ORIGINAL RESEARCH ARTICLE

In vitro antioxidant activity-guided fractionation of Daucus carota L. seed extract

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Abstract

Daucus carota Linn (carrot) seeds are medicinally useful in the management of diseases including diabetes mellitus. The present study investigates the in vitro antioxidant activities and phytochemical constituents of several fractions from aqueous seed extract of Daucus carota. D. carota seeds (78.8g) were pulverized and dissolved in 400 mL of distilled water for 24 hours. The crude extract obtained (16.4g, 20.8% yield) was partitioned in water/ethyl acetate (3:1) to yield ethyl acetate fraction (6.2g, 37.8% yield) and aqueous ethyl acetate fraction (7.6g, 46.3% yield) which was subjected to column chromatography. Thirteen (13) fractions obtained were evaluated for their in vitro antioxidant activities and screened for phytochemical constituents. The fractions exhibited in vitro antioxidant activities at 2 – 20 µg/mL with 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities (IC_{50} = 1.38 - 22.96 µg/mL), total antioxidant capacity (IC_{50} = 1.17 - 3.13 µg/mL) and nitric oxide scavenging activities (IC_{50} = 3.20 - 20.72 µg/mL). Fractions 4 – 8 expressed promising in vitro hydrogen peroxide (H_2O_2) antioxidant activities with IC_{50} comparable with that of ascorbic acid and butylated hydroxytoluene. The phytochemical screening of fractions 4 – 8 revealed the presence of alkaloids, flavonoids, and phenolics, which could be responsible for the antioxidant activities. The results suggest that fractions obtained from Daucus carota L. seed extracts possess significant antioxidant potential.

Life Sciences/Ethnobotanical: Keywords: In vitro, Antioxidants; Daucus carota; Fractionation; Seeds

Introduction

Oxidative stress refers to an imbalance between the production of oxidant and antioxidants as a result of significant decrease in antioxidant defense mechanisms in the biological systems (Urban et al., 1995). Antioxidants prevent oxidation of cell components, including lipids and proteins. They are thus, components capable of protecting cells against oxidative damage, playing beneficial roles in improving diseases conditions such as diabetes (Ametov et al., 2003; Munoz, and Castilla-Cortazar, 2012). Several sources are responsible for generating reactive oxygen

species (ROS), including radiation, chemicals, deep-fried or spicy foods, and physical stress (Agrawal et al., 2011), which might lead to oxidative stress.

Daucus carota L. (carrot) is an important root vegetable crop belonging to the apiacea family. Carrots are good sources of magnesium, phosphorus, calcium and carbohydrate (Gopalan et al., 1991) and are rich in α-carotene, β-carotene and lycopene (Olatunde et al., 2020). D. carota seeds are important in the management of diabetes mellitus (Pouraboli and Ranjbar, 2015), which is known to be associated with oxidative stress. Furthermore, the seeds have hepatoprotective (Singh et al., 2012), anti-inflammatory (Vasudevan et al., 2010), analgesic (Vasudevan et al., 2010), in vitro and in vivo antioxidant activities (Tijjani et al., 2020a; 2020b). The present study was designed to identify the active fractions and phytoconstituents responsible for the antioxidant properties of D. carota seed.

Materials and Methods

Reagent and Chemicals

Ascorbic acid, butylated hydroxytoluene and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals were of analytical grade.

Plant Material

Daucus carota L. seeds were purchased from Alheri Manoma, Musty Agroallied Nigeria, LTD and were identified by Mr Azila J. Joseph, a curator with the Federal College of Forestry, Jos, Plateau state and a voucher specimen deposited with the number FHJ 288.

Preparation of Extract

Daucus carota L. seeds were pulverized and 78.8g was dissolved in 400 mL of distilled water for 24 hours. The aqueous crude extract (16.4g, 20.8% yield) was concentrated at 40^{o}C to dryness and then re-dissolved in water and partitioned in ethyl acetate/water (3/1 v/v) using a separation funnel to yield ethyl acetate fraction (6.2g, 37.8% yield) and aqueous ethyl acetate fraction (7.6g, 46.3% yield) after concentration at 40^{o}C. The aqueous ethyl acetate fraction was further fractionated on a silica gel column using hexane, ethyl acetate and methanol starting with hexane (100%), followed by hexane (66%)/ethyl acetate (34%), hexane (50%)/ethyl acetate (50%), hexane (34%)/ethyl acetate (66%), ethyl acetate (100%) and methanol (100%) as mobile phases. The elution was monitored using a TLC plate developed using a solvent mixture containing hexane/ethyl acetate (1/3 v/v) and stained with vanillin to reveal the spots from chemical constituents. Thirteen (13) fractions were obtained which were concentrated and kept dry in a tight glass container until required for use.

