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Full Length Research Paper

Antiprotozoal and cytotoxic activities, and the acute toxicity of extracts from of *Brucea sumatrana* Roxb. (Simaroubaceae) leaves collected in Mai-Ndombe, Democratic Republic of Congo

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Results from the *in vitro* evaluation of the antiprotozoal activity of the aqueous extract, the 80% methanol extract and its fractions from the leaves of *Brucea sumatrana* against *Trypanosoma brucei brucei*, *Trypanosoma cruzi*, *Lesihmania infantum*, the multidrug-resistant K1 and chloroquine-sensitive NF54 strains of *Plasmodium falciparum* indicated that all samples from the leaves extract presented interesting antiprotozoal activity at different extents. The 80% methanol extract, its chloroform acid, petroleum ether and 80% methanol soluble fractions and the aqueous extract exhibited strong activity against *Trypanosoma b. brucei*, *T. cruzi*, *L. infantum* and the multidrug-resistant K1 strain of *P. falciparum* with IC_{50} values from < 0.25 to 4.35 µg/ml as well as against chloroquine-sensitive NF54 strain of *P. falciparum* with IC_{50} values ranging from < 0.02 to 2.0.4 µg/ml. Most samples were cytotoxic against MRC-5 cell lines (0.2 < cytotoxic concentration 50 (CC_{50}) < 24.5 µg/ml) and showed good selective effect against all tested parasites. In acute toxicity, the aqueous extract was found to be non-toxic and its LD_{50} was estimated to be greater than 5 g/kg. In addition, it did not significantly modify the concentration levels of some evaluated biochemical and hematological parameters in rats. These results constitute a scientific validation supporting and justifying the traditional use of the leaves of *B. sumatrana* for the treatment of malaria, sleeping sickness and at some extent Chaga disease.

Key words: *Brucea sumatrana*, Simaroubaceae, leaves, extracts, antiprotozoal activity, cytotoxic activity, acute toxicity.

INTRODUCTION

Brucea sumatrana Roxb. (synonyms: *Brucea amarissima* Desv. ex Gomes, *Brucea javanica* (L.) Merr., Gonusamarissimus Lour or Loussaamarissma O. Ktze) (Simaroubaceae) is a medicinal plant mainly growing in some Asian countries, such as Cambodia, China, Indonesia and Thailand. It is also found in Panama

(South America). In these countries, the fruit of this medicinal plant is used for the treatment of various ailments such as cancer, malaria and amoebic dysentery (O'Neill et al., 1987; Wright et al., 1988).

Different biological activities of extracts and quassinoids isolated the Asian *B. javanica* fruits including anti-plasmodial (O'Neill et al., 1985, 1987; Pavanand et al., 1986), antileukemic (Lee et al., 1979), antiamoebic (Wright et al., 1988), antiprotozoal (Wright et al., 1993; Bawm et al., 2008), antinematodal (Alen et al., 2000), antidiarrhoeal, (Sawangjaroen and Sawangjaroen, 2005), antibabesial (Subeki et al., 2007) activities were previously reported. This plant species is also found in some African countries and its seeds, leaves and stem bark are used for treatment of various ailments among them protozoal diseases such as malaria, amoebic dysentery and sleeping sickness (Biruniya, 1993; Ngoma and Bikengeli, 1993; Neuwinger, 2000).

In Democratic Republic of Congo (DR Congo), to treat malaria, 4 or 5 seeds are chewed 2 to 3 times per day for the treatment of malaria crisis, while an aqueous decoction of leaves or stem bark is drunk three times/day until the disappearance of fever (Biruniya, 1993). During our ethnopharmacological and ethnobotanical studies conducted in Mai-Ndombe, Bas-Congo province of Democratic republic of Congo (RD Congo) near traditional healers about their knowledge to treat parasitic diseases, the leaves of *B. sumatrana* were frequently cited as starting plant materials used to prepare traditional remedies to treat malaria, amoebiasis and sleeping sickness or human african Trypanosomiasis (HAT) (Musuyu Muganza et al., 2006).

On the basis of the aforementioned ethnopharmacological information and the lack of an investigation conducted in this field, it was decided to evaluate *in vitro* the antiprotozoal activity of aqueous extract, 80% methanol extract and its fractions from leaves of *B. sumatrana* against *T. brucei brucei*, *T. cruzi*, *L. infantum*, the multi-resistant K1 and chloroquine-sensitive NF54 strains of *P. falciparum*. The potential cytotoxic effects against MRC-5 cell line (human lung fibroblasts) of all samples as well as the acute toxicity of the aqueous extract which is the used traditional preparation were also assessed.

MATERIALS AND METHODS

Reagents

Methanol from Fischer Scientific (UK) was of high performance liquid chromatography (HPLC) grade. Chloroform and petroleum ether of HPLC grade were obtained from Across Organics (USA). Distilled water was used for the preparation of an aqueous decoction.

Plants

Leaves of *B. Sumatrana* Roxb. (Simaroubaceae) were collected in the district Mai-Ndombe's province Bandundu in DR Congo in December, 2009. The plant was identified by Mr. Bavukinina of the Institut de Recherches en Sciences de la Santé (I.R.S.S.) of Kinshasa, DR Congo. A voucher specimen (B 20122009BSL) was deposited in the herbarium of this institute. Leaves were dried at room temperature and reduced to powder.

Preparation of crude extracts, fractions and subfractions

A 150 g of dried powdered leaves of *B. sumatrana* were submitted to a Soxhlet extraction with 80% methanol (500 ml) for 2 h. The extractive solvent was evaporated *in vacuum* yielding corresponding dried 80% methanol extract denoted as ME-1 (12.53 g). 5 g of this dried 80% methanol extract were dissolved in 100 ml distilled water and fractionated according to the Mitscher's procedure (Figure 1) (Mitscher et al., 1978). The obtained fractions and sub-fractions were treated as described, yielding the corresponding dried extracts denoted as ME-1.1 to ME-1.6. On the other hand, 20 g of the powdered plant material were mixed with 150 ml distilled water and boiled at 100°C for 15 min. After cooling and filtration, the filtrate was treated as described, yielding the dried aqueous extract denoted as AE-1 (5.89 g). The total alkaloids extract (ME-2, 3.06 g) of plant part was obtained using the acid/base procedure described in the literature (Harborne, 1998).

Phytochemical screening

This study was performed by thin layer chromatography (TLC) on precoated silica gel plates F_{254} (thickness later 0.25, mm, Merck, Germany) using different reagents and mobile phases described in the literature to identify major chemical groups such as alkaloids, anthraquinones, coumarins, flavonoids, terpenes and steroids. Hydrochloride acid 0.2 N (aqueous solution of the sample anthocyanins. Froth test and Stiasny's reagent were used to detect saponins and tannins, respectively (Harborne, 1998).

Evaluation of biological activities

The antiprotozoal activity of all the samples obtained from the leaves of *B. sumatrana* was tested *in vitro* against *T. b. brucei*, *T. cruzi* and *L. infantum* from the laboratory of Microbiology, Parasitology and Hygiene of Prof. L. Maes,

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University of Antwerp, Belgium according to the respective procedures previously described by Kuypers et al. (2006) and Tshodi et al. (2012). The antiplasmodial activity against chloroquine and pyrimethamine-resistant K1 strain of *P. falciparum* obtained from the same laboratory, and chloroquine-sensitive NF54 from Tropical Medicine Institute of Antwerp, Belgium, was evaluated according to the lactate deshydrogenase procedure previously described by Makler et al. (1993) with some modifications (Kuypers et al., 2006). The cytotoxic effect against MRC-5 cell lines (human lung fibroblasts) was assessed using the MTT assay previously described by Kuypers et al. (2006). The selective index (SI) as a ratio cytotoxic concentration 50/inhibitory concentration 50 (CC_{50}/IC_{50}) was calculated for each sample to appreciate its effect against the tested parasites and MRC-5 cell lines. SI < 1 indicated a selective effect against the cell line while SI > 1 indicated a selective action against the tested parasite (Camacho et al., 2003; Tshodi et al., 2012). For the purpose of this *in vitro* antiprotozoal screening study, the following criteria were adopted: $IC_{50} \leq 5$ µg/ml: strong activity; $5 < IC_{50} \leq 10$ µg/ml: good activity; $10 < IC_{50} \leq 20$ µg/ml: moderate activity; $20 < IC_{50} \leq 40$ µg/ml: weak activity; $IC_{50} > 40$ µg/ml: inactive.

Acute toxicity

In the present study, only the acute toxicity of the aqueous extract was investigated in Wistar rats according to the procedure described by the Organization for Economic Co–operation and Development (OECD) guideline for testing chemicals, TG420 (OECD, 2001). Briefly, groups I (2 rats) orally received 5 ml distilled water and constituted the negative control groups. After, fifteen Wistar rats of either sex, (body weight: 130 to 150 g, aged 8 to 10 weeks) were divided in three groups of 5 rats each noted as groups II, III and IV which orally received a single oral dose of 500, 1000 and 5000 mg/kg body weight (bw), respectively. The animals were observed for toxic symptoms continuously for the first 4 h dosing and were daily weighted. Finally, all animals were then maintained in daily observation and the number of toxic effects and survivor were recorded for 14 days and further 28 days.

Biochemical and hematological parameters analysis

Blood from rats having received 5 g/kg of the aqueous extract (AE-1) was collected from tail vein on Day 28 for analysis. For biochemical parameters, blood was centrifuged at 4000 g for 5 min to obtain plasma, which was stored at –20°C. Glucose, creatinin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), serum glutamo pyruvate transferase (SGPT), serum glutamo oxalate transferase (SGOT), uric acid, total cholesterol, triglycerides, high-density lipoproteins (HDL), low-density lipoproteins (LDL), total and direct bilirubin were quantified using Architect (Abottâ) automation with Boehringen Ingelheim biochemical kits. Total proteins were estimated using Biuret's method. Hematological parameters analysis was carried out using an automatic hematological analyzer (Coulter STK, Beckam) with appropriated kits. The differential leucocyte count was performed with an optical microscopy after staining and, in each case, 100 cells were counted.

RESULTS AND DISCUSSION

Antiprotozoal and cytotoxic effects

Table 1 represents the antiprotozoal activity and the cytotoxicity of samples from the leaves of *B. sumatrana*. All samples were tested *in vitro* against *T. b. brucei*, *T. cruzi*, *L. infantum*, chloroquine and pyrimethamine-resistant K1 while Table 2 represents the activity of the samples against chloroquine-sensitive NF54 strains of *P. falciparum*. The cytotoxic effects of all samples against MRC-5 cell lines (human lung fibroblasts) (Table 1) and the acute toxicity only of the aqueous extract as the used traditional preparation were also evaluated.

The obtained results indicated that all samples exhibited the evaluated biological activities at different extents. The 80% methanol extract (ME-1) exhibited good activity against *T. cruzi* (6.15 µg/ml) and weak activity against *L. infantum* (24 µg/ml), while it showed strong activity against *T. b. brucei* and the multidrug-resistant K1 strain of *P. falciparum* with IC_{50} values of 4.35 and < 0.25 µg/ml, respectively (Table 1). Interestingly, its chloroform soluble fraction (ME-1.1) rich in waxes, steroids and terpenes and its petroleum ether soluble subfraction (ME-1.2) rich in waxes and lipids had the same antiprotozoal spectra of activity and presented strong activity against all selected protozoa with IC_{50} values ranging from < 0.25 to 1.70 µg/ml (Table 1). The 80% methanol soluble subfraction (ME-1.3) rich in steroids and terpenes exhibited strong activity against *T. b. brucei* and *T. cruzi* with IC_{50} value < 0.25 µg/ml for both parasites, weak activity against chloroquine and pyrimethamine-resistant K1 strain of *P. falciparum,* and was inactive against *L. infantum* (Table1).

The aqueous acid soluble fraction (ME-1.4), its chloroform base soluble subfraction (ME-1.5) rich in alkaloids and aqueous alkaline soluble subfraction (ME-1.6) rich in phenolic compounds had also the same antiprotozoal spectra of activity. All samples displayed good activity

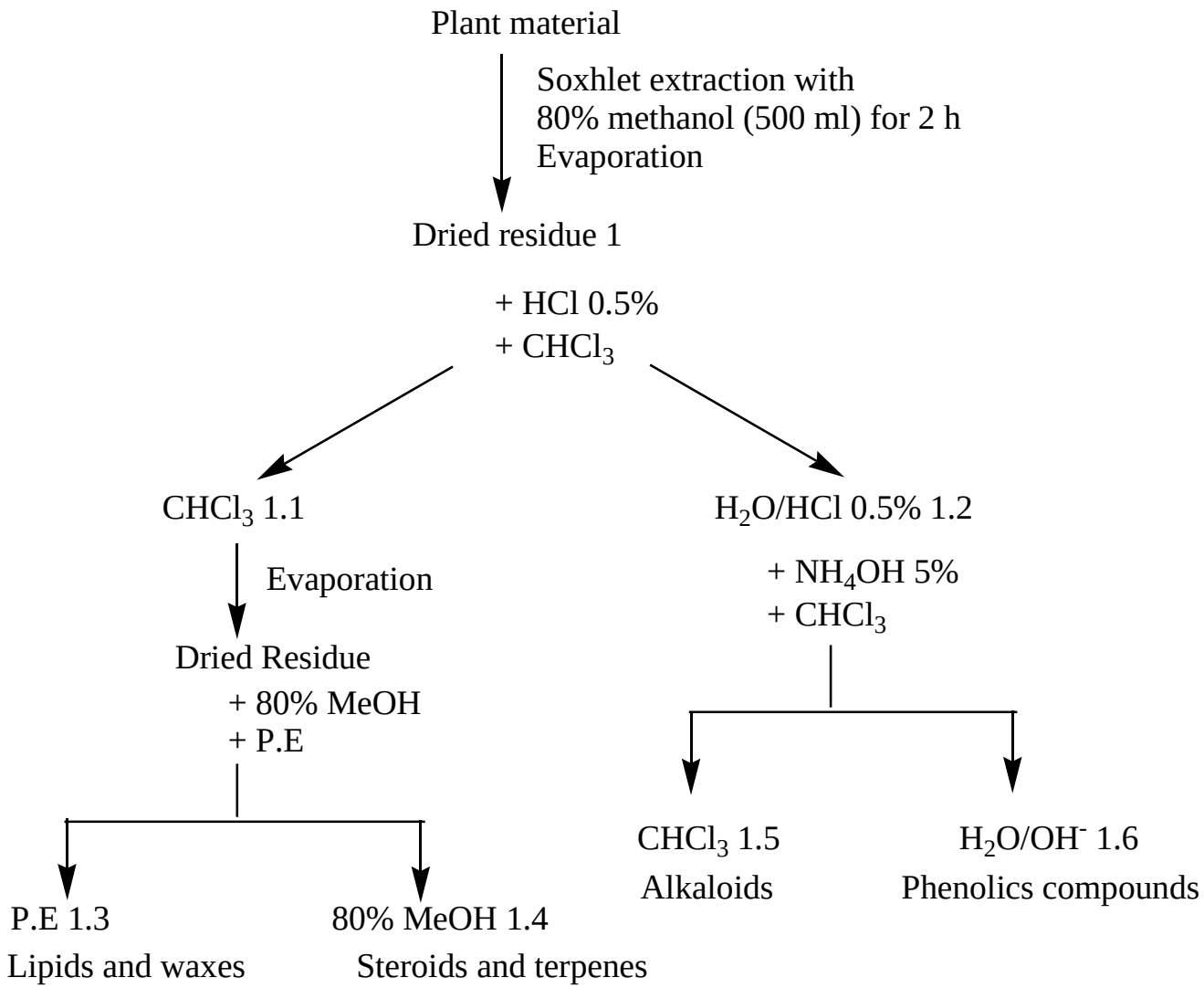

Figure 1. Fractionation of the 80% methanol extract.
Source: Mitcher et al. (1978).

against *T. b. brucei* and the multidrug-resistant K1 strain of *P. falciparum*, weak activity against *T. cruzi* and were inactive against *L. infantum* (Table 1). The aqueous extract (AE-1) which is the used traditional remedy exhibited strong activity against *T. b. brucei*, *T.cruzi* and the multidrug-resistant K1 strain of *P. falciparum* with IC_{50} values ranging from < 0.25 to 0.70 µg/ml (Table 1) and good activity against *L. infantum* (IC_{50} = 5.06 µg/ml). The total alkaloids extract (ME-2) showed good, moderate and weak activity against *T. b. brucei*, *T. cruzi*and *L.* infantum, respectively (Table 1). In addition, it however exhibited strong activity against the multidrug-resistant k1 strain of *P. falciparum* with IC_{50} value of 1.80 µg/ml.

All samples from *B. sumatrana* leaves exhibited strong activity against chloroquine-sensitive NF54 strain of *P. falciparum* with IC_{50} values from < 0.02 to 2.04 µg/ml. The most active samples were ME-1, ME-1.1, ME-1.4, ME-1.5, ME-2 and AE-1 inhibiting its growth with IC_{50} value < 0.25 µg/ml. In addition, ME-1.3 and ME-1.4 also showed strong activity against this *P. falciparum* strain with IC_{50} values of 1.04 and 1.55 µg/ml, respectively. In general, all samples showed strong antiplasmodial activity against this strain of *P. falciparum* (IC_{50} < 5 µg/ml) (Table 2).

With regards to the activity showed by the reference products, it is important to point out that ME-1.1, ME-1.2 and ME-1.5 exhibited higher activity ($0.25 \leq IC_{50} < 0.70$ µg/ml) than benznidazol against *T. cruzi* (IC_{50} = 2.65 µg/ml) whereas the antiplasmodial activity of ME-1, ME-1.1, ME-1.1 and AE-.1 (IC_{50} < 0.25 µg/ml) against the multidrug-resistant K1 strain of *P. falciparum* was

Table 1. Antiprotozoal and cytotoxic activities of samples from *Brucea sumatrana* leaves.

