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Article

Study of anti-diabetic, beta-carotene-bleaching inhibiting and iron chelating properties of *Carissa opaca* root extracts

Dildar Ahmed*, Wajeeha Malik, Maria Maqsood, Iqra Atique, Muhammad Tariq Qamar

Department of Chemistry, Forman Christian College (A Chartered University), Lahore, Pakistan

Degenerative diseases diabetes and oxidative stress constitute a major health concern worldwide. Medicinal plants are expected to provide effective and affordable remedies. The present research explored antidiabetic and antioxidant potential of extracts of *Carissa opaca* roots. Methanolic extract (ME) was prepared through maceration. Its fractions were obtained, sequentially, in hexane, chloroform, ethyl acetate and *n*-butanol. An aqueous decoction (AD) of the finely ground roots was obtained by boiling in distilled water. The leftover biomass with methanol was boiled in water to obtain biomass aqueous decoction (BAD). The extracts and fractions showed considerable porcine pancreatic α-amylase inhibitory activity with IC_{50} in the range of 5.38-7.12 mg/mL while acarbose had 0.31 mg/mL. The iron chelating activity in terms of EC_{50} was 0.2939, 0.3429, 0.1876, and 0.1099 mg/mL for AD, BAD, ME, and EDTA, respectively. The EC_{50} of beta-carotene bleaching activity for AD, BAD, ME, and standard BHA were 4.10, 4.71, 3.48, and 2.79 mg/mL, respectively. The total phenolic content (TPC) and total flavonoid content (TFC) of AD and BAD were also considerable. In general, ethyl acetate fraction proved to be the most potent. Thus, the *C. opaca* roots had excellent antioxidant activity while having moderate α-amylase inhibitory potential.

Keywords: *Carissa opaca*. Antidiabetic. α-Amylase. Oxidative stress. β-Carotene. Iron chelating.

INTRODUCTION

Deteriorating diseases including diabetes mellitus, cancer, cardiovascular disorders, ageing and skin wrinkles can be due to excessive release of ROS (reactive oxygen species) in the body (Halliwell, Gutteridge, 2015). Beside other activities, plant extracts have compounds that show antioxidant, anti-cancer, antidiabetic and anti-aging potential and can also retard the ageing, wrinkle formation and inhibit hyperpigmentation (Kim, Uyama, 2005). Studies revealed that the oxidative stress interferes in diabetes pathogenesis by the altering enzymatic systems, lipid peroxidation, decreasing Glutathione metabolism and reduced levels of vitamin C. Numerous biomarkers of oxidative stress in diabetes mellitus are lipids, proteins, DNA damage, glutathione, catalane and superoxide dismutase (Asmat, Abad, Ismail, 2016).

Presently, diabetes mellitus has become a major health issue. By 2035, about 592 million people would be diabetic patients, which will be 210 million higher than reported in 2013 (Shahid *et al*., 2016). Every year, the number of people affected by this lifelong health problem is increasing. More effective, safer and affordable disease controlling approaches could be plant based α-amylase inhibitors (Tysoe *et al*., 2016). α-Amylase inhibitors can also obstruct obesity, which is another growing global health concern. Diabetes can be controlled by reducing hydrolysis of diet starch and thus decreasing the production and then absorption of glucose into the body (Tucci, Boyland, Halford, 2010).

***Correspondence:** D. Ahmed, Department of Chemistry, Forman Christian College, (A Chartered University), Lahore, Pakistan. Email: dildarahmed@fccollege.edu.pk

