academicJournals

Vol. 9(12), pp. 490-497, December 2015
DOI: 10.5897/AJPS2015.1333
Article Number: 01BBF8E56043
ISSN 1996-0824

http://www.academicjournals.org/AJPS

African Journal of Plant Science

Full Length Research Paper

Cytotoxicity study on *Maerua pseudopetalosa* (Glig and Bened.) De Wolf tuber fractions

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Received 13 July, 2015; Accepted 12 October, 2015

Ethyl acetate and ethanol extracts of the tuber parts of *Maerua pseudopetalosa* were subjected to further separation by column chromatography technique and eight fractions were obtained for the former and twelve for the latter one. The brine shrimp lethality assay was used for assessment of the toxicity. Remarkable cytotoxicity against brine shrimp larvae was shown, for the first time, by the ethanol extract. The fractions F_8, F_9, F_{11} and F_{12}, with high cytotoxic values (1.25, 7.98, 0.185, 0.041 µg/ml, respectively), were subjected to gas chromatography/mass spectrometry analysis. Thirty three compounds were detected; which were not recorded in any previous work in the available literature. Fractions 8 and 9 were found to be cytotoxic due to the presence of oleate and linoleate compounds; with more cytotoxicity in fraction 8 as a result of the additional presence of decenoic acid. Also, fraction 12 was more cytotoxic than fraction 11 and this was attributed to the presence of a proline derivative (proline-N-methyl-butyl ester). This compound might be considered as the cause of the high toxicity of the fraction; since free proline was used as an inhibitor of breast cancer development. Surprisingly, *M. pseudopetalosa* tubers were used in the folkloric medicine by the natives of the South Blue Nile State for the treatment of breast cancer growth without any knowledge of its chemical constituents.

Key words: Capparaceae, brine shrimp larvae, bioactive compounds, column chromatography, GC/MS analysis, proline derivative.

INTRODUCTION

The use of plants in medicine is not limited or restricted to any region of the world. It is an old practice in various parts of the globe for both preventive and curative purposes. Dependence on herbs as medicine in the treatment of diseases is an adopted practice by a large proportion of the rural population; because of their availability and affordability (Sani et al., 2009).

The foliage parts of *M. pseudopetalosa* provide much relished browsing for goats in Somalia, while in parts of the Republic of South Sudan, the plant is eaten, but only

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as a famine-food, after careful preparations to remove any toxic component (Henry, 1948). The fruit is eaten in Sudan under the belief that it provides physical strength and the roots are used to make sweet drinks (Doka, 2001). However, the same parts are used in Nigeria in topical application to the chest for cure of cough (Rajesh et al., 2009). The roots may also be used to purify stored water in rural areas (Burkill, 1985). And its use to cure tumors is practiced in the southern part of the Blue Nile State-Sudan. The toxic principle caused by tetra-methyl ammonium iodide (known as tetra-amine for short) is reported for the tuberous roots, roots and leaves of *Maerua pseudoheptalosa* (Henry, 1948).

Pisutthanan et al. (2004) had shown that brine shrimp lethality test is a general and excellent bioassay for toxicity screening of medicinal plants popularly used for several purposes. The bioassay is also used for monitoring the isolation of biologically active compounds. The brine shrimp assay has been established as a safe, practical and economic method for the determination of the bioactivity of synthetic compounds (Almeida et al., 2002) as well as plant products (Meyer et al., 1982; McLaughlin et al., 1991). The brine shrimp assay had been successively employed for bioassay-guided fractionation of active cytotoxic and antitumor agents as reported by Parra et al. (2001); who also referred to a positive correlation between the lethality to brine shrimp and the corresponding lethal oral dose in mice. The plant kingdom represents an enormous reservoir of biologically active molecules and so far, only small fractions of plants with medicinal activity have been assayed. Nearly 50% of drugs used in medicine are of plant origin. There is therefore much current research devoted to the phytochemical investigation of higher plants that have ethanobotanical information associated with them. The phytochemicals (secondary metabolites) isolated are then screened for different types of biological activity (Harborne, 1998).

In addition, there will be need for the permanent search and development of new natural drugs. This need also arise from the advantage of certain natural products in controlling some diseases that chemicals fail to do. For example, the annonaceous acetogenins, extracted from pawpaw tree (*Asimina triloba Dunal*) were found to be the best effective against selected tumor type, e.g., squamtacin is selective against the human prostate carcinoma cell line (PC-3) and a series of 9-carbonyl compounds work best against the human pancreatic tumor cell line (PaCa-2) (Ahammadsahib et al., 1993).