Code sample	MRC-5	*T. b. brucei*	*T. cruzi*	*L. infantum*	*P. falc*. K1
ME-1	2.34	4.35	6.15	24.00	< 0.25
ME-1.1	< 0.25	< 0.25	< 0.25	0.32	< 0.25
ME-1.4	< 0.25	< 0.25	0.31	1.70	< 0.25
ME-1.3	> 64	< 0.25	< 0.25	> 64	35.33
ME-1.4	34.24	12.35	27.57	> 64	10.00
ME-1.5	10.32	8.11	21.22	> 64	7.05
ME-1.6	24.05	10.04	25.06	> 64	8.15
ME-2	7.00	8.30	12.70	24.00	1.80
AE-1	0.46	0.57	0.70	5.06	< 0.25
Tamoxifen	10.5				
Metarsoprol		0.02			
Benznidazol			2.65		
Multeforine				3.56	
Chloroquine	> 64				0.18

ME-1 : 80% methanol extract, ME-1.1 : chloroform acid soluble fraction, ME-1.2 : petroleum ether soluble subfraction, ME-1.3 : methanol 80% soluble subfraction, ME-1.4 : aqueous acid soluble fraction, ME-1.5 : chloroform base soluble subfraction, ME-1.6 : aqueous alkaline soluble subfraction, ME-2 : totum alkaloids extract,AE-1 : aqueous (decoction) extract, *T.b. brucei: Trypanosome bruceibruce, T. cruzi: Trypanosome cruzi, l. infantum: leishmania infantum, P. falc. K1: Plasmodium falciparum K1.*

comparable to that of chloroquine used as an antimalarial reference product (IC_{50} = 0.18 µg/ml). In addition, samples ME-1, ME-1.1, ME-1.2 and AE-1 were the most cytotoxic samples and were 4.49, < 42, < 42 and 23 times more cytotoxic, respectively (CC_{50} = 2.34, < 0.25, < 0.25 and 0.46 µg/ml, respectively) than tamoxifen (CC_{50} = 10.5 µg/ml) (Table 1).

Cytotoxic effects of samples of *B. sumatrana* leaves against MRC-5 cell line

In the cytotoxic studies, it was observed that except ME-1.3 and ME-1.4 samples devoid of cytotoxic effect against MRC-5 cell line (CC_{50} > 64 and 34.24 µg/ml, respectively) since their CC_{50} were greater than 32 µg/ml, the remaining samples were cytotoxic against this cell line with CC_{50} values ranging from < 0.25 to 24.05 µg/ml, since they were lower than 32 µg/ml (Kuypers et al., 2006). By assessing their respective selectivity index (SI), it was observed that the activities of ME-1, ME-1.1, ME-1.2, MS-2 and AE-1 against *T. b. brucei*, *T. cruzi* and *L. infantum* were correlated to their cytotoxic effect against MRC-5 cell line, since their SI were lower than 1 (Camacho et al., 2003; Tshodi et al., 2012). They however presented good selective action against the multidrug-resistant K1 strain of *P. falciparum,* since their respective SI were greater than 1 (Camacho et al., 2003; Tshodi et al., 2012) (Table 3). ME-1.3 had the highest selectivity index against *T. b. brucei* and *T. cr*uzi since its SI value was > 256. Its selective action against *L. infantum* and the strain K1 of *P. falciparum* was also appreciable (Table 3).

Against chloroquine-sensitive NF54 of *P. falciparum*, most samples showed good selective action. The best selective action was observed with ME-1.4 (SI = 1721), followed by ME-1.5 (SI = 516), ME-2 (SI = 350) and ME-1 (SI ≥ 117). The inhibitory effect of ME-1.2 on the growth of this parasite was correlated to its cytotoxic effect since it SI was lower than 1 (SI < 0.24) (Table 2) (Camacho et al., 2003; Tshodi et al., 2012).

Effects of the aqueous extract of *B. sumatrana* leaves on the concentration levels of haematological parameters

The hematological parameter profile is presented in Table 4. The reported results indicated that the oral administration of the aqueous extract of *B. sumatrana* leaves (AE-1) at the oral dose of 5 g/kg bw in acute toxicity, did not affect concentration levels of evaluated hematological parameters and no significant difference between the treated animals compared to untreated was deduced ($p > 0.005$). They remained all in normal limits and presented no sign of a particular pathologic state.

Table 2. Antiplasmodial activity of samples from *B. sumatrana* leaves against chloroquine-sensitive NF54 strain of *P. falciparum.*

Code sample	MRC-5	*P. falciparum. NF54*	*Selectivity index*
ME-1	2.34	< 0.02	> 117
ME-1.1	< 0.25	< 0.02	< 12.5
ME-1.4	< 0.25	1.04	< 0.24
ME-1.3	> 64	1.55	> 41.29
ME-1.4	34.24	< 0.02	> 1712
ME-1.5	10.32	< 0.02	> 516
ME-1.6	24.05	2.04	11.79
ME-2	7.00	< 0.02	> 350
AE-1	0.46	< 0.02	> 23
Chloroquine	> 64	0.15	> 426.67

ME-1 : 80% methanolextract, ME-1.1 : chloroform acid soluble fraction, ME-1.2 : petroleum ether soluble subfraction, ME-1.3 : methanol 80% soluble subfraction, ME-1.4 : aqueous acid soluble fraction, ME-1.5 : chloroform base soluble subfraction, ME-1.6 : aqueous alkaline soluble subfraction, ME-2 : totumalkaloidsextract,AE-1 : aqueous (decoction) extract, *T.b. brucei: Trypanosome bruceibruce, T. cruzi: Trypanosome cruzi, l. infantum: leishmania infantum, P. falc. K1: Plasmodium falciparum K1.*

Table 3. Selectivity index (SI= CC_{50}/IC_{50}) of samples from*Brucea sumatrana* leaves.

Code sample	MRC-5/*T.b.brucei*	MRC-5/*T. cruzi*	MRC-5/*L. infantum*	MRC-5/*P. falc*.K1
ME-1	0.53	0.38	0.10	> 9.36
ME-1.1	< 1	<1	< 0.80	< 1
ME-1.2	< 1	< 080	> 0.14	< 1
ME-1.3	> 256	> 256	ND	> 1.81
ME-1.4	2.77	1.24	ND	4.86
ME-1.5	1.27	0.48	ND	1.46
ME-1.6	2.40	0.96	ND	2.95
ME-2	0.84	0.55	0.29	3.89
AE-1	0.80	0.66	0.09	< 1.84
Chloroquine	> 64	-	-	> 355.55

ME-1 : 80% methanol extract, ME-1.1 : chloroform acid soluble fraction, ME-1.2 : petroleum ether soluble subfraction, ME-1.3 : methanol 80% soluble subfraction, ME-1.4 : aqueous acid soluble fraction, ME-1.5 : chloroform base soluble subfraction, ME-1.6 : aqueous alkaline soluble subfraction, ME-2 : totumalkaloidsextract,AE-1 : aqueous (decoction) extract, *T.b. brucei: Trypanosome bruceibruce, T. cruzi: Trypanosome cruzi, L. infantum: Leishmania infantum, P. falc. K1: Plasmodium falciparum K1.* ND: not determined because the tested sample was inactive.

Effects the aqueous extract of *B. sumatrana* leaves on the concentration levels of evaluated biochemical parameters

Table 5 shows the effects of the oral administration of the aqueous extract (decoction, AE-1) of *B. sumatrana* leaves on the concentration levels of some biochemical parameters of Wistar rats. The obtained results indicated that the oral administration of the extract at the highest oral dose of 5 g/kg bw in acute toxicity test produced significant decrease of the concentration level of glucose in treated groups compared to untreated groups ($p < 0.05$). This decrease may be due to the hypoglycaemic and antidiabetic properties of the extract as also previously reported for other plant extracts (Okoli et al., 2010; Luka et al., 2012).

Alanine amino transferase (ALAT) also called alanine transaminase (ALT) and aspartate transaminase (AST) also known as aspartate amino transferase (ASAT/AspAT/AAT) are two liver enzymes associated in the hepatocellular damages and thus considered as indicators of liver damages. ALAT is only specific for liver functions and ASAT is mostly found in the myocardium, skeletal muscle, kidneys and brain (Wasan et al., 2001; Crook et al., 2006). Although a slight decrease was observed, the results reported here indicated that

Table 4. Effects the aqueous extract of *B. sumatrana* leaves (AE-1) at oral dose of 5 g/kgbw on the concentration levels of haematological parameters.

Parameter	Negative control	*B. sumatrana*: 5g/kgbw	*Reference values*
RBC (×10^6 µME-1)	8.1 ± 0.8	8.4 ± 1.2	7.6-10.29
Hemoglobin (g/dl)	14.2 ± 0.2	16.4 ± 1.2	15-18.2
Hematocrit (%)	43.2 ± 0.1	47.2 ± 2.0	40.7-50
Platelets (×10^3 µME-1)	1421.0 ± 0.2	1404.2 ± 0.2	995-1713
WBC (x10^3 µME-1)	13.3 ± 0.3	16.0 ± 0.5	6.6-20.5
Neutrophils (%)	18.8 ± 0.7	23.2 ± 1.2	3-24.7
Basophils (%)	0.0	0.0	0.0
Eosinophils (%)	1.5 ± 0.1	1.5 ± 0.4	0-2
Lympocytes (%)	89.2 ± 1.1	88.3 ± 0.1	58.8-94
Monocytes (%)	3.4 ± 1.1	3.6 ± 1.2	0-4
Segmented leucocytes (%)	15.1 ± 0.6	19.3 ± 2.1	-

RBC; red blood cells, WBC : white blood cells

Table 5. Effects the aqueous extract of *B. sumatrana* leaves (AE-1) at oral dose of 5 g/kgbw on the concentration levels of biochemical parameters.

Parameters	Negative control	*B. sumatrana*: 5 g/kg bw
Glucose (mg/dl)	245.5 ± 0.4	206.3 ± 1.4
Creatinin (mg/dl)	0.90 ± 0.05	0.87 ± 0.02
AST (UI/L)	180.6 ± 0.3	178.2 ± 0.5
ALT (UI/L)	52.2 ± 2.2	53.5 ± 1.2
Totalcholesterol (mg/dl)	54.2 ± 1.1	53.3 ± 2.2
Triglycerides (mg/dl)	47.7 ± 1.8	46.3 ± 3.5
Total bilurbin (mg/dl)	0.6 ± 0.1	0.6 ± 0.1
Direct bilurbin (mg/dl)	0.2 ± 0.0	0.2 ± 0.0
Total proteins (g/dl)	8.0 ± 0.3	8.4 ± 1.1
Albumin (g/dl)	3.6 ± 0.5	3.5 ± 0.6
ALP (IU/L)	148.4 ± 1.6	146.3 ± 2.4
HDL (mg/dl)	65.3 ± 1.3	65.6 ± 1.3
LDL (mg/dl)	39.5 ± 2.1	38.5 ± 0.4
Uric acid (mg/dl)	1.8 ± 0.1	2.2 ± 0.5
SGOT (UI/L)	128.3 ± 1.6	126.4 ± 0.2
SGPT (UI/L)	32.7 ± 2.3	33.3 ± 1.2
Urea (mmol/L)	6.1 ± 0.8	6.6 ± 1.6

AST: aspartate transferase, ALT : alanine transferase, ALP : alkalinephospahte, HDL : hight-densitylipoproteins, LDL : low-densitylipoproteins, SGOT : serumglutamoxatetransferase, SGPT : serum glutamate transferase

concentration levels of these both enzymes in treated animals did not show a significant difference compared to the negative control ($p > 0.05$). This finding showed that the aqueous leaf extract at this tested oral dose may not cause liver, heart or kidney damages as also previously reported by Pieme et al. (2006) and Lima et al. (2009). Moreover, the hepatic function of these animals can be considered to be maintained (Arüjo et al., 2005). In addition, the concentration levels of creatinin and SGPT which did not show significant difference in treated rat groups compared to untreated rat groups ($p > 0.05$) well support this observation.

The slight decrease of the concentration levels of cholesterol and triglycerides in treated rat groups was not

significant ($p > 0.05$) (Table 5). This effect may be due to the hypolipidimic property of the aqueous leaves extract and to the increased secretion of thyroid hormones T3 and T4 (Arüjo et al., 2005). A slight decrease in concentrations of high density lipoprotein (HDL) and low density lipoprotein (LDL) in treated animals was also observed, but, it did not show significant difference compared to the control groups ($p > 0.05$). It can be considered as a consequence of the decrease of total cholesterol. These results suggest that the extract has some beneficial effects by reducing some risk factors related to cardiovascular diseases (Ameyaw and Owusu-Ansah, 1998).

Albumin is a protein with high concentration in plasma. Since it is produced in the liver, its decrease in serum may arise from liver and kidney diseases (Lima et al., 2009). Fortunately, a slight decrease of the concentration level of albumin was observed, but did not show significant difference compared to untreated groups ($p > 0.05$). In addition, although a slight decrease for the concentration levels of the total and direct bilirubin was observed, there was no significant difference of this biochemical parameter in treated animals compared to control groups ($p > 0.05$). The level of total proteins slightly increased ($p < 0.05$), suggesting an external supply. A slight increase of the concentration level of SGOT and SGPT in treated animals was observed. Nevertheless, it did not show significant difference compared to the control groups ($p > 0.05$) indicating that the heart and liver were not affected. No significant difference in the concentration level of ALP in treated rat groups compared to untreated groups was recorded although a slight decrease or increase in treated groups according to the case was observed ($p > 005$) (Table 5). As the presence of infiltrative diseases of the liver and all bones diseases is associated with osteoplastic activity, it is likely seen that the oral dose used in this study for the aqueous extract of *B. sumatrana* leaves did not abnormally interfere with the calcification or metabolic activities involving the liver. The intrahepatic and extrahepatic bile functions did not know an obstruction (Vasudevan and Sreekumari, 2005). This finding is in good agreement with results reported concerning the effect of other plant extracts on ALP concentration level in animals (Pieme et al., 2006; Eden and Usoh, 2009).

As urea production in mammals occurs specially in liver, its concentration level could also be used as an indicator of hepatic function. In our study, the urea concentration level significantly increased in treated groups compared to untreated groups ($p < 0.02$), but this observation was not found as a sign of insufficiency renal because its concentration level remained within the normal limits (2.5 to 7.5 mmol/L). Therefore, our results showed good hepatic function of treated animals for the aforementioned reasons (Arüjo et al., 2005).

In general, all concentration levels of biochemical and haematological parameters evaluated in the present study were within the normal ranges (Barry et al., 1995; Feldams et al., 1997).

Acute toxicity of the aqueous extract of *B. sumatrana* leaves in rats

Animals were treated with single oral doses of the aqueous extract (AE-1) (500, 1000 and 5000 mg/kg body weight, respectively). In this test, no sign of toxicity such as alteration of the locomotion activity and gastro-intestinal disturbances were observed. Rats received all tested oral doses significantly gained body weight compared to negative control groups. According to Pieme et al. (2006), the growth response effect could be considered as a result of increased food and water intake. On Day 21, one death (6.66%) was noted in the third group receiving 1000 mg/kg bw of the extract and on Day 22, 3 deaths (20%) were recorded in the fourth group having received 5000 mg/kg body weight of the extract. This last percentage of mortality is weaker than 50%. According to Kennedy et al. (1986), substances that present LD_{50} higher than 5 g/kg body weight via oral route, may be considered as practically non-toxic. Therefore, it may be suggested that the acute toxicity of *B. sumatrana* aqueous leaves extract is practically null via oral route. Therefore, the LD_{50} of the extract was estimated to be greater than 5000 mg/kg body weight. In addition, histopathological examination of vital organs of treated animals did not show any abnormality compared to untreated groups indicating that their state was well maintained at the administered oral dose.

On the other hand, the acute toxicity of leaves ethanol extract of *Brucea javanica* Merr. collected in Indonesia on mice was previously reported (Marissa et al., 2012). Results from this study indicated that the oral administration of the leaves ethanol extract at oral doses of 562.5, 1125, 2250 and 4500 mg/kg body weight, respectively, were unable to induce acute toxic effects in mice. But at the highest oral dose of 4500 mg/kg body weight, 26% mortality occurred after 14 days, and its DL_{50} was determined to be 1003.65 mg/kg body weight. This DL_{50} determined by these authors seems not to be correct because not only 26% of death observed at the highest oral dose of 4500 mg/kg bw was weaker than 50% of death, but also not percentage death was reported after the administration of 1125 and 2250 mg/kg bw, respectively, suggesting that these oral doses did not induce mortality of animals. Taking account of these important observations, the DL_{50} of the administered extract in this previous study must be greater than 4500 mg/kg bw and not 1003.65 mg/kg bw as reported. Interestingly, in this same investigation, it was reported

that vital organs and body average weights of treated animals did not show any difference compared to control groups. In addition, gross examination of the vital organs revealed no pathologic abnormality compared to control groups on the microscopic observation. These finding were in good agreement with our observations for the aqueous leaves extract tested in the present study. From these results of this study, these authors concluded that the extract can be considered to be slightly toxic since the damage caused by leaves ethanol extract is minor and not permanent considering the administered oral dose (Marissa et al., 2012).

The chemical composition of *B. sumatrana* leaves

Some plant parts of *B. javanica* growing in Asian regions were chemically previously investigated. Triterpenoids and quassinoids were isolated from the combined plant materials leaves, twigs and inflorescences. Flavonoids were also detected (Ismail et al., 2012) or isolated from the leaves (Dong et al., 2013). Results from our phytochemical screening conducted on *B. sumatrana* leaves collected in Mai-Ndombe, DR Congo, revealed the presence of alkaloids, steroids, terpenoids, flavonoids and tannins in the leaves. Anthraquinones, anthocyanins and coumarins were not detected in our experimental conditions. The presence mainly of alkaloids, flavonoids, steroids and terpenoids may largely contribute to the observed antiprotozoal activity because more chemicals belonging to these phytochemical groups present in other medicinal plant species had previously been reported to exhibit antiprotozoal activity at different extents (Wright et al., 1994; Camacho et al., 1998; Christensen and Kharazmi, 2001; Hoet et al., 2004: Bero et al., 2009; Bero and Quetin-Leclercq, 2011). Particularly, different terpenes belonging to the quassinoid groups mainly isolated from the seeds of *B. javanica* growing in Asian regions are known as active principles for various evaluated biological activities already mentioned earlier.