Carissa opaca, which belongs to family Apocynaceae, is a wild shrubby plant found in the Himalayan region (Ahmed *et al*., 2014; Kaunda, Zhang, 2017). It is known for its rich traditional medicinal applications (Sahreen, Khan, Khan, 2010). Many studies have recently been carried out on different parts of the plant. Fruits, seeds and leaves have been found to contain good amounts of organic nutrients and minerals (Ahmed *et al*., 2011). Antioxidant, anti-microbial, anti-cancer and many other pharmacological activities of *C. opaca* have been investigated using a variety of conditions (Izhar, Ahmed, 2016). The chemical substances isolated from the plant include terpenoids, flavonoids, tannins, coumarins, and glycosides (Sahreen, Khan, Khan, 2011; Saklani *et al*., 2012). Roots of the plant are known for healing wounds and injuries (Abbasi *et al*., 2010). They have been found to contain various flavonoids, terpenoids, sitosterols, vitamin E and a host of other phytochemicals (Izhar, Ahmed, 2016). Our group has been working on the roots for the past several years. The roots of the shrub exhibited antioxidative, antimicrobial and xanthine oxidase inhibitory potential (Ahmed *et al*., 2015; Saeed, Ahmed, 2015; Ahmed *et al*., 2016). The antioxidative properties of methanolic extract of the roots and its fractions were studied according to the total phenolic content, total flavonoid content, DPPH radical scavenging, reducing power, phosphomolybdate, ABTS, and lipid peroxidation inhibition assays (Ahmed *et al*., 2014). Alpha-amylase inhibitory activity was studied using the enzyme of bacterial origin (Saeed, Ahmed, 2015). Several chemical compounds have been isolated and identified from the extracts of *C. opaca* roots. They include lupeol, beta-sitosterol, limonene, vanillin, quercetin, rutin, vitamin E, 2-hydroxyacetophenone, 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanon, stigmasterol, campesterol, gamma-sitosterol, α-amyrin, (3β)-24-methylene-9,19-cyclolanostan-3-ol, lup-20(29)-en-3-one, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-2(1H) naphthalenone, 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone, and (3β)-lup-20(29)-en-3-ol, acetate (Saeed, Ahmed, 2015; Malik, Ahmed, Izhar, 2017).

As a continuation of our work on this plant, the present study was designed. Alpha-amylase inhibitory activity was conducted using the enzyme of porcine pancreatic origin. Antioxidant activity was evaluated according to β-carotene bleaching inhibitory and iron chelating potential. Aqueous decoction of the dried powdered roots was used for the study along with the methanolic extract and its fractions. The flavonoids and phenolics of the decoction were also evaluated. To our knowledge, such studies have not been conducted earlier. This work is necessary in order to discover new medicines for diabetes and degenerative disorders. The plant-based natural remedies are desirable because of their efficacy, safety and cost-effectiveness. In view of the fact that many chronic diseases still have no cures, continuous research for new drugs is indispensable. Since the medicinal plants constitute a rich reservoir of a vast array of molecular structures, research in the area holds immense hope for provision of safe and affordable remedies for different ailments.

MATERIAL AND METHODS

Chemicals

The solvents were of HPLC grade. Dimethyl sulfoxide (DMSO), sodium potassium tartrate, porcine pancreatic α-amylase, Folin-Ciocalteu reagent, sodium nitrite, aluminum chloride, EDTA, and anhydrous sodium carbonate were purchased from Merck (Germany). 3,5-Dinitrosalicylic acid, sodium hydroxide, Rutin, BHA (butylated hydroanisole), ferrozine, linoleic acid and β-carotene were acquired from Sigma-Aldrich (Germany). Acarbose was obtained from Daejung (Korea). Gallic acid was purchased from Scharlau (Spain). Dipotassium hydrogenphosphate and potassium dihydrogenphosphate were procured from Riedel-de Heän (Germany).

Collection of plant material

The *C. opaca* plant material was obtained from the hilly area near Abbottabad Hazara (Pakistan). The identification of the plant was done by the taxonomist Dr. Muhammad Ajaib of Government College University, Lahore, Pakistan, where a voucher specimen of the plant is deposited (GC-Herb Bot 2271). The roots were cleaned manually to remove mud and foreign particles.

Preparation of aqueous decoction (AD)

A method reported previously was used for extraction (Malik, Ahmed, Izhar, 2017). After washing

with distilled water, the roots were air dried in shade. The dried roots were crushed and ground, and the powder (50 g) so obtained was boiled in distilled water (500 mL) for 2 h to get a decoction, which was filtered using Whatman filter paper 41. The filtrate obtained this way was centrifuged (4000 rpm) for 10 min at 10 ºC, and the supernatant was collected.

Preparation of methanolic extract (ME) and its fractions

Two kilograms of the roots powder of *C. opaca* was macerated with 3 L methanol for 15 days at room temperature with frequent shaking. The extract was filtered and concentrated in vacuo to obtain ME as a gummy material and weighed. For fractionation into solvents of increasing polarity, the following procedure was used. In a separating funnel, 100 g ME was mixed in 200 mL distilled water. A suspension was obtained. It was successively fractionated into hexane, chloroform, ethyl acetate and *n*-butanol. The fractions so obtained were concentrated on a rotary evaporator.