In Vitro Antioxidant Studies

In vitro antioxidant activities were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method described by McCune and Johns (2002) and total antioxidant capacity (TAC) using the phosphomolybdenum assay described by Prieto et al. (1999). Nitric oxide (NO) scavenging capacity were evaluated according to the method of Fiorentino et al., (2008) and hydrogen peroxide (H_2O_2) scavenging activity according to the method described by Ruch et al. (1989).

Phytochemical Analysis
Phytochemical analysis was done using the method described by Odebiyi and Sofowora (1978).

Statistical Analysis
Data are presented as means and standard error of the mean (SEM) of triplicate determinations with significance considered at $p<0.05$ using Duncan multiple range tests (SPSS version 20, SPSS Inc., Chicago. IL, USA).

Results
The TLC profile of the aqueous extract of Daucus carota indicated three spots. The aqueous crude extract and the aqueous ethyl acetate and ethyl acetate fractions showed significant in vitro antioxidant activities which were significantly ($p<0.05$) higher when compared with butylated hydroxytoluene and ascorbic acid (Table 1). In order to separate the chemical compounds in the aqueous extract, column chromatography was performed on silica gel and eluted gradiently with mobile phases of different polarities namely hexane, ethyl acetate, methanol and mixtures thereof. A total of 13 fractions were obtained with various physical properties and Rf values (Table 2).

The fractions exhibited in vitro antioxidant activities at 2 – 20 µg/ mL with 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities having an IC_{50} ranging from 1.38 - 22.96 µg/mL with fraction 5 having the lowest IC_{50} values (1.38±0.01 µg/mL) (Table 3). Furthermore, the various fractions showed nitric oxide scavenging activities with an IC_{50} ranging from 3.20 - 20.72 µg/mL while total antioxidant capacity was expressed with an IC_{50} ranging from 1.17 - 3.13 µg/mL (Tables 4 and 5). Fractions 4, 5, 6 and 8 expressed significant H_2O_2 scavenging activities with IC_{50} ranging from 1.41 – 1.94 µg/mL when compared with butylated hydroxytoluene (BHT) and ascorbic acid IC_{50} values of 76 and 54 µg/mL, respectively (Table 6). The phytochemical screening of the most promising fractions (Fractions 4 – 8) indicated the presence of alkaloids, flavonoids and phenolics (Table 7).

Table 1: IC_{50} Values for DPPH Scavenging Activities of Aqueous Crude Extract and Partitioned Fractions of Daucus carota Linn Seed

Extracts	IC_{50} (µg/mL)
Aqueous crude	290.00 ± 30.00^{a}
Aqueous ethyl acetate	300.00 ± 70.00^{a}
Ethyl acetate	380.00 ± 50.00^{a}
Butylated hydroxytoluene	920.00 ± 150.00^{b}
Ascorbic acid	490.00 ± 120.00^{c}

Values are means ± SEM of triplicate determinations.

Values with different superscripts are significantly different at $p<0.05$.

IC_{50}= Half Maximal Inhibitory Concentration

Table 2: Rf Values for Various TLC Fractions Obtained from Aqueous Ethyl Acetate Extract of Daucus carota Linn Seed.

Fractions	Rf
Fraction 1	0.51
Fraction 3	0.51
Fraction 4	0.61
Fraction 6	0.72
Fraction 7	0.38
Fraction 9	0.53
Fraction 10	0.40
Fraction 11	0.40
Fraction 12	0.25
Fraction 13	0.24

Solvent mixture (Hexane: Ethyl acetate: 1/3 v/v)

Table 3: IC_{50} Values for DPPH Scavenging Activities of Daucus carota Linn Seed Fractions

Fractions	IC_{50} (µg/mL)
Fraction 1	>22.96[a]
Fraction 2	>21.71[a]
Fraction 3	>22.43[a]
Fraction 4	1.85±0.05[b]
Fraction 5	1.38±0.01[b]
Fraction 6	1.57±0.05[b]
Fraction 7	1.63±0.08[b]
Fraction 8	3.43±0.35[c]
Fraction 9	3.77±0.24[c]
Fraction 10	1.98±0.07[b]
Fraction 11	1.69±0.06[b]
Fraction 12	1.86±0.05[b]
Fraction 13	1.69±0.04[b]
Butylated hydroxytoluene	168.33±1.45[e]
Ascorbic acid	128.33±0.88[d]

Values are means ± SEM of triplicate determinations.

