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Antioxidant, haemolytic activities and GC-MS profiling of *Carissa carandas* roots.

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A B S T R A C T

The antioxidant potential of the plant *Carissa carandas* roots extracted with different polarity based solvents was assessed. The antioxidant activity was evaluated by the measurement of total phenolic contents (TPC), total flavonoid contents (TFC), reducing power, DPPH radical scavenging, IC_{50} and antioxidant activity in linoleic acid system. The plant material contained the TPC (1.79-4.35 GAE mg/g of dry extract), TFC (1.91-3.76 CE mg/g of dry extract), DPPH radical scavenging activity, IC_{50} (12.53-84.82 %) and % inhibition of peroxidation in linoleic acid (41.0-89.21 %) system respectively. Furthermore the antioxidant effectiveness of extracts was assessed using corn oil (CO) as the oxidation substrate. The oxidative alterations were evaluated by the measurement of conjugated diene (CD), conjugated triene (CT), *p*-anisidine, free fatty acid (FFA) and peroxide values (PV). The cytotoxicity of the plant extracts were assessed against human red blood cells (RBCs) and the % lysis was found to be in the range of 1.01-6.10 %. The GC-MS analysis of *n*-hexane extract was also profiled. It was concluded that extracts of plant roots may be used as potential source of antioxidant agents in food industries.

Keywords: Antioxidants, corn oil, *Carissa carandas*, roots, lipids.

Introduction

Carissa carandas is a flowering shrub belongs to family *Apocyanaceace*. Common names include Kronda, Christ's thorn or Bengal [1]. Different phytochemicals such as carotenoids, alkaloids, saponins [2], triterpenes and steroidal compounds [3] have already been reported. Plants considered being one of most valuable source drugs in various system of medicine and all the parts of this plant highly useful and among them roots seem to be much useful [4]. The whole plant usually used as antihelmintic and antidiarrheal and stem of plant is used to reinforce tendons, fruits are used in skin infections and leaves are remedy for fevers and syphilitic pain [1]. Alcoholic extract of root material reduces the blood pressure [5] and aqueous extract of roots exhibited various pharmacological activities like histamine releasing, antihelmintic, and sapsmolytic and cardio tonic [6, 7]. Analgesic, antiinflammatory and lipase activities of karonda fruit have also been reported [8, 9]. Chemical constituents such as carisone , carindone, carinol, lignin, oderoside H and 2-acetylphenol have been reported from roots of Karonda plant [7,10-13]. Triterpenes, tannins and carissic acid are reported from the leaves of this plant [14-16].

Plant materials

The roots of the plant *C. carandas* were collected from Botanical Garden University of Agriculture, Faisalabad (Pakistan) and were further identified by Taxonomist of Botany Department, University of Agriculture Faisalabad.

Preparation of extracts

For the removal of adhering dust particle fresh roots of plant washed with distilled water. They were shade dried. The grinded fine powder (1kg) was extracted with petroleum ether at room temperature. After filtering the extract was concentrated through rotary evaporator. This process was repeated thrice to obtain sufficient quantity of petroleum ether extract. The remaining plant residue was further extracted with other different polarity based solvents (Fig.1)

