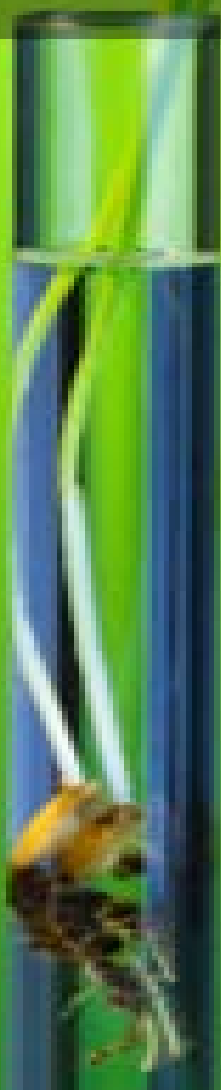


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## Full Length Research Paper

# Assessment of antioxidant capacity of ethanolic extract of *Portulaca oleracea* leaves *in vitro* and *in vivo*

Hefnawy Taha and Ali Osman

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The purpose of the present study was to prepare ethanol extract of *Portulaca oleracea* L. leaves (PLE) that is rich in phenolic compounds and investigate there *in vitro* and *in vivo* antioxidant activities using ethanol to induce oxidative stress in the liver. This study includes two phases. The first phase includes assessment of antioxidant capacity *in vitro* using 1,1- Diphenyl-2-picrylhydrazyl (DPPH) assay and 2,2-azinobis-(3-ethyl-benzothiazole-6-sulfonic acid) (ABTS) assay. In the second phase (assessment of antioxidant capacity *in vivo*), the levels of antioxidant parameters in the alcoholic liver disease rats were studied and the protective effect of ethanolic extract of *P. oleracea* L. in the alcoholic liver disease rats was examined to further understand their mechanisms. *In vitro* studies with DPPH and ABTS showed that PLE possesses antioxidant activity. *In vivo* administration of ethanol (7.9 g/kg body weight/day) for 45 days with PLE (100 mg/kg body weight/day) significantly decreased the activities of liver markers enzyme serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) and gamma glutamyl transferase in serum towards near normal level. In addition, PLE also significantly reduced the levels of lipid peroxidation and in addition, significantly restored the enzymatic and non-enzymatic antioxidants level in the liver of alcohol administration rats. Our study suggests that ethanol extract of *P. oleracea* leaves play a beneficial role in the treatment of alcohol induced tissue damage, which could be one of its therapeutic values.

**Key words:** Alcoholic liver disease, *Portulaca oleracea* L., oxidative stress, ethanolic extract, antioxidants, rats.

## INTRODUCTION

Oxidative stress is an important factor in the genesis of many pathologies, from cancer to cardiovascular and degenerative diseases (Gul et al., 2014; Zia-Ul-Haq et al., 2014 a,b; Galli et al., 1999; Parthasarathy et al., 2001; Cooke et al., 2003, Soltani et al., 2009). Oxidative stress can cause damage to lipids, proteins, and nucleic acids, resulting in changes in signal transduction pathways, gene expression, cell mutagenesis, and cell death

(Baratta, 2000). Alcoholic liver disease is a major cause of morbidity and mortality worldwide. Patients with cirrhosis caused by alcohol are at risk for developing complications associated with a failing liver. The long-term management of alcoholic liver disease stresses the following: Abstinence of alcohol (Grade 1A), with referral to an alcoholic rehabilitation program; Adequate nutritional support (Grade 1B), emphasizing multiple feedings

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and a referral to a nutritionist; Routine screening in alcoholic cirrhosis to prevent complications; Timely referral to a liver transplant program for those with decompensated cirrhosis; Avoid pharmacologic therapies, as these medications have shown no benefit.

Antioxidants play an important role in the human body by reducing oxidative reactions. Especially, endogenous antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic antioxidants such as vitamin C,  $\alpha$ -tocopherol and selenium protect internal organs and tissues from oxidative damage by various toxic reactive oxygen and nitrogen species (Ahn et al., 2004). In order to protect the body against the consequences of oxidative stress, an efficacious approach consists in improving the antioxidant nutrition. In this regard, scientific studies have shown that antioxidants from natural sources have a higher bioavailability and therefore higher protective efficacy than synthetic antioxidants (Gey, 1998). Focusing our attention on natural and bio-available sources of antioxidants, we undertook to investigate the antioxidant properties of *P. oleracea* L. leaves extract. *P. oleracea* has been shown to display radical scavenging activity (Lim and Quah, 2007; Oliveira et al., 2009; Siriamornpun and Suttajit, 2010).

*P. oleracea* L., belonging to the Portulacaceae family, is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution. It is eaten extensively as a potherb and added in soups and salads around the Mediterranean and tropical Asian countries and has been used as a folk medicine in many countries. Diverse compounds have been isolated from *P. oleracea*, such as flavonoids, alkaloids, polysaccharides, fatty acids, terpenoids, sterols, proteins vitamins and minerals. *P. oleracea* possesses a wide spectrum of pharmacological properties such as neuroprotective, antimicrobial, antidiabetic, antioxidant, anti-inflammatory, antiulcerogenic, and anticancer activities (Zhou et al., 2015).

*P. oleracea* is listed in the World Health Organization as one of the most used medicinal plants and it has been given the term 'Global Panacea' (Chu et al., 2002; Katalinic et al., 2006). Thus, the purpose of the present study was to prepare ethanol extract of *P. oleracea* L. leaves that is rich in phenolic compounds and investigate their *in vitro* and *in vivo* antioxidative activities using ethanol to induce oxidative stress in the liver.

## MATERIALS AND METHODS

### Chemicals and reagents

Ethanol, ascorbic acid and potassium persulfate were obtained from Merck (Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma (St. Louis, MO). 2,2-azino-bis-(3-ethylbenzothiazole-6-sulfonic acid) (ABTS) was obtained from Fluka (Fluka Chemie GmbH, Stenheim, Germany). All other reagents were of analytical grade.

### Plant

Fresh *P. oleracea* leaves were collected from the farm of Faculty Agricultural, Zagazig University, Egypt. The plant material was stored at room temperature in a dry place prior to use.

### Animals

Healthy male white albino rats (*Rattus norvegicus*), Wistar strain (160  $\pm$  10 g, body weight) were obtained from Organization of Biological Products and Vaccine (Helwan farm, Cairo, Egypt) and housed in plastic cages in groups of 5 animals/cage. The experimental animals were allowed to acclimatize under the laboratory conditions (temperature of 25  $\pm$  5°C; relative humidity 50 to 70% and normal light/dark cycle) for 2 weeks at least prior the experiment. They were provided with balanced pelleted diet (23% protein) and tap water *ad libitum* throughout the adaptation and experimental period.

### Sample preparation and extraction

Air dried leaves of *P. oleracea* L. were dried in a vacuum oven at 40°C and ground to a fine powder in a mill. Ground material (10 g) was extracted with ethanol 80% (100 ml) using magnetic stirrer at room temperature for 72 h followed by filtration through Whatman No.1 filter paper and concentrated to dryness with a rotary evaporator (BÜCH-water bath-B-480, Switzerland) and lyophilized by Freeze-Dryer (Thermo-Electron Corporation-Heto power dry LL300 Freeze Dryer, Czech Republic). The dried extract (PLE) after evaporation and lyophilization was stored at -20°C until further use.

### Assessment of antioxidant capacity *in vitro*

#### DPPH radical-scavenging activity assay

The electron donation ability of the obtained extract was measured by bleaching of the purple colored solution of DPPH according to the method of Hanato et al. (1988) with slight modification. One milliliter of *P. oleracea* leaves ethanolic extract (PLE) at different concentration (0, 50, 100, 150 and 200  $\mu$ g ml<sup>-1</sup>) was mixed with 4 ml of 0.15 mM DPPH (in 80% ethanol). The mixture was then shaken vigorously using a mixer. The reaction mixture was incubated for 30 min in the darkness at room temperature. The absorbance of the resulting solution was measured at 517 nm using JENWAY 6405 UV/visible spectrophotometer (UK). Ethanol 80% and ascorbic acid was used as a control and standard sample, respectively.

Percentage of antioxidant activity of free radical DPPH was calculated as follow:

$$\text{Antioxidant activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

The IC<sub>50</sub> value was defined as an effective concentration of extract that is required to scavenging 50% of radical activity. All experiments were carried out in triplicate.

#### ABTS<sup>•+</sup> radical-scavenging activity assay

For the ABTS<sup>•+</sup> assay, the method of Re et al. (1999) was adopted. The stock solutions were 7 mmol l<sup>-1</sup> ABTS<sup>•+</sup> solution and 2.4 mmol l<sup>-1</sup> potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 to 16 h at room temperature in the dark. One milliliter of the resulting ABTS<sup>•+</sup> solution was diluted with 60 ml of methanol. ABTS<sup>•+</sup> solution was freshly prepared for each

assay. Ten microliters of each extract (0, 50, 100, 150 and 200  $\mu\text{g ml}^{-1}$ ) was allowed to react with 5 ml of ABTS<sup>•+</sup> solution for 7 min, then the absorbance at 734 nm was recorded. A control with no added extract was also analyzed. Scavenging activity was calculated as follows:

$$\text{ABTS}^{\bullet+} \text{ radical-scavenging activity (\%)} = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} \times 100$$

where  $\text{Abs}_{\text{control}}$  is the absorbance of ABTS<sup>•+</sup> radical+methanol and  $\text{Abs}_{\text{sample}}$  is the absorbance of ABTS<sup>•+</sup> radical + extract/synthetic antioxidant.

The  $\text{IC}_{50}$  value was defined as an effective concentration of extract that is required to scavenging 50% of radical activity. All experiments were carried out in triplicate.

### Assessment of antioxidant capacity *in vivo*

In the present study, alcoholic liver disease rat's model is established. Furthermore, the levels of antioxidant parameters in the alcoholic liver disease rats were studied and the protective effect of ethanolic extract of *P. oleracea* L. leaves in the alcoholic liver disease rats was examined to further understand their mechanisms.

### Experimental design

The animals were randomly divided into six groups of six rats in each group. The extract and alcohol was administered as aqueous solution using intragastric tube daily for 45 days.

Group 1: Control rats (normal rats) treated with 30% glucose (isocaloric to ethanol) and 0.1% carboxymethylcellulose (CMC);

Group 2: Control rats orally received PLE (100 mg/kg body weight) suspended in 0.1% CMC; Group 3: Normal rats orally received 20% ethanol (3.95 g/kg body weight twice a day that is 7.9 g/kg/day) (Rajakrishnan et al., 1997); Group 4: Normal rats orally received 20% ethanol with PLE (25 mg/kg body weight); Group 5: Normal rats orally received 20% ethanol with PLE (50 mg/kg body weight); Group 6: Normal rats orally received 20% ethanol with PLE (100 mg/kg body weight).

### Preparation of serum, plasma and tissue homogenate

At the end of the experimental period, the animals were sacrificed by cervical decapitation. Blood was collected and centrifuged for serum separation. For plasma, blood was collected with anticoagulant and centrifuged (2000 g for 20 min) to separate plasma. The tissues were dissected out, weighed and washed using ice cold saline solution. Tissues were minced and homogenized (10% w/v) in Tris-HCl buffer (0.1M; pH 7.4) and centrifuged at 3000 g for 20 min at 4°C. The resulting supernatant was used for various biochemical assays.