Preparation of roots biomass aqueous decoction (BAD)

The residual biomass obtained after extraction with methanol was dried in an oven at 40 ºC. A weighed amount (19 g) of this biomass was soaked in distilled water (300 mL) and boiled for 2 h. The rest of the method was the same as used for AD.

α-Amylase inhibitory assay

For determination of porcine pancreatic α-amylase inhibitory activity, a reported method was used (Nickavar, Yousefian, 2010). The sample solutions were prepared in DMSO with serial concentrations (0.05- 0.5 mg/mL). Potato starch, employed as a substrate (0.5% w/v), was boiled in distilled water for 15 min to obtain its solution. The enzyme solution and coloring reagent DNS (3,5-dinitrosalicylic acid) solution were prepared according to the reported method.

A sample (0.5 mL) was combined with the equal volume of the enzyme solution, and placed as such for 30 min at 25 ºC. Then, 1 mL starch solution was added. After 3 min, DNS solution (1 mL) was introduced. The reaction mixture so obtained was heated at 85 ºC for 15 min. and diluted with water (9 mL). Absorbance was recorded at 540 nm. Acarbose was employed as a standard. For the blank, the method was the same except that the DNS solution was added before adding the starch solution. For negative control, the same volume of DMSO was taken instead of the sample.

The following equation was used to estimate percent enzymatic inhibitory activity of each sample:

$$\% \, Activity = \frac{A_2 \text{ x } A_1}{A_2} \text{ x } 100 \qquad (1)$$

where A_1 and A_2 are absorbance of a given sample and absorbance of negative control, respectively.

Total phenolic contents (TPC) assay

A reported procedure was employed to evaluate the total phenolic contents of AD and BAD of the roots of *C. opaca* with slight change (Ahmed *et al*., 2014). The concentration of the sample was 100 µg per 1 mL of methanol. The Folin-Ciocalteu (FC) reagent (200 µL) was combined with the 40 µL sample after diluting it with 3.16 mL distilled water. The solution was incubated at room temperature for 8 min. Sodium carbonate solution (600 µL, 20%) was added, after which the solution remained in the incubator at 40 ºC for 30 min. Absorbance was measured at 765 nm. The blank had 40 µL methanol instead of a plant sample. The total phenolic content was defined as µg/mL of gallic acid equivalents.

Total flavonoid contents (TFC) assay

The total flavonoid contents of AD and BAD were quantified according to a reported method (Baig *et al*., 2011). The samples were formulated in methanol (0.1 mg/mL). To 300 µL sample, 3.4 mL aqueous methanol (30%) was added. Then 150 µL $NaNO_2$ (0.5 M) was added. Finally, 150 µL $AlCl_3$ (0.3 M) was added. After an incubation at room temperature for 5 min, 1 mL sodium hydroxide (1 M) was combined. Absorbance was measured at 506 nm. Rutin was employed as a standard. The flavonoid content was stated as µg/mL of Rutin equivalents.

Iron chelating activity assay

The assay was conducted as per a reported procedure (Decker, Welch, 1990). The samples were made in distilled

water with serial dilutions. In a vial, 1 mL sample was combined with 100 µL $FeCl_2$ (1 mM). The solution was diluted with 3.7 mL distilled water following by the addition of 200 µL ferrozine (5 mM). It was incubated for 20 min at room temperature. Absorbance of the clear solution was measured at 562 nm. The negative control was prepared by mixing all reagents except the plant sample. EDTA was employed as a control.

The Fe^{2+}-chelating activity was estimated using the Equation 1.

β-Carotene bleaching inhibitory activity assay

Beta-carotene bleaching inhibitory ability of each of the samples was evaluated as per a reported method (Elzaawely *et al*., 2007). For the assay, β-carotene linoleate emulsion was prepared by combining a β-carotene solution in chloroform (2 mg/10 mL) with Tween-20 (200 mg) and linoleic acid (20 mg). The mixture was then allowed to stand for removal of chloroform. After adding distilled water (50 mL), the mixture was subjected to shaking, which resulted in the formation of emulsion.

The emulsion (250 µL) was combined with a sample (30 µL) in cuvette. The absorbance was determined at 470 nm at 0 time and after incubation for 2 h at 45 ºC. The β-carotene bleaching inhibitory activity of a sample was assessed as per the formula:

$$\% \, Activity = \frac{A_t}{A_0} \text{ x } 100 \quad (2)$$

where A_0 and A_t are absorbance at time zero and after 2 h, respectively. BHA (butylated hydroanisole) standard was the positive control.

