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Antioxidant Activities of Methyl Ricinoleate and Ricinoleic Acid Dominated *Ricinus communis* Seeds Extract Using Lipid Peroxidation and Free Radical Scavenging Methods

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ABSTRACT

The antioxidant activity of two methanolic fractions (C1, C2) of *R. communis* was determined by three methods: Lipid peroxidation by ferric thiocyanate method and free radical scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) and hydroxyl radical generated from hydrogen peroxide. C1 and C2 at various concentration possessed significant antioxidant activity ($p<0.05$) when compared with antioxidant standards Butylated Hydroxy Anisole (BHA), ascorbic acid and α-tocopherol used in the assay. C1 had percentage inhibition of 93.98% while C2 gave 90.10% inhibition at 0.8 mg mL^{-1} in the lipid peroxidation/ferric thiocyanate test. In the DPPH assay, C1 had inhibition of 73.71% while C2 gave 87.92% at 1.0 mg mL^{-1}. While in the hydroxyl radical scavenging assay, the inhibition of C1 was 85.07% while that of C2 was 94.91% at 0.1 mg mL^{-1}, both extracts therefore, showed comparative antioxidant activities at the concentrations used. C1 golden brown coloured oil was analysed using gas chromatography/gas chromatography-mass spectrometry. Four components were obtained, Methyl ricinoleate (46.68%), Ricinoleic acid (34.41%), (Z, Z)-9, 12-Octadecadienoic acid (12.99%) and (Z, Z)-9, 12-Octadecadienoic acid, methyl ester (5.92%). These chemical constituents were assumingly responsible for the observed antioxidant activities of methanolic extracts from *R. communis* seeds.

Key words: Antioxidant, *Ricinus communis,* 2, 2-diphenyl-1-picrylhydrazyl radical, hydrogen peroxide, ferric thiocyanate

INTRODUCTION

R. communis (Castor oil plant) of the family Euphorbiaceae is indigenous to the Southeastern Mediterranean Basin, Eastern and Western Africa, India and other tropical regions. It is sometimes grown as an ornamental plant. Castor seed is the source of castor oil containing triglycerides mainly ricinoleic acid and a very toxic albumin ricin. The oil has industrial and medicinal applications. In Brazil, castor oil is being used to produce biodiesel, used in body ointments, as an emotic, emollient, laxative, purgative, vermifuge, bactericide, larvicidal, in the treatment of cathartic cancer, arthritis, convulsion, epilepsy, venereal disease and tuberculosis (Brickell, 1996; Devendra and Raghavan, 1978; Duke, 1978; Duke and Wain, 1981; Gaydou *et al*., 1982). Methanolic extracts of the leaves of *R. communis* were used as antimicrobial agent and also found to possess analgesic activity and CNS stimulant in mice at low doses. Ethanolic extract of *R. communis* root bark was also found to have antihistamine and anti-inflammatory properties. The toxicity of raw castor beans due to the presence of ricin is well-known. The toxin provides the castor oil plant with some degree of natural protection from insect pests, such as aphids. The castor oil plant is also the source for undecylenic acid, a natural fungicide. Three terpenoids and a tocopherol-related compound have been found in the aerial parts and were isolated from the methanol extracts

(Reed, 1976; Duke, 1978; Devendra and Raghavan, 1978; Duke and Wain, 1981; Gaydou *et al.*, 1982; Wedin *et al.*, 1986; Cragg *et al.*, 1997; Boye and Ampofo, 1990; Simpson and Conner-Ogorzaly, 1995; Srivastava *et al.*, 1996; Heinrich *et al.*, 2004; Smith *et al.*, 1996; Andrew, 2009; Shuid *et al.*, 2011).

A comparative investigation of the antioxidant activity of *R. communis* is not yet in literature. Anti-oxidants from natural origin are being sourced for because they offer protective effect against oxygen reactive species which have been found to participate in a growing number of disorders, causing oxidative damage thus altering the structure and function of cells of biological macro-molecules. Diseases such as cancer, Parkinson's and liver diseases, amongst others have been implicated (Ansari *et al.*, 2011; Sawadogo *et al.*, 2006). Several substances; plant phenolics, flavonoids, polyamines, ascorbic acid, vitamin E as well as enzymes such as superoxide dismutase, catalase and various peroxidases have been proposed to act as antioxidant *in vivo* (Halliwell and Gutteridge, 1984; Bors and Saran, 1991; Sies, 1997; Egwuche *et al.*, 2011).