Values with different superscripts are significantly different at $p<0.05$.

IC_{50}= Half Maximal Inhibitory Concentration

Table 4: IC_{50} Values for Total Antioxidant Capacity of Daucus carota Linn Seed Fractions

Fractions	IC_{50} (µg/mL)
Fraction 1	2.45 ± 0.59^{a}
Fraction 2	1.31 ± 0.08^{b}
Fraction 3	1.26 ± 0.05^{b}
Fraction 4	1.42 ± 0.01^{b}
Fraction 5	2.07 ± 0.60^{a}
Fraction 6	1.17 ± 0.05^{b}
Fraction 7	1.83 ± 0.20^{b}
Fraction 8	1.99 ± 0.06^{b}
Fraction 9	1.50 ± 0.17^{b}
Fraction 10	1.83 ± 0.03^{b}
Fraction 11	2.20 ± 0.05^{a}
Fraction 12	1.99 ± 0.04^{b}
Fraction 13	3.13 ± 0.09^{c}
Butylated hydroxytoluene	1.92 ± 0.27^{b}
Ascorbic acid	19.96 ± 0.04^{d}

Values are means ± SEM of triplicate determinations.

Values with different superscripts are significantly different at $p<0.05$.

IC_{50}= Half Maximal Inhibitory Concentration

Table 5: IC_{50} Values for Nitric Oxide Scavenging Properties of Daucus carota Linn Seed Fractions

Fractions	IC_{50} (µg/mL)
Fraction 1	3.20±0.08[a]
Fraction 2	10.67±4.75[b]
Fraction 3	8.60±0.90[b]
Fraction 4	10.93±4.55[b]
Fraction 5	4.13±0.14[a]
Fraction 6	3.51±0.02[a]
Fraction 7	5.03±0.43[a]
Fraction 8	5.07±0.22[a]
Fraction 9	12.33±4.39[b]
Fraction 10	9.81±1.79[b]
Fraction 11	20.72±0.72[c]
Fraction 12	15.54±1.65[b]
Fraction 13	14.93±3.76[b]
Butylated hydroxytoluene	16.06±1.81[b]
Ascorbic acid	3.22±0.70[a]

Values are means ± SEM of triplicate determinations.

Values with different superscripts are significantly different at $p<0.05$.

IC_{50}= Half Maximal Inhibitory Concentration

Table 6: IC_{50} Values for H_2O_2 Decomposing Activity of Active Fractions from Daucus carota Linn Seed.

Samples	IC_{50} (µg/mL)
Fraction 4	1.41 ± 0.01^{a}
Fraction 5	1.94 ± 0.04^{a}
Fraction 6	1.79 ± 0.02^{a}
Fraction 8	1.64 ± 0.01^{a}
Butylated hydroxytoluene	76.00 ± 6.03^{c}
Ascorbic acid	54.00 ± 1.53^{b}

Values are means ± SEM of triplicate determinations.

Values with different superscripts are significantly different at $p<0.05$.

IC_{50}= Half Maximal Inhibitory Concentration

Table 7: Phytochemical Status of Antioxidant-Active Fractions from Aqueous Seed Extracts of Daucus carota

Phytochemicals	Alkaloids	Phenols	Flavonoids
Fraction 2	+	++	+++
Fraction 5	+	++	+++
Fraction 8	++	+	+++
Fraction 11	+++	++	+++

+++ = Strongly present ++ = Moderately Present + = Slightly Present

Discussion

Several plants have been reported with beneficial biological activities. These activities have been linked with the active compounds in the plants against a disease. Diabetes mellitus and malaria are reported to be associated with oxidative stress and may require multiple approaches to their treatment. Daucus carota seed has been reported as an active antidiabetic agent (Khaki, 2011; Pouraboli and Ranjbar, 2015), possessing in vitro and in vivo antioxidant protective effects (Tijjani et al., 2020a; 2020b). The aqueous crude extract and partitioned aqueous ethyl acetate and ethyl acetate fractions exhibited significant in vitro antioxidant activities when compared with butylated hydroxytoluene and ascorbic acid used as reference compounds (Table 1). Furthermore, the various fractions obtained from chromatography showed in vitro antioxidant activities towards DPPH, TAC, NO and H_2O_2 assays.