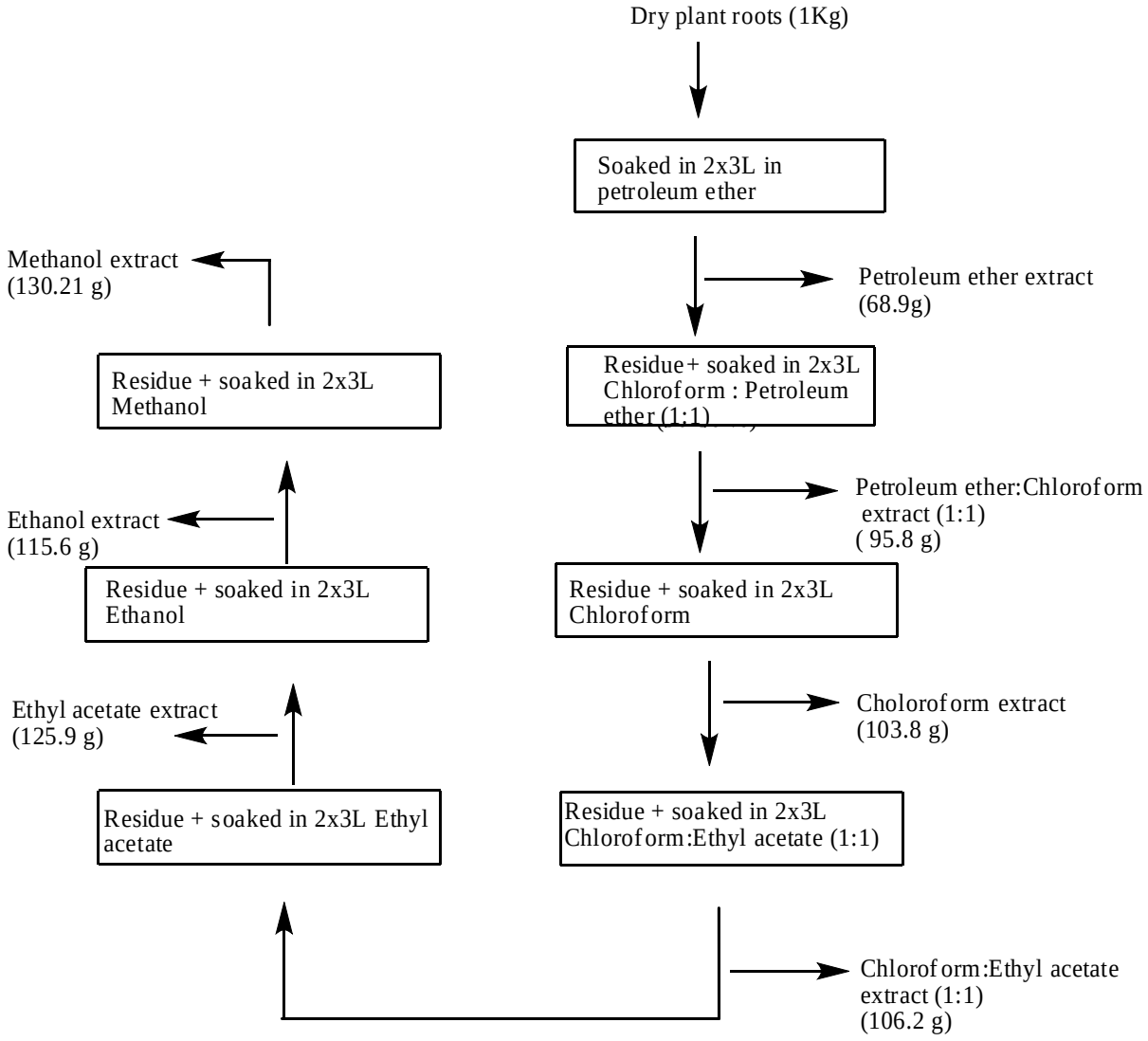

Fig.1 Schematic flow sheet diagram for the preparation of plant extracts

Phytochemical analysis

Powdered roots of the plant were subjected to qualitative and quantitative phytochemical screening to analyze the presence of alkaloids, tannins, saponins, steroids, by using standard phytochemical procedures [17].

Evaluation of antioxidant activity

The total phenolics and flavonoids contents were assessed as method described [18, 19]. DPPH free radical scavenging and % inhibition of linoliec acid peroxidation of sample extracts was determined as method described [20, 21] respectively. The reducing potential of the plant extracts was evaluated by using already reported method [22].

Haemolytic activity

The haemolytic activity against the human RBCs was evaluated as method described [23]. 3 mL of freshly obtained heparinized human blood was gently mixed, poured into a sterile 15 mL falcon tube and centrifuged for 5 minute at 850 x*g*. The supernatant was poured off and viscous pellet washed three additional times with 5 mL of chilled (4°C) sterile isotonic phosphate-buffered saline (PBS) solution, adjusted to pH 7.4, to stabilize the pH mix it almost for half an hour at room temperature (25–30°C). The washed cells were suspended in the 20 mL chilled, saline PBS buffer. Erythrocytes were counted on haemacytometer. The blood cell suspension was maintained on wet ice and diluted with sterile (PBS), the cell count was maintained 7.068 x10^8 cell per mL for each assay. Plant extract (20 µL) in five different solvent was taken in 2 mL eppendorfs for each assay, 0.1% triton X-100 was taken as a positive control and PBS as negative control. In each eppendorfs already contain 20 µL samples and add 180 µL diluted blood cell suspension and mixed it with the help of pipette tip. Tubes were incubated for 35 minute at 37°C. Immediately after incubation, the tubes was placed on ice for 5 minute then centrifuged for 5 minute at 1310 x*g*. After centrifugation 100 µL supernatant was taken from the tubes, and diluted with 900 µL chilled PBS. All tubes were maintained on wet ice after dilution. Then 200 µL diluted RBCs were added into 96 well plates, after this absorbance at 576 nm was taken at μ Quant (spectrophotometer). The experiments were done in triplicate.