The objective of this research work was to subject the fractions obtained from *R. communis* to *in vitro* lipid peroxidation by ferric thiocyanate method and free radical scavenging activities using scavenging effect on 2,2-diphenyl-1-Picrylhydrazyl Radical (DPPH) and hydroxyl radical generated by hydrogen peroxide. The effectiveness and mode of action of the antioxidant effect of *R. communis* will also be determined in these assays. These three antioxidant screening methods have not yet been reported in literature for this plant. Butylated hydroxy anisole (BHA), ascorbic acid and α-tocopherol are used as reference standards (Yen and Duh, 1994; Soare *et al.*, 1997; Koleva *et al.*, 2002; Gupta and Sharma, 2010; Sharma *et al.*, 2011; Krishna *et al.*, 2009; Onocha *et al.*, 2011). Also the chemical constituents of the oil will be determined by Gas Chromatography and Gas Chromatography-Mass Spectrometry analysis (Ganesh and Vennila, 2011).

MATERIALS AND METHODS

Chemicals and reagents: Ethyl acetate, chloroform, hexane, methanol butanol, hydrochloric acid, ammonia solution, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, copper acetate, NaOH, sodium chloride, copper sulphate pentahydrate, ferric chloride, conc. tetraoxosulphate (VI) acid, Conc. HCl, ammonia solution, sodium potassium tartrate, linoleic acid, ammonium thiocyanate, ethanol, ferrous chloride, hydrochloric acid, potassium chloride, glacial acetic acid, disodium hydrogen phosphate and dihydrogen potassium phosphate were all BDH general purpose chemicals and distilled prior to use. Dimethyl sulfoxide (M and B, England), hydrogen peroxide (Merck, Germany) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid butylated hydroxy anisole (BHA) and α-tocopherol were obtained from Sigma Chemical Co (St Louis, MO).

Equipment and apparatus: Aspirator bottles (for cold extraction), Mettler analytical balance H80 (UK), Water Bath (Gallenkamp), Rotavapor RII0 (Buchi, England), silica gel GF_{254} (precoated aluminium sheets-Merck Germany), Oven (Gallempkamp), pH meter (Jenway model), UV-Visible spectrophotometer (Unico1200 and Perkin Elmer lambda 25 models, Germany). GC-Mass spectrophotometer (Agilent technologies).

Sample collection/preparation: Seeds of *R. communis* weighing 2 kg were collected in Ibadan, Oyo State in August, 2010 and air-dried for 5 weeks until the weight were constant and then pulverized using mill machine. The pulverized samples were weighed and kept for further analysis.

Reference standards: Ascorbic acid, Butylated hydroxy anisole (BHA) and α-tocopherol for antioxidant activity.

Extraction/partitioning procedure: The powdered seeds (1.95 kg) were extracted with 5 L of methanol by cold extraction. The sample was soaked for 72 h. The extract obtained separated into three layers and was separated using separating funnel to give C1 (golden coloured oily substance; 368.9 g), C2 (dusty brown substance; 55 g) and C3 (chocolate brown substance; 5 g), respectively. The extracts were collected and concentrated with the aid of a Buchi rotavapor at 37°C and stored at 0°C prior to further analysis. Thin Layer Chromatography (TLC) was employed using silica gel 60 F_{254} precoated plates and solvent system: Ethyl acetate/methanol (8:2) and (7:3) to detect antioxidant activity by using 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) as a spray reagent. Instant yellow coloration on the spots on the TLC plates indicates that C1 and C2 have antioxidant activity. C3 did not give satisfactory screening result. Quantitative antioxidant screening; lipid peroxidation and free radical scavenging activity tests were carried out on C1 and C2 using the following spectrophotometric experiments; peroxide oxidation by ferric thiocyanate method and scavenging effect on DPPH and scavenging effect on hydroxy radical generated by hydrogen peroxide.