In this regard, a simple bioassay was used for screening purposes (Hostettmann, 1991). Thus *Artemia salina* larvae (brine shrimps nauplii) has been used as a target organism to detect bioactive compounds in plant extracts and toxicity to this crustacean has a good correlation with anti-tumor activities in man (McLaughlin, 1991) since the brine shrimp responds similarly to the corresponding mammalian system (Solis et al., 1993).

Cytotoxicity via the brine shrimp test is studied in order to reveal new anticancer compounds (Harborne, 1998). Therefore, this study aimed to evaluate the cytotoxicity of tuber fractions of *M. pseudopetalosa* against brine shrimp larvae as a new potential source of natural anti-tumor agent and the bioactive fractions were subjected to GC/MS analysis.

MATERIALS AND METHODS

Plant collection and extraction

The investigated plant (*M. pseudopetalosa*) was collected from Upper Nile State (Republic of South Sudan). Its geographical coordinates are: 9° 32' 13" North, 31° 39' 22" East. The plant was authenticated at the Department of Botany by Prof. Hatil H. Alkamali, Omdurman Islamic University. The dried ground tubers (1 kg) of *M. pseudopetalosa* were soaked for 3 days in 1500 ml ethyl acetate and ethanol, consecutively. They were subjected to silica gel (230 -400 mesh) column chromatography separation; using stepwise gradient elution of n-hexane to chloroform, and chloroform to ethyl acetate and finally washing with pure methanol. Using suitable solvent systems, portions of 100 ml were collected, concentrated and combined according to their similarity in spectrometric and TLC separation behaviors. Ethyl acetate gave eight fractions while ethanol gave twelve fractions (Harborne, 1998).

Brine shrimp lethality test

Brine shrimp lethality bio-assay was carried out to investigate the cytotoxicity of the plant extracts. *A. salina* (Leach) eggs (50 mg) were added to a hatching chamber containing sea water (45 ml). The hatching chamber was kept under an inflorescent bulb for 48 h for the eggs to hatch into shrimp larvae. Test fractions (20 mg) were separately dissolved in 2 ml of methanol; then 5, 50 and 500 µl of each solution were transferred into vials corresponding to 10, 100 and 1000 µg/ml, respectively. Each dosage was tested in triplicates. The vials (9 for each test) and one control containing 500 µl of the solvent were allowed to evaporate to dryness in 48 h at room temperature (Meyer et al., 1982).

Ten larvae of *A. salina* Leach (taken 48-72h after the initiation of hatching) were added to each vial and the final volume of the solution in each vial was adjusted to 5 ml with sea water; immediately after adding the shrimps. One drop of dimethyl sulphoxide (DMSO) was added to the test and control vials before the addition of the shrimps to enhance the solubility of the plant extract (Meyer et al., 1982). The LC_{50} values of the brine shrimps obtained for the tested plant extracts were recorded. The reference cytotoxic drug (Etoposide) was used as a positive control with LC_{50} (7.465 µg/ml) (Ahmad et al., 2009).

Gas chromatography/mass spectrometry technique (GC/MS)

The GC/MS analysis was done on a thermo-gas chromatograph /mass spectrometer (model Shimadzu 2010) equipped with DB-5 capillary column (30 m long, 0.25 mm in diameter, film thickness 0.25 µm). The carrier gas was helium and the maximum usable temperature was 325°C. The separated compounds were identified by computer searches in commercial libraries of NIST and WILEY

Table 1. Brine shrimps lethality of plant fractions.

S/N*	LC_{50} (µg/ml)	
	Ethanol fraction	Ethyl acetate fraction
1	>1000	37.80 (0.00-298.16)
2	>1000	883 (186.5-12552)
3	>1000	>1000
4	102 (50.43-206.05)	375.4 (205.2-840.8)
5	807 (7.85-142.1)	>1000
6	>1000	744.3 (236.1-16738.7)
7	89.9 (22.5-326.7)	299.7 (72.7-14875.1)
8	1.25 (18.71429988)	520.5 (199.8-3591.6)
9	7.98 (.9082-20.12)	
10	30.69 (2.79-95.82)	
11	0.1853 (7.6-445180	
12	0.0413 (2.95-9.32)	

*****Number of fractions.

(Ronald, 1997).

Statistical analyses

LC_{50} values were determined at 95% confidence intervals by analyzing the data on a computer loaded with a Finney Program (Mclaughin et al., 1991).