Determination of antioxidant activity using corn oil as oxidation substrate

Corn oil was used as an oxidation substrate. The antioxidant activity of plant roots was determined by using of refined, bleached and deodorized corn oil. The crude concentrated different extracts of the plant material were separately added to preheated (50°C), the refined, bleached and deodorized (RBD) corn oil (CO) at concentrations of 400 ppm (w/w). The CO samples were stirred for 30 minute at 50 °C for uniform dispersion. Synthetic antioxidant (BHT) was employed at its legal limit of 200 ppm to compare the efficacy of extracts. Stabilized and control oil samples (100 mL) were placed in dark brown airtight glass bottles with narrow necks and subjected to accelerated storage in an electric oven at 60 °C for 28 days. All oil samples were examined in triplicate. A corn oil sample, without antioxidant, was used as the control. Oil samples were taken after every seven days interval for analysis [24].

Measurement of lipid oxidation

The oxidative deterioration level was assessed by the measurement of peroxide value (PV), Conjugated dienes (CD), conjugated trienes (CT) and *p*-anisidine values. The peroxide value , the free fatty acid contents and *para*- anisidine values of corn oil sample were determined as the method described [25, 26]. Conjugated diene and triene product were evaluated as the method described [27].

GC-MS analysis and identification of compounds

The dried and ground 100 g plant was extracted with *n*-haxane through soxhlet apparatus for the

GC-MS analysis. The sample was analyzed using GCMS-QP2010 (Shimadzu, Japan) [28, 29].

Results and Discussion

Yield of extracts

The yield of plant roots extracts was within the range of 6.89-13.21 g/100g of dry plant. The maximum yield was observed with absolute methanol while the minimum with petroleum ether. The yield of extracts in various solvents such as in absolute methanol, absolute petroleum ether, petroleum ether: chloroform (1:1) absolute chloroform, chloroform: Ethyl acetate (1:1), absolute ethyl acetate, absolute ethanol was 13.21, 6.89, 9.58, 10.38, 10.62, 12.59, 11.56 g/100g respectively.

Phytochemical study

Preliminary phytochemical analysis of the plant indicated the presence of various constituents like alkaloids, steroids, flavonoids, tannins and saponins. In plant these secondary metabolites were present in ethanol, methanol, ethyl acetate extracts while alkaloids were absent in ethyl acetate:chloroform extract. Alkaloids and flavonoids were absent in chloroform extract whereas in chloroform: petroleum ether (1:1) extract, saponins and flavonoids were present. Petroleum ether extract contained terpenoids. The quantitative estimation of chemical constituents in plant roots were as alkaloids (0.34±0.02), flavonoids (0.24±0.020), tannins (17.10±0.14), saponins (0.20±0.02) and total phenolic components (0.30±0.03) % respectively. Presence of secondary metabolites such as alkaloids, steroids, flavonoids, saponins and terpenoids in ethanol extract of *C. carandas* roots have been already reported [30, 31]. Edeoga and coworkers reported the crude percentage of secondary metabolites such as alkaloids, phenolics, tannins, flavonoids and saponins in *C. rutidosperma* [17].

Antioxidant studies

Antioxidant results of all the tests are documented in Table 1. Total phenolic contents observed in plant roots were in the range of 1.79 to 4.35 GAE mg/g of dry plant extract. The decreasing order of total phenolic contents found with different solvent extracts of plant was methanol (4.35) >ethanol (3.55) >ethyl acetate:chloroform (3.34) >ethyl acetate (2.84) >petroleum ether:chloroform (2.60) >chloroform (2.38) >petroleum ether (1.79) GAE mg/g of dry plant extract. The total phenolic contents in ethanol extract of *Carissa carandas* has been already reported [4].

Total flavonoid contents in the plant ranged from 1.91 to 3.76 CE mg/ g of dry plant extract. Methanol was efficient to extract TPC and TFC whereas petroleum ether was less. Bhaskar and Balakrishnan reported the quantity of TFC in ethanol extract of *C. carandas* roots [4]. Here again absolute methanol extract showed good results and its antioxidant activity was maximum. The absorbance increases as the concentration of tested plant extracts increases. The maximum reducing power observed with methanol extract 0.471, 0.592, 0.754 and 0.98 nm at the concentration of 2.5, 5, 7.5 and 10.0 mg/ml (Fig. 2) respectively. The decreasing order of reducing power was as followed. Methanol > ethanol > ethyl acetate > ethyl acetate: chloroform > chloroform > petroleum ether: chloroform > petroleum ether. Bhaskar and Balakrishnan reported the reducing potential of *C. carandas* roots ethanolic extract (0.25 nm) at the concentration of 10 µg/ml [4]. % inhibition of linoleic acid per oxidation ranged from 41.01 to 72.11 %. The highest antioxidant activity in term of % inhibition was observed with methanol extract whereas BHT showed 89.21% inhibition peroxidation. The decreasing order of percentage inhibition was; methanol (72.10) >ethanol (68.05) >ethyl acetate (61.02) >ethyl acetate:chloroform (59.15) >chloroform (53.2) > petroleum ether (53.02) > petroleum ether: chloroform (41.00). In DPPH free radical scavenging $IC_{50,}$ the petroleum ether extract showed the maximum IC_{50} (84.8 µg/mL) and minimum value obtained with methanol (46.1 µg/mL) extract. The least value of IC_{50} represents the better antioxidant and high value for less antioxidant activity. In this assay BHT was used as positive control (IC_{50}=12.53 µg/mL). The IC_{50}

