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Chemical Constituents of *Carissa opaca* Extracts and Their Evaluation as Antioxidants and Preservatives in Edible Oils

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Carissa opaca (Stapf ex Haines) leaves were extracted using Soxhlet apparatus with petroleum ether (COE-PE), chloroform (COE-CH), ethyl acetate (COE-EA), acetone (COE-AC), methanol (COE-ME), *n*-butanol (COE-BU) and water (COE-WA). Antioxidant potential of *Carissa opaca* extracts (COEs) was evaluated by four methods: ferric thiocyanate, thiobarbituric acid, scavenging of DPPH$^{\bullet}$ and total antioxidant capacity along with determination of total phenolics and antioxidant potential of COEs in peanut and sunflower oils. Results revealed that COEs had significant antioxidant potential (inhibition of lipid peroxidation > 50 %) and DPPH· scavenging potential (scavenging > 80 %). Moreover, total phenolic content was also higher in the extracts: COE-AC = 940 and COE-BU = 600 mg of gallic acid equivalents (GAE). Application of COEs as natural antioxidants in peanut and sunflower oils significantly reduced lipid peroxidation (inhibition > 60 %). A strong correlation of total phenols was observed with DPPH$^{\bullet}$ and total antioxidant capacity assays (R^2 = 0.9776 and R^2 = 0.8104, respectively).

Key Words: Constituents, *Carissa opaca*, Peanut oil, Sunflower oil, Antioxidant activity.

INTRODUCTION

Carissa (family: *Apocynaceae*, subfamily: *Plumerioideae*) is mostly referred to a genus of about 20-30 species of shrubs or small trees native to tropical and subtropical regions of Africa, Australia and Asia. Only two species: *C. carandas* and *C. opaca* are found in Pakistan[1]. Pickled fruits from *Carissa* are widely used for flavouring sweet dishes throughout the subcontinent. Fruits and leaves of *Carissa opaca* are cardiac and stimulant[2]. Medicinally they are used for curing fever and eye disorders while fruit of the plant mixed with roots of *Mimosa pudica* is taken as aphrodisiac[3]. Previously carissone, cardiac glycosides, caffeic acid, fatty acids and tannins from *C. opaca* were reported[4-7] while alcoholic extract was found to lower the blood pressure in cats[8].

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Lipid peroxidation is the oxidative deterioration of lipids containing any number of carbon-carbon double bonds. Oxygen-dependent deterioration of lipids may be initiated by different agents such as heat, light or metal ions. Lipid hydroperoxides which are primary products of peroxidation of unsaturated fatty acid moieties gradually decompose to secondary products such as aldehydes, alcohols, carboxylic acids, epoxides and hydrocarbons[9] which are purportedly associated with carcinogenesis, mutagenesis and ageing[10,11]. Therefore, it is obvious that the prevention of lipid peroxidation in food is required not only for the stability of the nutritional content but also the extension of the best before date. In order to discover natural antioxidants which offer significant advantage over synthetic ones, much attention has been paid to them because they are expected to prevent food and living systems from peroxidative damage. Number of synthetic antioxidants such as BHA, BHT and TBHQ, have been added to foodstuffs[12]. Although these synthetic antioxidants are efficient and relatively cheap, there are some disadvantages, because they are suspected of having some toxic properties. Therefore, search for natural antioxidants has received much attention and efforts have been made to identify natural compounds that can act as suitable antioxidants to replace synthetic ones[13]. Plant constituents have become an important source of active natural products which differ widely in terms of structure and biological properties. In recent years, the prevention of many disorders such as cancer and cardiovascular diseases has been found associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants[14-16]. The antioxidant potential of plant products is due to the presence of several components which have distinct mechanisms of action; some are enzymes and proteins and others are low molecular weight compounds such as vitamins[17,18], carotenoids[19], flavonoids/anthocyanins[20] and other phenolic compounds[21].

The aim of the present study is to determine possible use of the extracts of *Carissa opaca* as natural antioxidants and identificaiton of their major chemical constituent classes.