RESULT AND DISCUSSION

Brine shrimp toxicity assay

Two solvent fractions, namely ethanol and ethyl acetate of *M. pseudopetalosa* were used to test their cytotoxic effects against the brine shrimp *A. salina.* The mortality end point (LC_{50}) was calculated after 24 h; according to the method described by Meyer et al. (1982). The results of this study are classified as: LC_{50} less than 20 µg/ml were considered as highly toxic, LC_{50} from 20 to 100 µg/ml as toxic, LC_{50} from 100 to 500 µg/ml as moderately toxic and from 500 to 1000 µg/ml was weakly toxic according to Padmaja et al. (2002). However, Meyer et al. (1982) considered the LC_{50} values >1000 µg/ml as non-toxic or safe.

As a matter of fact, the results in Table 1 revealed that the ethanol extract is a very promising one with remarkable toxicity against brine shrimp larvae. The four fractions f_8, f_9, f_{11} and f_{12} have the highest cytotoxic effects with values equal to 1.25, 7.98, 0.185 and 0.041 µg/ml, respectively.

This is a clear indication of a first time achievement which was not preceded by any other reports in the available literature. The importance of the cytotoxicity stems from the fact that it is linked with the discovery of anticancer compounds (Moshi et al., 2004, 2006).

From a pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect antitumoral compounds in terrestrial plant extracts (Mackeen et al., 2000; Zani et al., 1995). The significant correlation between the brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines were demonstrated by the National Cancer Institute (NCI, USA). It is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research (Anderson et al., 1991). Not only that there is positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal car-cinoma) cytotoxicity ($p = 0.036$ and kappa = 0.56). The brine shrimp test was being used as a prescreen for a panel of six human solid tumor cell lines at the Cell Culture Laboratory of the Purdue Cancer Center (*McLaughin* and Rogers, 1998). This is an internationally accepted bioassay for screening of antitumor compounds (Meyer et al., 1982).

Some of the other fractions including F_7 with a value equal to 89.9 µg/ml and F_{10} (LC_{50} 30.6 µg/ml) are considered to be toxic, whereas F_5 (LC_{50} 807 µg/ml) was weakly toxic. However, the other fractions showed non-toxic effects on brine shrimp larvae. Similar results were obtained by Adoum (2009) after application of an aqueous and ethyl acetate extracts of roots of *Cochlospermum tinctorium* and the chloroform fraction of stem bark of *Entada sudanica* which had exhibited very high lethality on brine shrimps at LC_{50} values of 8, 10 and 6 µg/ml.

Moderate toxicity for fractions 7 and 4 was shown by ethyl acetate fractions at LC_{50} (299.7 and 375.4 µg/ml), whereas F_2, F_6 and F_8 were weakly toxic. Moreover, F_3 and F_5 (LC_{50}>1000) gave non-toxic effects and were considered to be inactive or safe. F_1 is the only fraction which exhibited high toxic effect. However, the other fractions showed non-toxic effects on brine shrimp larvae. Bastos et al. (2009) examined four fractions of *Zeyheria tuberculosa* and reported that two of them were not toxic, while the other two were weakly toxic. Bose et al. (2011) studied the cytotoxicity of a member of the Capperaceae family (*Cleome viscose*) on the brine shrimp *A. salina* and had shown that its crude extract produced the most prominent cytotoxicity (LC_{50} 28.18 µg/ml).

Cytotoxicity may be linked to anticancer activity; since compounds with high toxicity (7.46 µg/ml LC_{50} and less), are considered as anticancer agents. The United States National Cancer Institute had a fixed standard level for cytotoxicity which is set according to the type of drug used (Mojica and Jose, 2007).

Gas chromatography/mass spectrometric (GC/MS) analysis

The GC/MS chromatographic separation technique was

Table 2. Molecular weights, retention times, formulae and peak areas of compounds present in F_8 as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt. time	Formula	Peak area (%)
1	Octadecanoic acid /stearic acid	284	37.425	$C_{18}H_{36}O_2$	0.95
2	Hexadecanoic acid, ethyl ester (ethyl palmitate)	284	38.083	$C_{18}H_{36}O_2$	7.08
3	7-Tetradecyne	194	40.067	$C_{14}H_{26}$	1.20
4	9-Octadecenoicacid,(Z)-methyl oleate	296	40.175	$C_{19}H_{36}O_2$	0.55
5	9,12-Octadecadienoic acid	280	40.83	$C_{18}H_{32}O_2$	3.07
6	Cis-9-Hexadecenal	238	40.925	$C_{16}H_{30}O$	2.94
7	Ethyl linoleate	308	41.325	$C_{20}H_{38}O_2$	45.53
8	Ethyl oleate	310	41.417	$C_{20}H_{38}O_2$	37.06
9	Ethyl-9-hexadecenoate	282	41.525	$C_{18}H_{34}O_2$	0.85
10	Heptadecanoic, ethyl ester/ ethyl -n-heptadecanaote	298	41.875	$C_{19}H_{38}O_2$	0.78

Mw. = Molecular weight; Rt. Time = retention time.