of *C. carandas* roots ethanolic extract at different concentrations have been reported [4].

Table.1 The total phenolic contents (TPC), total flavonoid conents (TFC) and antioxidant potential.

Samples	TPC (GAE mg/g of dry extract)	TFC (CE mg/g of dry extract)	DPPH (IC_{50} µg/mL)	% Inhibition of linoleic acid oxidation
Petroleum ether	1.79±0.06	1.91±0.05	84.82±1.71	53.02±0.81
Petroleum ether-chloroform (1:1)	2.60±0.08	2.84±0.09	81.27±1.52	41.00±0.64
Chloroform	2.38±0.13	3.00±0.13	68.73±1.32	53.2±0.74
Chloroform- ethyl acetate (1:1)	3.34±0.14	2.33±0.05	67.76±1.42	59.15±0.91
Ethyl acetate	2.84±0.09	3.51±0.09	54.91±0.81	61.02±1.21
Ethanol	3.55±0.15	2.68±0.04	49.40±0.10	68.05±1.82
Methanol	4.35±0.21	3.76±0.10	46.15±0.93	72.10±1.45
BHT	-	-	12.53±0.21	89.21±0.84

The results are the average of triplicate samples (n=3) ± S.D., ($p < 0.05$)

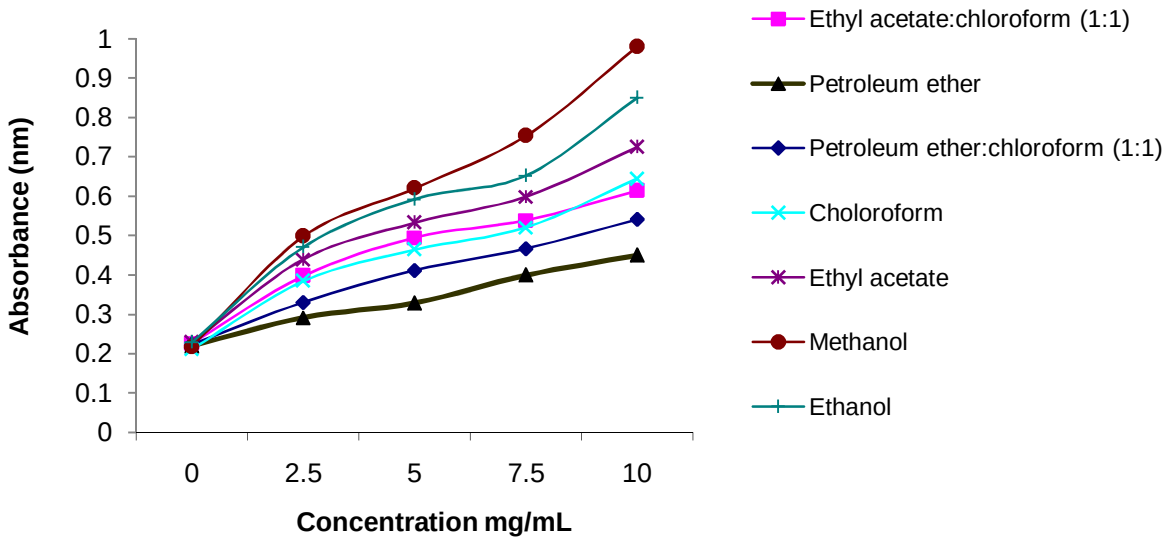

Fig. 2 Reducing potential of different extracts of *C. carandas* roots.