### Biomarkers of oxidative stress

The activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits. Gamma glutamyl transferase (GGT) activity was determined by the method of Rosalki and Rau (1972). Superoxide dismutase (SOD) content was measured using the technique of Kakkar et al. (1984). A single unite of enzyme was expressed as 50% inhibition of nitroblue tetrazolium (NBT) reduction/min/mg protein. The catalase (CAT) activity was measured calorimetrically at 620 nm and expressed as

1 mol of  $\text{H}_2\text{O}_2$  consumed/min/mg protein as described by Sinha (1972). The glutathione peroxidase (GPx) activity was measured using the Ellman (1959).

Ascorbic acid (vitamin C) concentration was measured using Omaye et al. (1979) method. Vitamin E concentration was measured using Desai (1984) method. Reduced glutathione (GSH) activity was determined using Ellman (1959) method. Lipid hydroperoxides as evidenced by formation of TBARS and hydroperoxides (HP) were measured using Niehius and Samuelsson (1968) and Jiang et al. (1992) methods, respectively.

### Histopathological investigation

The liver samples were fixed for 48 h in 10% formal-saline, and were dehydrated by passing successfully in different mixture of ethyl alcohol-water, cleaned in xylene and embedded in paraffin. Section of liver (5 to 6  $\mu\text{m}$  thick) were prepared and then stained with hematoxylin and eosin dye (H&E), which was mounted in neutral distyrene-dibutyl phthalate-xylene (DPX) medium for microscopic observations.

### Statistical analysis

The data for various biochemical parameters were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using statistics software package (SPSS for Windows, V. 13.0, Chicago, USA). P values <0.05 were considered as statistically significant.

## RESULTS

### Assessment of antioxidant capacity *in vitro*

Figure 1 shows the antioxidant activities of ethanolic *P. oleracea* leaves extract at different concentrations (50, 100, 150 and 200  $\mu\text{g ml}^{-1}$ ) against DPPH and ABTS radicals. The ethanolic extract of *P. oleracea* leaves exhibited a significant inhibition on DPPH and ABTS radicals in a dose dependent manner. The 50% inhibition ( $\text{IC}_{50}$ ) of DPPH and ABTS radicals were shown in a concentration of  $116.25 \pm 2.74$  and  $89.73 \pm 5.56 \mu\text{g ml}^{-1}$ , respectively. The  $\text{IC}_{50}$  values of standard ascorbic acid are  $46.17 \pm 1.74$  and  $29.72 \pm 2.53 \mu\text{g ml}^{-1}$ .

### Assessment of antioxidant capacity *in vivo*

#### Liver AST, ALT, ALP and GGT activities

The results of the changes in activities of the serum enzymes, AST, ALT, ALP and GGT are represented as shown in Table 1. The result shows that ethanol administration significantly ( $P < 0.05$ ) increased the activities of AST, ALT, ALP and GGT ( $165.91 \pm 12.43$ ,  $65.02 \pm 5.47$ ,  $130.92 \pm 9.28$ , and  $5.85 \pm 0.41 \text{ IU l}^{-1}$ ), respectively. Administration of PLE along with alcohol significantly ( $P < 0.05$ ) reversed these functional markers towards near normal in a dose dependent manner. PLE at a dose of 100 mg/kg body weight was more effective ( $80.81 \pm 6.08$ ,  $25.60 \pm 1.99$ ,  $83.47 \pm 7.38$  and  $2.18 \pm 0.28 \text{ IU l}^{-1}$ ),



**Table 1.** Liver AST, ALT, ALP and GGT after daily administration for 45 days of ethanolic extract of *P. oleracea* L. leaves (PLE) at different concentration (25, 50 and 100 mg/kg body weight) in normal and alcoholic liver disease rats (20% ethanol) compared to control.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	GGT (IU/L)
Control	74.29 ± 5.44	23.89 ± 2.39	77.79 ± 4.71	2.14 ± 0.19
Control + PLE (100 mg/kg)	73.56 ± 4.68	20.71 ± 2.16	77.85 ± 5.41	1.83 ± 0.12
20% Ethanol	165.91 ± 12.43	65.02 ± 5.47	130.92 ± 9.28	5.85 ± 0.41
20% Ethanol + PLE (25 mg/kg)	153.07 ± 10.09	50.16 ± 5.23	115.92 ± 8.40	4.68 ± 0.49
20% Ethanol + PLE (50 mg/kg)	120.53 ± 11.26	40.60 ± 3.72	95.40 ± 5.52	3.63 ± 0.33
20% Ethanol + PLE (100 mg/kg)	80.81 ± 6.08	25.60 ± 1.99	83.47 ± 7.38	2.18 ± 0.28

Values are given as mean ± SD for 6 rats in each group. Values not sharing a common superscript letter differ significantly at  $P < 0.05$  (DMRT).

**Table 2.** Levels of lipid peroxidation markers (TBARS and lipid hydroperoxides) in liver tissue after daily administration for 45 days of ethanolic extract of *P. oleracea* L. leaves (PLE) at 100 mg/kg body weight in normal and alcoholic liver disease rats (20% ethanol) compared to control.

Group	TBARS (mM/100 g tissue)	Lipid hydroperoxides (mM/100 g tissue)
Control	0.77 ± 0.05	91.92 ± 8.24
Control + PLE (100 mg/kg body weight)	0.78 ± 0.05	83.31 ± 5.27
20% Ethanol	2.02 ± 0.14	160.27 ± 11.26
20% Ethanol + PLE (100 mg/kg body weight)	0.84 ± 0.08	103.26 ± 9.01

Values are given as mean ± SD for 6 rats in each group. Values not sharing a common superscript letter differ significantly at  $P < 0.05$  (DMRT).

**Table 3.** Levels of non-enzymatic antioxidants (Vitamin C, Vitamin E and GSH) in liver tissue after daily administration for 45 days of ethanolic extract of *P. oleracea* L. leaves (PLE) at 100 mg/kg body weight in normal and alcoholic liver disease rats (20% ethanol) compared to control.

Group	Vitamin C (µmol/mg tissue)	Vitamin E (µmol/mg tissue)	GSH (µmol/mg tissue)
Control	1.91 ± 0.11	2.21 ± 0.11	50.55 ± 3.39
Control + PLE (100mg /kg bw)	1.71 ± 0.11	1.59 ± 0.12	49.59 ± 3.62
20% Ethanol	0.91 ± 0.71	1.04 ± 0.06	27.95 ± 1.99
20% Ethanol + PLE (100 mg/kg bw)	1.35 ± 0.10	1.51 ± 0.10	40.16 ± 2.91

Values are given as mean ± SD for 6 rats in each group. Values not sharing a common superscript letter differ significantly at  $P < 0.05$  (DMRT).

respectively when compared with two other doses (25 and 50 mg/kg body weight). Hence, 100 mg/kg body weight was used for further studies.

Table 2 shows that the level of peroxidation products such as TBARS and lipids hydroperoxide in liver tissue of control and experimental rats. A significant elevation in the levels of lipid peroxidation markers [TBARS (2.02 ± 0.14 mM/100 g tissue) and lipids hydroperoxide (160.27 ± 11.26 mM/100 g tissue)] were observed in ethanol fed group when compared with control rats (0.77 ± 0.05 and 91.92 ± 8.24 mM/100 g tissue), respectively. Administration of PLE (100 mg/kg body weight) along

with ethanol caused a significant decrease in the level of TBARS (0.84 ± 0.08 mM/100 g tissue) and lipid hydroperoxide (103.26 ± 9.01 mM/100 g tissue) in liver when compared with alcohol administrated rats.

Table 3 represents the levels of non-enzymatic antioxidants (vitamin E, vitamin C and GSH) status in tissues. The levels of vitamin C, vitamin E and GSH were significantly ( $P < 0.05$ ) reduced in alcohol treated rats (0.91 ± 0.71, 1.04 ± 0.06 and 27.95 ± 1.99 µmol/mg tissue), respectively when compared with control rats (1.91 ± 0.11, 2.21 ± 0.11 and 50.55 ± 3.39 µmol/mg tissue), respectively. Administration of PLE (100 mg/kg

**Table 4.** The activities of antioxidant enzymes (SOD, CAT, GPx and GST) in liver tissue after daily administration for 45 days of ethanolic extract of *P. oleracea* L. leaves (PLE) at 100 mg/kg body weight in normal and alcoholic liver disease rats (20% ethanol) compared to control.

Group	SOD (U <sup>A</sup> /mg protein)	CAT (U <sup>A</sup> /mg protein)	GPx (U <sup>A</sup> /mg protein)	GST (U <sup>A</sup> /mg protein)
Control	9.57 ± 0.73	78.88 ± 6.56	9.95 ± 0.85	6.81 ± 0.44
Control + PLE(100 mg/kg bw)	9.89 ± 0.87	84.65 ± 6.59	10.07 ± 0.80	6.78 ± 0.46
20% Ethanol	4.77 ± 0.39	56.41 ± 5.47	5.82 ± 0.44	4.26 ± 0.26
20% Ethanol + PLE (100 mg/kg bw)	9.02 ± 0.73	74.65 ± 7.40	9.46 ± 0.55	5.75 ± 0.36

Values are given as mean ± SD for 6 rats in each group. Values not sharing a common superscript letter differ significantly at  $P < 0.05$  (DMRT). <sup>A</sup>Units of enzyme activities are expressed as: SOD – One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min. CAT –  $\mu\text{mol}$  of hydrogen peroxide consumed/min. GPx –  $\mu\text{g}$  of glutathione consumed/min. GST –  $\mu\text{mol}$  of CDNB – GSH conjugate formed/ min.

body weight) to alcohol treated rats significant ( $P < 0.05$ ) restored the levels of non-enzymatic antioxidants in tissues.

The activities of antioxidant enzymes, namely, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) in liver were given as shown in Table 4. A significant ( $P < 0.05$ ) decrease in the activities of enzymatic antioxidants were observed in alcohol treated rats ( $4.77 \pm 0.39$ ,  $56.41 \pm 5.47$ ,  $5.82 \pm 0.44$  and  $4.26 \pm 0.26$  U/mg protein), respectively when compared with control rats ( $9.57 \pm 0.73$ ,  $78.88 \pm 6.56$ ,  $9.95 \pm 0.85$  and  $6.81 \pm 0.44$  U/mg protein), respectively. Administration of PLE (100 mg/kg body weight) to alcohol treated rats significantly ( $P < 0.05$ ) increased the activities of enzymatic antioxidants ( $9.02 \pm 0.73$ ,  $74.65 \pm 7.40$ ,  $9.46 \pm 0.55$  and  $5.75 \pm 0.36$  U/mg protein), respectively when compared with alcohol treated rats.

Histological slices of rats liver were examined at the end of the experimental period. Results are as shown in Figure 2. The liver samples of alcohol-administered rats showed the focal hepatocytes damage and degeneration (Figure 2C). The administration of alcohol along with PLE (100 mg/kg body weight) showed near normal appearance hepatocytes (Figure 2D). The control (Figure 2A) and PLE (Figure 2B) alone treated rats showed normal appearance of liver.