Conclusion

The present investigation has described for the first time the antiprotozoal and cytotoxic activities, and acute toxicity of extracts, fractions and sub-fractions from *B. sumatrana* leaves from RD Congo on a large spectrum of protozoa. The reported results showed that all samples possessed interesting *in vitro* antiprotozoal activity at different extents. The aqueous extract (AE-1), which is the used traditional preparation, was found to be non-toxic and did not affect the concentration levels of evaluated biochemical and hematological parameters, and can be considered as a sign of no appearance of pathologic abnormality. The reported results can partly justify and support the use of this plant part of *B. sumatrana* as raw material for the preparation of traditional remedy for the treatment of parasitic diseases such as malaria, sleeping sickness, leishmaniosis and in some extents American trypanosomiasis named Chagas disease, with no apparent toxic effects in patients. Further studies are in progress on the most active fractions, sub-fractions and the total alkaloids extract, leading to the isolation and structural elucidation of active constituents.

Conflict of interest

Authors declare that there are no conflicts of interests

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Full Length Research Paper

New pentacyclic triterpene ester and flavone glycoside from the biologically active extract of *Carduus pycnocephalus* L.

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Bioassay guided phytochemical investigation of petroleum ether, chloroform and butanol fractions obtained from biologically active total alcoholic extract of *Carduus pycnocephalus* led to isolation of two new compounds, 3-*O*-acetyl-ursolic acid-28-ethyl ester (compound 1) and diosmetin-7-*O*-*α*-L-arabinopyransyl (1```→4``)-*β*-D- glucopyranoside (compound 5) along with three known compounds, *bis* (2-ethylhexyl) benzene-1,2-dicarboxylate) (compound 2), 3α, 24 dihydroxyolean-12-en-28, 30-dioic acid dimethyl ester (compound 3) and kaempferol (compound 4). Their structures were established on the basis of spectroscopic methods (UV, MS, ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC) and by comparison with published data. Compounds 2 and 3 were isolated for the first time from genus *Carduus*. The cytotoxic, antioxidant, and antimicrobial activities of the ethanolic extract were evaluated and the extract showed variable degrees of activities. It showed significant cytotoxic activity against MCF-7, A-549 and HepG-2 cell lines with IC_{50}, 17.9, 17.5 and 21.8 µg, respectively. The extract displayed weak antioxidant activity by scavenging of DPPH with SC_{50} 554.2 µg. In agar diffusion assay, the extract exhibited strong antibacterial activities against Gram negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* and strong antifungal activity against *Syncephalastrum racemosum*.

Key words: *Carduus pycnocephalus* L.; new triterpene ester, flavonoidal glycoside, antioxidant, cytotoxic, antimicrobial activity.

INTRODUCTION

Genus *Carduus* which belongs to the family Asteraceae includes approximately 100 species worldwide (Chaudary, 2000) and is widely distributed around the Mediterranean. In Chinese folk medicine, the plants of genus *Carduus* are used for the treatment of various human diseases such as cold, stomach ache as well as rheumatism (Esmaieli, 2005). Genus *Carduus* was found to possess a wide range of biological activities such as liver tonic, anti-inflammatory, antispasmodic, anticancer, antiviral and antibacterial (Esmaeili, 2005; Jordon Thaden and Louda, 2003; Orhan and Ozcelik, 2009). Phytochemical studies on several *Carduus* species were

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Figure 1. *Carduus pycnocephalus* L.

carried out and revealed that this genus is rich in secondary metabolites as lignans (Fernandez et al., 1991), flavonoids, flavonoidal glycosides (Jordon - Thaden and Louda, 2003; El-Lakany et al., 1995, 1997; Amer et al., 1985; Abdallah et al., 1989; Bain and Desrochers, 1988; Abdel Salam et al., 1982; Liu, et al., 2013), coumarins (Jordon Thaden and Louda, 2003; Cardona et al., 1992), alkaloids (Zanng, 2002), sterols and triterpenes (Abdel Salam et al., 1982, 1983). Concerning the phytoconstituents of *Carduus pycnocephalus*, the literatures survey revealed the presence of flavonoids (El-Lakany et al., 1995, 1997; Amer et al., 1985), essential oils (Esmaeili, 2005; Al-Shammari, 2012), sterols and triterpenes (Gallo and Sarachine, 2009). In our previous work on Saudi plant, the isolation of seven flavonoidal compounds were reported, apigenin, kaempferol-3-*O* *β* – D-glucoside, kaempferol-3- *O* – *α* – L- rhamnoside, kaempferol-7-methoxy-3- *O* – *α* – L- rhamnoside, diosmetin-7-O-*β*-D-xylosyl-(1```→6``)-*β*-D-glucopyranoside, diosmetin- 7- *O* - *α* – L- arabinosyl (1``` →6``) – *β* – D – glucopyranoside, kaempferol-3-*O*-α-L-rhamnosyl—(1``` →2``) -α-L rhamnoside, in addition to, lupeol, *β*-sitosterol and *β*-sitosterol-3-*O*-*β*-D- glucoside were isolated from the aerial parts of *C. pycnocephalus* L. Anti-inflammatory, antispasmodic and hypotensive activities were assessed for all extracts which showed variable activities (Al-Shammari, et al., 2012). In continuation of our phytochemical and biological studies on *C. pycnocephalus* which grows in Saudi Arabia, we report here the isolation and structure elucidation of two new compounds 1, 5 and three known compounds 2 to 4. Compounds 2 and 3 were reported for the first time from genus *Carduus*. In addition, the cytotoxic, antioxidant and antimicrobial activities for the total alcoholic extract of *C. pycnocephalus* L. were evaluated.

MATERIALS AND METHODS

General experimental section

Melting points were determined on a Mettler FP 80 Central Processor supplied with a Mettler FP 81 MBC Cell Apparatus, and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 on a Bruker Avance DRX – 500 instruments (Central Lab. at the College of Pharmacy, KSU) at 500 MHz for protons and 125 MHz for carbons using the residual solvent signal as an internal standard. All 1D and 2D spectra were obtained using the standard Bruker software; EI and FAB mass spectra on a Jeol JMS, Ax 500, 5890 series II (Tokyo, Japan), Ultraviolet spectra were obtained in methanol using the shifting reagents $AlCl_3$, NaOAc, NaOMe, and H_3BO_3 for flavonoids were obtained using a Hewlett-Packard HP-845 UV-Vis spectrophotometer. The ultraviolet lamp used in visualizing TLC plates was a Mineralight® device, multiband UV-254/366 nm obtained from UVP, Inc., USA. Column chromatography was performed on silica gel (60-230 mesh, Merck) and TLC was carried out with silica gel 60 pre-coated plates F-254 (Merck).

Plant

The fresh plant (Figure 1) was collected from Al-Hada (Saudi Arabia) on March, 2008 and was kindly identified by Dr. Jakob Thomas, Professor in College of Science, KSU. A voucher specimen (#15106) was deposited at the herbarium in the College of Pharmacy at King Saud University (KSU).

Extraction and isolation

The air dried aerial parts of *C. pycnocephalus* L. (2.0 kg) were exhaustively extracted with 95% ethanol. The concentrated ethanol extract (210 g) was suspended in methanol: water (1:9) and fractionated by extraction with petroleum ether (40 to 60°C), chloroform, ethyl acetate and water saturated butanol, each (0.5 L ×

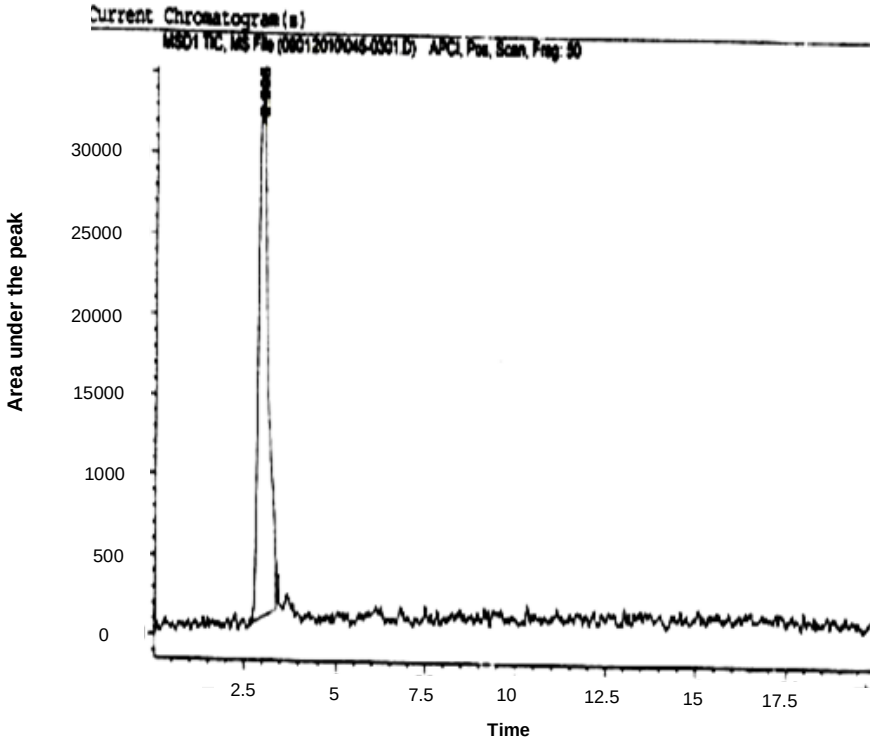

Figure 2. HPLC system used in isolation and chromatogram showing the purity of compound 5.

3), to give petroleum ether (47.3 g), chloroform (23.5 g), ethyl acetate (8.9 g), butanol (19.2 g) and aqueous (100 g) extracts.

Isolation of compounds 1 to 3 from petroleum ether extract

The petroleum ether extract (25 g) was subjected to silica gel column eluted with *n*-hexane and increasing the polarity with chloroform till 100% chloroform then with methanol, 60 fractions were collected (250 ml each), the important fractions were re-chromatographed using silica gel column eluted with *n*-hexane as eluent, then increasing the polarity by using chloroform and methanol to give compounds 1 (8 mg), 2 (1.37 g) and 3 (6 mg).

Isolation of compound 4 from chloroform extract

The chloroform fraction (2.5 g) was chromatographed on silica gel column. Elution was started with petroleum ether/chloroform (9:1) and the polarity was increased by methanol. Fifty fractions were collected, 100 ml each. The fractions were monitored by TLC and similar fractions were pooled together. From fraction 40 to 41 (120 mg) compound 4 (8 mg) was isolated by preparative TLC using chloroform: methanol (9.5:0.5) as solvent system.

Isolation of compound 5 from butanol extract

The butanol fraction (10 g) was chromatographed on silica gel column eluted with *n*-hexane/EtOAc (1:1) and increasing the polarity by ethyl acetate then by methanol. 70 Fractions were collected, 200 ml each. The fractions were monitored by TLC and similar fractions were combined together. Fractions 39 to 55 (750 mg) was subjected to preparative HPLC to yield 20 mg of highly pure compound 5 (Figure 2).

Acid hydrolysis of compound 5

Compound 5 (5 mg) was dissolved in MeOH (5 ml), to which an equal volume of 10% sulfuric acid was added. The mixture was refluxed on a boiling water bath for 3 h, and then cooled. The hydrolysate was shaken with ethyl acetate (3 × 50 ml). The combined extract was distilled off and subjected to TLC; the acidic mother liquor containing the sugar moiety (s) was neutralized with sodium carbonate, concentrated and separately spotted alongside with authentic sugars using chloroform:methanol (6:4) as solvent system and anisaldehyde sulfuric as spraying reagent. It showed the presence of *β*-D-glucose and *α*-L-arabinose as sugar moieties.

Compound 1: White powder (8 mg); R_f =0.59 [(*n*-hexane: $CHCL_3$) (2: 1)]; EI-MS *m/z* = 526 [M^+], 483 [M^+-$COCH_3$], 454 [M^+-$COCH_3$-C_2H_5], 411, 396, 382, 278, 248, 218; ^{1}H NMR and ^{13}C NMR (500 MHz, 125 MHz, $CDCl_3$, TMS) (Table 1).

Compound 2: Yellowish oily substance (1.37 g); ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ_H (ppm) 7.72 (2H, m, H-3, 6), 7.54 (2H, m, H-4, 5), 4.22 (4H, d, *J*= 6.4 Hz, H-3`, 3``), 1.70 (2H, m, H-4`, 4``), 1.35 (14H,m, H-5`, 5``, 6`, 6``, 7`, 7``), 0.88 (4H, m, H-8`, 8``), 1.35 (14 H, m, H-9`, 9``) and 0.90 (6H, t) H-10`, 10``); ^{13}C NMR (125 MHz, $CDCl_3$, TMS) δ_C 132.6 (s, C-1, 2), 129.0 (d, C-3, 6), 131.1 (d, C-4, 5), 167.9 (s, C-1`, 1``), 68.3 (t, C-3`, 3``), 38.9 (d, C-4`, 4``), 31.2 (t, C-5`, 5``), 29.6 (t, C-6`, 6``), 22.4 (t, C-7`, 7``), 14.4 (q, C-8`, 8``), 22.8 (t, C-9`, 9``) and 11.6 (q, C-10`, 10``); EI-MS: *m/z* = 390 [M^+,

Table 1. ^{1}H and ^{13}C NMR data of compound 1 in $CDCl_3$.

Position	δ_H	δ_C (DEPT)	Position	δ_H	δ_C (DEPT)
1	1.59, 1.0 (2H, m)	38.3 t	18	2.12 (1H, d, J=7.4 Hz)	55.3 d
2	1.57 (1H, m)	24.9 t	19	1.16 (1H, m)	40.9 d
3	4.45 (1H, t, *J* =7.0 Hz)	80.3 d	20	0.88 (1H, m)	39.8 d
4	-	37.8 s	21	1.36 (1H, m)	32.6 t
5	0.76 (1H, br)	55.3 d	22	1.26, 1.51 (2H, m)	31.9 t
6	1.32, 1.45 (2H, m)	18.3 t	23	0.80 (3H, s)	28.4 q
7	1.26, 1.46 (2H, m)	33.3 t	24	0.78 (3H, s)	16.8 q
8	-	39.8 s	25	1.25 (3H, s)	14.1 q
9	1.50 br	47.6 d	26	0.89 (3H, s)	16.8 q
10	-	37.1 s	27	1.08 (3H, s)	23.7 q
11	2.20 d, *J*= 7 Hz)	23.7 t	28	-	173.9 s
12	5.12 (1H, t, *J* =3.6 Hz)	121.7 d	29	0.90 (3H, d, *J*=5.5 Hz)	14.3 q
13	-	145.2 s	30	0.82 (3H, d, *J*=4.5 Hz)	23.5 q
14	-	41.7 s	31	-	173.7 s
15	1.62, 0.92 (2H, m)	26.1 t	32	2.20 (3H, s)	23.0 q
16	1.15, 1.50 (2H, m)	23.6 t	33	4.05 (2H, q, *J*=7.2 Hz)	60.1 t
17	-	47.2 s	34	1.20 (3H, t, *J*=5.0 Hz)	15.5 q

36%] , 149 [100%].

Compound 3: White powder (6 mg); ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ_H (ppm) 5.19 (1H, broad t, *J*= 7.6 Hz, H-12), 4.10 (1H, m, H-3), 3.50 (6H, s, two OCH_3- 31 and 32), 2.30 (1H, broad t , *J*=7.0 Hz, C-18), 1.26 (9H, s, for three methyl groups), 0.85 (6H, s, two methyl groups) ; ^{13}C NMR (125 MHz, $CDCl_3$, TMS) δ_C (ppm) 38.2 (C-1), 24.4 (C-2), 80.6 (C-3), 37.8 (C-4), 55.2 (C-5), 18.2 (C-6), 32.7 (C-7), 39.2 (C-9), 36.6 (C-10), 23.2 (C-11), 121.6 (C-12), 145.2 (C-13), 42.9 (C-14), 28.3 (C-15), 23.2 (C-16),46.7 9C-17), 37.8 (C-18), 41.8 (C-19), 47.2 (C-20), 31.1 (C-21), 34.7 (C22), 60.1 (C-23), 14.2 (C-24), 15.5 (C-25), 16.5 (C-26), 25.8 (C-27), 173.7 (CO-28), 50.3 (OCH_3-31), 29.7 (C-29), 174.0 (CO-30) and 50.3 (OCH_3-32). EI-MS: *m/z* 530 [M^+], 512, 470 and 247.

Compound 4: Pale yellow powder (8 mg); R_f =0.45 [$CHCl_3$:MeOH, 9:1]; UV λ max; MeOH (266, 338); ^{1}H NMR (500 MHz, DMSO-*d*$_6$, TMS) δ_H 6.05 (1H, *s*, H-6), 6.40 (1H, *s*, H-8), 6.85 (2H, *d*, *J* = 8.0 Hz, H-3`and 5`), 7.80 (2H, *d*, *J*= 8.0 Hz, H-2`and 6`), 5.50 (1H, s, OH-4`), 13.10 (1H, br s, OH-5); EI-MS: *m/z* = 286 [M^+], 134, 152.

Compound 5: Gray to greenish powder (20 mg); mp: 187-188 ^{0}C; R_f = 0.62 [butanol : acetic acid : water, 24:10:1]; UV λ $_{max}$ MeOH: (nm) 269, 344; (NaOMe): 269 , 300 sh, 280; ($AlCl_3$): 273, 362, 390; ($AlCl_3$/HCl): 276, 297, 361, 383; (NaOAc): 271, 365 (NaOAc/H_3BO_3): 269, 348; ^{1}H NMR (500 MHz, DMSO-*d*$_6$, TMS); ^{13}C NMR (125 MHz, DMSO-*d*6, TMS) (Table 2); FAB-MS (Positive ion mode) *m/z* = 633 [M^++K] for $C_{27}H_{30}O_{15}$, 595 [M^++H], 463 [M^+-arab+H], 301 [M^+-glu-arab], 152.

Biological activities

All the biological activities were carried out at Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University, Cairo, Egypt.

Cytotoxic activity

The cytotoxic effect of total alcoholic extract of *C. pycnocephalus* L. (Figure 6) was investigated at different concentrations, 50, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/ml. The tested cell lines, breast carcinoma (MCF-7), lung carcinoma (A-549) and hepatocellular carcinoma (HepG-2) were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown as monolayers in growth RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/ml gentamycin. The monolayers of 10,000 cells adhered at the bottom of the wells in a 96-well microtitre plate incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate filtered saline (0.01 M, pH 7.2) and simultaneously the cells were treated with 100 µl from different dilutions of the test sample in fresh maintenance medium and incubated at 37°C. A control of untreated cells was made in the absence of the test sample. Six wells were used for each concentration of the test sample. Every 24 h, the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and read the absorbance at λmax 490 nm using ELISA reader (SunRise TECAN, Inc, USA) after well mixing. The absorbance values from untreated cells were considered as 100% proliferation. The number of viable cells was determined using ELISA reader as previously mentioned and the percentage of viability was calculated as:

[1- (ODt / ODc) × 100%]

where ODt: optical density of wells treated with the test sample. ODc: optical density of untreated cells. The IC_{50} value which reduces the cells number by 50%, was determined from dose response curve.