Antioxidant activities of *R. communis* extracts

Peroxide oxidation by ferric thiocyanate (FTC) method: The antioxidant activities of C1 and C2 obtained from methanolic extract of *R. communis* were determined by ferric thiocyanate method (Oloyede *et al*., 2010a). Ten milligram of each extract was dissolved separately in 99.5% of ethanol and various concentrations (0.00625-0.8 mg mL^{-1}) were prepared. A mixture of a 2 mL of sample in 99.5% ethanol, 2.0 mL of 2.51% linoleic acid in 99.5% ethanol, 4 mL of 0.05 M phosphate buffer (pH 7.0) and 2 mL of water was placed in a vial with a screw cap and placed in an oven at 60°C in the dark. To 0.1 mL of this sample solution, 10 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate was added. After the addition of 0.1 mL of 2×10^{-2} M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the red colour that developed was measured in 3 min at 500 nm. The control and standards were subjected to the same procedures as the sample, except that for the control, only solvent was added and for the standard, sample was replaced with the same amount of Butylated hydroxy anisole (BHA), ascorbic acid and α-tocopherol (reference compounds) (Oloyede *et al*., 2010a). All test and analysis were run in triplicates and the results obtained were averaged. The inhibition of lipid peroxidation in percentage was calculated using this equation:

$$\text{Inhibition } (\%) = 1 - \frac{A1}{A2} \times 100$$

where, A1 was the absorbance of the test sample and A2 was the absorbance of control reaction.

Scavenging effect on DPPH: The antioxidant activity or the capacity to scavenge the stable free radical DPPH was determined using the DPPH free-radical scavenging method. The stable radical 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), (3.94 mg) was dissolved in methanol (100 mL) to give a 100 μM solution. To 3.0 mL of the methanolic solutions of DPPH was added 0.5 mL of each of the fractions with doses ranging from 1.0 to 0.0625 mg mL^{-1} (Gulcin *et al*., 2002; Mutee *et al*.,

2010; Oloyede *et al*., 2010b). The decrease in absorption at 517 nm of DPPH was measured 10 min later. The actual decrease in absorption was measured against that of the control and the percentage inhibition was also calculated. The same experiment was carried out on butylated hydroxy anisole (BHA), α-tocopherol and ascorbic acid which are known antioxidants. All test and analysis were run in triplicates and the results obtained were averaged. The Radical Scavenging Activity (RSA) was calculated as the percentage inhibition of DPPH discoloration using the equation below:

$$\text{Inhibition } (\%) = \frac{A_{DPPH}-A_S}{A_{DPPH}} \times 100$$

where, A_S is the absorbance of the solution and A_{DPPH} is the absorbance of the DPPH solution.

Scavenging effect on hydrogen peroxide: The ability to scavenge hydroxyl radical generated from hydrogen peroxide was spectrophotometrically determined at 285 nm. A solution of 2 mM hydrogen peroxide was prepared in Phosphate Buffered-Saline (PBS) pH 7.4. The fractions (C1, C2) at the following concentrations; 0.1-0.00625 mg mL^{-1} was added to the H_2O_2 solution. Decrease in absorbance of H_2O_2 at 285 nm was determined spectrophotometrically 10 min later against a blank solution containing the test extract in PBS without H_2O_2. All tests were run in triplicates and averaged (Soare *et al*., 1997; Oloyede and Farombi, 2010). The same experiment was carried out on Butylated hydroxy anisole (BHA), ascorbic acid and α-tocopherol.

Analysis of the oil: Gas chromatography: GC-MS analysis of the oil (C1) was carried out on an Agilent technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. An Agilent (9091)-413:325°C HP-5 column (30×320×0.25 μm) was used with helium as carrier gas at a flow rate of 3.3245 mL min^{-1}. The GC oven temperature was initially programmed at 50°C (hold for 1 min) and finally at 300°C (hold for 5 min) at a rate of 80°C min^{-1}, while the trial temperature was 37.25°C. The column heater was set at 250°C and was a split less mode while the pressure was 10.153 psi with an average velocity of 66.45 cm sec^{-1} and a hold-up time of 0.75245 min was recorded. Mass spectrometry was run in the electron impact mode (EI) at 70 eV. The percentage compositions were obtained from electronic integration measurements using Flame Ionization Detector (FID), set at 250°C. The peak numbers and relative percentages of the characterized components are given in Table 4.

Gas chromatography-mass spectrometry: The oil were analysed by GC-MS on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683 B series device. An Agilent (9091)-413:325°C HP-5 column (30×320×0.25 μm) was used with helium as carrier gas at a flow rate of 3.3245 mL min^{-1}. GC oven temperature and conditions were as described above. The injector temperature was at 250°C. Mass spectra were recorded at 70 eV. Mass range was from m/z 30 to 500.

Identification of oil components: The individual constituents of the oil were identified on the basis of their retention indices determined with a reference to a homologous series of n-alkanes and by comparison of their mass spectral fragmentation patterns (NIST 08.L database/chemStation data system) with data previously reported in literature (Ganesh and Vennila, 2011).