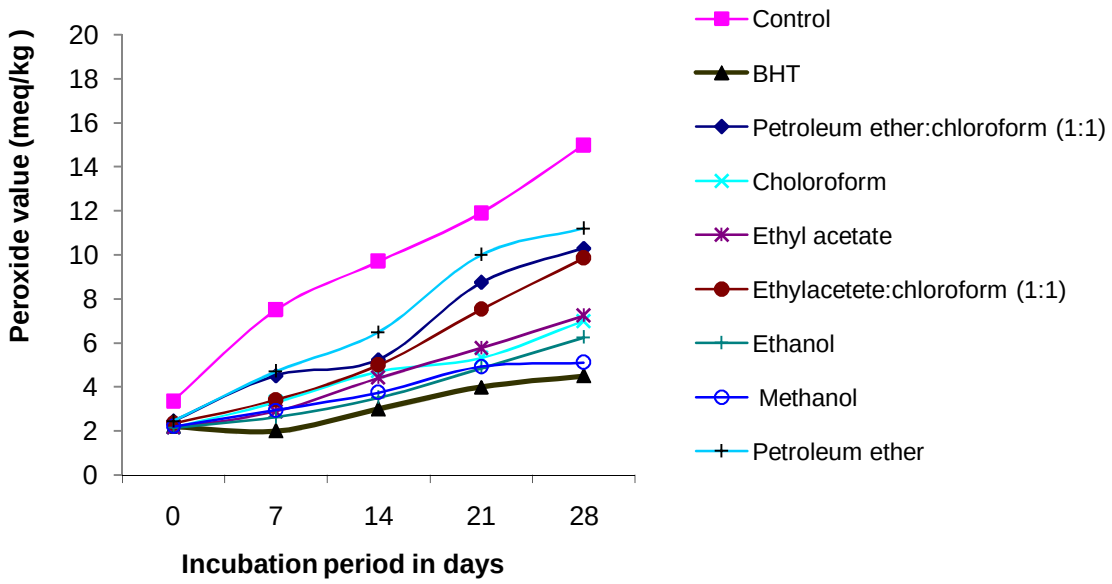

Fig. 3 Peroxide value of corn oil stabilized with *C. carandas* roots extracts

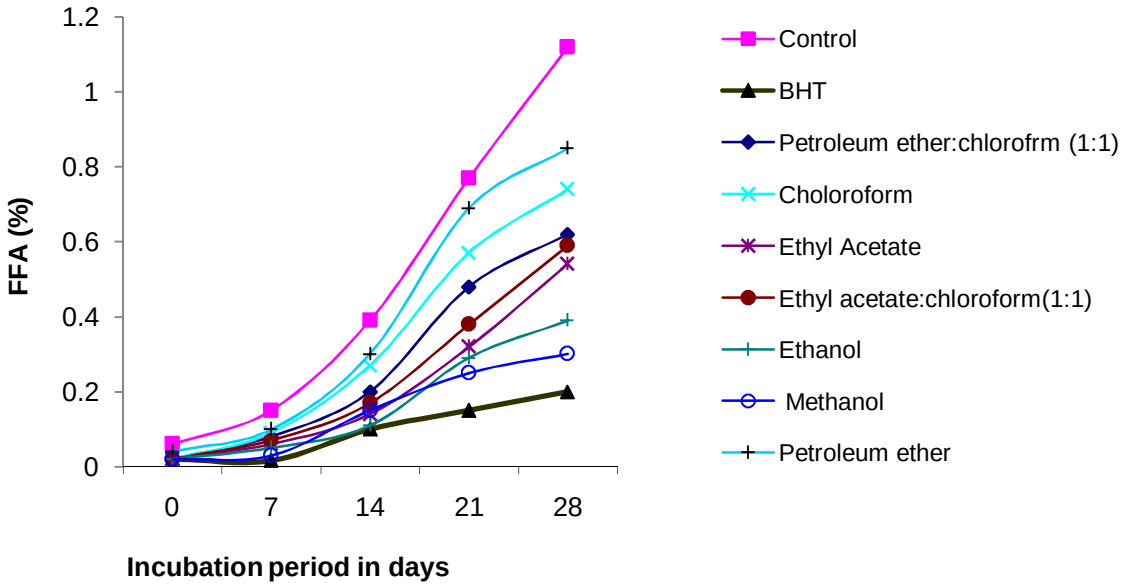

Fig. 4 Free fatty acid contents (%) of corn oil stabilized with *C. carandas* roots extracts