Antioxidant activity

Radical scavenging activity of total alcoholic extract of *C.*

Table 2. NMR data of compound 5 (500, 125 MHz, DMSO-d_6, TMS).

No.	δ_H (δ ppm)	δ_C (DEPT)	HSQC	HMBC	HHCOSY
2	-	164.7 s	-	-	-
3	6.84 s	104.3 d	C-3	4, 10	-
4	-	182.5 s	-	-	-
5	12.90 (OH, s)	161.6 s	-	-	-
6	6.48 (1H, d, *J*=2.0 Hz)	100.1 d	C-6	8, 10	8
7	-	163.4 s	-	-	-
8	6.81 (1H, d, *J*=2.0 Hz)	95.3 d	C-8	6, 10	6
9	-	157.5 s	-	-	-
10	-	105.9 s	-	-	-
1`	-	123.4 s	-	-	-
2`	7.46 (1H, d, *J*=8.8 Hz)	113.7 d	C-2`	4`, 6`, 2	6`
3`	9.24 (OH, s)	147.3 s	-	-	-
4`	OCH_3 3.87 (3H, brs)	151.8 s; 55.8 q	OCH_3	4`	-
5`	7.16 (1H, d, *J*=8.8 Hz)	112.7 d	C-5`	1`, 3`	6`
6`	7.58 (1H, dd, *J*=2.0, 8.8 Hz)	119.4 d	C-6`	2`, 4`	2`, 5`
1``	5.06 (1H, d, *J*=7.2 Hz)	100.4 d	C-1``	7	2``
2``	3.65 (1H, m)	75.9 d	C-2``	-	1``, 3``
3``	3.25 (1H, m)	76.7 d	C-3``	-	2``, 4``
4``	3.32 (1H, m)	78.9 d	C-4``	-	3``, 5``
5``	3.10 (1H, m)	76.1 d	C-5``	-	4``, 6``
6``	3.40 , 4.10 (2H, m)	60.1 t	C-6``	-	5``
1```	4.15 (1H, d, *J*= 4.8 Hz)	103.3 d	C-1```	-	2```
2```	3.33 (1H, m)	70.9 d	C-2```	-	1```, 3```
3```	3.24 (1H, m)	72.9 d	C-3```	-	2```, 4```
4```	3.60 (1H, m)	67.6 d	C-4```	-	3```, 5```
5```	3.20, 3.65 (2H, m)	64.5 t	C-5```	-	4```

pycnocephalus L. was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University by the DPPH free radical scavenging assay in triplicate and average values were considered (Figure 7).

DPPH radical scavenging activity (Yen and Duh, 1994): Methanol solution (0.004% w/v) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was freshly prepared and stored at 10°C in the dark. A methanol solution of the test extract was prepared. A 40 µl aliquot of the methanol solution was added to 3 ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula:

DPPH scavenging activity % [PI] = [{(*A*C- *A*T)/*A*C} × 100] (1)

where *A*C = Absorbance of the control and *A*T = absorbance of the sample + DPPH.

Antimicrobial activity

Agar diffusion method (Perez et al., 1990): The total alcoholic extract of *C. pycnocephalus* L. was dissolved in DMSO and evaluated by agar diffusion method at a dose of 50 µg/ml and the results were reported as shown in Table 3. The tested fungi were *Asperagillus fumigates* (RCMB 02568), *Syncephalastrum racemosum* (RCMB 05922), *Geotricum candidum* (RCMB 05097) and *Candida albicans* (RCMB 05036); Gram positive bacteria *were Streptococcus pneumonia* (RCMB 010010) and *Bacillus subtillis* (RCMB 010067) while Gram negative bacteria were *Pseudomonas aeruginosa* (RCMB 010043) and *Escherichia coli* (RCMB 010052). Amphotericin B, ampicillin and gentamicin were used as standard antifungal and antimicrobial agents; the diameter of zone of inhibition was measured in mm. The microorganisms were obtained from Regional Centre of Mycology and Biotechnology, Al-Azhar University.

RESULTS AND DISCUSSION

Phytochemical results

Characterisation of the isolated compounds

Petroleum ether, chloroform and butanol extracts of air dried aerial parts of *C. pycnocephalus* L. were subjected to chromatographic separation followed by purification and gave five pure compounds 1 to 5. The identification

Table 3. Antimicrobial activity of total alcoholic extract of *C. pycnocephalus* L.

Microorganism	Inhibition zone diameter (mm) of	
	Total alcoholic extract	Standards
Fungi		**Amphotericin B**
Asperagillus fumigates	16.8 ± 0.58	23.7 ± 0.1
Syncephalastrum racemosum	15.2 ± 0.58	19.7 ± 0.2
Geotricum candidum	18.6 ± 0.63	28.7 ± 0.2
Candida albicans	***NA**	25.4 ± 0.1
Gram positive bacteria		**Ampicillin**
Streptococcus pneumonia	17.3 ± 0.63	23.8 ± 0.2
Bacillus subtilis	18.6 ± 0.72	32.4 ± 0.3
Gram negative bacteria		**Gentamicin**
Pseudomonas aeruginosa	15.3 ± 0.58	17.3 ± 0.1
Escherichia coli	17.2 ± 0.63	19.9 ± 0.3

*NA: No activity; Extract and used standards were tested at a dose of 50 µg.

of these compounds was carried out on the basis of spectroscopic methods (UV, IR, ^{1}H NMR, ^{13}C NMR, DEPT 135, COSY, HMBC, HSQC and MS spectroscopy).

Compound 1, [3-*O*-acetyl ursolic acid-28-ethyl ester]: The EI-MS of compound 1 exhibited a distinct molecular ion peak at *m/z* 526 [M^+] corresponding molecular formula $C_{34}H_{54}O_4$. The ^{13}C NMR and DEPT data of compound exhibited 34 carbons. These carbons are attributed to nine methyls, ten methylenes, seven methines and eight quaternary carbon atoms. The ^{1}H-NMR data, (Table 1), revealed the presence of nine methyl signals, six appeared as singlet at δ_H 0.80, 0.78, 1.25, 0.89, 1.08, 2.20 and connected, via HSQC to C-23 (δ_C 28.4), C-24 (δ_C 16.8), C-25 (δ_C 14.1), C-26 (δ_C 16.8), C-27 (δ_C 23.7) and C-32 (δ_C 23.0), respectively; and two doublets at δ_H 0.90 (3H, d, *J*=5.5 Hz) and 0.82 (3H, d, *J*= 4.5 Hz) as well as one triplet at δ_H 1.20 (3H, t, *J*= 5.0 Hz) and connected to C-29 (δ_C 14.3), C-30 (δ_C 23.5) and C-34 (δ_C 15.5), respectively. In addition, the ^{1}H-NMR data showed a triplet signal at δ_H 5.12 (1H, t, *J*= 3.6 Hz) connected via HSQC to C-12 (δ_C 121.7). These information along with the carbon chemical shift of C-12 and C-13 (δ_C 121.7/145.2 and proton shift of H-12 (δ_H 5.12) suggested that compound 1 was a triterpene carrying a $(\Delta^{12})^{13}$ double bond (Ahmad and Rahman, 1994).

The presence of two douplet methyl groups at 0.90 (3H, d, *J* =5.5 Hz) and 0.82 (3H, d, *J*=4.5 Hz) for CH_3-29 and CH_3-30, respectively suggested the presence of ursolic acid nucleus. This suggestion was further confirmed by HMBC experiment (Figure 4) in which there are three bond correlations between CH_3-29 with C-18 and C-20; CH_3-30 with C-19 and C-21. Further HMBC correlations were detected between methine proton at C-12 (δ_C 121.7) and C-9 (δ_C 47.6), C-14 (δ_C 41.7), and two bonds correlations with C-11 (δ_C 23.7). The methine proton at C-3 appearing at δ_H 4.45 (1H, t, *J*=7.0 Hz) showed two bond correlation with C-2 (δ_C 24.9) and C-4 (δ_C 37.8). Also the ^{1}H NMR spectrum showed extra signals, quartet signal appear at δ_H 4.05 (2H, q, *J*= 7.2 Hz) and triplet δ_H 1.20 (3H, t, *J*=5.0 Hz) connected to C-33 (δ_C 60.1, CH_2) and C-34 (δ_C 15.5, CH_3) in HSQC spectrum, respectively which indicated the presence of ethyl group. The position of it was established from HMBC correlation of methylene group at δ_H 4.05 (2H, q, *J*= 7.2 Hz) with C=O -28 at δ_C 173.9. Further confirmation was obtained from the upfield shift of carbonyl group at 28 position to 173.9 ppm compared to that of ursolic acid 180.1 (Babalola and Shode, 2013). Other extra ^{1}H NMR signal at δ_H 2.20 (3H, s) and two ^{13}C NMR signals at δ_C 23.1 and 173.7 were observed which indicated the presence of acetyl group. The position of acetyl group was proved through HMBC correlation by three bonds of H-3 (δ_H 4.45) with carbonyl (C-31) (δ_C 173.7). The EI-MS confirmed the presence of acetyl and ethyl moieties in compound 1 where it showed fragments at *m/z* 483 [M^+-$COCH_3$] and 454 [M^+-$COCH_3$-C_2H_5]. From the aforementioned data and through comparison with reported literatures for related compounds (Ahmad and Rahman, 1994), the structure of compound 1 was established to be 3-*O*-acetyl ursolic acid-28-ethyl ester and it is the first report of this compound from nature.

Compound 2, [Bis (2-ethylhexyl) benzene-1, 2-dicarboxylate]: Compound 2 (1.37 g), was isolated as pale yellow oil, R_f 0.7 [(10% EtOAc in *n*-hexane)]. The EI/MS spectrum displayed a pseudo molecular ion [$M]^+$ at *m/z* 390, which is in a good agreement with the molecular formula $C_{24}H_{38}O_4$ (Figure 3). ^{1}H NMR and ^{13}C NMR data

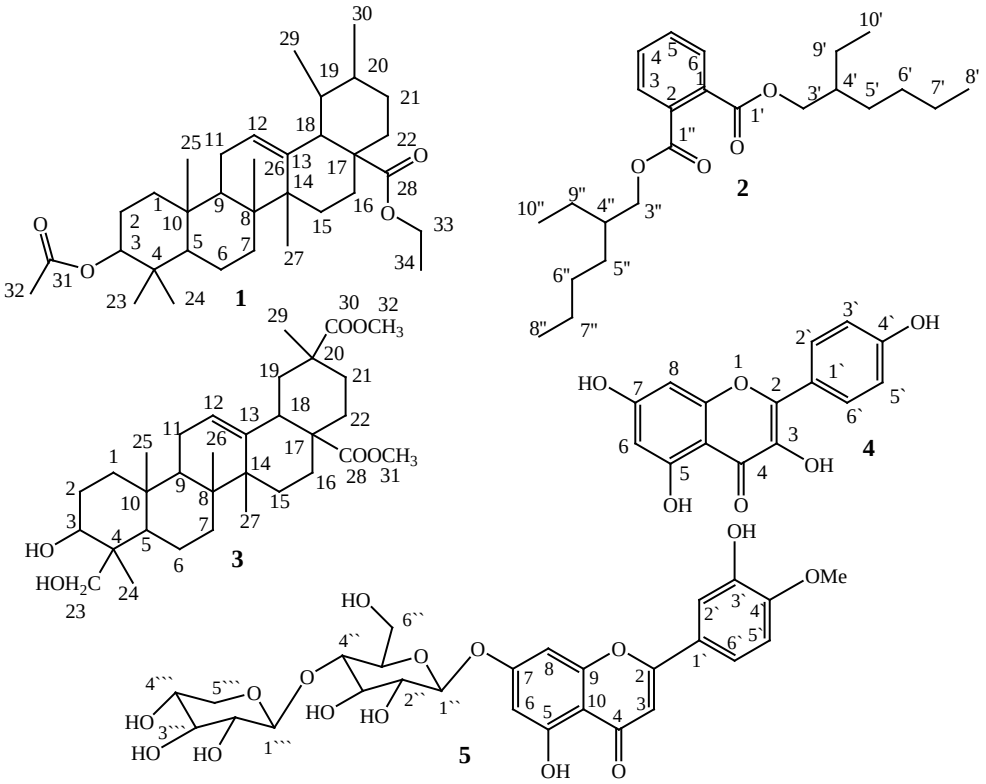

Figure 3. The chemical structures of compounds 1 to 5.

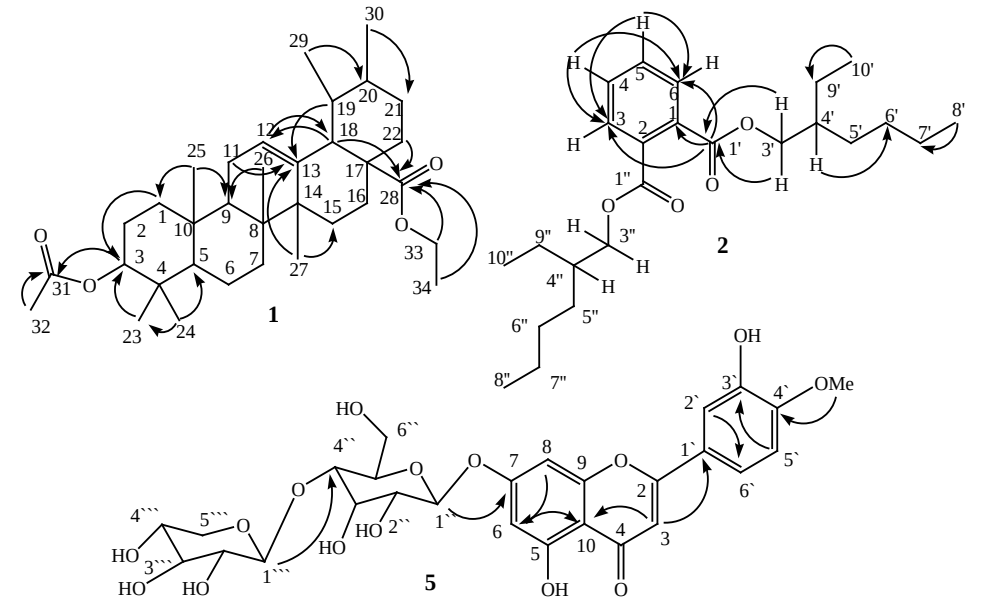

Figure 4. Important HMBC correlations of compounds **1**, **2** and **5**.

demonstrated the presence of 12 signals attributed to two methyls, five methylenes, three methines and two quaternary carbons indicated the symmetry of the compound since the number of carbons and protons measured by mass is 24 and 38, respectively. Two of the four quaternary carbons appeared at δ_C 167.9, indicated

the presence of two esters carbonyl moiety in the compound. The ^{1}H NMR data exhibited two aromatic protons appeared at δ_H 7.72 (2H, *m*, H-3, 6) and 7.54 (2H, *m*, H-4, 5), two methylene protons at δ_H 4.22 (4H, *d*, *J* = 6.4 Hz, H-3`, 3``), two methine protons at $\delta_H$ 1.70 (2H, *m*, H-4`, 4``), four methyl resonances at δ_H 0.90 (4H, 10`, 10``) and 0.88 (4H, 8`, 8``). The spectrum also showed multiplets around δ_H 1.35 accounts for eight methylenes (16 H, 5`, 5``, 6`, 6``, 7`, 7``, 9`, 9``). Combined studies of 2D NMR experiments, notable COSY, HSQC and HMBC allowed to complete assignment of the spectral data. The existence of only two aromatic peaks in ^{1}H NMR spectrum (integrated to 2 protons each) suggested that the compound must have an *ortho*-disubstituted benzene ring bearing the same substituent in both positions (Silverstein et al., 1997). The aromatic protons at δ_H 7.72 (H-3/ H-6) showed direct coupling to a carbon at δ_C 129.0 in the HSQC experiment, in addition to long range HMBC correlations with carbons at δ_C 131.1, 132.6 and the carbonyl group at δ_C 167.9. Similarly, H-4/H-5 (δ_H 7.54) revealed direct coupling to carbon at δ_C 132.6 and long range correlations to carbons at δ_C 129.0. From the aforementioned correlations, the quaternary carbon at δ_C 132.6 was assigned as C-1/C-2 and the methine carbons at δ_C 131.1 and 129.0 to C-4/C-5 and C-3/C-6, respectively. The carbonyl quaternary carbon at δ_C 167.9 (C=O) showed correlation with H-3/ H-6. The methylene protons at δ_H 4.22 showed direct coupling to an oxygenated carbon at δ_C 68.3 and long range correlation with the carbonyl group at δ_C 167.9. Therefore, these protons were assigned as H-3'/H-3''. The multiplet (integrated for 2 protons) at δ_H 1.70 exhibited coupling with H-3'/H-3'' in COSY experiment which, in turn, showed direct coupling at δ_C 38.9 (C-4'/C-4'') in the HSQC experiment and long range correlations with carbons at δ_C 68.3 (C-3'/C-3''), 31.2 (C-5'/C-5''), 29.6 (C-6'/C-6''), 22.8 (C-9'/C-9'') and 11.6 (C-10'/C-10'') in HMBC (Figure 4). The methyls at δ_H 0.90 and 0.88 exhibited direct coupling to methyl carbons at δ_C 11.6 (C-10'/C-10'') and 14.4 (C-8'/C-8''), respectively. A common long range correlations by H-4'/H-4'' and the methyls at δ_H 0.88 (H-8'/H-8'') to δ_C 29.6 confirmed their assignment as C-6'/C-6''. These methyls were also connected to C-7'/C-7'' at δc 22.4. The other two methyl groups (δ_H 0.90, H-10'/H-10'') showed long range correlations to carbons at δ_C 38.9 (C-4'/C-4''). A multiplet at δ_H 1.35 showed long range correlations to C-10'/C-10'' (δ 11.6), C-4'/C-4'' (δ_C 38.9) and C-3'/C-3'' (δ 68.3). As a result, these protons must be H-9'/H-9'', which showed direct coupling with carbons at δ_C 22.8 (C-9'/C-9''). On the basis of the aforementioned data, compound 2 was identified as bis (2-ethylhexyl) benzene-1,2-dicarboxylate). This compound is a synthetic polypropylene derivative, which is used as a plasticizer for polyvinyl chloride (PVP) (Beeler et al., 1976). Bis (2-ethylhexyl) benzene-1,2-dicarboxylate) was previously isolated and reported as natural product from *Mentha longifolia* (Ertaş et al., 2015) and *Ziziphora persica* (Nadaf et al., 2013) and it showed larvicidal activity (Katadea et al., 2006). This is the first report to isolate *bis* (2-ethylhexyl) benzene-1, 2-dicarboxylate) from genus *Carduus*.