Table 3. Molecular weights, retention times, formulae and peak areas of compounds present in F_9 as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt.time	Formula	Peak area (%)
1	Furan-3-carboxaldehyde	96	6.667	$C_5H_4O_2$	2.12
2	Propanol,2,3-dihydroxy/ Glycerose	96	8.492	$C_3H_6O_3$	5.06
3	3-Pyridinecarboxylic acid 1,2,5,6-tetrahydro-1-methyl-methyl ester	155	14.808	$C_8H_{13}NO_2$	8.01
4	2-Furancarboxaldehyde,5-(hydroxyl- methyl)	126	19.633	$C_6H_6O_3$	58.54
5	Octanoic acid, 2-methyl,methyl ester/methyl2-methyl octanoate	172	38.075	$C_{10}H_{20}O_2$	0.80
6	(Z,Z)-heptadeca-8,11-dien-1-yl bromide	314	40.067	$C_{17}H_{31}Br$	0.61
7	Ethyl linoleate	308	41.317	$C_{20}H_{36}O_2$	12.27
8	Ethyl oleate	310	41.408	$C_{20}H_{38}O_2$	9.60

Mw. = Molecular weight; Rt. Time = retention time.

used for identification of the four ethanol fractions (8, 9, 11 and 12) which were selected according to their cytotoxic effects against brine shrimp larvae.

The detection of 33 different compounds found in the four fractions in this study is considered as a pioneer achievement which has not been reported before. F_8 and F_9 are composed of ten and eight compounds, respectively (Tables 2 and 3, Figures 1 and 2). Out of these compounds, two were found to be common in the two fractions; these are ethyl linolate and ethyl oleate derivatives of the corresponding unsaturated fatty acids. These two fatty acids are possible causes of the cytotoxicity observed for the two fractions (F_8 and F_9); since Ortsater (2011) reported that the ethyl derivatives of unsaturated fatty acids oleate and linoleate are known to display some toxicity. However, he also referred to the potent toxicity of the palmitate derivatives which may provide another explanation for the increased cytotoxicity of fraction 8; since their presence is restricted to this fraction. A further possible explanation for the increased cytotoxicity of fraction 8 might be as a result of the presence of the ethyl derivatives of the additional fatty acids (other than oleate and linoleate ethyl derivatives) present in this fraction which are replaced by the methyl derivatives in fraction 9.

Furthermore, the presence of decenoic derivative in fraction 8 might also be taken as another proof for the increased cytotoxicity of the fraction as compared to fraction 9 which lacks this derivative. Also, hexadecenoic acid, which was also reported in the royal jelly, of the nurse bees is known to have bactericide, anti- inflamematory and anticancer activities (Isidrov et al., 2011).

As for F_{11} and F_{12} and despite the fact that they have five compounds in common (Tables 4 and 5, Figures 3 and 4), it was found that F_{11} has more detectable compounds (15 compounds) and less cytotoxicity than F_{12} which has got only 9 compounds.

The only compound which bears some kind of resemblance (not exactly similar) is the compound proline, N-methyl- butyl ester, in fraction 12, as compared to proline betaine ethyl ester identified from the same *Maerua sp*. by William et al. (1996).

One of the compounds present in fraction 12 is a derivative of proline (proline-N-methyl- butyl ester). This