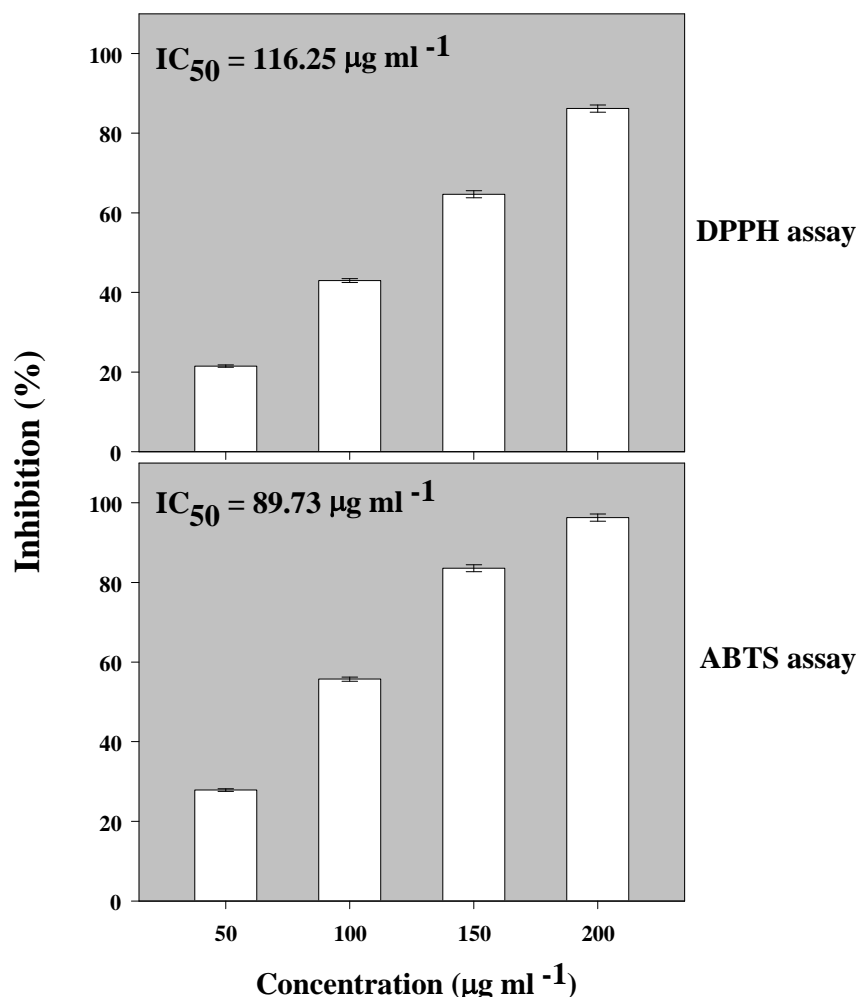
## DISCUSSION

The antioxidant activity of the plant extracts and standard were assessed on the basis of the radical scavenging effect on the stable DPPH and ABTS free radicals. A wide variety of *in vitro* methods to assessed radical scavenging ability of certain agents from natural and synthetic source. DPPH free radical has been used to assess the ability of phenolic compounds to transfer labile hydrogen atoms to radicals (Goupy et al., 2003). Our results showed the capacity of PLE had effective DPPH and ABTS radical scavenging activity in a

concentration dependent manner. There is a significant decrease ( $P < 0.05$ ) in the concentration of DPPH and ABTS due to the scavenging capacity of PLE. Several polyphenol including flavonoids are found in *P. oleracea* leaves (Brand-Williams et al., 1995). Generally, the chemical structure of flavan-3-ol family has good antioxidants response towards DPPH and ABTS radical. The hydrogen donating substituents (hydroxyl groups), attached to the aromatic ring structures of flavonoids, which enable the flavonoids to undergo a redox reaction that helps them to scavenge the free radicals (Brand-Williams et al., 1995).

AST and ALT are the reliable makers for liver function. It is established that AST can be found in the liver, cardiac muscle, skeletal muscle, kidney, brain, pancreas, lungs, leukocytes and erythrocytes whereas ALT presence in liver (Rej, 1997). The increased levels of serum enzyme such as AST and ALT indicate the increased permeability and damage and/or necrosis of hepatocytes (Goldberg and Watts, 1965). The membrane bound enzymes like ALP and GGT are released unequally into bloodstream depending on the pathological phenomenon (Sillanaukee, 1996). In our study, we have found that chronic ethanol consumption caused a significant increased in the activities of AST, ALT, ALP and GGT, which could be a severe damage to tissue membrane. The decreased activities of these enzymes on PLE administrated rats indicate the hepatoprotective effect of *P. oleracea* leaves extract.

Administration of PLE significantly decreased the level of lipid peroxidation when compared with alcohol treated rats, which may be due to the scavenging of free radicals generated by ethanol. It is known that the flavonoids found in *P. oleracea* leaves had inhibitory effect in lipid peroxidation by its free radical scavenging nature (Kravchenko et al., 2003). The flavonoids found in grape leaves such as anthocyanins, catechin and non-flavonoids (stilbene) has been reported to possess the capability to prevent the  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$  induced lipid peroxidation (Fauconneau et al., 1997). Since the transition metals play a central role in lipid peroxidation process,



**Figure 1.** Scavenging activity of ethanolic *P. oleracea* L. leaves extract at different concentrations (50, 100, 150 and 200 µg ml<sup>-1</sup>) against DPPH and ABTS radicals.

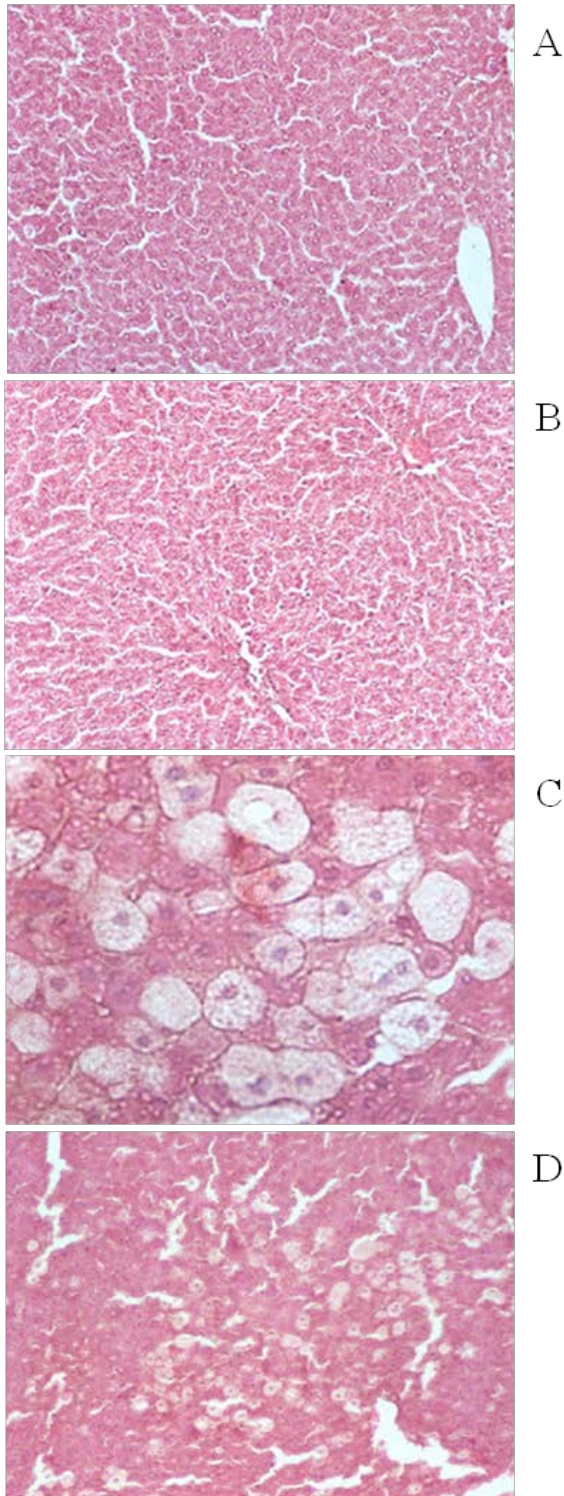
our results obviously indicate that purslane leaves extract has radical scavenging activity and inhibit the lipid peroxidation damage in alcohol toxicity.

Non-enzymatic antioxidants such as GSH, vitamin C and vitamin E are closely interlinked to each other and play an excellent role in protecting the cell from lipid peroxidation. The depleted level of GSH in alcohol toxicity may be due to scavenging of toxic radicals and inhibition of the synthesis and increased rates of turnover (Lieber, 1997). In addition to GSH, we have also observed a decrease in the levels of antioxidants such as vitamin C and E in tissue of alcohol treated rats. Supplementation of PLE to alcohol treated rats restored the non-enzymatic antioxidants levels in liver. The biologically active antioxidants found in *P. oleracea* leaves sparing the antioxidant activity and reduced the consumption of endogenous antioxidants, which could be responsible for the reduction of oxidative stress during ethanol toxicity. The active constituents such as

flavanols, flavanol oligomers and proanthocyanidins were found in *P. oleracea* leaves (Parry et al., 1993) and it has been reported as powerful antioxidants (Lim and Quah, 2007). This could be responsible for the reversal of antioxidants levels in tissues of alcohol fed rats treated with PLE.

Free radical scavenging enzymes such as SOD, CAT, GPx and GST are the first line of defense against oxidative injury. The inhibition of antioxidant system may cause the accumulation of H<sub>2</sub>O<sub>2</sub> or products of its decomposition (Halliwell, 1994). SOD catalyzes the conversion of superoxide anion into H<sub>2</sub>O<sub>2</sub>. The primary role of catalase is to scavenge H<sub>2</sub>O<sub>2</sub> that has been generated by free radical or by SOD in removal of superoxide anions.

The GSTs are a multigene family of isozymes that catalyze the conjugation of GSH to a variety of electrophilic compounds, and thereby exert a critical role in cellular protection against ROS (Hayes and Pulford, 1995). The



**Figure 2.** Histo-pathological examination of H&E stained sections of liver of male Albino rats after daily administration for 45 days of ethanolic extract of *P. oleracea* L. leaves (PLE) at 100 mg/kg body weight in normal and alcoholic liver disease rats (20% ethanol) compared to control. (A) control rat liver, (B) Control + PLE (100mg /kg body weight) rat liver, (C) alcohol treated rat liver and (D) 20% Ethanol + PLE (100 mg/kg body weight) rat liver.

detoxification of 4-hydroxynonenal, a toxic aldehyde generated from ethanol metabolism is compromised when GST activity is reduced. Thus, ethanol or its metabolic products might specifically target GST isoenzymes and the reduction in enzyme activity or expression may contribute to ethanol hepatotoxicity (Alin et al., 1985). The ethanol oxidation by CYP2E1 produces 1-hydroxy ethyl radicals, which have been shown to inactivate several proteins including antioxidant enzyme system (Epstein, 1996). In consistent with these reports, our results also showed that decreased activities of SOD, CAT, GPx and GST in tissues on the chronic alcohol treatment in rats. Administration of PLE restored the activities of enzymatic antioxidant in liver and kidney. Polyphenolic compounds are present in *P. oleracea* leaves, which are powerful antioxidant properties, that are free radical scavenging activity (Lim and Quah, 2007).