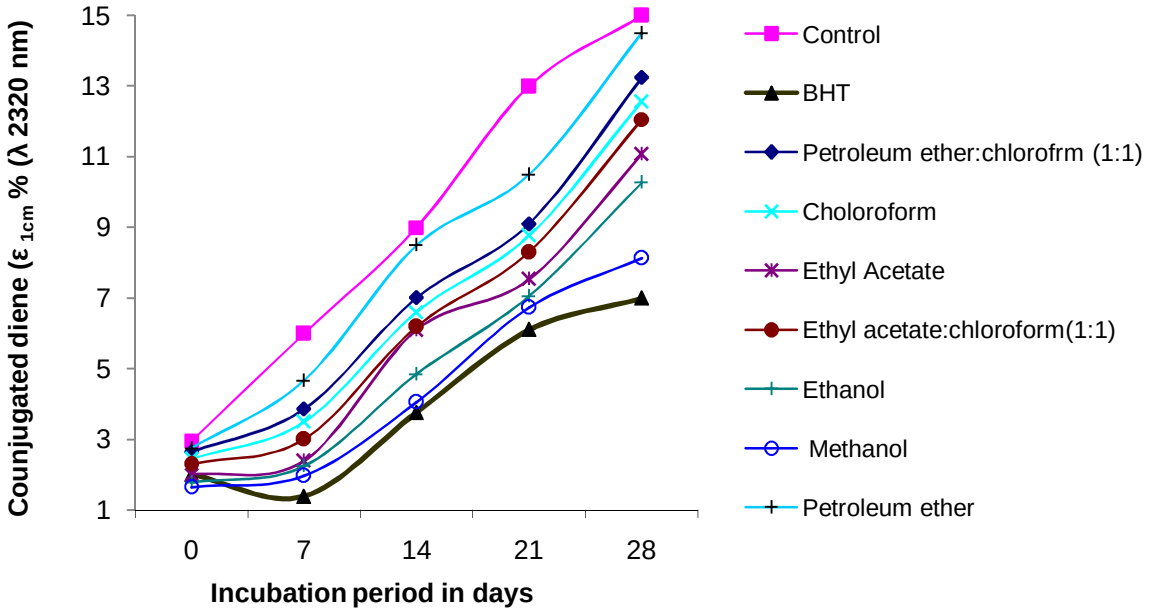

Fig. 5 Conjugated diene values of corn oil stabilized with *C. carandas* roots extracts

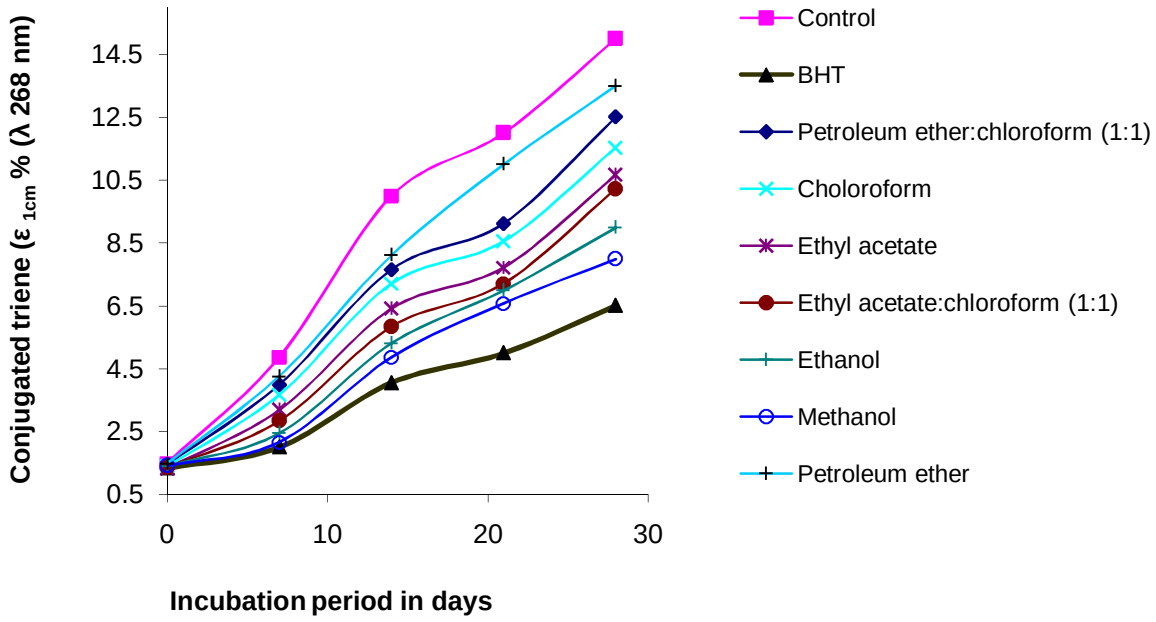

Fig. 6 Conjugated triene values of corn oil stabilized with *C. carandas* roots extracts