RESULTS AND DISCUSSION

Antioxidant activity by ferric thiocyanate method (FTC): The FTC method was used to determine the amount of peroxide which oxidized ferrous chloride ($FeCl_2$) to a reddish ferric chloride ($FeCl_3$) pigment. Peroxide is known to be involved in free radical chain reaction thereby causing damages to biological macromolecules. C1 and C2 at various concentrations (0.00625-0.8 mg mL^{-1}), showed antioxidant activities in a concentration dependent manner. The concentration of peroxide decreases as the antioxidant activity increases for C1 while for C2, though there was decrease in concentration of peroxide, the reverse is the case (Table 1). Also, as a function percentage inhibition, at 0.8 mg mL^{-1}, C1 had percentage inhibition of 93.98% while C2 gave 90.10% inhibition but at the lowest concentration 0.00625 mg mL^{-1}, the inhibition was 91.30%. These activities were better than those of the standards used, ascorbic acid (76.98%), BHA (56.51%) and α-tocopherol (82.23%) at the same concentration (Fig. 1).

Scavenging effects on DPPH: Methanolic solution of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) is used in this assay. DPPH is known to be a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soare *et al*., 1997). The reduction in absorbance of DPPH at 517 nm caused by the samples was measured in triplicate after 10 min. At 517 nm, the absorbance of the DPPH solution was 0.933 nm. The tested samples showed very good activity when compared to the standard used (Table 2). At the entire concentration tested, there was decrease in absorption at 517 nm indicating that the fractions have hydrogen donating ability

Table 1: Peroxide oxidation of fractions (C1, C2) from *R. communis* methanol extract at 500 nm using the ferric thiocyanate method*

Conc. (mg mL^{-1})	C1	C2	Ascorbic acid	BHA	Alpha tocopherol
0.8	0.045±0.001	0.074±0.002	0.173±0.008	0.326±0.006	0.133±0.004
0.4	0.063±0.004	0.074±0.004	0.173±0.008	0.375±0.008	0.164±0.006
0.2	0.074±0.000	0.074±0.001	0.245±0.008	0.431±0.008	0.184±0.009
0.1	0.094±0.005	0.071±0.003	0.275±0.006	0.616±0.005	0.195±0.023
0.05	0.128±0.002	0.069±0.012	0.287±0.050	0.647±0.004	0.294±0.004
0.025	0.202±0.002	0.067±0.003	0.367±0.004	0.653±0.008	0.340±0.069
0.0125	0.208±0.011	0.065±0.001	0.516±0.008	0.747±0.003	0.360±0.005
0.00625	0.247±0.007	0.049±0.006	0.668±0.002	0.750±0.001	0.377±0.008

*Absorbance measurement of C1, C2, ascorbic acid, BHA and α-tocopherol at 500 nm

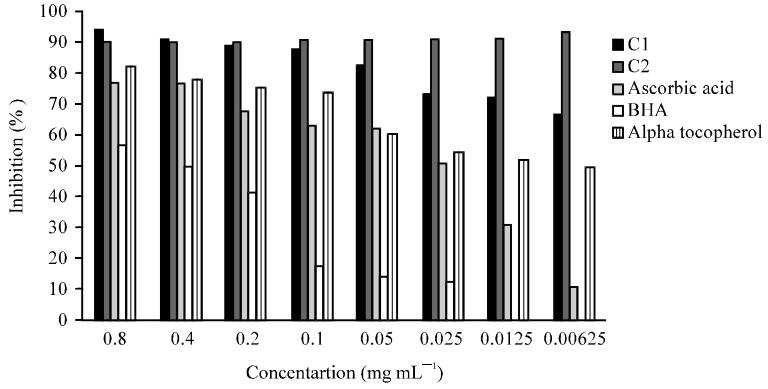

Fig. 1: Peroxide oxidation of the extracts from *R. communis* seeds and standards at 500 nm measured in triplicate

Table 2: Absorbance values from scavenging effect of C1 and C2 from *R. communis* methanol extract on DPPH at 517 (nm)*

Conc. (mg mL^{-1})	C1	C2	Ascorbic acid	BHA	Alpha tocopherol
1.0	0.033±0.102	0.015±0.020	0.195±0.001	0.043±0.016	0.032±0.045
0.5	0.036±0.200	0.006±0.112	0.208±0.012	0.062±0.019	0.063±0.032
0.25	0.042±0.042	0.015±0.243	1.265±0.119	0.074±0.015	0.155±0.061
0.125	0.046±0.100	0.013±0.275	2.759±0.049	0.095±0.003	0.181±0.015
0.0625	0.042±0.002	0.006±0.162	2.924±0.211	0.113±0.014	0.494±0.017