Several in vitro antioxidant activities are available for assessing plants and plant extracts. DPPH is a stable organic free radical, that accept an electron or free radicals and then absorbs spectrum

at a particular wavelength (Chithiraikumar et al., 2017). The stable colors formed in DPPH assay are monitored by their changes from a purple-coloured to a yellow-coloured radical species (Chang et al., 2002). Fractions 4 – 13 expressed significant DPPH scavenging activities (Table 3) with IC_{50} values < 4 µg/mL, and significantly higher than the reference compounds. These fractions were obtained from the solvent mixture of hexane: ethyl acetate (1/3 v/v).

The total antioxidant capacity (TAC) was also used to assess the antioxidant activities of Daucus carota seed fractions (Table 4). TAC involves the reduction of hexavalent molybdenum Mo (VI) to pentavalent molybdenum Mo(V) after accepting an electron from an electron donor which leads to formation of a green-colored molybdenum complex in acidic pH ensured by tetraoxosulphate (VI) acid (Keffous et al., 2016). The IC_{50} values were < 3.1 µg/mL and lower than that of ascorbic acid. The presence of polyphenolic compounds in plant extracts increases antioxidant capacity (Belboukhari et al., 2014). Fractions 4 – 8 contains phenolic compounds in appreciable concentrations (Table 7).

Nitric oxide (NO) is a free radical due to the unpaired electron and their reactions with proteins and other free radicals (Nagmoti et al., 2011). NO is generated from L-arginine in vascular endothelial cells, brain cells and phagocytes. NO generation is implicated in vascular disease, sepsis and trauma, undernutrition and other conditions (Luiking et al., 2010). NO scavenging activity was lower in all fractions obtained compared with ascorbic acid except for fraction 1 (Table 5). This suggests that the fractions could prevent adverse NO toxicity, since their toxicity increases with NO reaction with superoxide radicals and forming high reactive peroxynitrite anion ($ONOO^-$) (Nagmoti et al., 2011). Hydrogen peroxide (H_2O_2) are strong oxidizing agents, released during the detoxification of hydroxyl radicals, by several biological enzymes including superoxide dismutase (Buettner, 2011). It is activated during signaling pathways to stimulate differentiation and cellular proliferation (Geiszt and Leto, 2004; Li et al., 2006). Increased levels of H_2O_2 are markers of oxidative stress and inflammation reactions, in disease conditions including cancer, cardiovascular, and diabetes diseases (Mahmoud et al., 2011; DeGracia et al., 2012, Tijjani et al., 2020a). The H_2O_2 scavenging activities of the most promising fractions were higher when compared with ascorbic acid and BHT.

The strong in vitro antioxidant activities expressed by the various fractions of aqueous seed extract of Daucus carota indicates that the plant has the potential to prevent or contribute to antioxidant defense system in disease conditions. More so, prolong oxidative stress could results in damage to vital organs of the body leading to further complications in disease such as acquired immunodeficiency syndrome, cancer, cirrhosis, heart diseases, malaria, neurodegenerative diseases and premature aging (Nagmoti et al., 2011; Agarwal et al., 2012).

Phenolics have been shown to have a remarkable range of biological and pharmacological properties, such as antioxidant, antidiabetics, anti-inflammatory, anti-proliferative, antiviral, anticancer and anti-allergic effects (Liu, 2003; Scalbert et al., 2005). The use of phenolic constituents in food and some therapeutic medication are due to their antioxidant and other health endorsing benefits (Ou et al 2002). Flavonoids are plant secondary metabolites derived from the shikimate acid pathway (Tijjani et al., 2018). They play beneficial roles in health due to their antioxidant effects. They are found in a variety of seeds, fruits and vegetables. In addition,

antioxidant, anti-inflammatory, antiallergenic, anti-carcinogenic, antimicrobial and anti-viral properties have been linked to the presence of flavonoids rich compounds in plant extracts (O'Neil et al., 2000). Flavonoids help to prevent platelets sicknesses and hence platelet aggregation (Okwu and Emenike, 2006). The observed antioxidant activities could be due to the presence of various phytochemicals detected in the fractions.

Conclusion

Fractions from Daucus carota seed possess potent in vitro antioxidant properties, which are comparable with that of butylated hydroxytoluene and ascorbic acid. The most active fractions contain high concentrations of alkaloids and flavonoids, and the latter are likely to contribute to the antioxidant property of the seeds reported herein, given the close relationship between antioxidant potential and the content of polyphenols and alkaloids.

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