Statistical analysis

The inhibitory concentration (IC_{50}) and effective concentration (EC_{50}) values were calculated from linear regression analysis of the percent activity as a function of concentration by using The Microsoft Excel 2010, and the data obtained were subjected to analysis of variance (ANOVA) to determine the significant difference ($P<0.05$) using Minitab 17 statistical software (Minitab Ltd., Coventry, UK).

RESULTS

α-Amylase inhibitory activity

The porcine pancreatic α-amylase inhibitory effect of AD, BAD and ME of roots of *C. opaca* and its fractions was evaluated. The results are presented in I and Figure 1. The samples showed efficacy in a dose dependent style.

TABLE I - α-Amylase inhibitory, β-carotene bleaching inhibitory and iron chelating activities of aqueous decoction (AD) and biomass aqueous decoction (BAD) of *Carissa opaca* roots and their total phenolic (TPC) and flavonoid (TFC) contents

Sample	α-Amylase inhibitory activity IC_{50} (mg/mL)	β-Carotene bleaching inhibitory activity IC_{50} (mg/mL)	Iron chelating effect EC_{50} (mg/mL)	TPC (µg/mL GAE)	TFC (µg/mL RE)
AD	5.38	4.10	0.2939	279.57	53.82
BAD	6.20	4.71	0.3429	245.71	49.39
Standard	0.31 (Acarbose)	2.79 (BHA)	0.1099 (EDTA)	-	-

BHA butylated hydroanisole; EDTA ethylene diamine tetraacetate; GAE Gallic acid equivalent; RE Rutin equivalent.

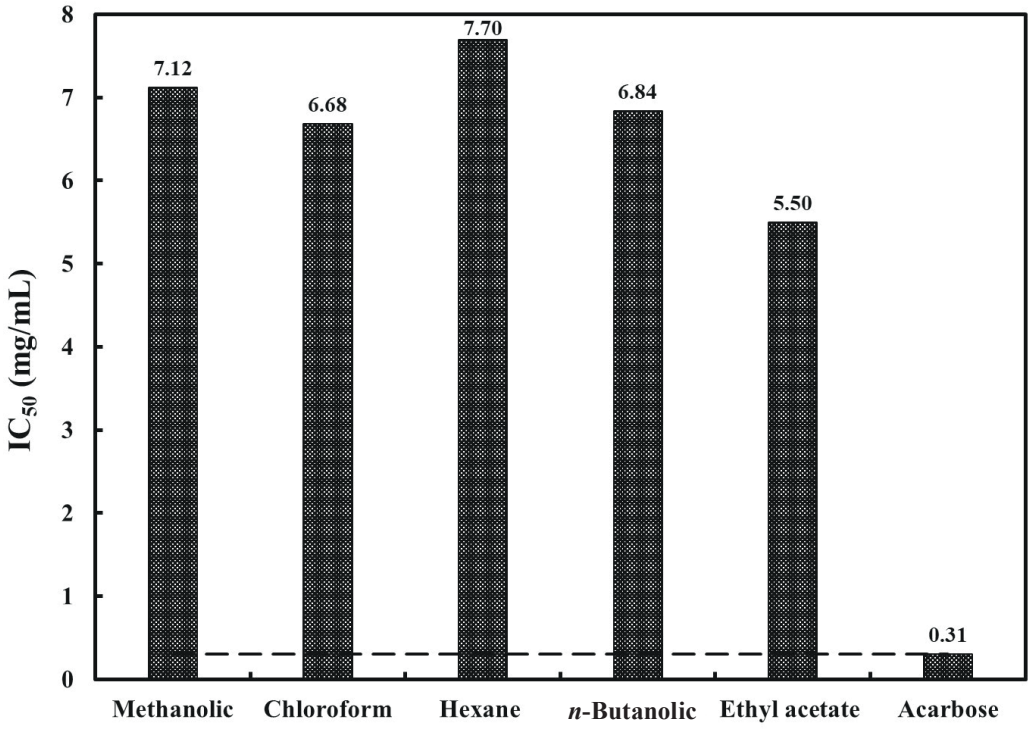

FIGURE 1 - The comparison of porcine pancreatic α-amylase inhibitory activity (IC_{50}) of *Carissa opaca* roots methanolic extract and its fractions in hexane, chloroform, ethyl acetate and *n*-butanolic with the standard Acarbose. The reference line indicates the difference from standard Acarbose significantly with $P<0.05$.