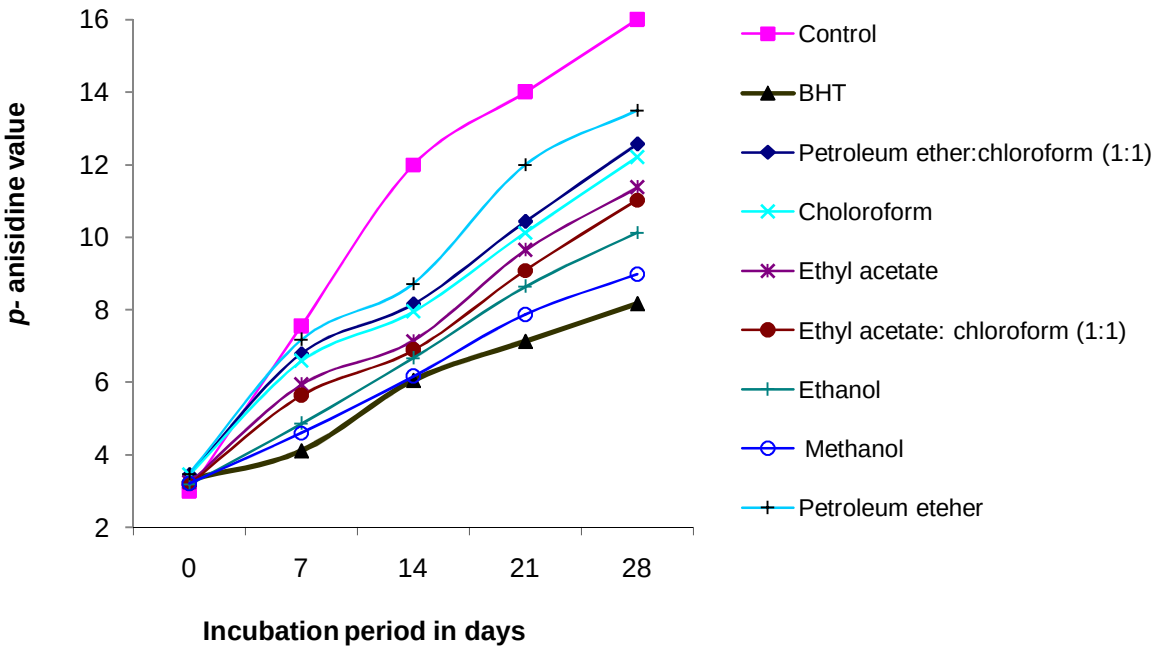

Fig. 7 *p*-anisidine values of corn oil stabilized with *Carissa carandas* roots extracts

Antioxidant potential of *C. carandas* roots extracts for stabilization of corn oil.

To check the stabilization of corn oil with plant extracts five tests were performed such as PV, FFA, CT, CD and *p*-anisidine value. The peroxide value of corn oil stabilized with different extracts of plant at 60ºC storage for 28 days at the concentration of 400 ppm, The peroxide values trend is shown in Fig. 3. The oil sample without extract was used as control. Maximum peroxide value showed with control (3.35-14.98 meq/kg) and minimum with BHT (2.2-4.5 meq/kg). Methanol extract showed the minimum PV value (2.2-5.11 meq/kg) than petroleum ether extract (2.45-11.2 meq/kg) which showed maximum value after control. In all tests control showed highest value and BHT showed lowest value. In FFA % value with different extracts was as followed, Methanol (0.02-0.31 %), ethanol (0.02-0.39%), ethyl acetate (0.02-0.54%), and ethyl acetate: chloroform (0.02-0.59), chloroform (0.02-0.74%), petroleum ether: chloroform (0.02-0.62%) petroleum ether (0.04-0.85%). Fig. 4 illustrates different trends in FFA value.

The specific extinction at 232 nm evaluated in terms of conjugated dienes (Fig. 5). This revealed the oxidative deterioration of the corn oil stabilized with extract of plant roots. The contents of conjugated diene are also a good indicator of the degree of deterioration of oils. The control had greater rise in conjuable oxidation products as compared with those of treated oil samples. In CD the initial value was 1.68 with methanol extract which jumped to 8.14 after 28 days. Fig.6 represents the whole change in values with different solvents. The measurement of specific extinction was done at 268 nm in conjugated trienes. In CT the highest value after control observed with petroleum ether extract (1.49-13.5). Fig.7 showed the rise in CT value with different solvents from initial value to final value after 28 days. The relative increase in *para*-anisidine values of corn oil sample

Table. 2 GC-MS analysis of *n*- hexane extract of *C. carandas* roots.