*Absorbance measurement for C1, C2, ascorbic acid, BHA and α-tocopherol at 517 nm

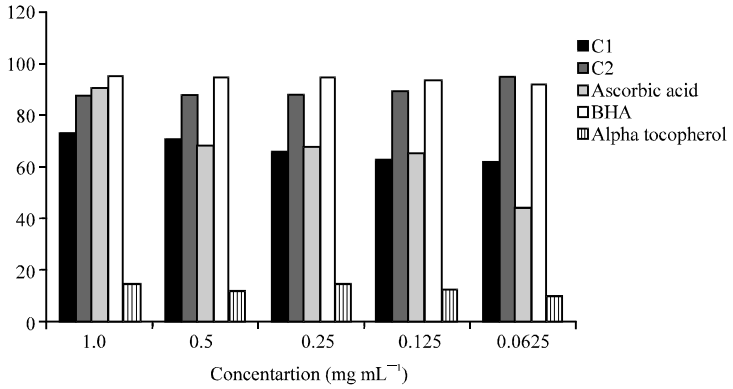

Fig. 2: DPPH free radical scavenging activity of extracts from *R. communis*

or can scavenge free radical. The observed result was also supported by the calculated percentage inhibition. At 1.0 mg mL^{-1}, C1 had% inhibition of 73.71% while C2 gave 87.92% inhibition but at the lowest concentration of 0.0625 mg mL^{-1}, C1 gave 61.32% and C2 gave 94.84% inhibition, respectively, these inhibitions though higher than that of ascorbic acid and α-tocopherol was lower than percentage inhibition of BHA. Therefore, fractions from *R. communis* have good activities as free radical scavengers when compared with controls, ascorbic acid butylated hydroxy anisole (BHA) and α-tocopherol (Table 2, Fig. 2).

Scavenging effects on hydrogen peroxide (H_2O_2): C1 and C2 and the reference antioxidants; ascorbic acid, Butylated hydroxy anisole (BHA) and α-tocopherol were subjected to hydroxyl radical scavenging activity and the result is shown Table 3. Scavenging effects on H_2O_2 was measured in triplicates after 10 min of incubation at 285 nm. Hydrogen peroxide is an active-oxygen specie and has potential to produce the highly reactive hydroxyl radical which is often involved in free radical chain reactions (Namiki, 1990; Lugasi *et al*., 1999).

Hydroxyl radical scavenging ability of the fractions from *R. communis* is seen in Table 3 and Fig. 3, respectively. The two fractions at concentration of 0.1-0.0065 mg mL^{-1} scavenged hydroxyl radical in a concentration dependent manner and activities were significant at $p<0.05$ when compared to standards. Fractions C1 and C2 showed increase in absorption measurement as the concentration decreases but the standards showed a reverse relationship. The% inhibition of C1 was 85.07% while C2 gave 94.91% inhibition at 0.1 mg mL^{-1}. Inhibition increases as the concentration is decreased (Fig. 3). C2 therefore, showed better activity than C1 but same activity as ascorbic acid (94.91%) and lower than activity of BHA (98.90%) and α-tocopherol (99.15%). *R. communis*, therefore has ability to scavenge the highly reactive hydroxyl radicals and could be a source of antioxidant compounds.

Table 3: Scavenging effect of hydroxyl radical generated by H_2O_2 of the *R. communis* extracts at 285 nm*

Conc. (mg mL^{-1})	C1	C2	Ascorbic acid	BHA	Alpha tocopherol
0.1	3.662±0.095	0.981±0.002	0.1952±0.001	0.0413±0.016	0.0321±0.045
0.05	2.873±0.009	0.451±0.002	0.2078±0.012	0.0617±0.019	0.0633±0.032
0.025	1.056±0.007	0.310±0.001	1.2645±0.119	0.0740±0.015	0.1552±0.061
0.0125	0.793±0.002	0.221±0.001	2.7586±0.049	0.0947±0.003	0.1807±0.015
0.00625	0.563±0.005	0.192±0.000	2.9236±0.211	0.1126±0.014	0.4940±0.017

*Absorbance measurement of C1, C2, ascorbic acid, BHA and α-tocopherol at 285 nm

Table 4: Chemical composition of C1 from *R. communis* seeds from GC-MS analysis*