Compound 3, [3α, 24 -dihydroxyolean-12-en-28, 30-dioic acid dimethyl ester]: The ^{1}H NMR spectrum of compound 3 showed signals at δ (ppm) 1.26 (9H, s), 0.85 (6H, s) which in addition to the five ^{13}C NMR signals at δ_C 14.2 (C-24), 15.5 (C-25), 16.5 (C-26), 25.8 (C-27) and 29.7 (C-29) indicated the presence of five methyl groups in this compound. The other signal at δ_H 5.19 (1H, broad t, *J*= 7.6 Hz, H-12) in ^{1}H NMR spectrum with two signals at δ_C121.6 (C-12), 145.2 (C-13) in ^{13}C NMR spectrum revealed the presence of (Δ^{12})^{13} triterpenoidal compound (Ahmad and Rahman, 1994). The ^{1}H NMR signals at δ_H 4.10 (1H, *m*), 3.50 (6H, s), 2.30 (1H, broad t, *J*= 7.0 Hz) with ^{13}C NMR signals at 80.6, 50.3 and 37.8 were interpreted for CH-3, two OCH_3 groups at 31 and 32 and CH-18, respectively. The presence of CH_2OH was established from the ^{1}H NMR and ^{13}C NMR signals at δ_H 3.70 and 4.10 each for one proton with signal at δ_C 60.1 for CH_2OH at position 24 (Ahmad and Rahman, 1994). The aforementioned data with the remaining signals reported in experimental section and EI-MS spectrum at *m/z* 530, in addition to comparison with literature (Ahmad and Rahman, 1994) confirmed the structure of compound 3 as 3-*α*, 24- dihydroxyolean-12-en-28, 30-dioic acid dimethyl ester. This is the first report of this compound in genus *Carduus*.

Compound 4 [kaempferol]: The UV spectral data of compound 4 showed UV λ max; MeOH (266, 338 nm) which indicated the presence of flavonol compound. The ^{1}H NMR spectrum showed an A_2B_2 spin system for ring B at δ_H 6.85 (2H, *d*, *J*= 8.0 Hz, H-3`and 5`), 7.80 (2H, *d*, *J*= 8.0 Hz, H-2`and 6`). In addition, the presence of singlet signal at δ_H 6.40 for H-6, and other signal at δ 6.05 (1H, *s*, H-8) for H-8 indicated the presence of 3, 5, 7, 4` tetrahydroxy flavone nucleus. Also, the EI-MS spectrum showed a molecular ion peak at *m/z* 286 [M^+ , 100%] which are in a good agreement with the molecular formula $C_{15}H_{10}O_6$ from the aforementioned data and through comparison with literatures (Mabry et al., 1970; Markham et al., 1978) compound 4 was identified as kaempferol, it was previously reported in genus *Carduus*.

Compound 5 [Diosmetin- 7-*O* -*α*- L- arabinopyransyl -(1'''→ 4'') -*β* - D - glucopyranoside]: The UV spectral data of compound 5 showed absorption bands at 269 and 344 nm indicating the flavones nature of the compound. The different shifting reagents confirmed the presence of flavone nucleus with free hydroxyl group at 5 position and the absence of free hydroxyl group at 7 and 4` positions or presence of occupied ones. The FAB-MS spectrum(Figure 5), exhibited a molecular ion peak at *m/z* 633 [M^++ K] and *m/z* 595 [M^+ +H], which is in agreement

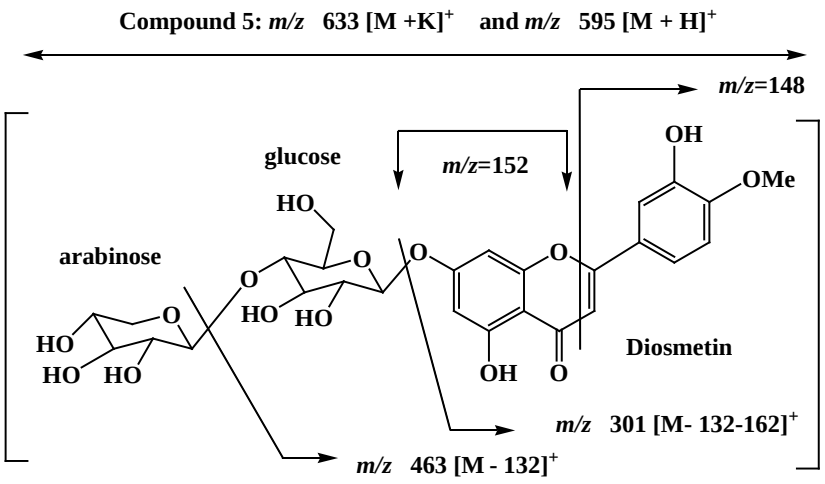

Figure 5. Fragmentation pattern of compound 5.

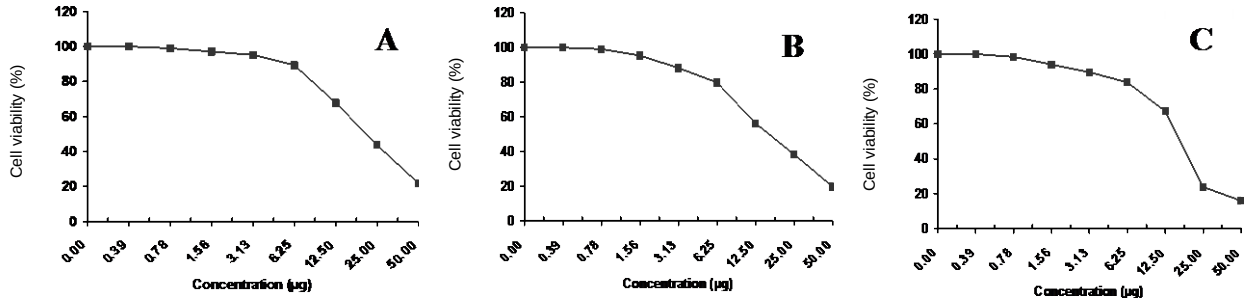

Figure 6. Cytotoxic effect of total alcoholic extract of *C. pycnocephalus* L. against HepG-2 (**A**), A-549 (**B**) and MCF-7 (**C**) cells.

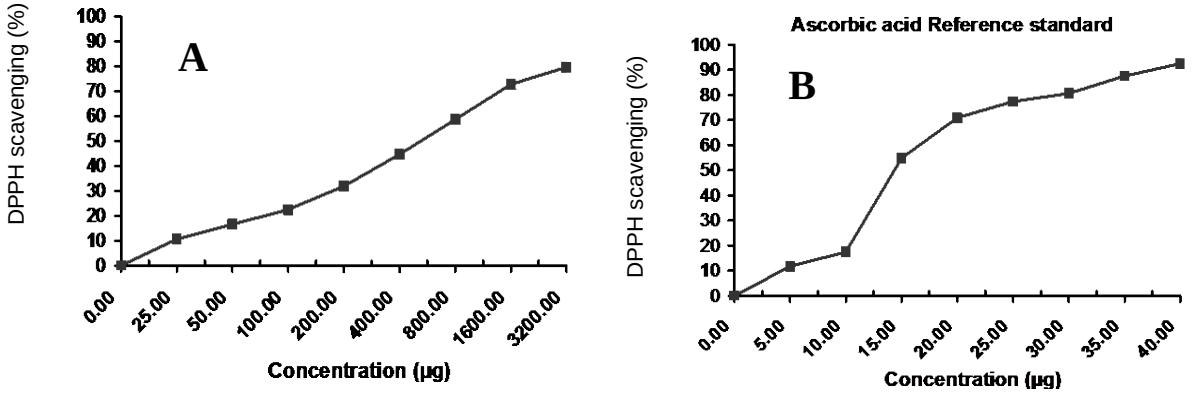

Figure 7. Antioxidant activity of total alcoholic extract of *C. pycnocephalus* (**A**) using DPPH free radical scavenging test and ascorbic acid as a reference compound (**B**).

with molecular formula $C_{27}H_{30}O_{15}$. The ^{1}H and ^{13}C NMR spectral data of compound 5 (Table 2) were close to that of compounds reported (El-Lakany et al., 1997; Saeidnia et al., 2011) suggesting that 5 had diosmetin as an aglycon. This was confirmed from the ^{13}C NMR spectral data of compound 5 which showed 27 carbons, 16 of which were very similar to those for diosmetin. Further confirmation for the presence of diosmetin was the ^{1}H NMR which showed the presence of methoxy group appeared as singlet signal at δ_H 3.88.

This methoxy group connected via HMBC correlation to C-4` at δ_C 151.8 (Figure 4). Furthermore, the ^{1}H NMR

and H-HCOSY data showed ABX spin system for ring B appearing at δ_H 7.58 (1H, dd, *J* = 2.0, 8.8 Hz, H-6`), 7.46 (1H, d, *J* = 2.0 Hz, H-2`), 7.16 (1H, d, *J* = 8.8 Hz, H-5`). In addition to the presence of two meta coupled signals at δ_H 6.48 and 6.81 (*J* = 2.0 Hz) suggested the 5, 7-disubstituted A ring of flavonoid and assigned to H-6 and H-8, respectively. This was confirmed from the HMBC experiment that exhibited correlations of H-2' (δ_H 7.46) with C-2 (δ_C 164.7), H-6' at δ_H 7.58 with C-2 (δ_C 164.7) and C-4' (δ_C 151.8) and H-5' (δ_H 7.16) with C-1` (δ_C 123.4) and C-3' (δ_C 147.3). Further HMBC correlations of H-6 (δ_H 6.48) with C-10 (δ_C 105.9) and H-8 (δ_H 6.81) with C-6 (δ_C 100.1) and C-10 (δ_C 105.9) were detected. These data are in a good agreement with diosmetin (El-Lakany et al., 1997; Saeidnia et al., 2011). The remaining ^{1}H NMR signals and the other eleven ^{13}C NMR signals, with ^{1}H ^{1}H COSY spectrum (Table 2) of compound 5 suggested the presence of two sugar moieties (hexose and pentose), the sugar moieties were identified by acid hydroplysis of compound 5 as *β*-D-glucose and *α*-L-arabinose. The FABMS fragments at *m/z* 463 [M^+-ara] and *m/z* 301 [M^+- glu - ara + H] confirmed the presence of glucose and arabinose. The linkage of glucose moiety was found to be at C-7 from HMBC correlations (Figure 4), as the anomeric proton of glucose at δ_H 5.09 showed three bond correlation with C-7 (δ_C 163.4). The position of arabinose was deduced from the downfield shift of C-4`` ($\delta_C$ 78.9) of glucose and this confirmed the connection of arabinose moiety at C-4`` of glucose. From the earlier discussed NMR data, compound 5 was identified as diosmetin- 7-*O*-*α*-L- arabinopyransyl - (1'''→4'') -*β* - D - glucopyranoside. This is the first report of this compound in nature.

Cytotoxic activity

The cytotoxic effect of total alcoholic extract of *C. pycnocephalus* was assessed against breast carcinoma (MCF-7), lung carcinoma (A-549) and hepatocellular carcinoma (HepG-2) cell lines at different concentrations, 50, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/ml, and the extract showed strong activity against the tested cell lines with IC_{50} 16.9, 17.5 and 21.8 µg/ml, respectively as shown in Figure 6.

Antioxidant activity

The antioxidant effect of total alcoholic extract of *C. pycnocephalaus* using DPPH free radical scavenging method was measured at eight different concentrations and the extract showed weak antioxidant activity in comparison with ascorbic acid with SC_{50} 554.2 and 14.2 µg/ml, respectively as shown in Figure 7.

Antimicrobial activity

The antimicrobial activity against fungi, *A. fumigates* (RCMB 02568), *S. racemosum* (RCMB 05922), *G. candidum* (RCMB 05097), *C. albicans* (RCMB 05036); Gram positive bacteria, *S. pneumonia* (RCMB 010010), *B. subtillis* (RCMB 010067) and Gram negative bacteria *P. aeruginosa* (RCMB 010043) and *E. coli* (RCMB 010052) was evaluated using agar diffusion technique. The extract exhibited strong antifungal activity against *S. racemosum* with inhibition zone diameter of 15.2 mm compared to that of amphotericin B 19.7 mm. In addition, a wide range of inhibitory activity against Gram-negative bacteria was observed. The extract showed strong anti-bacterial activity against *P. aeruginosa* and *E. coli* with inhibition zone diameter of 15.3 and 17.2 mm compared to that of gentamicin 17.3 and 19.9 mm, respectively. The extract showed also variable activities against other tested microorganisms as shown in Table 3.

Conclusion

Conclusively, this work represented the isolation of two new compounds 1 and 5 from *C. pycnocephalus* extract with three known compounds 2 to 4. In addition, the plant extract showed highly significant anticancer and antimicrobial activity; so it is considered as a good source for strong anticancer and antimicrobial principles.

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Conflict of interest

Authors declare that there are no conflicts of interests

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Characterization of alkaloid constitution and evaluation of antimicrobial activity of *Solanum nigrum* using gas chromatography mass spectrometry (GC-MS)

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In this study, the alkaloid compounds of *Solanum nigrum* have been evaluated. The chemical compositions of the leaf methanol extract of *S. nigrum* were investigated using gas chromatography-mass specroscopy (GC-MS). GC-MS analysis of *S. nigrum* alkaloid leaf methanol extract revealed the existence of the cyclopentasiloxane-decamethyl, L-proline, ethylester, 2-ethyl-1-butanol, methyl ether, cyclopentasiloxane-ocamethyl, betanedioic acid, hydroxyl, diethyl, ester, 1.1.3.3.5.5.7.7-octamethy-l-7-(2methyl-propoxy) tetrasiloxane-1, dodecanoic acid, 3-hydroxy-, ethyl ester, cyclopentasiloxane-ocamethyl, dodecanedoic acid, bistert-butyldimethylsilyl ester, 2-pyrrolidinecarboxylic acid-5-oxo, ethyl ester, 1-dodecanamine, N.N-dimethyl, cyclooctasiloxane, hexadecamethyl, 5-keto-2, 2-dimethylheptanoic acid, ethyl ester, cyclodecasiloxane, eicosamethyl, 9.12.15-octadecatrienoic acid, octadecanoic acid, octadecenal, 9-octadecenamide, octadecane, 3-ethyl-5-(ethylbutyl), N-acetyl-L-tryptophan ethyl ester, ethyl iso-allocholate, phthalic acid, di(2-propylpentyl)ester and 17-(1.5-Dimethylhexyl)-10. 13-dimethyl-2.3.4.7.8.9.10.11. 12.13.14.15.16.17-tetradecahydro-1H. Alkaloids extract from leaf of *S. nigrum* were assayed for *in vitro* antibacterial activity against *Escherichia coli*, *Proteus mirabilis, Staphylococcus aureus, Pseudomonas aerogenosa* and *Klebsiella pneumonia* using the diffusion method in agar. The zone of inhibition was compared with different standard antibiotics. The diameters of inhibition zones ranged from 0.8 to 2.01 mm for all treatments.

Key words: Alkaloids, antibacterial activity, gas chromatography-mass specroscopy (GC-MS) analysis, *Solanum nigrum*.

INTRODUCTION

Plants are rich source of secondary metabolites with interesting biological activities (Koduru et al., 2006). Several plant products have been shown to exert a protective role against the formation of free radicals and playing a beneficial role in maintaining disease condition (Ajitha et al., 2001). *Solanum nigrum* is a common weed in gardens, fields and waste-land throughout the country (up to 1500 m altitude) (Figure 1). It is found in Baghdad,

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Basrah, Kut, Tal-Kaif, and Sulaimaniya.Leaves of *S. nigrum* contain solanin; solanidin are poisonous to cattle, sheep, horses and goat. The effects of poison are necrosis, paralysis, salivation, vomiting and diarrhoea. The medicinal value of drug plants is due to the presence of some chemical substances in the plant tissues which produce a definite physiological action on the human body. These chemicals include alkaloids, flavanoids, glucosides, tannins, gums, resins, essential oils, fatty oils, carbon compounds, hydrogen, oxygen, nitrogen salts of some chemicals, etc. Very few of these chemicals are toxic also (Haraguchi et al., 1999; Sashikumar et al., 2003).

The photochemicals with adequate antibacterial activity will be used for the treatment of bacterial infections (Iwu et al., 1999; World Health Organization, 2002; Purohit and Vyas, 2004; Krishnaraju et al., 2005). Successful extraction is largely dependent on the type of solvent used in the extraction procedure. The most often tested extracts are water extract as a sample of extract that is primarily used in traditional medicine and extracts from organic solvents, such as methanol, as well as ethyl acetate, acetone, chloroform, and dichlormethane. Diffusion and dilution method are two types of susceptibility test used to determine the antibacterial efficacy of plant extracts. Diffusion method is a qualitative test which allows classification of bacteria as susceptible or resistant to the tested plant extract according to the size of diameter of the zone of inhibition (Alves et al., 2000; Palombo and Semple, 2001; Uzun et al., 2004; Cos et al., 2006; Ncube et al., 2008; Stanojević et al., 2010). Considering the high economical and pharmacological importance of secondary plant metabolites, industries are deeply interested in utilizing plant tissue culture technique for large scale production of these substances (Misawa, 1994). The aim of this study is to assess the antibacterial activity of alkaloids extracts from the leaves of *S. nigrum*, which can be the basis for the synthesis of new antibiotics. This is because of increase in the emergence of bacterial strains resistant to multiple clinical disease.