The microscopic changes in the liver of alcohol treated rats predominant in the centrilobular region. Hepatic damage observed in the present study may be partially attributed to cytochrome P<sub>450</sub> (Pieffer et al., 1979). Administration of PLE to alcohol treated rats reduced the liver cell damage and improved the histomorphology of the liver near to normal.

The results of functional tests together with histological observations suggest that alcohol leads to serious changes in histology of liver. The increased formation of lipid peroxides and associated reactive oxygen species leads to damage in membrane integrity and other pathological changes in liver. The efficacy of any protective drug is essentially dependent on its capacity of either reducing the harmful effects or in maintaining the normal physiology of cells and tissues, which have been attributed by toxins. The membrane protective properties and antioxidant nature of PLE might be helpful to alleviate the pathological changes caused by alcohol in liver and kidney.

## Conclusion

Our data indicate that PLE (100 mg/kg body weight) has a protective action against alcohol-induced toxicity as evidenced by the lowered tissue lipid peroxidation and elevated levels of the enzymatic and non-enzymatic antioxidants in liver. Hence, our study suggests that PLE play a beneficial role in the treatment of alcohol induced tissue damage, which could be one of its therapeutic values.

## Conflict of interests

The authors declared that there is no conflict of interests.

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## Full Length Research Paper

# Antimicrobial and efflux pumps inhibitory activities of *Eucalyptus grandis* essential oil against respiratory tract infectious bacteria

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Essential oils obtained by hydrodistillation from the fresh and dry leaves of *Eucalyptus grandis* were analyzed by gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS). The main components of the fresh leaf oil were  $\alpha$ -pinene (29.6%), *p*-cymene (19.8%), 1,8-cineole (12.8%) and  $\alpha$ -terpineol (6.4%). While, the dry leaf oil had 1,8-cineole (47.4%), limonene (13.3%),  $\alpha$ -pinene (7.5%) and spathulenol (7.1%). The antimicrobial activities of the essential oils were tested against respiratory tract infectious microorganisms (*Klebsiella pneumoniae*, *Staphylococcus aureus* and *Moraxella catarrhalis*) using the microdilution-broth methods. The minimum inhibitory concentration and minimum bactericidal concentration values of the oils ranged between (0.31 to 1.25) mg/ml and (0.63 - >5) mg/ml respectively. The minimum bactericidal concentration values caused the release of cytosolic lactate dehydrogenase (membrane damage) which ranges from 8 to 24% in comparison with Triton.X-100. The accumulation of rhodamine 6G in bacterial cells showed that the essential oils were effective as efflux pump inhibitors. The results of this study support the use of the plant in folk medicine.

**Key words:** *Eucalyptus grandis*, myrtaceae, essential oil, antimicrobial activity, efflux pump, R6G.

## INTRODUCTION

*Eucalyptus* (Myrtaceae), previously native to Australia, now grows in both tropical and subtropical climates round the world. Different species of these plants are known, but *Eucalyptus globulus* is the most studied (Nagpal et al., 2012). In many countries around the world, traditional healers reportedly use the leaves (fresh and dry) of different species of the genus *Eucalyptus* for asthma,

cough colds, flu, sore throats, bronchitis, pneumonia, aching, stiffness, neuralgia, and as an antibiotic (Bajpai et al., 2008; Hutchings et al., 2006; Hopkins-Broyles et al., 2004). The dry leaves are also consumed as teas or used in bathing (Chen et al., 2006). Sisay (2010), reported that the essential oils of *E. globules* and *Corymbia citriodora*, which have 70% of their constituent to be 1.8 cineole

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are used for the management of bronchitis, asthma, catarrh, sinusitis and throat infections. Among the *Eucalyptus* species, *E. grandis* is the most widely distributed in KwaZulu-Natal Province, South Africa. Traditional healers in this region uses both the fresh and dry leaves of *E. grandis* to treat many illnesses such as infections, colds, flu, sore throats, bronchitis, pneumonia, aching, stiffness, neuralgia, and as an antibiotic (Hutchings et al., 2006; Hopkins-Broyles et al., 2004). Bajpai et al. (2008), reported its use as an anti-fungal agent for some skin infections.

Respiratory tract diseases are diseases that affect the air passages (the nasal passages, bronchi and the lungs) and it includes pneumonia, bronchitis, asthma and pulmonary diseases (Nunez et al., 2000; Nester et al., 2001; van Wyk and Wink 2004; lindell et al., 2005). The respiratory pathogens which affect the respiratory tracts that have been observed to trigger asthma attacks include *Cryptococcus neoformans*, a fungal infection that causes cough and chest pains (Nunez et al., 2000; lindell et al., 2005). *Klebsiella pneumonia*, a gram negative bacterium also known to cause pneumonia with chills, fever and the development of mucoid sputum, coughing and chest pain (Nester et al., 2001). *Moraxella catarrhalis* is a fastidious and nonmotile gram-negative bacteria that causes lower respiratory infection, leading to otitis coupled with sinusitis, shortness of breath, chronic bronchitis and cough (Nester et al., 2001, van Wyk and Wink, 2004).

*Mycobacterium tuberculosis* is a bacterium that causes tuberculosis, chronic cough, fever and bloody sputum (Nester et al., 2001) and *Staphylococcus aureus* a bacterium responsible for lower respiratory tract infections like *K. pneumonia* (Nester et al., 2001). Despite the availability of variety of antibiotics, the pathogens are becoming more resistant to currently used drugs. There is a need to develop new antibacterials, either by improving the molecular design of old antibiotics or by developing efflux pump inhibitors (EPIs) (Nikaido, 1996; Zechini and Versace, 2009). EPIs can become active against multidrug resistance (MDR) pumps by binding directly to the pump and blocking it, in a competitive or non-competitive manner (Mahamoud et al., 2007; Lomovskaya and Bostian, 2006). As a result, there is need to find a plant based therapy to manage these pathogens. The aim of this study was to investigate the antimicrobial and efflux pumps inhibitory activities of *E. grandis* essential oil against respiratory tract infectious bacteria.

## MATERIALS AND METHODS

### Plant material

*E. grandis* Hill ex Maiden was collected from the Mbakanathubana area of Eshowe, KwaZulu-Natal Province, South Africa. The plant sample was identified at the Department of Botany, University of Zululand, KwaDlangezwa and voucher specimens (OS.01UZ) was

deposited at the University Herbarium.

### Extraction of essential oils

Fresh and air-dry leaves of *E. grandis* (300 g) were separately subjected to more than three hours of hydrodistillation, using a Clevenger-type apparatus, according to the standard method recommended by the British Pharmacopoeia (1988). The essential oil obtained was dried over anhydrous sodium sulfate, dissolved in methanol and then stored at - 4°C until required.

### Gas chromatography (GC)

GC essential oils were carried out using an Agilent Gas Chromatography (7890A) equipped with an Agilent 190915 (30 m x 250 µm x 0.25 µm calibrated). The oven temperature was programmed from 50°C (after 2 min) to 240°C at 5°C/ min and the final temperature was held for 10 min. Injection and detector temperatures were 200°C and 240°C respectively. Hydrogen was the carrier gas at flow rate of 1 ml/min. 0.5 µl of the diluted oil was injected into the GC. Peaks were measured by electronic integration. *n*-Alkanes were run at the same condition for retention indices determination.

### Gas chromatography/mass spectrometer (GC/MS)

GC/MS essential oils were carried out using an Agilent Gas Chromatography (7890A) equipped with an Agilent 190915 (30 m x 250 µm x 0.25 µm calibrated ) attached with an Agilent mass spectrometer system (5975C VL MSD with Triple Axis Detector). The oven temperature was programmed from 45°C to 310°C. Helium was used as the carrier gas at a flow rate of 5 ml/min with a split ratio of 1:200. The essential oil (1 µl) was diluted in hexane and 0.5 µl of the solution was manually injected into the GC/MS. The chemical compositions of the essential oil of the leaves of *E. grandis* were determined according to their retention time, and spectrometric electronic libraries (WILEY, NIST).

### Bacteria strains

Bacteria strains used in the study were collected from the Water Department, Umhlatuze municipality and Nkonjeni Hospital, Nongoma, both in KwaZulu-Natal Province, South Africa. These microbes were *K. pneumonia* (ATCC 31488), *S. aureus* (ATCC 25925) and *M. catarrhalis*. The stock cultures were maintained at 4°C in Müeller-Hinton agar (Oxoid, Germany).

### Antimicrobial assay

The minimum inhibitory concentration (MIC) of the essential oils was determined by the method described by Eloff (1998). Nutrient broth (50 µl) was added to all wells of the microtitre plate; 50 µl of the essential oils (10 mg/ml) in 1% DMSO was added to the well in row A and then serially diluted down the rows from row A. The remaining 50 µl was discarded. Bacteria culture (50 µl) of McFarland standard was then added to all the wells and then incubated at 37°C for 24 h. P-iodonitrotetrazolium violet (INT) solution (20 µl of 0.2 mg/ml) was then added to each well and incubated at 37°C for 30 min. The MIC is the lowest concentration at which no visible microbial growth is observed. The minimum bactericidal concentration (MBC) is the lowest concentration of the sample at which inoculated bacterial strains are completely killed. This was confirmed by re-inoculating 10 µl of each culture medium

from the microtiter plates on nutrient agar plates and incubating at 37°C for 24 h. Bacteria treated with ampicillin and neomycin were used as positive controls.

#### Lactate dehydrogenase (LDH) release assay (Membrane Damage)

The cytosolic lactate dehydrogenase release assay was carried out according to the method described by Korzeniewski and Callewaert, (1983) and modified by Badovinac et al. (2000) and Tadić et al. (2012). The susceptible organisms were grown and incubated with the MBC concentrations of the essential oils overnight. The microbial cultures were then centrifuged (5000 g; 5 min). The supernatant (100 µl) was then mixed with 100 µl of lactic acid dehydrogenase substrate mixture of 54 mM lactic acid, 0.28 mM of phenazinemetosulfate, 0.66 mM p-iodonitrotetrazolium violet and 1.3 mM NAD. The pyruvate-mediated conversion of 2,4-dinitrophenyl-hydrazine into visible hydrazone precipitate was measured on an auto microplate reader (BiotekELx 808) at 492 nm. The total loss of membrane integrity resulting in complete loss of cell viability was determined by lysing the cells of untreated organisms with 3% Triton X-100 and using this sample as a positive control. The cytotoxicity in the LDH release test was calculated using the formula:

$$(E-C) / (T-C) \times 100$$

Where E is the experimental absorbance of the cell cultures, C is the control absorbance of the cell medium, and T is the absorbance corresponding to the maximal (100%) LDH release of Triton X-100 lysed cells (positive control).