EXPERIMENTAL

Carissa opaca (Stapf ex Haines) leaves were collected from district Kotli, by the mid of June 2007. The plant was identified at Department of Botany and a voucher specimen (GCU-Bot-123-07) was submitted at Dr. Sultan Ahmad Herbarium, GC University, Lahore.

Plant material and extracts: The dried powdered leaves (200 g) were extracted in soxhlet extractor successively for 12 h each with petroleum ether, chloroform, ethyl acetate, acetone, methanol, *n*-butanol and water to yield COE-PE (8.5 g), COE-CH (4.5 g), COE-EA (3.5 g), COE-AC (3.0 g), COE-ME (5.5 g), COE-BU (4.0 g) and COE-WA (6.5 g), respectively.

UV-Vis spectroscopic studies: UV-Visible spectral studies of COEs in methanol (0.2 mg/mL) were carried out in the range 200 nm to 700 nm (Cecil CE-7200, England) to evaluate major class/es of phytochemicals present in the extract.

Ferric thiocyanate (FTC) assay: The antioxidant activity of COEs on inhibition of linoleic acid peroxidation was assayed by thiocyanate method[22]. 0.1 mL of each of COEs solutions (0.5 mg/mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated for 7 days at 40 ºC. The mixture without extract was used as control. 0.1 mL of the mixture was taken and mixed with 5.0 mL of 75 % ethanol, 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5 % HCl and allowed to stand at room temperature. Precisely 3 min after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as percentage inhibition of peroxidation (IP %) [IP % = {1-(abs. of sample)/(abs. of control)} × 100]. The antioxidant activities of BHT, gallic acid and α-tocopherol were also assayed for comparison as reference standards.

Thiobarbituric acid (TBA) method: TBA reactive species (carbonyl compounds) were determined by the method of Onay-Uncar *et al.*[23]. 2.0 mL of 20 % trichloroacetic acid and 1.0 mL of 0.67 % 2-thiobarbituric acid were added to 1.0 mL of incubated sample solution (above sample solution, prepared for FTC method). The mixture was placed in boiling water bath for 10 min and centrifuged at 3000 rpm for 10 min after cooling. Absorbance of supernatant was measured at 532 nm.

Scavenging activity on DPPH free radical: Radical scavenging activity of COEs was established by measuring the decrease in absorbance of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical[23]. Equal volume (2 mL) of 10^{-4} M DPPH (in MeOH) and COEs (0.2 mg/mL) or reference solution [0.2 mg/mL, gallic acid and BHT] were mixed and incubated at 30 °C for 15 min in dark. Control solution containing equal volume of DPPH and MeOH was used as blank (A_{blank}). Absorbance of COEs solutions at 517 nm was also measured to avoid the inaccurate data (excess values) caused from the absorbing materials without antioxidant activity, which can exist in extracts. Actual absorbance (A_{sample}) originated from the inhibition of DPPH (A_{sample}) was evaluated by subtracting the absorbance of methanolic extracts (or reference compounds), from absorbance of corresponding DPPH extracts (or reference compound) at 517 nm. Decrease in absorbance indicated the antioxidant activity. Radical scavenging activity was expressed as percentage inhibition of DPPH and estimated by the following formula.

$$\% \text{ Inhibition of DPPH} = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Total antioxidant capacity assay (TAOC): Total antioxidant activity of COEs was evaluated according to the method of Prieto *et al.*[24]. 0.1 mL of each sample (0.5 mg/mL) was added to 1.9 mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate). The blank solution contained 2.0 mL reagent solution only. The mixtures were incubated at 95 ºC for 150 min. After the mixture had cooled to room temperature, absorbance was measured at 695 nm. The

antioxidant activity was expressed as the absorbance of the sample. The antioxidant activities of BHT and gallic acid were also assayed for comparison as standards.

Determination of total phenolic content (TP): Total phenolics of COEs were determined by the method of Makkar *et al.*[25]. 0.1 mL of the sample (0.5 mg/mL) was combined with 2.8 mL of 10 % Na_2CO_3 and 0.1 mL of 2 N Folin-Ciocalteu reagent. After 40 min absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenols were determined as milligrams of gallic acid equivalents (GAE) per gram of extract by computing with standard calibration curve constructed for different concentrations of gallic acid.