Phenolic and flavonoid contents

Total phenolic contents (TPC) and total flavonoid contents (TFC) of AD and BAD of roots of *C. opaca* were estimated and are given in I.

Iron chelating activity

The ability of AD, BAD and ME of roots of *C. opaca* and its different fractions to chelate with iron was assessed and the results are shown in I and Figure 2.

β-Carotene bleaching inhibitory activity

The potential of AD, BAD and ME of roots of *C. opaca* and its different fractions to inhibit bleaching of β-carotene was assessed and presented in I and Figure 3.

DISCUSSION

Carissa opaca is well-known as a medicinal plant (Izhar, Ahmed, 2016). In the recent years, many studies, *in vitro* and *in vivo*, have been conducted on the aerial parts of the plant to explore various bioactivities. Studies on its roots, however, are limited. Moreover, their aqueous decoction has not been investigated for any activity so far. The ME of the roots have been studied for some activities recently, however, the data is still limited.

α-Amylase inhibitory activity

The present work is a part of our ongoing effort to explore natural remedies for diabetes type-II. Previously our group studied α-amylase inhibitory activities of the methanolic extract and its fractions against the enzyme from bacterial source (Bacillus subtilis) (Saeed, Ahmed, 2015). In this study, porcine pancreatic α-amylase was used because of its closer resemblance to its human's counterpart. The study expected to have a better idea about the efficacy of *C. opaca* roots to control postprandial blood glucose level. Alpha-amylase converts the diet starch into maltose and other oligosaccharides that subsequently changes into glucose

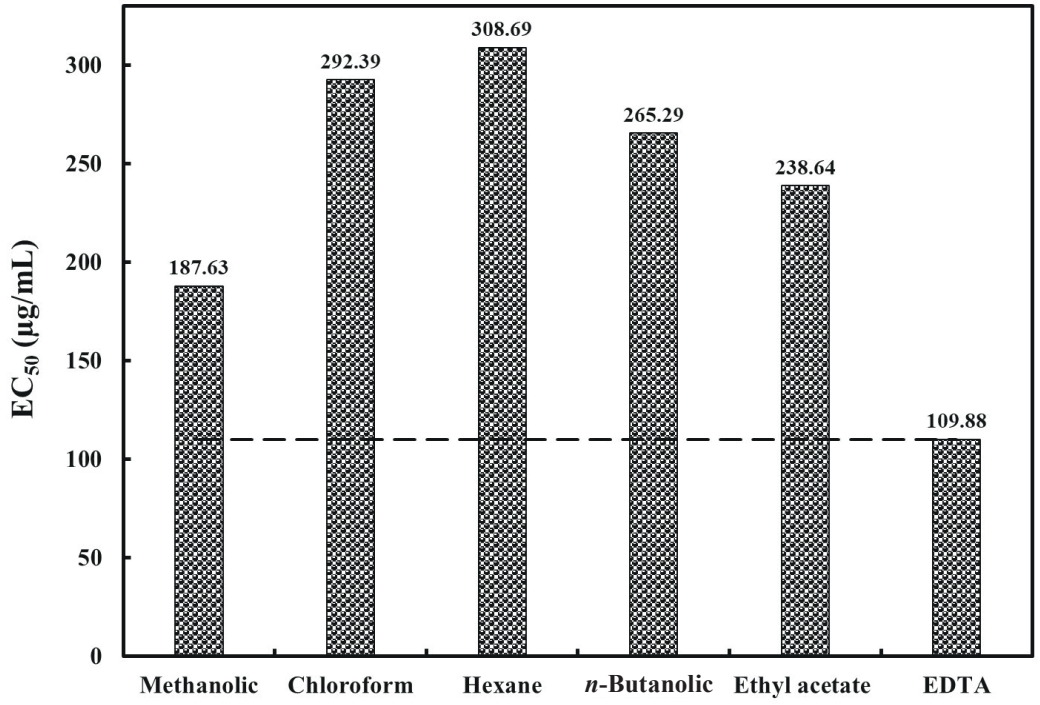

FIGURE 2 - The comparison of iron chelating activity (EC_{50}) of *Carissa opaca* roots methanolic extract and its fractions in hexane, chloroform, ethyl acetate and *n*-butanol with the standard EDTA. The reference line illustrates the significant difference ($P<0.05$) in activities from standard EDTA.