#### Rhodamine 6G uptake

The activities of the essential oils were tested for their MDR inhibition of Rhodamine 6G (R6G) accumulation using the method of Maesaki et al. (1999) with some modifications. Bacteria were cultured overnight at 37°C with shaking (110 rpm). After 24 h, cells were centrifuged at 4000xg for 5 min and washed twice with phosphate buffer saline (PBS, pH 7.2). Cells were centrifuged again and re-suspended at 40 mg/ml in PBS containing 10 mM sodium azide (NaN<sub>3</sub>). R6G was added to a final concentration of 10 µM, and cells placed in an incubator for 1 h. Cells were then divided into two aliquots, tube 1 and tube 2. Cells were centrifuged for 5 min at 4000 rpm. Cells in tube 1 were re-suspended in PBS containing 1 M glucose while the cells in tube 2 were re-suspended in PBS alone. Essential oils were then added to the cells containing glucose to a final concentration of 100 µM. Both tubes were then placed in an incubator with agitation for 30 min at 37°C. Cells were centrifuged and the supernatant discarded. The remaining pellet was re-suspended in 0.1M glycine HCl, pH 3 and placed in the shaking incubator overnight. After 24 h, cells were centrifuged for 10 min at 4000xg and the supernatant collected, and absorbance read at 527 nm. The accumulation of the R6G was expressed as percentage accumulation in the cells. The percentage accumulation of R6G inside cells after exposure to glucose, essential oil and standards was calculated using this formula:

$$(1-A_t / A_o) \times 100$$

Where A<sub>t</sub> is the absorbance of the test compound, and A<sub>o</sub> is the absorbance of the control in the presence of glucose only.

#### Statistical analysis

The mean and standard error of four experiments for accumulation

of R6G inside cells and controls were determined. Statistical analyses of the differences between mean values obtained were calculated using Graphpad Prism 6. Data were subjected to one way analysis of variance (ANOVA). *P* values ≤ 0.05 were regarded as significant and *P* values ≤ 0.01 as very significant.

## RESULTS

The chemical composition Table 1 of the essential oil from fresh leaves revealed 31 compounds which were about 99.3% of the essential oil. The most abundant compounds found in the oil were α-pinene (29.6%), p-cymene (19.8%) and 1, 8-cineole (12.8%). In the essential oil from the dry leaves, 13 compounds were identified which together constituted 89.2% of the total oil. The major compounds were 1, 8-cineole (47.4%), limonene (13.3%), α-pinene (7.5%) and spathulenol (7.1%). The minimum inhibitory and minimum bactericidal concentrations of the essential oils from the fresh and dry leaves of *E. grandis* against the microorganisms tested are shown in Table 2. The results obtained from the MIC and MBC for the fresh oil revealed *K. pneumonia* to be the most sensitive microorganism with the lowest MIC and MBC values of (0.31 and 0.63) mg/ml, respectively. While, *S. aureus* (1.25 and 2.5) mg/ml and *M. catarrhalis* (1.25) mg/ml, respectively exhibit high MIC and MBC values for the fresh leaf oil. On the contrary, the dry leaf oil displays highest MIC and MBC values, expect for the MIC of *S. aureus* (1.25) mg/ml. % LDH release in comparison to Triton X-100 presented in Table 2 shows low level release of cytosolic LDH from bacteria cells (8 to 24%). The percentage accumulation of R6G inside cells after exposure to glucose, essential oils and the standard inhibitor (beberine) were summarized in Table 2. While, the accumulation over time were displayed in Table 3. The result shows that R6G was bacteria strain specific as observed in Table 2.

## DISCUSSION

Although, drying of plant material has been reported to increase essential oil yields and accelerate distillation by improving the heat transfer (Whish and Willam, 1998). Other advantages of drying include the reduction of microbial growth and the inhibition of some biochemical reactions in the dried materials (Baritiaux et al., 1992; Combrinck et al., 2006). Concentrations of various volatile substances, when leaves are dried, have been observed to increase in numerous species of plants and have been attributed to the breakdown of glycosylated forms, dehydration reactions, and oxidation reactions (Moyler, 1994; Bartley and Jacob, 2000) or due to ruptures in plant cells where the volatile compounds are stored. Some compounds also arise from dehydration of oxygenated compounds which could have occurred during the process of drying (Combrinck et al., 2006). It is



**Table 1.** Chemical composition of essential oils of *E. grandis*.

Compound	KI <sup>a</sup>	KI <sup>b</sup>	Percentage composition	
			Fresh	Dry
$\alpha$ - Pinene	8.10	936	29.6	7.5
Camphene	8.50	950	1.5	-
$\beta$ - Pinene	9.31	964	-	0.2
$\beta$ -Myrcene	9.69	993	-	0.3
<i>p</i> -Cymene	10.72	1025	19.8	5.4
Limonene	10.84	1029	3.1	13.3
1,8-cineole	10.92	1031	12.8	47.4
$\gamma$ -Terpinene	11.71	1060	2.1	1.5
Terpinolene	12.60	1089	0.3	1.6
Carene	12.90	1148	0.2	-
$\beta$ -Fenchol	13.35	1122	1.2	-
<i>trans</i> -Pinocarveol	13.73	1139	0.6	-
Camphor	14.11	1146	2.5	-
Sabinyl acetate	14.82	1166	0.5	-
Borneol	14.90	1169	3.4	-
Terpinen-4-ol	15.22	1177	0.8	-
$\alpha$ -Terpineol	15.62	1189	6.4	1.2
<i>cis</i> -Carveol	16.38	1231	0.4	-
Thymol	18.19	1290	0.2	-
Carvacrol	18.63	1299	0.3	-
Terpinyl acetate	19.97	1349	1.6	-
Caryophyllene	21.90	1419	1.8	-
Alloaromadendrene	26.12	1441	0.2	-
$\gamma$ -Gurjunene	25.53	1447	0.3	-
Viridiflorene	22.83	1497	1.5	-
$\alpha$ -Calacorene	24.87	1546	0.4	-
Spathulenol	25.76	1578	1.4	7.1
Caryophyllene oxide	25.92	1583	1.5	2.7
$\alpha$ -Eudesmol	26.35	1632	0.7	0.3
<i>cis</i> -Cadin-4-en-7-ol	26.65	1637	1.7	-
Epoxy-allo- alloaromadendrene	26.82	1641	1.0	-
Cadine-1,4-diene	26.82	1646	0.4	0.5
Amiteol	27.13	1660	0.1	-
Monoterpene hydrocarbons	-	-	56.6	29.9
Oxygenated monoterpenes	-	-	31.0	48.7
Sesquiterpene hydrocarbons	-	-	4.9	0.5
Oxygenated sesquiterpenes	-	-	6.8	10.1
Total identified	-	-	99.3	89.2

\*Kovats index on a DB-5 column in reference to *n*-alkanes (Adams 1995, 2001). MS, NIST and Wiley libraries spectra and the literature; KI, Kovats index; RT, Retention time.

obvious that the drying process does not only affect the composition of the oils, but the concentration of the components as well. For example, while the concentrations of  $\alpha$ -pinene and *p*-cymene decreased in dry leaf, the concentration of 1,8- cineole and limonene increased in the dry leaf.

However, this study shows that the fresh leaf oil possessed significant antibacterial properties than the dry leaf oil. These compounds have been reported to possess antimicrobial properties (Raju and Maridas, 2011). In addition, readily react with air and heat sources thereby reducing their antimicrobial activity of the dry leaf

**Table 2.** Antibacterial, cytosolic lactate dehydrogenase assay and R6G inside the cell after exposure to *E. grandis* essential oils and Berberine.

Microorganism	MIC <sup>a</sup>		MBC <sup>b</sup>		Percentage accumulation and release				
	Fresh	Dry	Fresh	Dry	LDH <sup>c</sup>		R6G <sup>d</sup>		Beberine
					Fresh	Dry	Fresh	Dry	
<i>S. aureus</i> <sup>e</sup>	1.25	1.25	2.5	> 5	11	8	53	11	98
<i>K. pneumonia</i> <sup>e</sup>	0.31	2.5	0.63	> 5	13	24	9	3	74
<i>M. catarrhalis</i> <sup>f</sup>	1.25	2.5	1.25	> 5	11	20	25	4	67

<sup>a</sup>MIC and <sup>b</sup>MBC values are given as mg/ml for essential oils; <sup>c</sup>LDH release (membrane damage) activity % LDH Release in comparison to Triton X-100; <sup>d</sup>Values of drug accumulation in the presence of glucose were taken as the control; <sup>e</sup> - American Type Culture Collection (ATCC), USA; <sup>f</sup> - Clinical isolates.

**Table 3.** Accumulation of R6G against respiratory tract infectious bacteri<sup>a</sup>.

Microorganism	FLEO	DLEO	Plus Glucose	No Glucose	Beberine
<i>S. aureus</i>	2.01 ± 0.50**	2.43 ± 0.75**	0.75 ± 0.20****	4.30 ± 1.00	2.50 ± 0.80**
<i>K. pneumonia</i>	3.24 ± 0.50*	2.30 ± 0.5***	0.89 ± 1.00****	4.75 ± 0.90	2.37 ± 0.60***
<i>M. catarrhalis</i>	2.84 ± 1.00	2.22 ± 1.00*	1.38 ± 0.50**	4.00 ± 0.50	2.75 ± 1.00

<sup>a</sup>(n = 3, mean ± S.D); FLEO – fresh leaf essential oil; DLEO – dry leaf essential oil.

(Abdelmajeed et al., 2012).

The low levels of cytosolic LDH released (8 to 24%, Table 2) does suggest that, microbial cell membrane damage contributes very little to microbial death. Living cells (including bacteria) have mechanisms that expel toxic substances. These systems (such as resistant-nodulation-division pump) are mostly found in bacteria in which a pump structure (efflux pump) is anchored to the inner membrane to release noxious substances, including antibiotics which are aimed to kill the bacteria (Wexler, 2012). The efflux pump confers bacterial resistance (Amusan et al., 2007). It is therefore important that new antibiotics, specifically efflux pump inhibitors (EPIs) are developed to reduce the emergence of multidrug resistance (MDR). In this study, the essential oils of *E. grandis* were able to increase the accumulation of rhodamine 6G inside bacterial cells, which revealed that the essential oils can apparently be used as efflux pump inhibitors.

The R6G absorption was greater for *K. pneumoniae* and *M. catarrhalis* than *S. aureus* in the presence of the essential oil for the fresh leaves. On the other hand, *S. aureus* had a high accumulation with the dry leaf oil. The uptake of R6G by oil of the fresh leaf was even higher than that of the standard used, while for *Staphylococcus aureus*, the essential oils from the dry leaves were more effective, which showed that plants extracts increase R6G concentration. It is also noted that *S. aureus* had the highest percentage accumulation than the other organisms (Table 3), because gram positive organism have a single layer of cell wall (which make them to be more susceptible to antibiotics) than gram negative which

have a double membrane, making them less susceptible to antimicrobial agents (Kaur and Arora, 2009).

## Conclusion

The essential oils of both the fresh and dry leaves of *E. grandis* showed considerable activities against respiratory tract bacteria. Various respiratory pathogens affect the respiratory tracts which lead to oxidative stress which in turn triggers asthmatic attack (Soyingbe et al. 2013) by destroying the membrane integrity of the bacteria and also blocking the efflux pump mechanism of the bacteria. It is concluded that the essential oils could be used as part of the existing anti-asthma therapy, and also justify its rationale use by traditional healer in the treatment of asthma.