Antioxidant potential of COEs in peanut and sunflower oil: The oxidative deterioration of samples was studied using sachaal oven test method[26]. 5.0 mg of the extract was added to 50 mL each of peanut and sunflower oils, homogenized and incubated in open 100 mL beakers at 60 ± 5 ºC for one week. Butylated hydroxy toluene was used as a reference for comparison. A blank sample was also prepared under the same conditions. The rate of auto oxidation of the oils was estimated according to the increase of 2-thiobarbituric acid-reactive substances (TBARS) using the classical TBA procedure as described above (TBA method). The TBARS values of untreated and treated samples were used to calculate the inhibition of lipid oxidation [% inhibition = {(control-treated)/control × 100}].

Correlation of antioxidant assays: To evaluate the compatibility and reliability of the methods used to determine the antioxidant potential of the seven extracts of *C. opaca*, the authors performed linear regression and correlation analysis of the results obtained by these methods. The coefficients of determination (R^2) were calculated.

Statistical analysis: All the determinations were done in triplicate. The correlation coefficients (R^2) were determined by Microsoft excel 2003.

RESULTS AND DISCUSSION

UV-Visible spectrum of petroleum ether extract (COE-PE) exhibited a single peak at 215 nm which is proposed to be related with diterpenes[27]. The COE-CH demonstrated two peaks at 209 and 315 nm which suggested the presence of derivatives of coumarin and a third peak at 401 nm indicating the presence of aurone type flavonoids[27]. COE-EA (268 nm, 324 nm), COE-ME (278 nm, 326 nm) and COE-BU (280 nm, 326 nm) exhibited two absorption bands in the range of 240-285 nm and 300-400 nm indicating the presence of flavonoids with flavonol and dihydroflavonol structure. COE-AC showed strong absorption at 280 nm indicating the presence of hydrolysable tannins[28].

Ferric thiocyanate method has been used to determine oxidative deterioration (hydroperoxides) of linoleic acid. Among all the extracts petroleum ether extract (COE-PE) exhibited the highest IP % (55 %) and was found more effective than α-tocopherol (53 %). COE-CH (47.81 %), COE-EA (42.5 %), COE-ME (41.2 %) and COE-BU (37.3 %) also showed significant results. The inhibitory effects of COEs on peroxidation of linoleic acid emulsion are shown in Fig. 1.

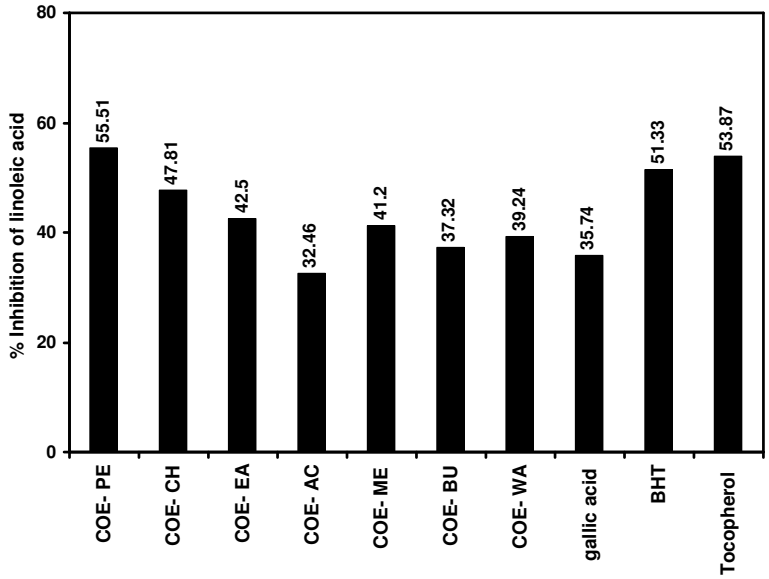

Fig. 1. Inhibition of linoleic acid peroxidation by COEs, BHT, gallic acid and α-tocopherol, using FTC method

Determination of TBARS (carbonyl compounds) was carried out by measuring the absorbance of malonaldehyde on the 7th day of linoleic acid incubation (Fig. 2). The results suggested that this process ran almost parallel to the formation of conjugated dienes in FTC method. The antioxidant activity of 0.05 % sample of petroleum ether was higher than the standard α-tocopherol at the same concentration. None of the COEs showed absorbance higher than the negative control.