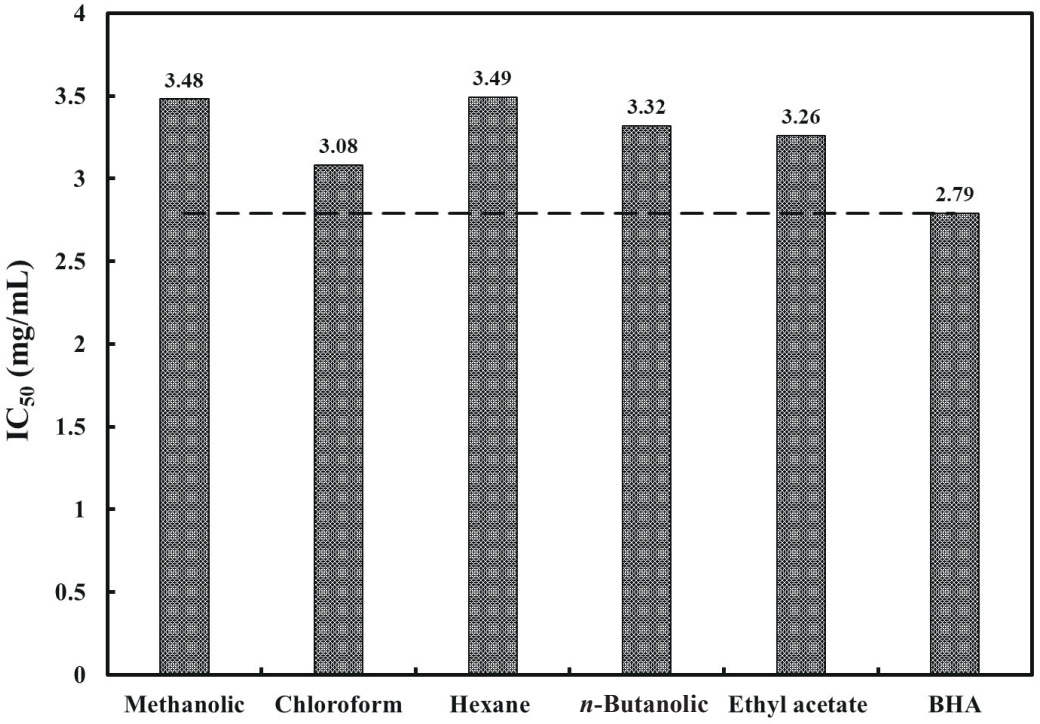

FIGURE 3 - The comparison of β-carotene bleaching inhibitory activities (IC_{50}) of *Carissa opaca* roots methanolic extract and its fraction in hexane, chloroform, ethyl acetate and *n*-butanol with the standard BHA. The reference line illustrates the difference in activities from standard BHA with P value less than 0.05.

by other enzymes. The inhibitors of this enzyme thus avert the digestion of starch thereby preventing the formation of glucose and its entry into the body. The strategy is used to control postprandial glucose level in diabetic patients (Koukiekolo *et al*., 2001). The antidiabetic drug Acarbose follows the same strategy. In the current work, the aqueous decoction (AD) exhibited highest α-amylase inhibitory activity, which was dose dependent. The IC_{50} values of the decoction and Acarbose were 5.38 and 0.31 mg/mL, respectively.

The α-amylase inhibitory activity of ME and its fractions was also no. The IC_{50} of ME was 7.12 mg/mL. The ethyl acetate fraction displayed maximal inhibitory effect with IC_{50} 5.50 mg/mL. The chloroform fraction and biomass aqueous decoction (BAD) came next with IC_{50} 6.68 mg/mL and 6.20 mg/mL, respectively. The hexane and *n*-butanolic fractions had activity in the same range with IC_{50} of 7.70 and 6.84 mg/mL, respectively. Quite interestingly, the efficacy of the ME and its fractions against porcine pancreatic α-amylase was almost in the same range as displayed by the bacterial α-amylase reported earlier (Saeed, Ahmed, 2015). Thus, the present study provided a further evidence for the usefulness of *C. opaca* roots to provide treatment for diabetes type II. It offers a strong support for researchers of natural products, pharmacognosy and pharmacology to focus their effort on this plant for more conclusive results and possible product development.