Peak No.	Compound name	Retention time	% composition
1	*n*-octane	5.462	0.78
2	2-4-Dimethy hexane	5.508	0.44
3	*n*-nonane	9.002	2.95
4	*n*-decane	12.758	0.62
5	*n*-undecane	16.302	1.11
6	Acetophenone	18.267	0.70
7	*n*-dodecane	19.587	1.34
8	*n*-tetradecane	22.636	2.24
9	Phenol, 2-methoxy-4-(2-propenyl)-	24.141	0.60
10	*n*-penta decane	27.975	0.55
11	Phenol, 2,4-bis(1,1-dimethyl ethyl)-	28.067	0.72
12	*n*-eicosane	30.058	2.54
13	*n*-heptadecane	31.850	0.78
14	Hyristic acid	32.808	1.17
15	*n*-heneicosane	33.483	5.41
16	*n*-nanodecane	34.958	0.66
17	7,9-di-test-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-diene	35.067	0.57
18	Hexadecanoic acid, methyl ester	35.308	0.42
19	Hexadecane,1,1-bis(dodecyloxy)-	35.492	0.59
20	1-(+)-Ascorbic acid 2,6-dihexa decanoate	35.767	12.08
21	Hexa decanoic acid , ethyl ester	36.242	0.26
22	*n*-hexatriacontane	37.275	0.31
23	2-H,8-H, Benzo[1,2-b:5,4-b] dipyran-2-one,8,6 dimethyl	37.525	2.05
24	7-Octa decadienoic acid, methyl ester	37.633	1.71
25	9,12-Octa decenoic	38.000	5.11
26	6- Octa decenoic acid (z)-	38.117	29.61
27	Stearic acid	38.358	1.95
28	9-Octa decenoic acid	38.467	0.80
29	*n*-tetrasocane	38.842	1.7
30	2-H-1-Benzopyran-2-one, 7-methoxy-6-(3-methyl-2-butyl)	39.117	2.75
31	1,2 Benzenecarboxylic acid mono, (3-ethylhexyl)ester	42.517	2.67
32	z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	42.842	0.57
33	Stearaldehyde	44.667	0.64
34	Gamma-sitosterol	49.125	0.81
35	4-Pyrimidinecarboxylic acid, 2,6 bis [(tet-butyl dimethylsilyl)oxy]	49.500	0.40
36	Beta-amylin	49.600	1.23
37	beta-viscol	50.075	11.18

treatments stabilized with extracts of plant documented in Fig. 7. The control (without any extract) had the highest *para*-anisidine value (2.99-15.98) and thus indicated a higher rate of oxidation. *Para*-anisidine value is used to measure the secondary product of lipid oxidation. The proposed reaction of *para*-anisidine with aldehyde formed a yellowish product. The highest value after control was observed with petroleum ether (3.48-13.5) and lowest with methanol (3.21-8.98).

Haemolytic activity

The percentage haemolysis of *C. carandas* roots extracts was observed. Chloroform extract showed highest amount of toxicity (6.10±0.210) and the lowest observed with methanol extract (1.011±0.07).The toxicity value in absolute ethanol, ethyl acetate, chloroform:ethyl acetate (1:1), chloroform:petroleum ether (1:1) and in petroleum ether were found to be 1.33, 3.49, 2.68, 3.93 and 3.11 respectively. The order of decreasing toxicity in plant is given below: chloroform > petroleum ether:chloroform (1:1) > ethyl acetate > petroleum ether > ethyl acetate:chloroform (1:1)> ethanol>methanol

GC-MS analysis of *n*-hexane extract and identification of compounds

The total 37 compounds were identified by GC-MS analysis (Table. 2) of *n*-hexane extract. The mass spectrum of each compound was compared with that in NIST 05 library. The major compounds present in the *n*-hexane extract as identified by GC-MS were 6- octa decanoic acid (z)- , 1-(+)-Ascorbic acid 2,6-dihexa decanoate and beta-viscol found in 29.61, 12.08 and 11.18 % respectively. Some of the compounds were not identified and all of these were present in traces or less concentration as compared to other identified compounds. The *n*-hexane extract may have some fatty acids/methyl esters which may be implicated in some other antioxidant activities.

Conclusions

The result of present analysis showed that methanol and ethanol extracts of *C. carandas* roots were rich in phenolics and flavonoids constituents and exhibited good antioxidant activity. Various oxidation parameters of corn oil stabilization, qualitative and quantitative analysis of major chemical constituents in *C. carandas* plant could be helpful for explaining the relationships between total phenolics and flavonoids contents of extracts. The ctyotoxicity evaluation of this plant extracts showed minor cytotoxic effect on RBCs. So the palnt extract may be used as an antioxidant.

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