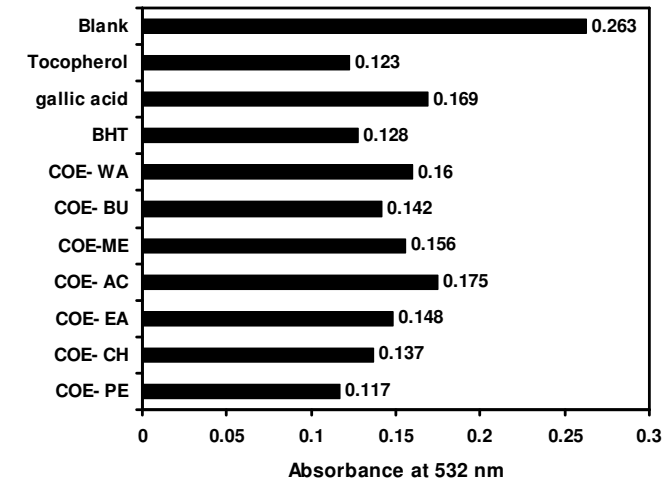

Fig. 2. Antioxidant activities of COEs determined by TBA method

The DPPH• assay is based on the measurement of reducing ability of antioxidants towards DPPH• which can be determined by decrease in absorbance. A flat concentration assay was carried out with COEs and the results are presented in Fig. 3. These results provide a direct comparison of the antioxidant activities of COEs with BHT and gallic acid. The acetone extract(COE-AC) demonstrated the highest % scavenging (90.6 %) followed by COE-BU (78.8 %) and COE-EA (71.0 %), while COE-PE and COE-CH were almost inactive with 1.5 and 12.3 % scavenging of DPPH radical, respectively.

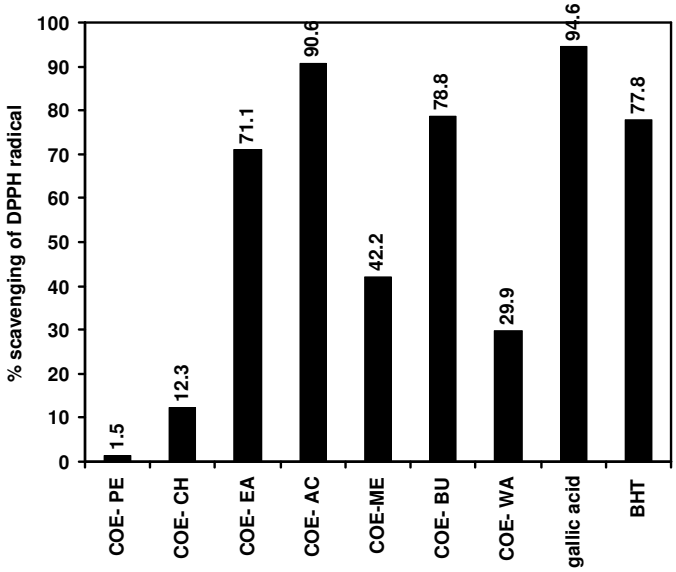

Fig. 3. DPPH free radical scavenging activity of COEs, gallic acid and BHT

TAOC assay determines the reducing ability of the antioxidants through the generation of green phosphate/Mo(V) complex formed by the reduction of Mo(VI) to Mo(V). COE-AC exhibited the highest absorbance; 0.53 at a concentration of 0.5 mg/mL comparable with the standard gallic acid (absorbance = 0.73 at 0.5 mg/mL). The total antioxidant activity was measured and compared with that of BHT, gallic acid and control. The high absorbance values indicated that the samples COE-EA, COE-AC and COE-BU possessed significant antioxidant activity (Fig. 4).