## ACKNOWLEDGEMENTS

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## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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## Full Length Research Paper

# Determination of alkaloid compounds of *Ricinus communis* by using gas chromatography- mass spectroscopy (GC-MS)

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In this study, the alkaloid compounds of *Ricinus communis* have been evaluated. The chemical compositions of the leaf ethanol extract of *R. communis* were investigated using gas chromatography-mass spectroscopy (GC-MS). GC-MS analysis of *R. communis* alkaloid leaf ethanol extract revealed the existence of the n-hexadecanoic acid, octadecanoic acid, 1-hexadecanol, 2-Methyl, gibb-3-ene-1, 10decarboxylic acid, 2,4a, 7trihydroxy-1-methyl-8-methylene, 1.4a-lactone, 10-methyl, L-valine, ethyl ester, hexadecamethyl, tetradecamethyl, octadecamethyl, butanedioic acid, hydroxyl, diethyl ester, 1.1.3.3.5.5.7.7.9.9.11.11.13.13.15.15 hexadecamethyl, triethyl citrate, diethyl phthalate, and 3-octadecene.

**Key words:** Alkaloids, ethanol, gas chromatography-mass spectroscopy (GC-MS) analysis, *Ricinus communis*.

## INTRODUCTION

The castor oil plant *Ricinus communis*, also known as *Palma(e) Christi* or wonder tree (Figure 1), is a perennial scrub of the spurge family Euphorbiaceae. *R. communis* probably originates from Africa and was used in ancient Egypt and by the Romans and Greeks (Waller and Skursky, 1972). Apart from the highly toxic ricin and the less toxic *R. communis* agglutinin, the plant contains another toxic compound, the low molecular weight alkaloid ricinine (MW = 164.2 g/mol). Ricinine or 3-cyano-4-methoxy-N-methyl-2-pyridone (CAS 524-40-3) belongs to the group of piperidine alkaloids. It was first discovered and named by Tuson in the seeds of *R. communis*, while searching for its medically active compounds even before ricin was known. Subsequently, its chemical structure

was identified and its biosynthesis and metabolism was studied (Waller and Skursky, 1972; Mann and Byerrum, 1974).

Ricinine can be found in all parts of the plant and it is a quite strong insecticide. The castor seeds contain approximately 0.2% of the alkaloid. *R. communis* contains a complex cocktail of toxic substances including the type II ribosome-inactivating protein (RIP) ricin, the haemagglutinin RCA120 and the alkaloid ricinine. Furthermore, other compounds like fatty acids, flavonoids and saponins have been found to exhibit deleterious effects on bacteria, virus, fungi, invertebrates and higher animals, seemingly giving the plant some sort of protection in a hostile environment (Sitton and West,

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**Figure 1.** Plant of *Ricinus communis*.

1975; Taylor et al., 1994; Upasani et al., 2003; Bigi et al., 2004; De Assis et al., 2011) Furthermore, allergenic reactions against *R. communis*, in particular the seed dust, were realized. Low molecular proteins, 2S albumins, have been identified as the main allergenic compounds (Thorpe et al., 1988; Bashir et al., 1998; Deus-de-Oliveira et al., 2011). Experimental intoxication studies underline the major contribution of ricin as compared to other hazardous compounds found in the seeds (He, 2010). The oil and seed have been used as folk remedies for warts, cold tumors, indurations of the abdominal organs, whitlows, lacteal tumors, indurations of the mammary gland, corns, and moles, etc. Castor-oil is a cathartic and has labor-inducing properties. Ricinoleic acid has served in contraceptive jellies (Allardice, 1993). Ricin, a toxic protein in the seeds, acts as a blood coagulant. The oil is used externally for dermatitis and eye ailments. The seeds, which yield 45 to 50% of a fixed oil, also contain the alkaloids ricinine and toxalbumin ricin, and is considered purgative, counter-irritant in scorpion-sting and fish poison. The leaves, applied to the head, is used to relieve headache and as a poultice for boil (Foster, 1990).

Phytochemical interactions of poisons lead to injury or death of living tissues. Toxicology is like science and an art like medicine. It includes observational data gathering

and data utilization to predict outcome of exposure in human and animals. The ancient humans categorized some plants as harmful and some as safe. The aim of the present work is to study the toxic nature of the powder of *R. communis* leaves.

## **MATERIALS AND METHODS**

### **Collection and preparation of plant**

In this research, the leaves were dried at room temperature for 10 days and when properly dried the leaves were powdered using clean pestle and mortar, and the powdered plant was size reduced with a sieve. The fine powder was then packed in airtight container to avoid the effect of humidity and then stored at room temperature.

### **Extraction and identification of alkaloids**

The powdered leaves (2 g) were boiled in a water bath with 20 ml of 5% sulphuric acid in 50% ethanol. The mixture was cooled and filtered. A portion was reserved. Another portion of the filtrate was put in 100 ml of separating funnel and the solution was made alkaline by adding two drops of concentrated ammonia solution. Equal volume of chloroform was added and shaken gently to allow the layer to separate. The lower chloroform layer was run off into a second separating funnel. The ammoniacal layer was reserved. The chloroform layer was extracted with two quantities each of 5 ml

of dilute sulphuric acid. The various extracts were then used for the following test.

#### **Mayer's test**

To the filtrate in test tube I, 1 ml of Mayer's reagent was added drop by drop. Formation of a greenish coloured or cream precipitate indicates the presence of alkaloids (Evans, 2002).

#### **Dragendoff's test**

To the filtrate in test tube II, 1 ml of Dragendoff's reagent was added drop by drop. Formation of a reddish-brown precipitate indicates the presence of alkaloids (Evans, 2002).

#### **Wagner's test**

To the filtrate in tube III, 1 ml of Wagner's reagent was added drop by drop. Formation of a reddish-brown precipitate indicates the presence of alkaloids (Evans, 2002).

#### **Gas chromatography-mass spectroscopy (GC-MS) analysis**

GC-MS analysis of the methanol extract of *R. communis* was carried out using a Clarus 500 Perkin Elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold-Perkin Elmer Turbo mass 5.1 spectrometer with an Elite – 1 (100% dimethyl poly siloxane), 30 m × 0.25 mm ID × 1 µm of capillary column. For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization system which was operated in electron impact mode with ionization energy of 70 eV (Imad et al., 2014, 2015a; Muhanned et al., 2015). The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised up to 280°C at the rate of an increase of 5°C min<sup>-1</sup>, and maintained for 9 min. Helium gas (99.999%) was used as carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 µl was employed (split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed at 110°C (isothermal for 2 min), with an increase of 100°C min<sup>-1</sup> to 200°C, then 5°C min<sup>-1</sup> to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min and the total GC-MS running time was 36 min. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45 to 450 (m/z). The mass detector used in this analysis was Turbo Mass Gold-Perkin Elmer and the software adopted to handle mass spectra and chromatograms was a Turbo Mass ver 5.2 (Imad et al., 2015b; Mohammed et al., 2015).

## **RESULTS**

GC-MS analysis of alkaloid compound clearly showed the presence of nine compounds. The alkaloid compound, formula, molecular weight and exact mass are as shown in Table 1. The GC-MS chromatogram of the nine peaks of the compounds detected are as shown in Figure 2-4. Chromatogram GC-MS analysis of the methanol extract of *R. communis* showed the presence of nine major peaks and the components corresponding to the peaks

were determined as follows. The first set up peaks were determined to be n-hexadecanoic acid. The second peaks were indicated to be octadecanoic acid. The next peaks were considered to be 1-hexadecanol, 2-methyl, Gibb-3-ene-1, 10decarboxylic acid, 2,4a, 7trihydroxy-1-methyl—8-methylene, 1,4a-lactone, 10-methyl, L-Valine, ethyl ester, hexadecamethyl, tetradecamethyl, octadecamethyl, Butanedioic acid hydroxyl, Diethyl ester, 1.1.3.3.5.5.7.7.9.9.11.11.13.13.15.15 hexadecamethyl, Triethyl citrate, Diethyl Phthalate, and 3-Octadecene (Figures 5 to 11). The identified phytocompounds have the property of antioxidant and antimicrobial activities (He, 2010; Deus-de-Oliveira, 2011).

## **DISCUSSION**

Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects. Continued further exploration of plant derived antimicrobials is needed today.

Castor bean may become a weed in neglected crop land and pasture. It is not difficult to control through cultivation and mowing. Of greater concern than its weedy potential is the high toxicity of its seeds, which contain ricin, a water-soluble protein. Even a small amount of masticated seed is likely to cause death. Humans and horses are especially vulnerable. Fatal doses are from 2.5 to 6 seeds for humans and about 6 seeds for horses (CISR, 1972). The symptoms are stomach irritation, diarrhea, abdominal pain, increased heart rate, profuse sweating, collapse, and convulsions. Broken seeds can cause skin irritation. The foliage is only slightly toxic (Anonymous, 2000). It is advisable to completely eliminate castor bean from pastures, especially horse pastures, and pinch off flowers of ornamental plants to prevent possible poisoning of children. When assessing the numerous reports on intoxications with ricin, *R. communis* seeds or *R. communis*-containing feed and fertilizer, some general aspects have to be considered. The term ricin in any toxicological publication suggests a degree of homogeneity or a lack of variability that might be expected for pure chemicals (Despeyroux et al., 2000; Thullier and Griffiths, 2009).

Furthermore, other compounds like fatty acids, flavonoids and saponins have been found to exhibit deleterious effects on bacteria, virus, fungi, invertebrates and higher animals, seemingly giving the plant some sort of protection in a hostile environment (Upasani et al., 2003; De Assis et al., 2011). Furthermore, allergenic reactions against *R. communis*, in particular the seed dust, were realized.