Retention time (min)	Chemical composition	Total (%)
15.369	(Z, Z)-9, 12-octadecadienoic acid, methyl ester	05.92
15.666	(Z, Z)-9, 12-octadecadienoic acid	12.99
16.626	Methyl ricinoleate	46.68
17.049	Ricinoleic acid	34.41
		100

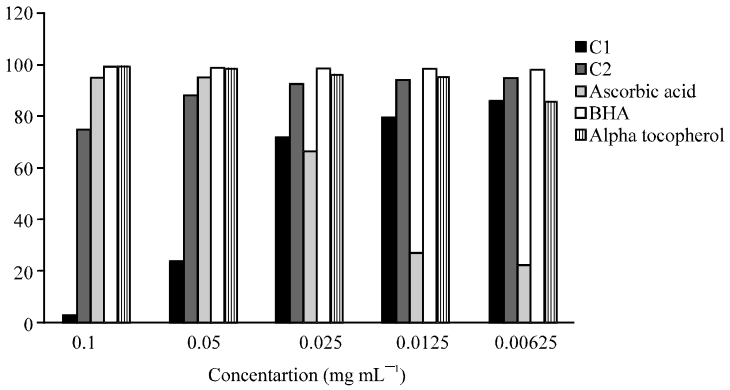

Fig. 3: H_2O_2 Free radical scavenging activity of the extracts from *R. communis* and standards at 285 nm measured in triplicate

GC-MS analysis of the oil: The methanolic fraction C1, golden brown oil from seeds of *R. communis,* analyzed by GC and GC/MS systems using a polar column gave 4 constituents representing 100% of the total chemical components identified in the oil. The result of the analysis is presented in Table 4.

The presence of these compounds: (Z, Z)-9, 12-Octadecadienoic acid, methyl ester, (Z, Z)-9, 12-Octadecadienoic acid, Methyl ricinoleate and Ricinoleic acid supports the ethno-medicinal application of *R. communis* seeds extracts in medicine (Fig. 4). Linoleic acid or (Z, Z)-9, 12-Octadecadienoic acid is an unsaturated n-6 fatty acid that is essential for good health and has been found to have many physiological activities among which are; acne reductive, anti-inflammatory and moisture retentive properties when applied topically on the skin. The industrial application has also been documented; especially in making oil paints and varnishes. Methyl ricinoleate is used as a plasticiser, lubricant, emollients and in skin conditioning or as a fragrance (Letawe *et al*., 1998; Darmstadt *et al*., 2002). Ricinoleic acid (12-hydroxy-9-cis-octadecenoic acid) is also an unsaturated omega-9 fatty acid that occurs naturally in mature Castor plant and has been shown to exert anti-inflammatory and analgesic effects (Vieira *et al*., 2000).

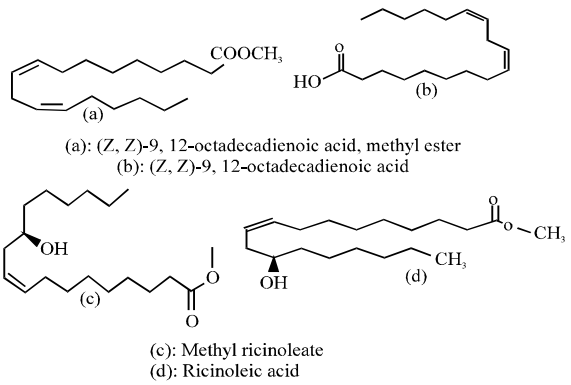

Fig. 4: Structures of the chemical constituents of *R. communis* seeds extract obtained from GC/GC-MS analysis

CONCLUSION

Antioxidant activity of *R. communis* seed extracts were investigated using Lipid peroxidation by ferric thiocyanate method and free radical scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) and hydroxyl radical generated from hydrogen peroxide. It was revealed the fractions possessed significant antioxidant activity when compared with antioxidant standards Butylated Hydroxy Anisole (BHA), ascorbic acid and α-tocopherol used in the assay. The high antioxidant activity of the seed of *R. communis* at low concentration shows that it could be very useful for the treatment of ailments resulting from oxidative stress. Also the four major chemical constituents have been shown to be responsible for the observed antioxidant activities and they are Methyl ricinoleate, Ricinoleic acid, (Z, Z)-9, 12-octadecadienoic acid and (Z, Z)-9, 12-octadecadienoic acid, methyl ester.

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