In order to assess the extent of oxidation that occurs in biological systems, the amount of 2-thiobarbituric acid-reactive substances (TBARS) is often measured[29]. Percentage inhibition of lipid peroxidation of peanut and sunflower oils is shown in Figs. 5 and 6, respectively. Oxidation levels decreased in all oil samples in the presence of COEs and standards. COE-PE, COE-CH and COE-BU which were more active in FTC & TBA methods, exhibited higher % inhibition in both the oils. Over all rate of peroxidation of peanut oil was found slower than sunflower oil, it seems to be due to the lower content of unsaturated fatty acids in peanut oil[30].

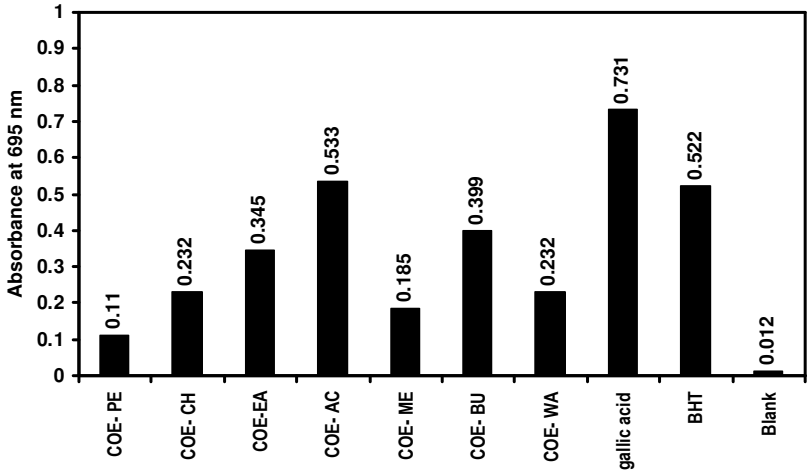

Fig. 4. Total antioxidant activity of COEs, BHT and gallic acid

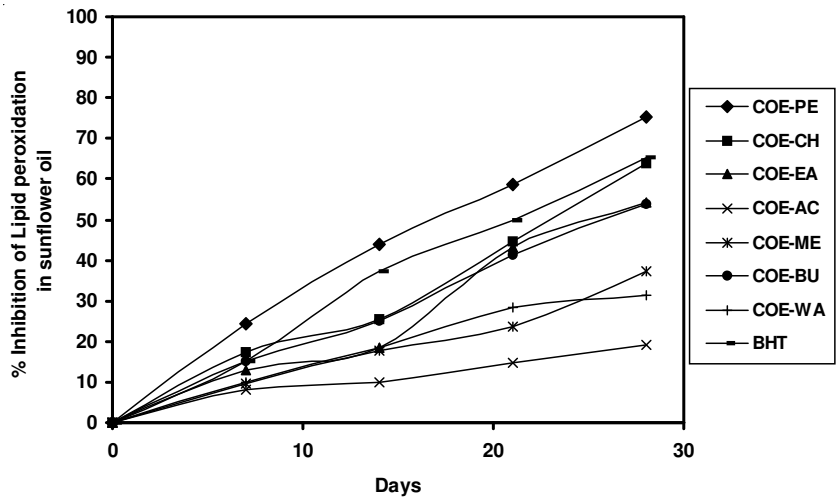

Fig. 5. Lipid peroxidation inhibited by COEs in sunflower oil

A correlation study of all the six experiments suggested that FTC and TBA methods have strong correlation ($R^2 = 0.8801$), in the same way results of DPPH assay and total antioxidant activity by phosphomolybdenum method showed good correlation ($R^2 = 0.7899$). A strong correlation was also found between total phenols, DPPH assay and total antioxidant capacity assay with $R^2 = 0.9776$ and $R^2 = 0.8104$, respectively. Relationship of results of FTC assay with results of DPPH assay ($R^2 = 0.4225$) and total antioxidant assay ($R^2 = 0.2686$) showed poor correlation among themselves. This stands also true for the correlation of results of TBA assay with results of DPPH assay ($R^2 = 0.3045$) and total antioxidant assay ($R^2 = 0.2487$).

