylamine solution (5%) was added, mixed, and allowed to stand for 30 min to form pink chromophores. The absorbance was then measured at 540 nm against the corresponding blank solution. All samples were prepared and assayed in triplicate and averaged. The antioxidant activity was measured using the formulae:

$$\%\,Inhibition = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c- is the absorbance of the control; A_s- is the absorbance of sample/standard.

Hydrogen peroxide scavenging assay

Hydrogen peroxide is the most stable ROS and may be generated directly by divalent reduction of O_2 or indirectly by univalent reduction of O_2 by numerous oxidases, such as xanthine oxidase, uricase, and α-hydroxy acid oxidase localized in the peroxisome. H_2O_2 is decomposed to H_2O and O which can induce cell injury and cause DNA damage in the form of chromosomal aberrations rather than superoxide ions.[9]

The antioxidant activity of the extracts and the standard were assessed on the basis of their hydrogen peroxide scavenging ability.[9] The standard, ascorbic acid, and the extract were prepared in phosphate buffer, pH 7.4. Sample and standard solutions (0.5 mL) were taken in different test tubes and to each test tube, 0.6 mL hydrogen peroxide solution (2 mM hydrogen peroxide in phosphate buffer, pH 7.4) was added. A control was prepared by replacing the sample/standard with phosphate buffer. These solutions were then kept at room temperature for ten minutes. The absorbance was measured at 230 nm against the blank solution containing phosphate buffer without hydrogen peroxide. All samples were prepared and assayed in triplicate and averaged. The antioxidant activity was measured using the formulae:

$$\% \text{Inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c- is the absorbance of the control; A_s-is the absorbance of sample/standard; 50% Inhibitory Concentration (IC50).

The concentration (μg/mL) of the various extracts required to scavenge 50% of the radicals was obtained for both the nitric oxide radical-scavenging assay and hydrogen peroxide scavenging assay by extrapolating their graphs of concentration *vs* % scavenging ability.

Data and statistical analysis

Data obtained for extracts were statistically analyzed with the standard using two-way ANOVA in GraphPad® IV software.

RESULTS

Yield

The alcoholic extract was found to have the highest yield, followed by the aqueous extract, and finally the hexane extract [Table 1].

Estimations

The total phenolic and flavonoid content of the alcoholic extract was found to be the highest, followed by the hexane extract and finally, the aqueous extract [Table 1].

Antioxidant study

Antioxidant studies by the beta-carotene bleaching method showed that all the extracts were comparable to the standard drug, propylgallate, with the alcoholic extract having the best activity [Table 2]. The % inhibition obtained in the presence of different concentrations of the extracts (Hexane extract (HE), Alcoholic extract (AE), Aqueous extract (AQE) and Standard (STD)) were calculated and depicted in Figure 1 and the IC_{50} values calculated. Antioxidant studies by the nitric oxide radical scavenging assay showed that all the extracts have IC_{50} values comparable to that of the standard, with the alcoholic extract having the best activity [Table 3]. The % inhibition of hydrogen peroxide obtained in the presence of different concentrations of the extracts (Hexane extract (HE), Alcoholic extract (AE), Aqueous extract (AQE) and Standard (STD)) were calculated and depicted in Figure 2 and the IC_{50} values calculated. Antioxidant studies by using the hydrogen peroxide scavenging assay showed that all the extracts have IC_{50} values comparable to that of the standard, with the hexane extract having the best activity [Table 3]. The isolation of therapeutically active constituent(s) from these extracts will probably give better antioxidant activity than even the standard at lower concentrations.

The percentage inhibition obtained for each extract was statistically compared with that of the standard and found to have $P < 0.001$ significance.

Table 1: Comparative evaluation of extracts for its phenolic and flavonoid content

Extracts	Percentage yield (%)	Total phenolic content* (μg/ mL)	Total flavonoid content* (μg/mL)
Hexane extract	0.4	35.00	27.5
Alcoholic extract	5.87	51.25	38.75
Aqueousextract	2.8	13.75	6.25

* Average of three determinations

DISCUSSION

Citrus fruits are considered to be common, rich, dietary sources of phenolics and are hence, considered to be beneficial for many neurodegenerative disorders. The present study estimated the phenolic and flavonoid contents of extracts of *C. limon* peels and evaluated their antioxidant activity. The alcoholic extract was found to have a good yield for both phenolics and flavonoids compared to the hexane and aqueous extracts. Phenols and polyphenols, including flavanoids in wine, fruits, and vegetables, have been reported to exhibit a wide range of biological activities, and their effects are mainly attributed to their antioxidant property that prevents free radical-mediated cytotoxicity, lipid peroxidation, and oxidation of low-density lipoproteins.