MATERIALS AND METHODS

Collection and preparation of plant

In this research, the leaves were dried at room temperature for 13 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 specroscopy (GC-MS) analysis

GC-MS analysis of the methanol extract of *S. nigrum* 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 Turbomass 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 operated in electron impact mode with ionization energy of 70 eV. 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, 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 to 200°C, then 5°C/min 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.

Measurement of antibacterial activity

The antibacterial activity of alkaloids was determined using agar well diffusion method. Wells of 5 mm diameter were punched in the agar medium with sterile cork borer and filled with plant alkaloid extract. Standard antibiotics, penicillin, kanamycin, cefotoxime, streptomycin and refampin (1 mg/ml) were also tested for their antibacterial activity. The plates were incubated at 370°C for

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

Serial No.	Phytochemical compound	Formula	Molecular weight	Exact mass	Structure
1	Cyclopentasiloxane. decamethyl	$C_{10}H_{30}O_5Si_5$	370	370.093956	Figure 3
2	L-Proline, ethylester	$C_7H_{13}NO_2$	143	143.094628	Figure 4
3	2-Ethyl-1-butanol, methyl ether	$C_7H_{16}O$	116	116.1201153	Figure 5
4	Cyclopentasiloxane. ocamethyl	$C_8H_{24}O_4Si_4$	296	296.075165	Figure 6
5	Betanedioic acid , hydroxyl,diethyl, ester	$C_8H_{14}O_5$	190	190.084124	Figure 7
6	1.1.3.3.5.5.7.7-Octamethy-l-7-(2methyl-propoxy) tetrasiloxane-1	$C_{12}H_{34}O_5Si_4$	370	370.14833	Figure 8
7	Dodecanoic acid, 3-hydroxy-, ethyl ester	$C_{14}H_{28}O_3$	244	244.203845	Figure 9
9	Dodecanedoic acid. Bistert-butyldimethylsilyl ester	$C_{24}H_{50}O_4Si_2$	458	458.324762	Figure 10
10	2-Pyrrolidinecarboxylic acid-5-oxo, ethyl ester	$C_7H_{11}NO_3$	157	157.073894	Figure 11
11	1-Dodecanamine, N.N-dimethyl	$C_{14}H_{31}N$	213	213.24565	Figure 12
12	Cyclooctasiloxane, hexadecamethyl	$C_{16}H_{48}O_8Si_8$	592	592.15033	Figure 13
13	5-keto-2, 2-dimethylheptanoic acid, ethyl ester	$C_{11}H_{20}O_3$	200	200.141245	Figure 14
14	Cyclodecasiloxane, eicosamethyl	$C_{20}H_{60}O_{10}Si_{10}$	740	740.187912	Figure 15
15	9.12.15-Octadecatrienoic acid	$C_{18}H_{30}O_2$	278	278.22458	Figure 16
16	Octadecanoic acid	$C_{18}H_{36}O_2$	284	284.27153	Figure 17
17	Octadecenal	$C_{18}H_{34}O$	266	266.260965	Figure 18
18	9-Octadecenamide	$C_{18}H_{35}NO$	281	281.271864	Figure 19
19	Octadecane, 3-ethyl-5-(ethylbutyl)	$C_{26}H_{54}$	366	366.422552	Figure 20
20	N-Acetyl-L-tryptophan ethyl ester	$C_{15}H_{18}N_2O_3$	274	274.131742	Figure 21
21	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	436.318874	Figure 22
22	Phthalic acid, di(2-propylpentyl)ester	$C_{24}H_{38}O_4$	390	390.27701	Figure 23
23	17-(1.5-Dimethylhexyl)-10. 13-dimethyl-2.3.4.7.8.9.10.11. 12.13.14.15.16.17-tetradecahydro-1H	$C_{27}H_{46}O$	386	386.354866	Figure 24

The negative control was added without adding the cultures to know the sterile conditions. Then Petri dishes were placed in the refrigerator at 4°C or at room temperature for 1 h for diffusion, then incubate at 37°C for 24 h. Observation was done on zone of inhibition which produced different antibiotics. Measurement was done using a scale and the average of two diameters of each zone of inhibition was recorded.

RESULTS AND DISCUSSION

GC-MS analysis of alkaloid compound clearly showed the presence of twenty three compounds. The alkaloid compound, formula, molecular weight and exact mass are as shown in Table 1. The GC-MS chromatogram of the 23 peak of the compounds detected are as shown in Figure 2. Chromatogram GC-MS analysis of the methanol extract of *S. nigrum* showed the presence of twenty three major peaks and the components corresponding to the peaks were determined as follows. The first setup peaks were determined to be cyclopentasiloxane-decamethyl (Figure 3). The second peaks were indicated to be L-proline, ethylester (Figure 4). The next peaks was considered to be 2-ethyl-1-butanol, methyl ether, cyclopentasiloxane-ocamethyl, betanedioic acid, hydroxyl, diethyl, ester, 1.1.3.3.5.5.7.7-Octamethy-l-7-(2methyl-propoxy) tetrasiloxane-1, dodecanoic acid, 3-hydroxy-, ethyl ester, cyclopentasiloxane-ocamethyl, dodecanedoic acid, bistert-butyldimethylsilyl ester, 2-pyrrolidinecarboxylic acid-5-oxo, ethyl ester, 1-dodecanamine, N.N-dimethyl, cyclooctasiloxane, hexadecamethyl, 5-keto-2, 2-dimethylheptanoic acid, ethyl ester, cyclodecasiloxane,

Table 2. Zone of inhibition (mm) of test bacterial strains to alkaloid leaf extracts of *Solanum nigrum* (L.) and standard antibiotics.

Alkaloid antibiotic	*K. pneumonia*	*P. aeruginosa*	*S. aureus*	*P. mirabilis*	*E. coli*
Alkaloid	1.8±0.42	1.4±0.59	1.9±0.61	2.01±0.51	1.7±0.62
Kanamycin	0.8±0.3	0.5±0.4	0.6±0.2	0.4±0.1	0.9±0.1
Cefotoxime	1.3±0.5	1.5±0.1	1.2±0.1	1.2±0.6	1.2±0.3
Penicillin	1.1±0.2	1±0.5	1±0.4	1±0.2	1.6±0.1
Streptomycin	1.2±0.3	1.1±0.3	1.3±0.5	1.7±0.2	1.3±0.6
Rifampin	1.1±0.1	1.1±0.1	1.2±0.5	0.6±0.1	0.8±0.2

Figure 1. Leaves of *Solanum nigrum*.

eicosamethyl, 9.12.15-octadecatrienoic acid, octadecanoic acid, octadecenal, 9-octadecenamide, octadecane, 3-ethyl-5-(ethylbutyl), N-Acetyl-L-tryptophan ethyl ester, ethyl iso-allocholate, phthalic acid, di(2-propylpentyl)ester and 17-(1.5-Dimethylhexyl)-10. 13-dimethyl-2.3.4.7.8.9.10.11.12.13.14.15.16.17-tetradecahydro-1H (Figures 5 to 23). Among the identified phyto-compounds are the property of anti-oxidant and antimicrobial activities (Stainer et al., 1986; Singh et al., 1998; Prescott et al., 1999; Kumar et al., 2001; Purohit and Vyas, 2004; John and Senthilkumar, 2005; Venkatesan et al., 2005; Santh et al., 2006; Sazada et al., 2009). Plant based antimicrobials have enormous therapeutic potential as they can serve the

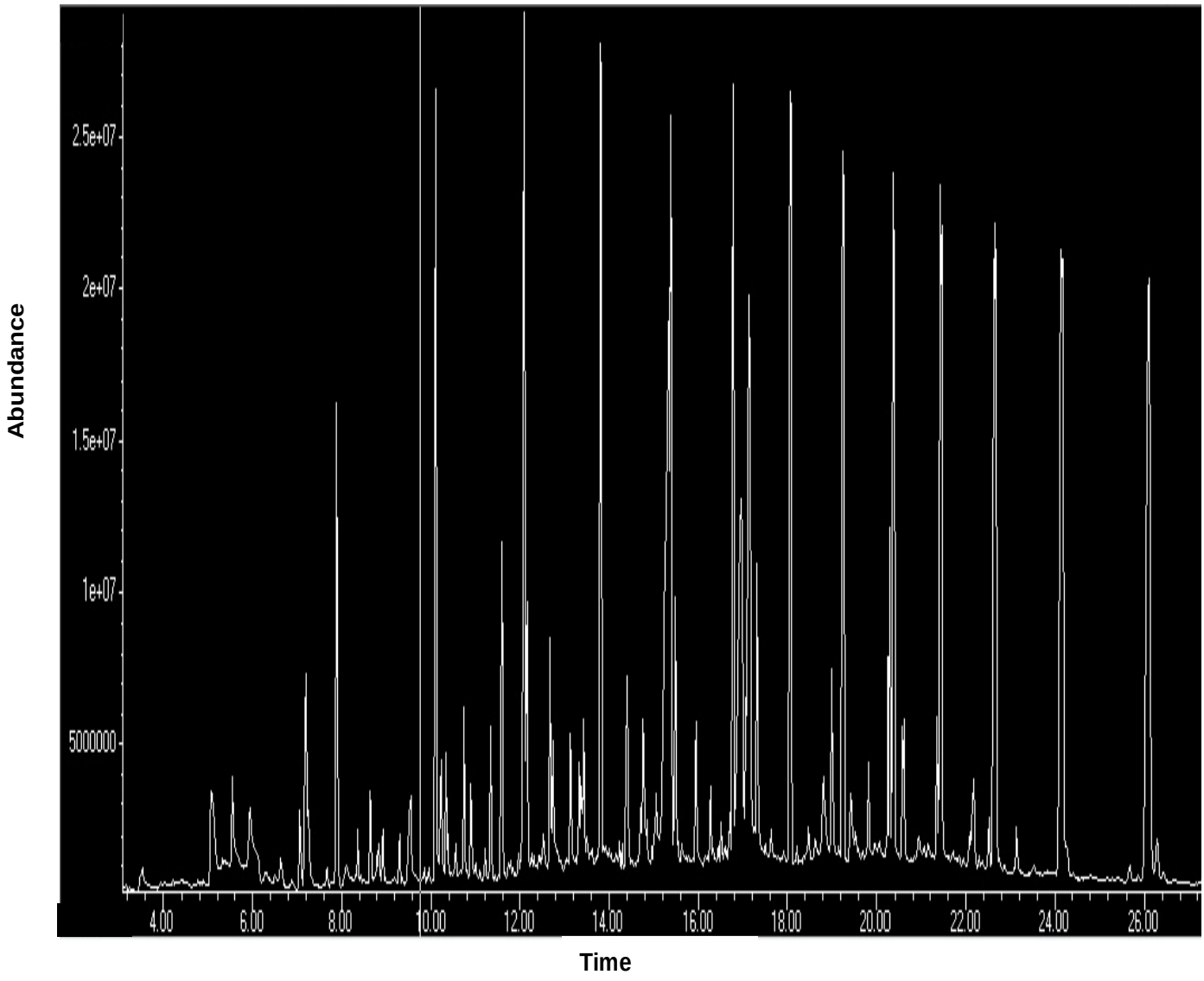

Figure 2. GC-MS Profile of leaves extract of *Solanum nigrum*.

purpose with lesser side effects. Continued further exploration of plant derived antimicrobials is needed today.

The results of the antimicrobial activity of the extracts of leaves of *S. nigrum* are as shown in Table 2. It was observed that the sensitivity tests show the effect of crude extracted alkaloids from seeds and roots of different bacterial strains, giving varying diameters depending on the tested strains.

The clear zone of growth inhibition was noted around the well due to diffusion of alkaloid compound. The diameter of the zone denotes the relative susceptibility of the test microorganism to a particular antimicrobial. The obtained results of the crude extracts were compared with the standard antibiotics such as penicillin, kanamycin, cefotoxime, streptomycin and refampin. All the tested organisms are highly sensitive to the methanol leaf extract (1.4 to 2 mm) than the standard antibiotics which showed more or less activity (0.4 to 1.7 mm).

The presence of antimicrobial substances in the higher plants is well established. Plants have provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health (Walton and Brown, 1999). Further works on the types of phytoconstituents and purification of individual groups of bioactive components can reveal the exact potential of the plant to inhibit several pathogenic microbes. *S. nigrum* is the most potent plant against pathogenic microorganisms. However, further studies are needed, including toxicity evaluation and purification of active antibacterial constituents from *S. nigrum* extracts looking toward a pharmaceutical use.

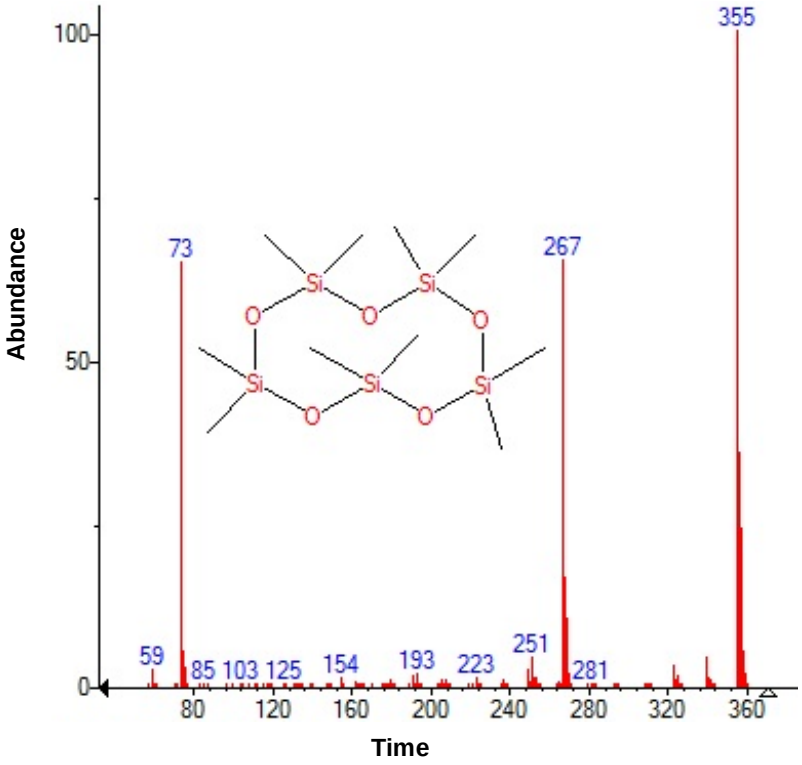

Figure 3. Structure of cyclopentasiloxane-decamethyl present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

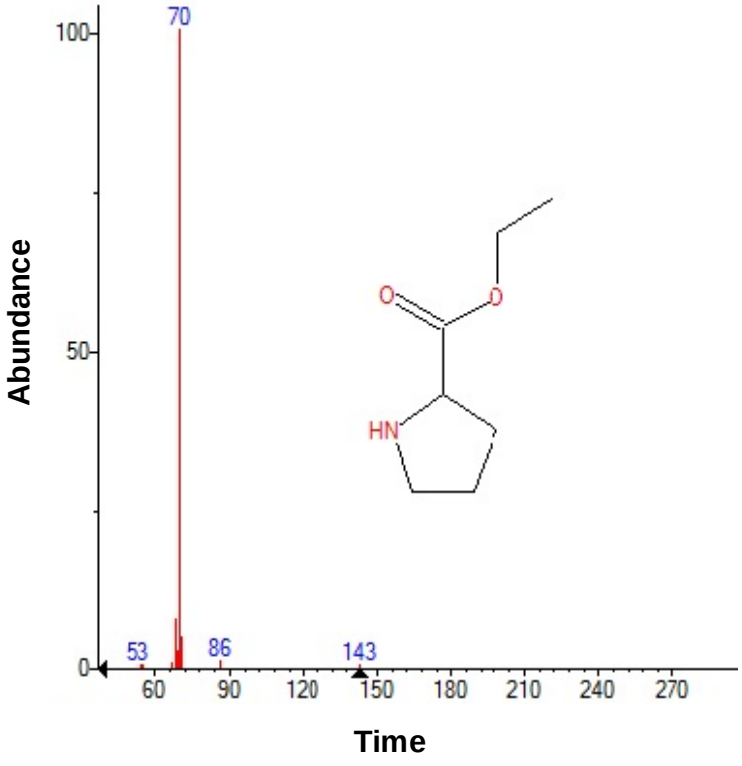

Figure 4. Structure of L-Proline, ethylester present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

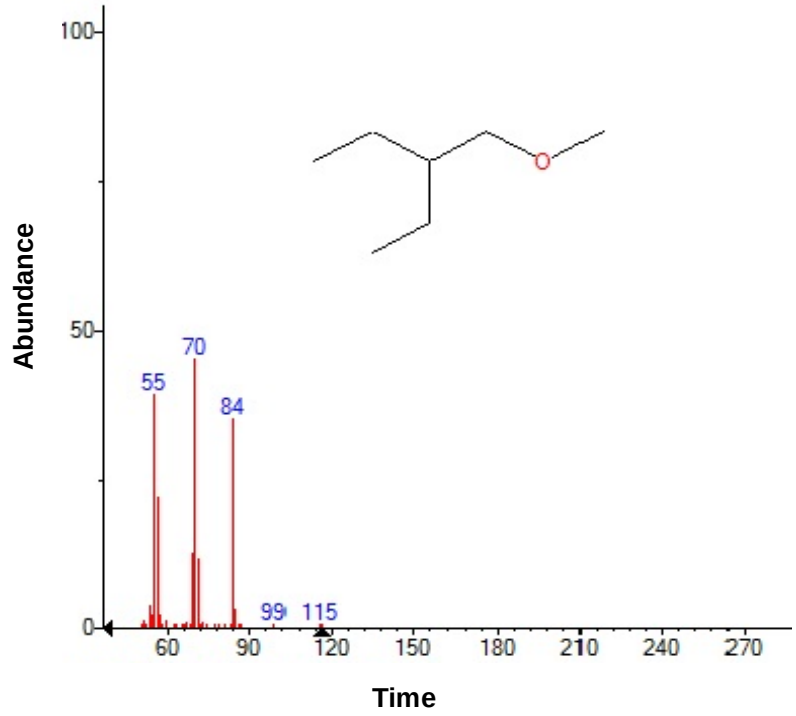

Figure 5. Structure of 2-ethyl-1-butanol, methyl ether present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