## **Conclusion**

*R. communis* is a native plant of Iraq. It contains chemical

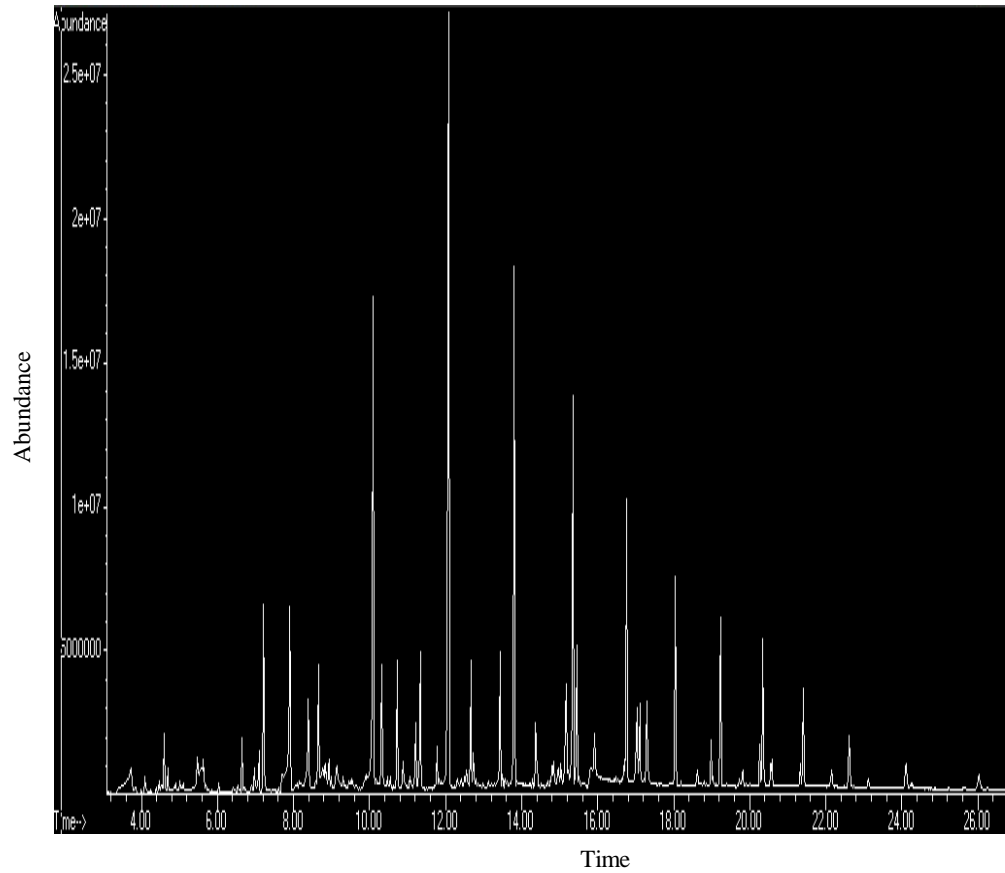


Figure 2. GC-MS Profile of leaves extract of *Ricinus communis*.

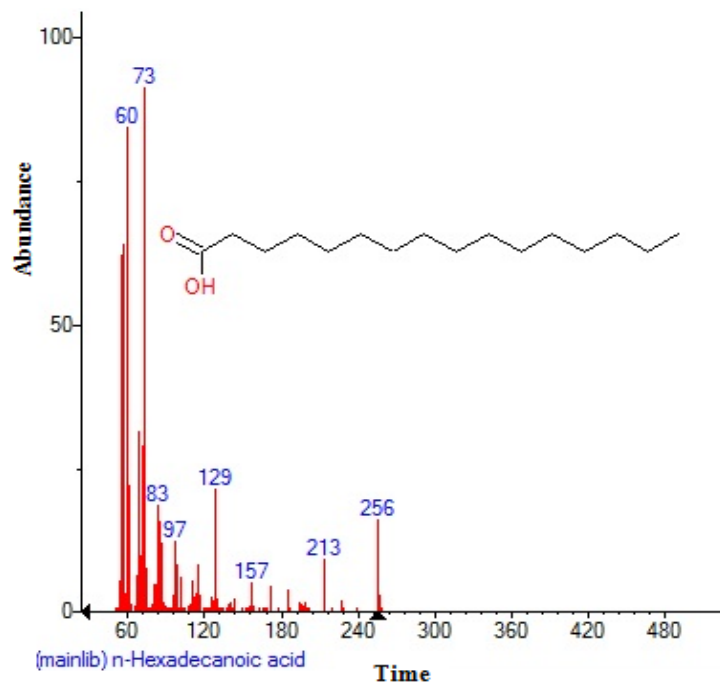
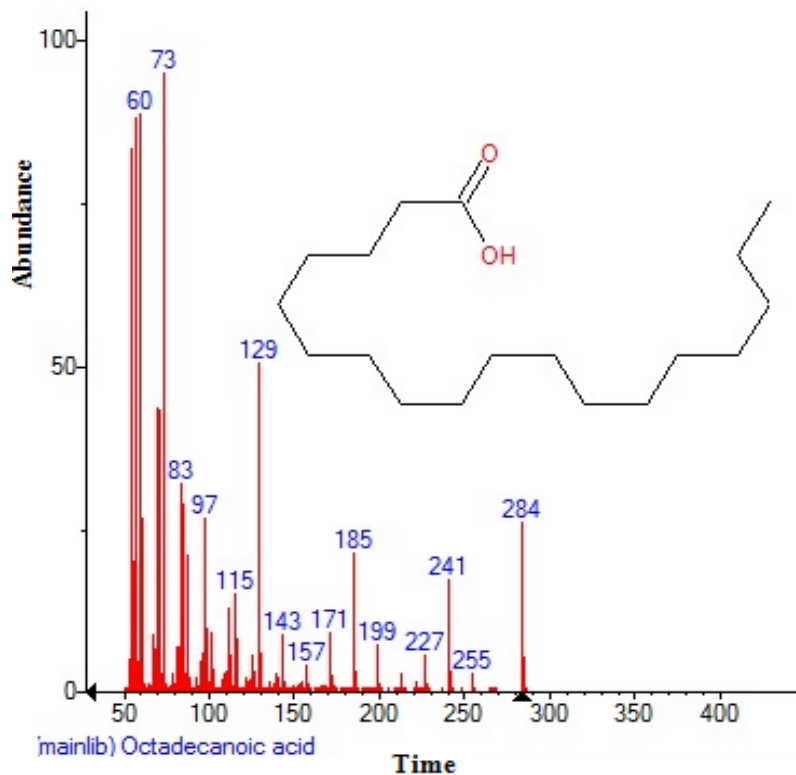
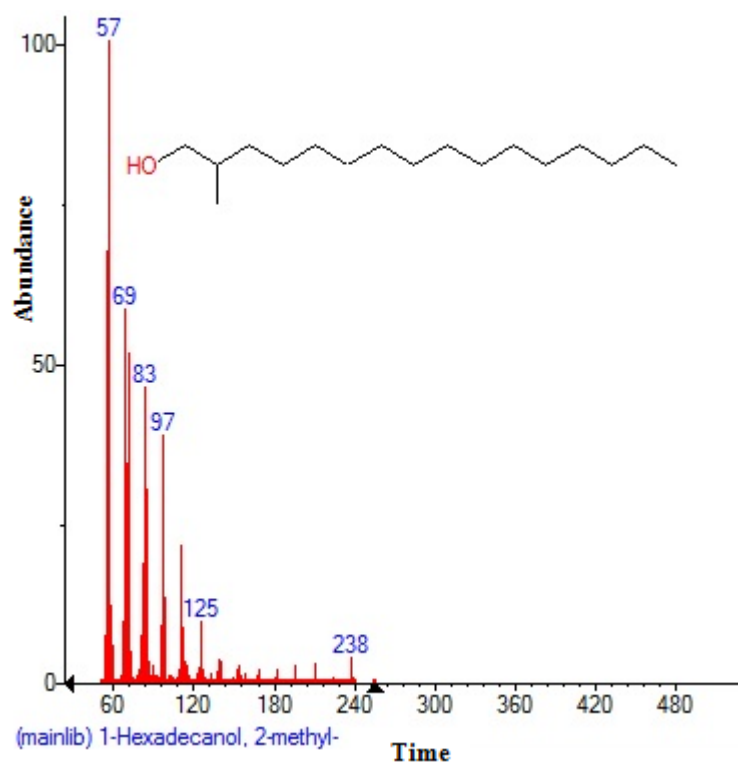


Figure 3. Structure of n-haxadecanoic acid present in the leaves extract of *Ricinus communis* using GC-MS analysis.

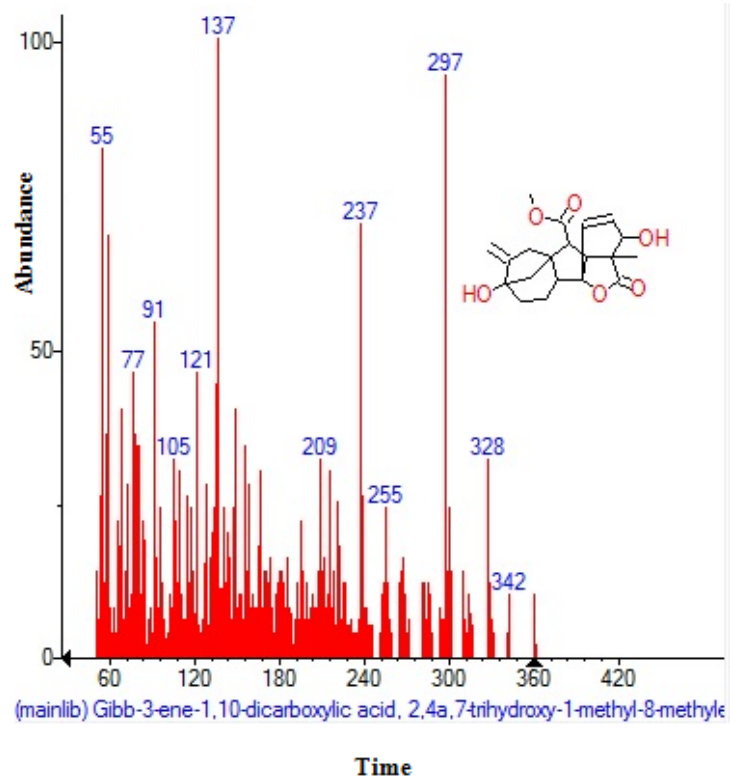


**Figure 4.** Structure of Octadecanoic acid present in the leaves extract of *Ricinus communis* using GC-MS analysis.

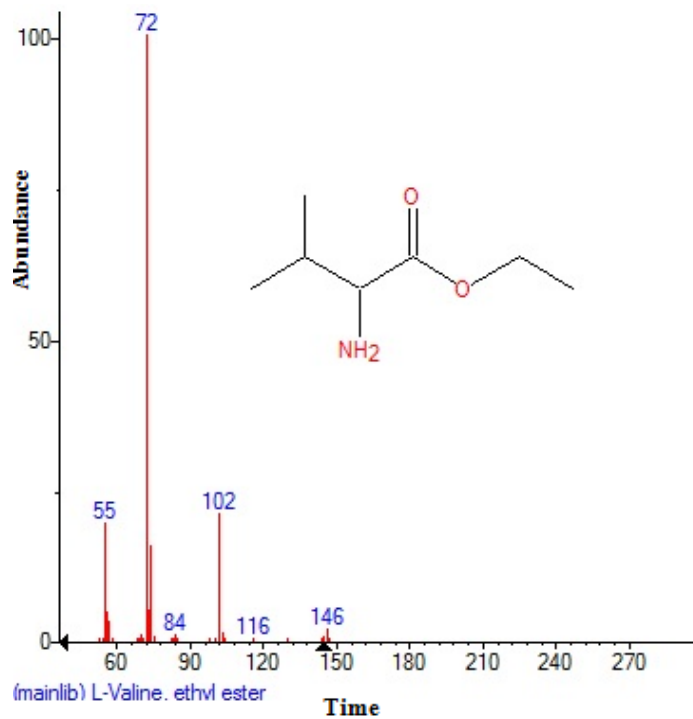


**Figure 5.** Structure of 1-hexadecanol, 2-methyl present in the leaves extract of *Ricinus communis* using GC-MS analysis.