Table 2: Comparative evaluation of extracts of *Citrus limon* burm peel for antioxidant activity using the beta-carotene bleaching model

Extracts	Absorbance (nm) at			% antioxidant activity* at	
	0 time	30th min	60th min	30th min	60th min
Control	0.143±0.002	0.128±0.0026	0.119±0.001		
Hexane extract	0.4±0.0017	0.395±0.0034	0.391±0.0028	66.67	62.5
Alcoholicextract	0.295±0.001	0.291±0.001	0.288±0.0026	73.34	70.84
Aqueous extract	0.397±0.002	0.390±0.002	0.385±0.0036	53.34	50
Standard drug	0.205±0.0026	0.202±0.0026	0.200±0.001	80.4	79.17

* Average of three determinations; standard drug: Propylgallate

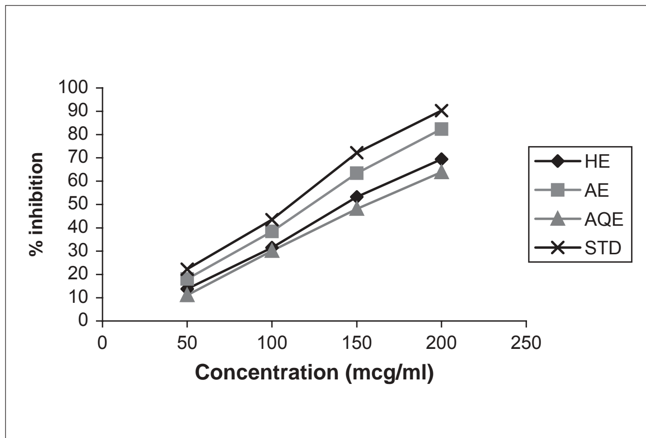

Figure 1: Calculation of IC_{50} for the extracts of *C. limon* peel and the standard drug (quercetin) using the Nitric oxide scavenging model; HE: Hexane extract etc

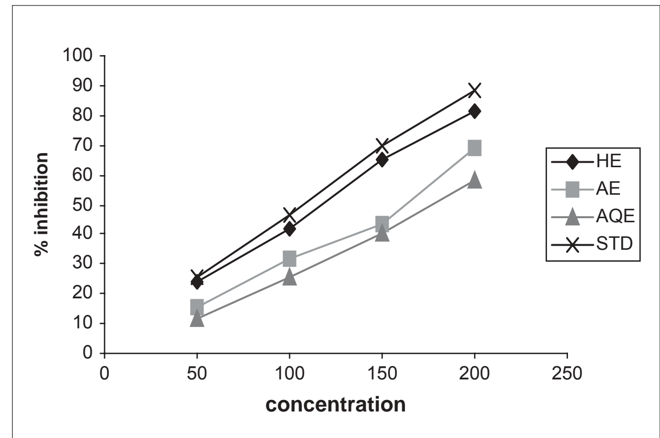

Figure 2: Calculation of IC_{50} for the extracts of *C. limon* peel and the standard drug (ascorbic acid) using the Hydrogen peroxide-scavenging model; HE: Hexane extract etc

Table 3: Comparative evaluation of extracts of *Citrus limon* Burm peel for antioxidant activity using the Nitric oxide radical-scavenging and hydrogen peroxide-scavenging models

Extracts	IC_{50} values	
	Nitric oxide-scavenging assay* (µg/mL)	Hydrogen peroxide-scavenging assay* (µg/mL)
Hexane extract	142.5	117.5
Alcoholic extract	127.5	165
Aqueous extract	157.5	180
Quercetin	112.5	
Tocopherol		110

* Average of three determinations

Our antioxidant studies revealed that the alcoholic extract with the higher concentrations of phenolics and flavonoids did have significant free radical-scavenging and nitric oxide radical-scavenging activities. The hexane extract that was rich in citrus terpenoids, was found to have significant hydrogen peroxide-scavenging activity.

CONCLUSION

The alcoholic and hexane extracts of *Citrus limon* peel showed significant antioxidant activity in all the three antioxidant models when compared to the respective standards. Further phytochemical studies can be done for the isolation of compound(s) from these alcoholic and hexane extracts, which could have wide therapeutic utility against various diseases.

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