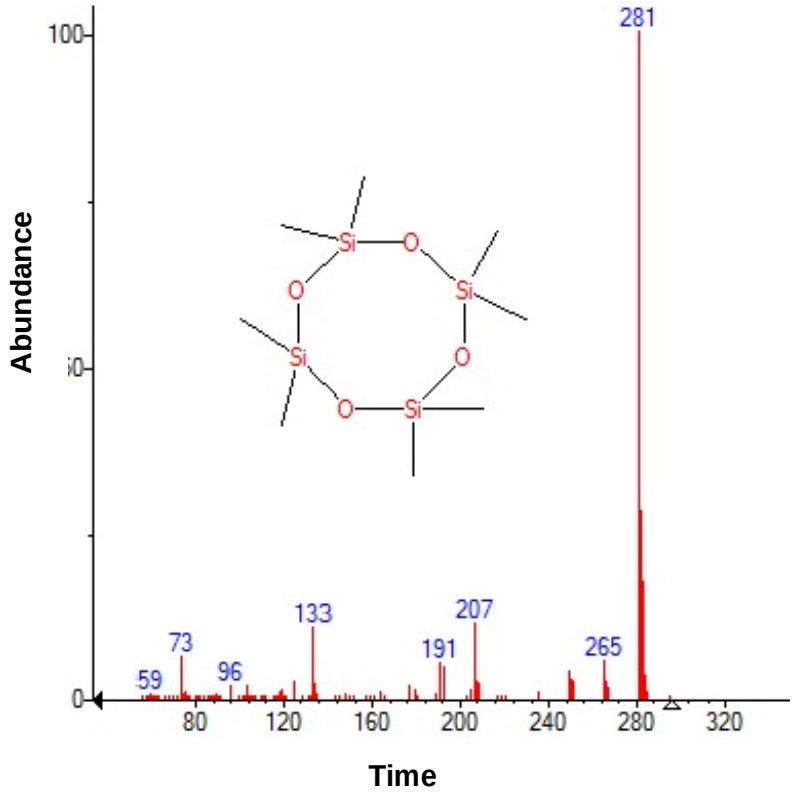

Figure 6. Structure of cyclopentasiloxane-ocamethyl present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

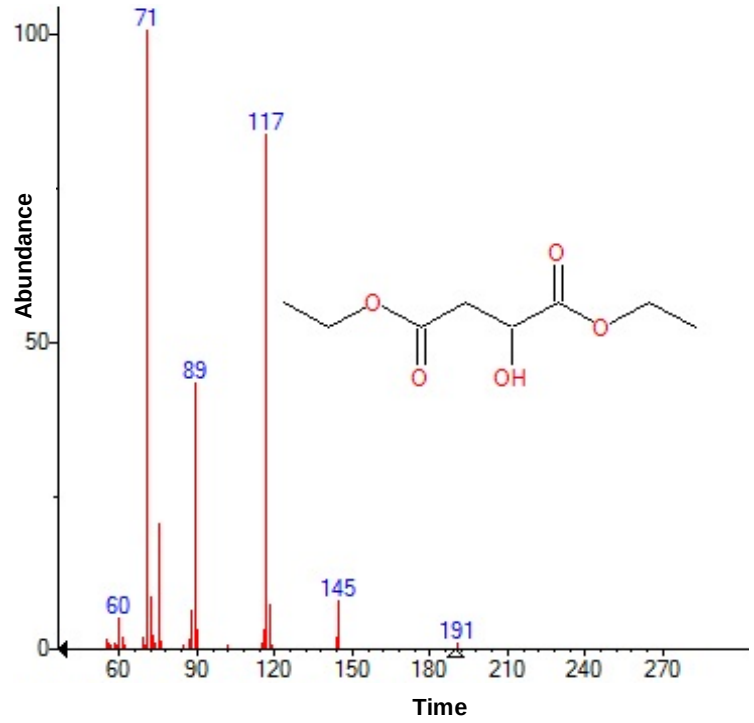

Figure 7. Structure of betanedioic acid, hydroxyl, diethyl, ester present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

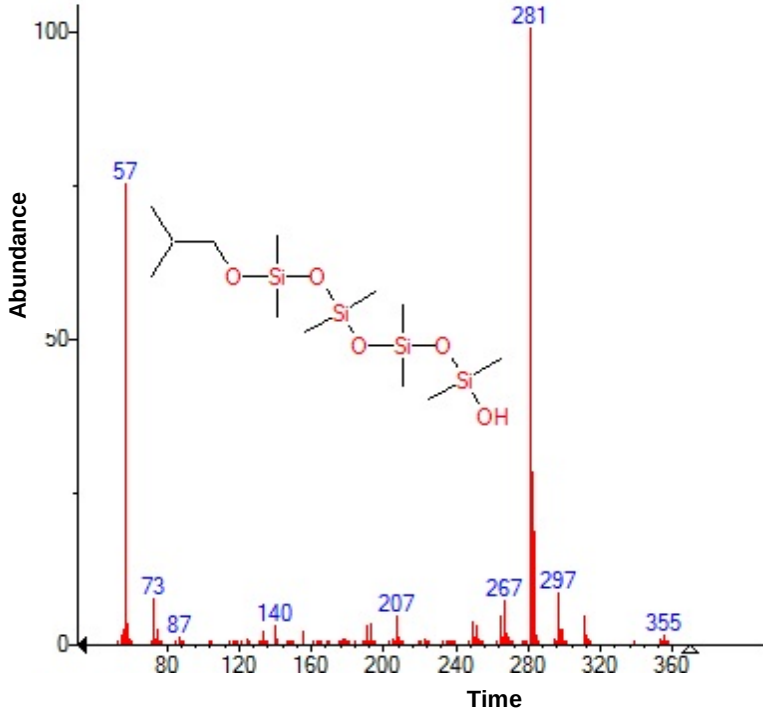

Figure 8. Structure of 1.1.3.3.5.5.7.7-Octamethy-l-7-(2methyl-propoxy) tetrasiloxane-1 present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

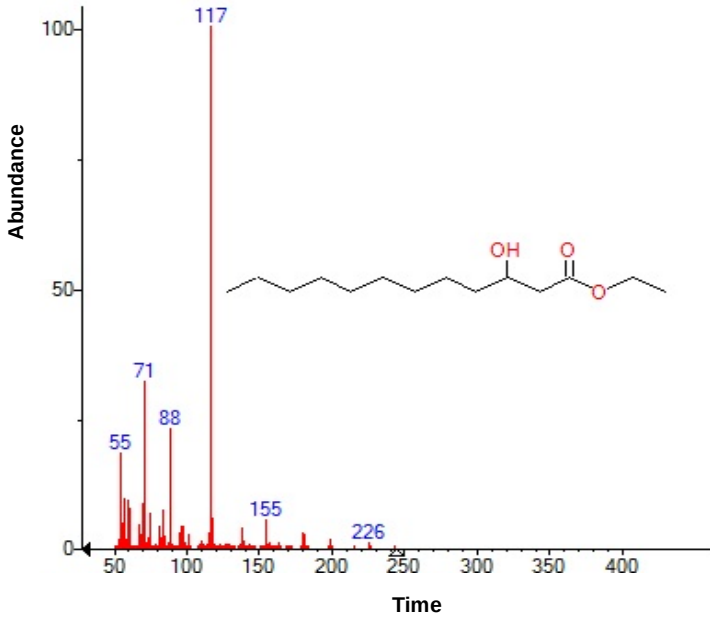

Figure 9. Structure of dodecanoic acid, 3-hydroxy-, ethyl ester present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

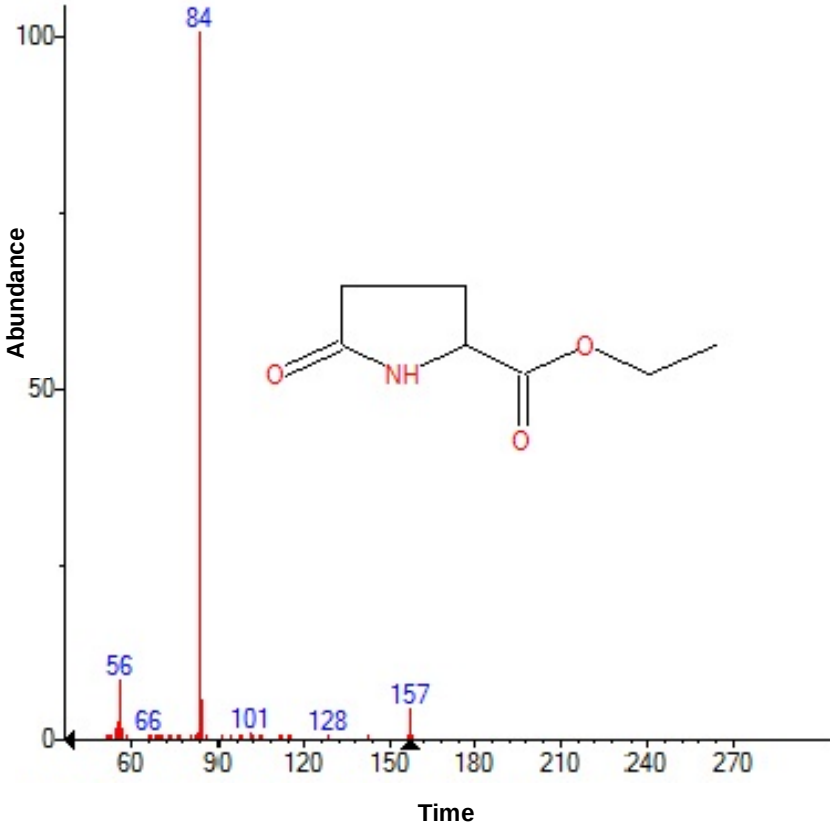

Figure 10. Structure of 2-pyrrolidinecarboxylic acid-5-oxo, ethyl ester present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

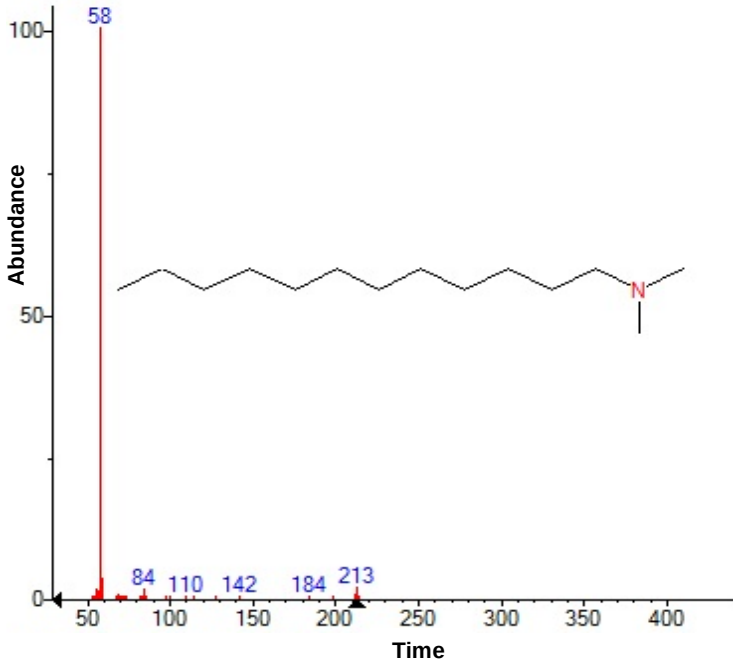

Figure 11. Structure of 1-dodecanamine, N.N-dimethyl present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

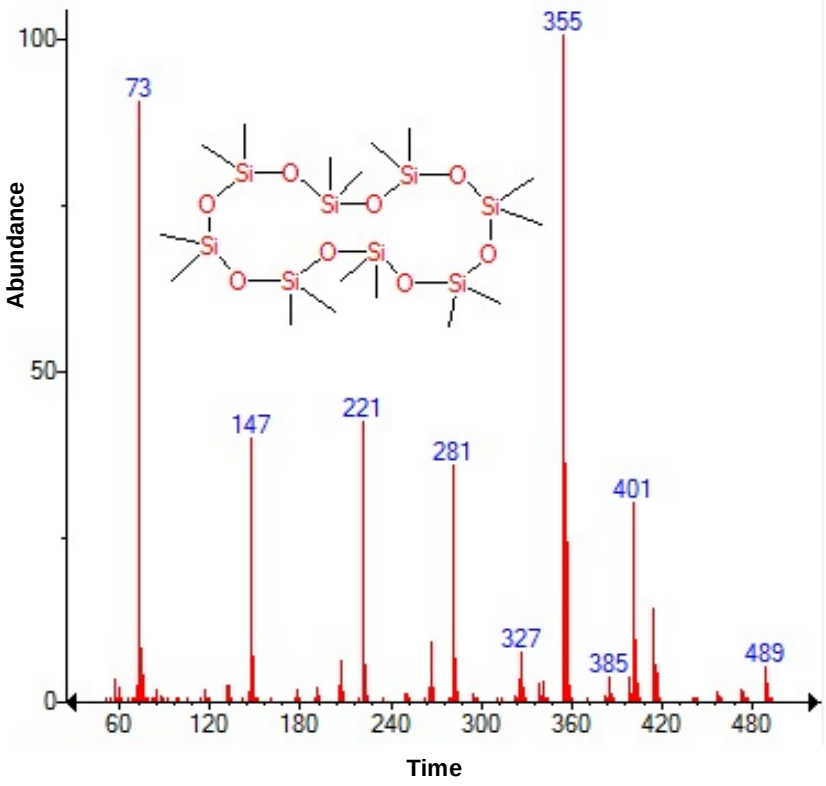

Figure 12. Structure of cyclooctasiloxane, hexadecamethyl present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

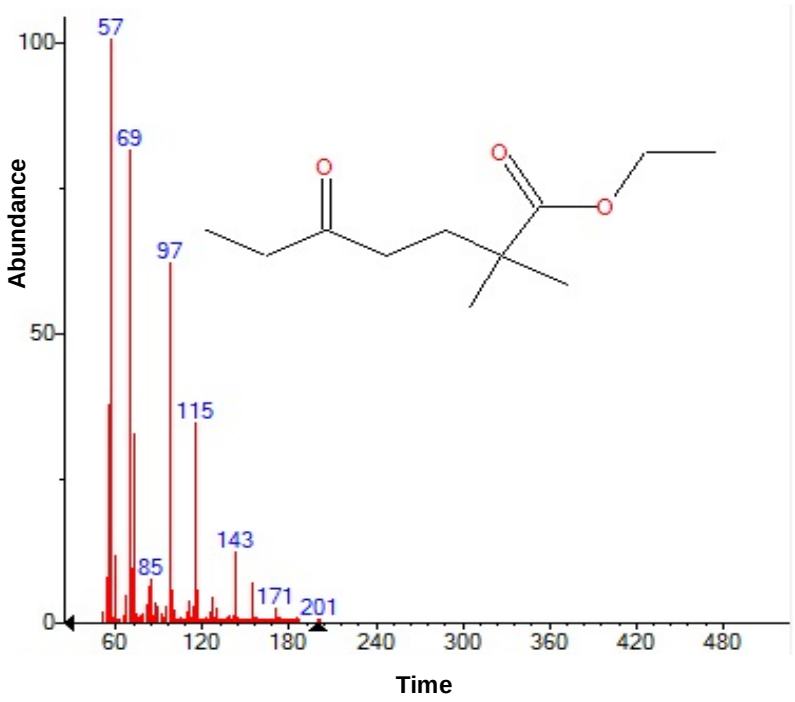

Figure 13. Structure of 5-keto-2, 2-dimethylheptanoic acid, ethyl ester present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

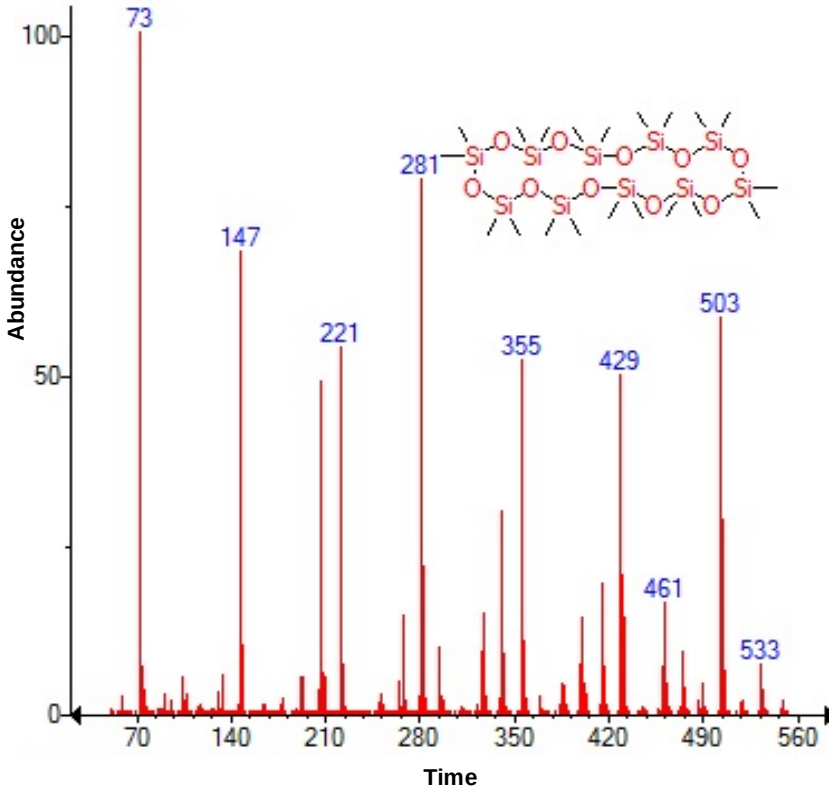

Figure 14. Structure of cyclodecasiloxane, eicosamethyl present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