Iron chelating activity

The Fe(II) chelating assay is used to assess the reducing ability of a sample (Decker, Welch, 1990). In the assay, ferrozine is used to chelate with iron(II) forming a complex with maximum absorbance at 562 nm. The iron(II) accelerates, in the body, the production of hydroxyl radicals by way of Fenton reaction, leading to a range of degenerative diseases (Sharma *et al*., 2012). The substances capable to bind with Fe(II), therefore, inhibit the formation of toxic radicals.

The iron chelating potential of the extracts/fractions of *C. opaca* roots was studied over a range of concentrations (50-500 µg/mL). The EC_{50} value of the chelating activity of the aqueous decoction was 293.92 µg/mL while EDTA, used as reference standard, had EC_{50} 109.88 µg/mL. The ME was more potent than the aqueous decoction with EC_{50} of 187.63 µg/mL. Chloroform, *n*-butanolic and ethyl acetate fractions had moderate chelating effect and the EC_{50} values of these fractions were 292.39, 265.29 and 238.64 µg/mL, respectively. Hexane fraction had the lowest chelating potential (EC_{50} 308.69 µg/mL).

β-Carotene bleaching inhibitory activity

β-Carotene bleaching assay was employed to explore the antioxidant potential of the plant samples. The assay involves "bleaching" or loss of orange color of β-carotene when its conjugation is interrupted by the action of a free radicals produced by the oxidation of linoleic acid, such as lipid (L•) or lipid peroxyl (LOO•) (Shah *et al*., 2013). However, when an antioxidant is present in the reaction medium, it retards the bleaching by scavenging the free radicals attacking β-carotene molecule. The β-carotene bleaching inhibitory activity of aqueous decoction *C. opaca* roots was comparable to the standard BHA with IC_{50} of 4.06 and 3.34 mg/mL, respectively.

Chloroform and ethyl acetate fractions displayed high efficacy with IC_{50} of 3.08 and 3.26 mg/mL, respectively as compared to the other fractions and reference standard BHA that had the IC_{50} of 2.79 mg/mL. The *n*-butanolic fraction had IC_{50} as 3.32 mg/mL which is almost equal to the standard. Hexane fraction, biomass aqueous decoction and ME showed low antioxidant potential with IC_{50} values 3.64, 4.71 mg/mL and 3.41 mg/mL, respectively. The results suggest that *C. opaca* roots have a substantial capacity to transmute the free radicals into non-toxic products. The conclusion is also supported by the previous work done on the plant using other assays (Ahmed *et al*., 2014). As the antioxidant assays work through a variety of mechanisms, no single assay is conclusive (Huang, Ou, Prior, 2005). Therefore, a number of assays are routinely used to measure the antioxidant or free radical scavenging activity of an analyte, natural or synthetic, for maximum substantiation. The beta-carotene bleaching assay is being reported here for the first time. Its results provide additional confirmation of potential antioxidant application of the plant.

Phenolics and flavonoids

In one of our previous studies on this plant, we have reported TPC and TFC of ME and its fractions (Ahmed *et al*., 2014). The present work reports these quantities of AD and BAD. The TPC of AD and BD was 279.57 and 245.71 µg/mL of GAE, which was higher than ME of roots of

C. opaca, which was 211.95 µg/mL GAE. The TFC of AD and BD was 53.82 and 49.39 µg/mL of RE which was much higher than that of ME (8.35 µg/mL RE). As the phenolic and flavonoids are known to possess antioxidant properties, it is useful to know their quantity in a plant sample. They at least partly explain bioactivities that a sample shows under the experimental conditions.

The present work has a number of limitations. The analyses have been conducted on extracts and fractions, and not on the pure compounds. While they provide very useful insight in the efficacy of the plant for chronic diseases, they are inconclusive from the standpoint of the single molecule-based therapies. Furthermore, *in vivo* studies will be required to substantiate the finding of the *in vitro* analyses carried out in the present work. Our plans include isolation of chemical constituents from the roots of this plant and evaluation of their bioactivities.

CONCLUSION

The present study demonstrated that *Carissa opaca* roots are a useful source of antidiabetic and antioxidative agents. The methanolic extract and its fractions and aqueous decoctions showed promising results in iron chelating, β-carotene, and α-amylase inhibitory assays which prove the plant to be a potent candidate for further pharmacological studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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