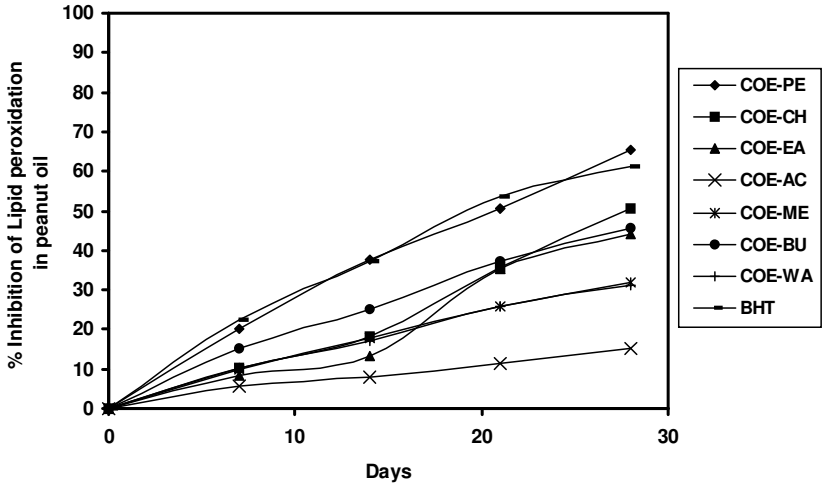

Fig. 6. Lipid peroxidation inhibited by COEs in peanut oil

Conclusion

In present studies, the correlation in between the antioxidant assays and the type of chemical constituents in different extracts of *C. opaca* are reported. The results of antioxidant capacity of plant extracts determined by different methods depend upon the mechanism of reaction in the selected method. Usually hydrogen atom transfer (HAT) mechanism operates to quench the peroxyl radical in lipid peroxidation in foods and biological systems. Many antioxidants that react quickly with peroxyl radical may remain inert towards DPPH, either due to stability of DPPH radical or steric inaccessibility. Therefore, it can be suggested that the antioxidant efficiency towards stable radicals depends upon the structure and redox potential of the compound under study. There are several reports on the reducing ability of polyphenols which indictates the free radical scavenging capacity of these compounds[31]. This study indicated that polyphenols are responsible in the acetone (COE-AC) and *n*-butanol (COE-BU) extracts of *C. opaca* for strong radical scavenging ability in DPPH and phosphomolybdenum assays. The two absorption bands in the range of 240-285 and 300-400 nm in the UV-Visible spectra were also in agreement with the presence of flavonoids in ethyl acetate, methanol and *n*-butanol extracts of *C. opaca*[27]. On the other hand, a different trend was observed in FTC and TBA methods. The experimental results led to the conclusion that petroleum ether extracts of *C. opaca* which showed highest antioxidant activity in FTC and TBA methods contain different type of antioxidants. UV-Vis spectrometry of petroleum ether extract (COE-PE) in methanol suggested the presence of diterpenens with a single absorption band at 215 nm[27]. Phytochemical analysis of COE-PE with ceric sulphate reagent was also in agreement with the presence of terpenoids[32]. The suitability of COEs as antioxidant was also determined in peanut and sunflower oils. COEs treatment significantly reduced lipid oxidation in both the oils, compared to

the control. In case of COE-PE and COE-AC it was found almost equal to that of standards. Thus this study gives a strong impact for expanding the investigations of natural antioxidants for use in health cares and food industry.

Even though the antioxidant activity of the compounds present in different extracts was strong, the overall antioxidant effect could be higher by the combined and synergistic effects of further purified/isolated individual compounds. Therefore isolation and identification of individual active compounds, their *in vivo* antioxidant activities as well as different antioxidant mechanisms *in vitro* are still needed.

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