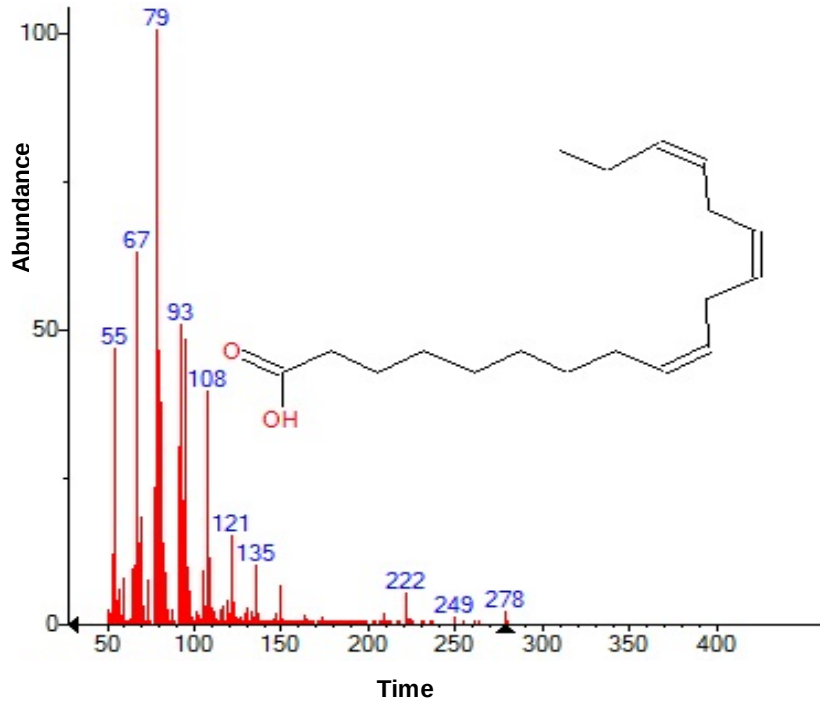

Figure 15. Structure of 9.12.15-octadecatrienoic acid present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

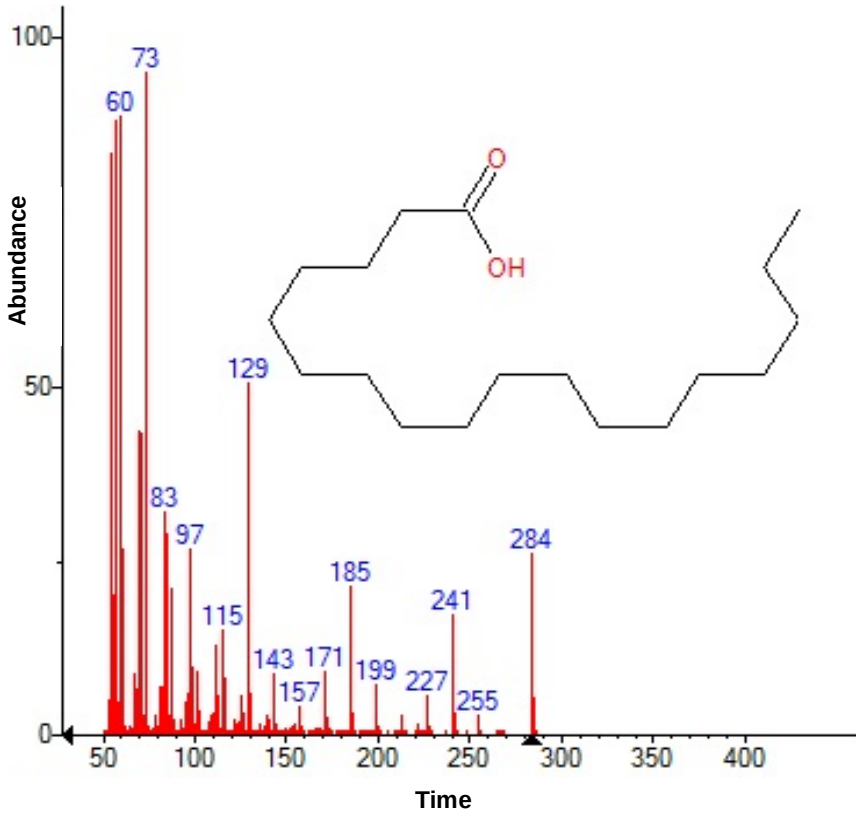

Figure 16. Structure of octadecanoic acid present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

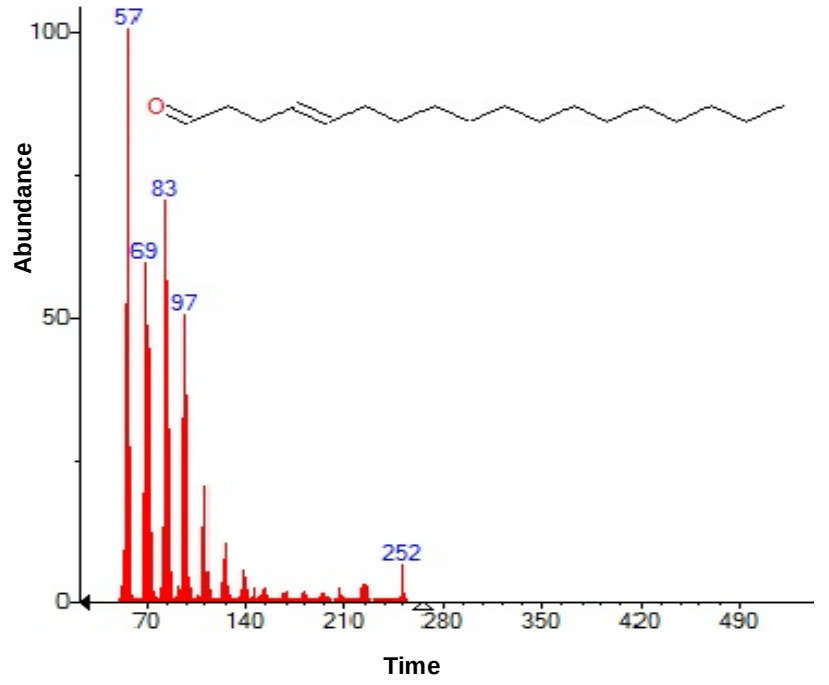

Figure 17. Structure of octadecenal present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

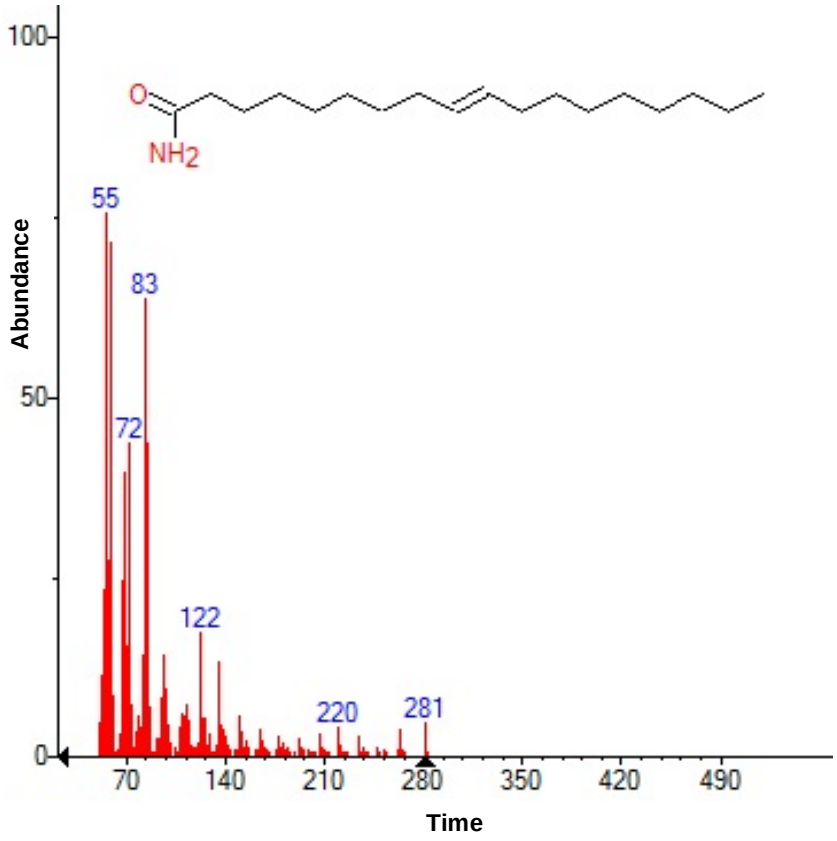

Figure 18. Structure of 9-octadecenamide present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

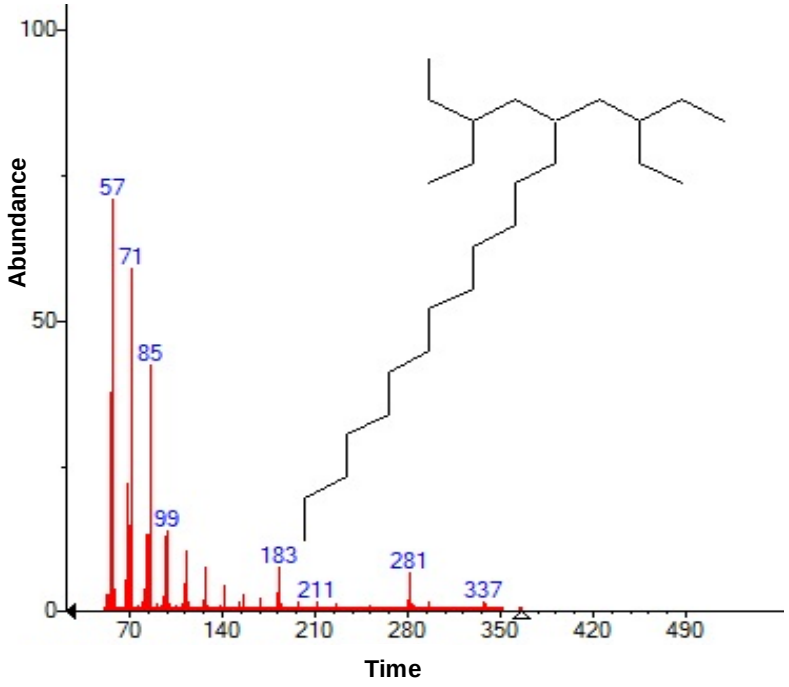

Figure 19. Structure of octadecane, 3-ethyl-5-(ethylbutyl) present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

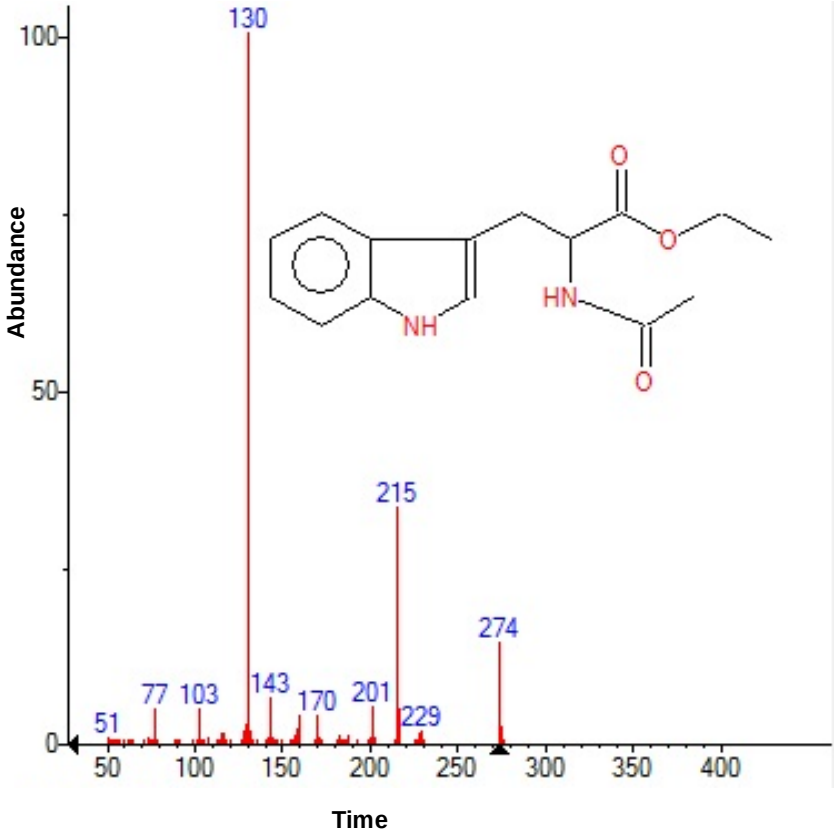

Figure 20. Structure of N-acetyl-L-tryptophan ethyl ester present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

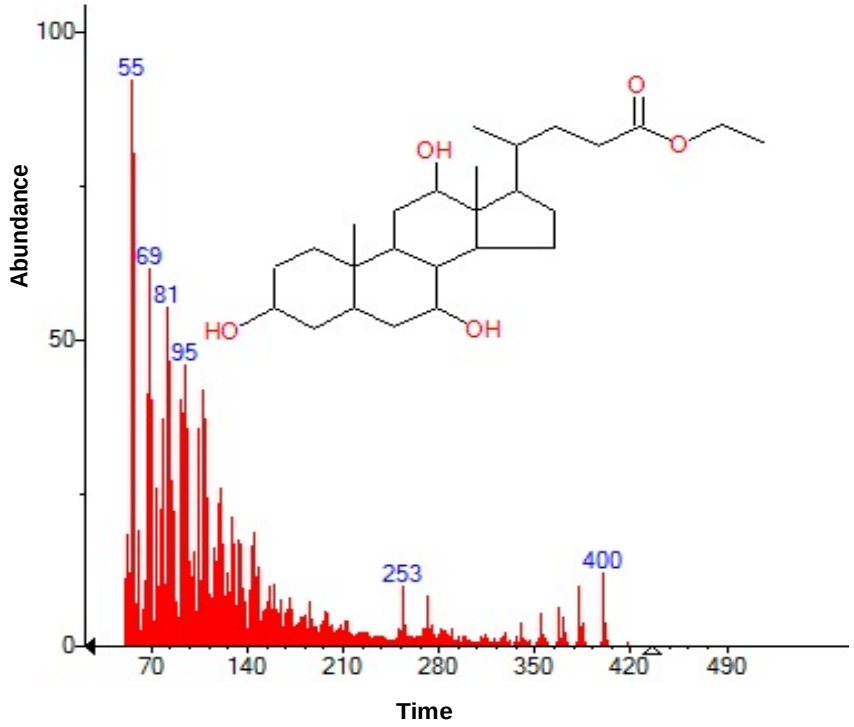

Figure 21. Structure of ethyl iso-allocholate present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

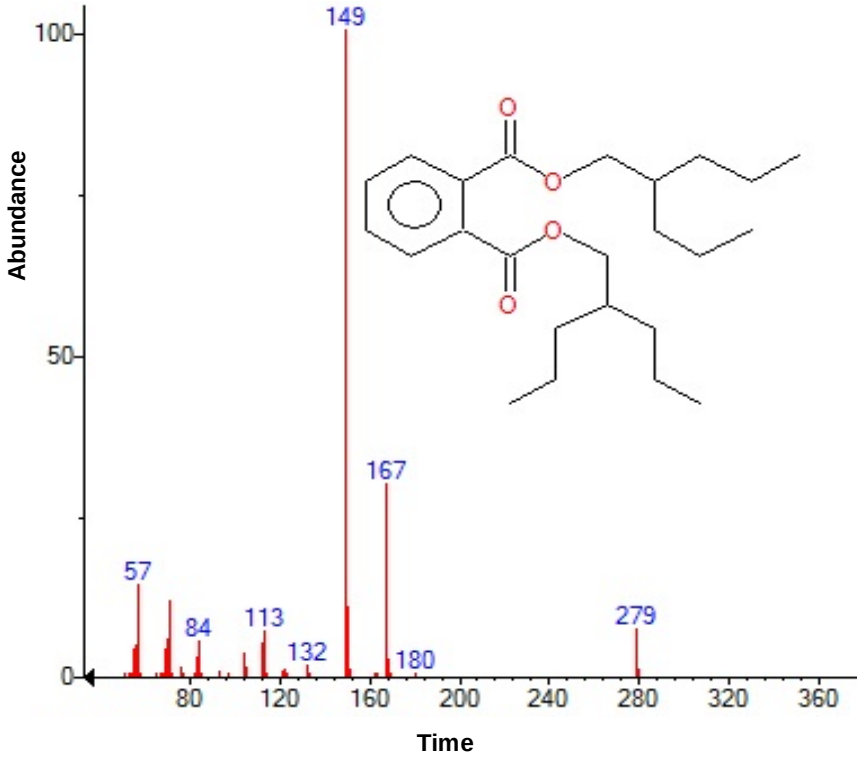

Figure 22. Structure of phthalic acid, di(2-propylpentyl)ester present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

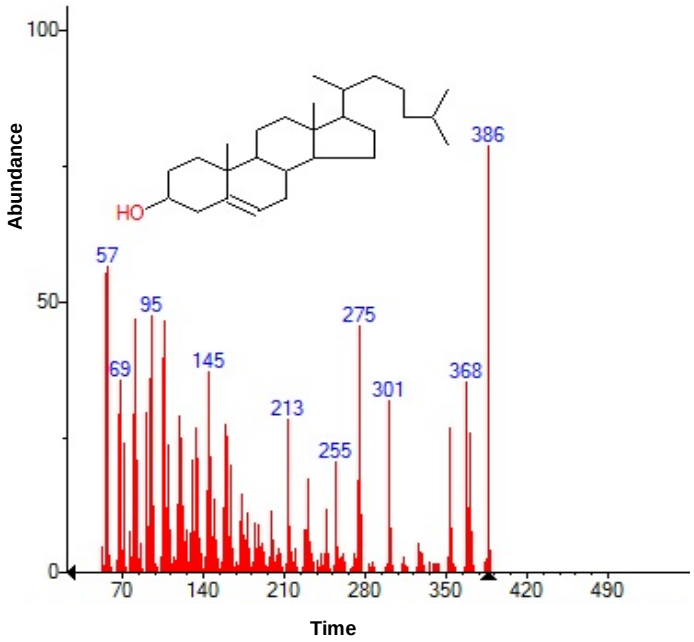

Figure 23. Structure of 17-(1.5-Dimethylhexyl)-10. 13-dimethyl-2.3.4.7.8.9.10.11. 12.13.14.15.16.17-tetradecahydro-1H present in the leaves extract of *Solanum nigrum* using GC-MS analysis.

Conclusion

Twenty three chemical alkaloids constituents have been identified from ethanolic extract of the *S. nigrum* by GC-MS. *In vitro* antibacterial evaluation of *S. nigrum* forms a primary platform for further phytochemical and pharmacological investigation for the development of new potential antimicrobial compounds.

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Conflict of interest

Authors declare that there are no conflicts of interests

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