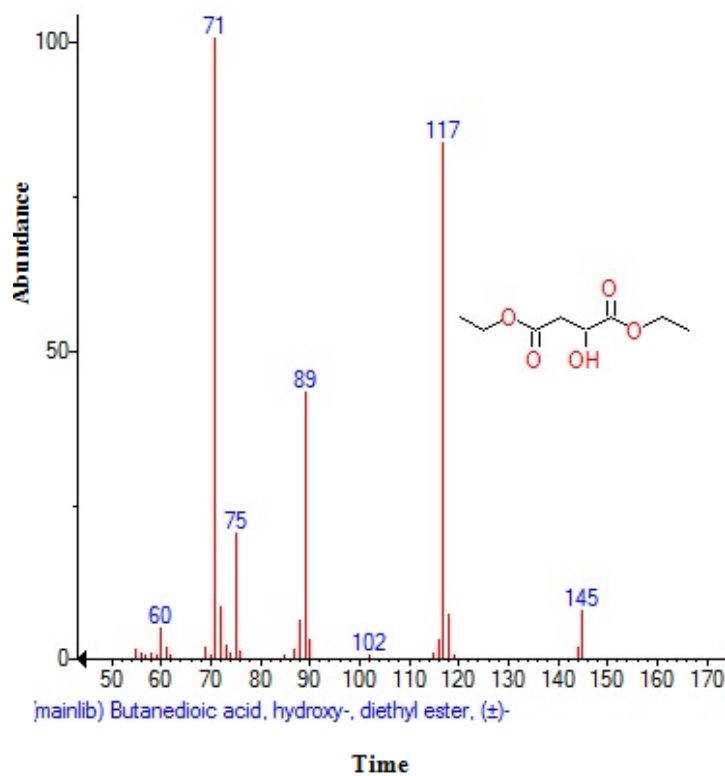




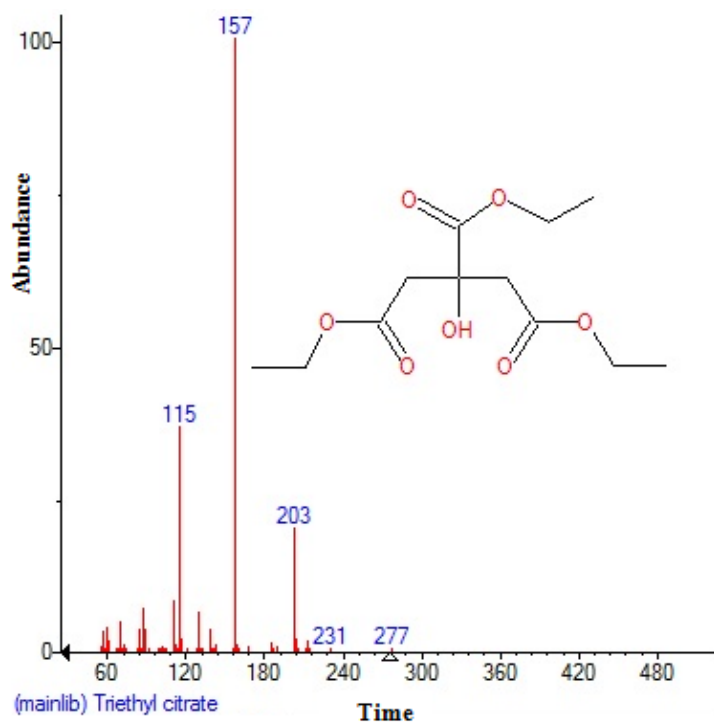
**Figure 6.** Structure of Gibb-3-ene-1, 10decarboxylic acid,2,4a, 7trihydroxy-1-methyl -8-methylene, 1.4a-lactone, 10-methyl present in the leaves extract of *Ricinus communis* using GC-MS analysis.



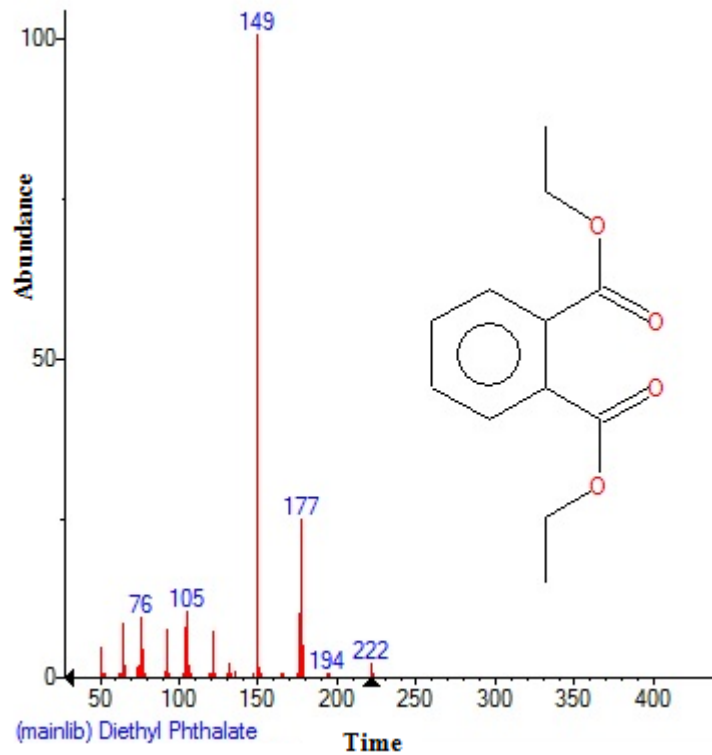
**Figure 7.** Structure of L-Valine, ethyl ester present in the leaves extract of *Ricinus communis* using GC-MS analysis.



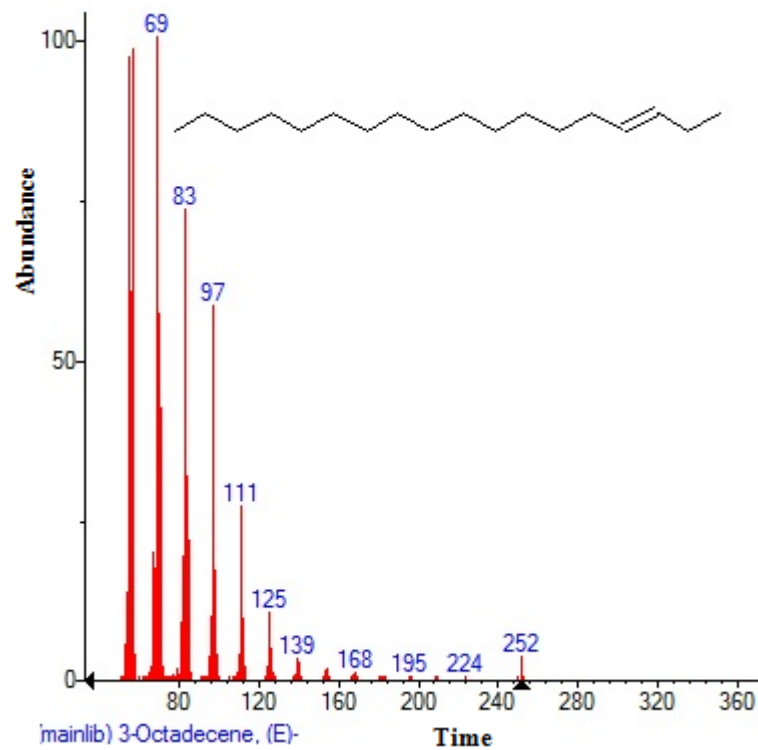
**Figure 8.** Structure of Butanedioic acid hydroxyl diethyl ester present in the leaves extract of *Ricinus communis* using GC-MS analysis.



**Figure 9.** Structure of Triethyl citrate present in the leaves extract of *Ricinus communis* using GC-MS analysis.



**Figure 10.** Structure of diethyl phthalate present in the leaves extract of *Ricinus communis* using GC-MS analysis.



**Figure 11.** Structure of 3-octadecene present in the leaves extract of *Ricinus communis* using GC-MS analysis.

**Table 1.** Compounds present in the leaves extract of *Ricinus communis* using GC-MS analysis.


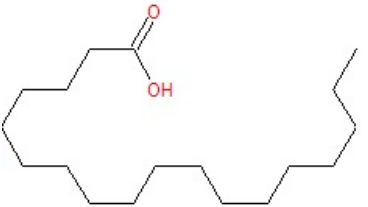
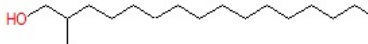
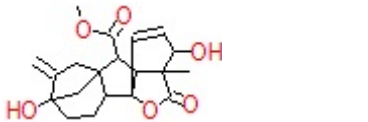
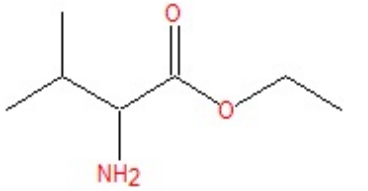
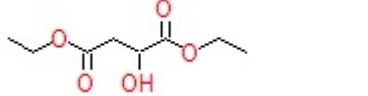
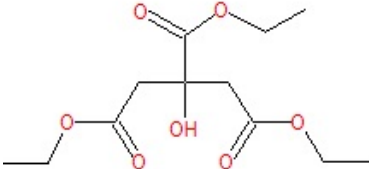
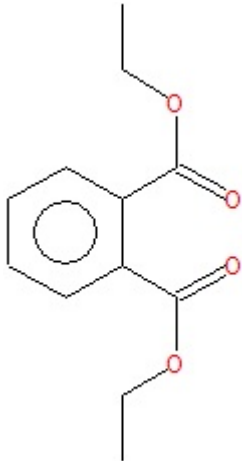

Serial No.	Phytochemical compound	RT (min)	Formula	Molecular weight	Exact mass	Chemical structure
1	n-haxadecanoic acid	15.177	C <sub>16</sub> H <sub>23</sub> O <sub>2</sub>	256	256.24023	
2	Octadecanoic acid	17.043	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	284	284.27153	
3	1-hexadecanol. 2-methyl	17.300	C <sub>17</sub> H <sub>36</sub> O	256	256.276615	
4	Gibb-3-ene-1. 10decarboxylic acid ,2,4a. 7trihydroxy-1-methyl —8-methylene, 1.4a-lactone. 10-methyl	18.628	C <sub>20</sub> H <sub>24</sub> O <sub>6</sub>	360	360.157288	
5	L-Valine, ethyl ester	4.088	C <sub>7</sub> H <sub>15</sub> NO <sub>2</sub>	145	145.110279	
6	Butanedioic acid hydroxyl. Diethyl ester	7.207	C <sub>8</sub> H <sub>14</sub> O <sub>5</sub>	190	190.084124	

Table 1. Contd.

7	Triethyl citrate	11.778	C <sub>12</sub> H <sub>20</sub> O <sub>7</sub>	276	276.120903	
8	Diethyl Phthalate	11.344	C <sub>12</sub> H <sub>14</sub> O <sub>4</sub>	222	222.089209	
9	3-Octadecene	13.348	C <sub>18</sub> H <sub>36</sub>	252	252.281701	

constitutions which may be useful for various herbal formulation as anti-inflammatory, analgesic, antipyretic, cardiac tonic and antiasthmatic. So, it may be concluded that *R. communis* is a very important indigenous medicinal plant which requires more exploration to utilize its medicinal property.

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#### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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