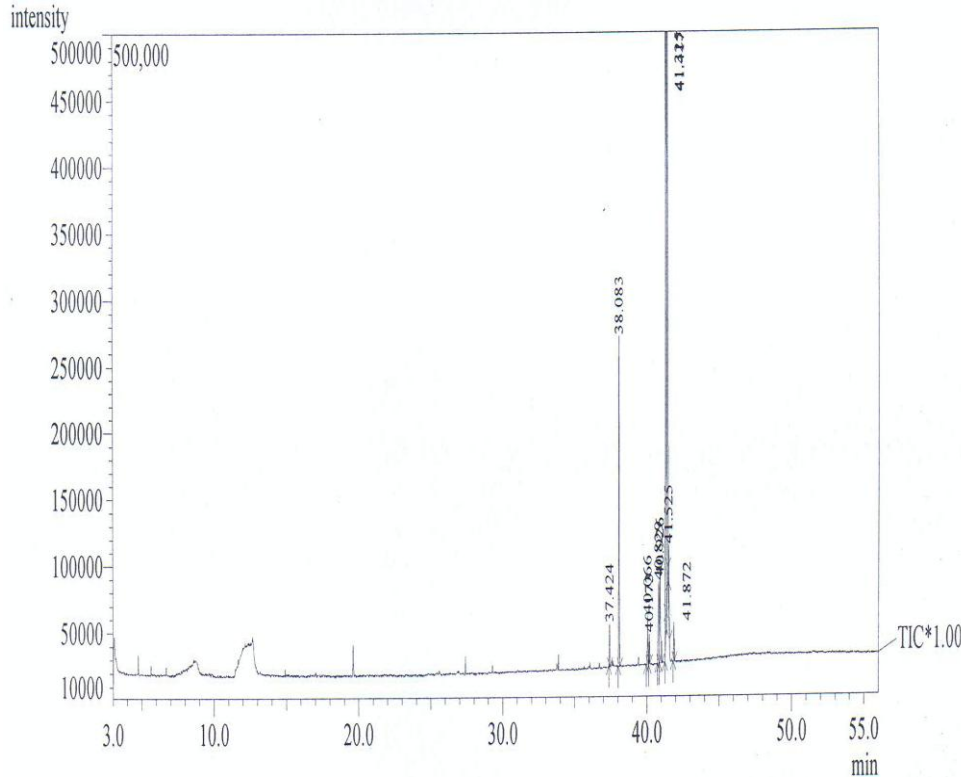

Figure 1. GC/MS total chromatogram of compounds detected in fraction (8).

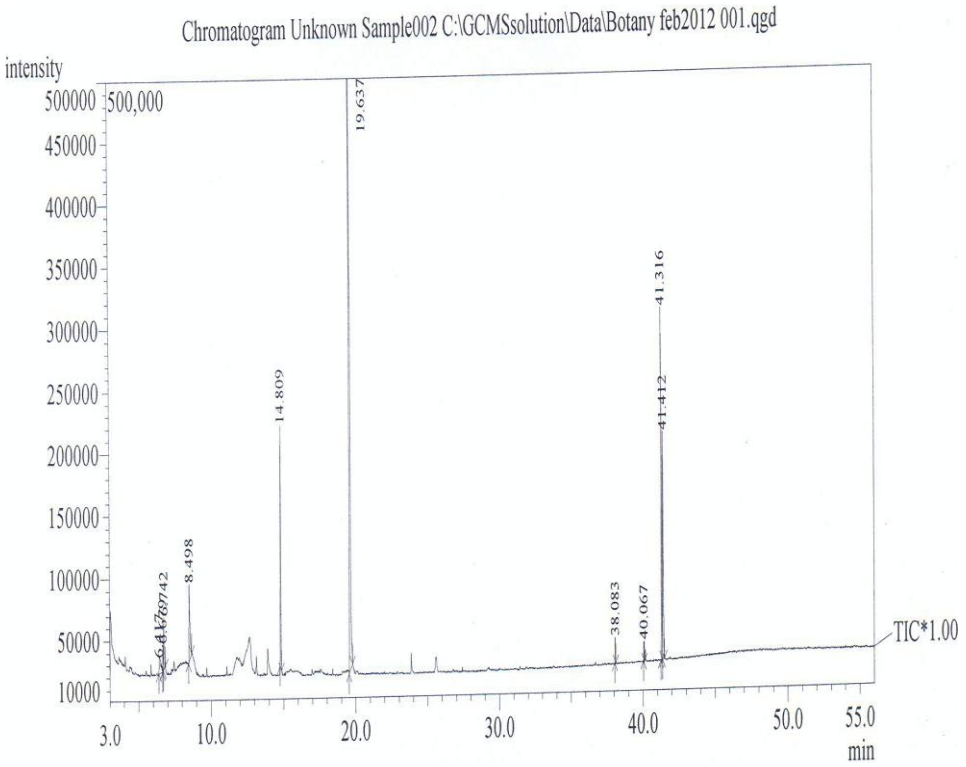

Figure 2. GC/MS total chromatogram of compounds detected in fraction (9).

Table 4. Molecular weights, retention times, formulae and peak areas of compounds present in F_{11} as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt. time	Formula	Peak area (%)
1	Methanamine,N-(3-methyl-2-butenylidene	97	6.725	$C_6H_{11}N$	15.26
2	N,N-Dimethyl-1,3-butadien-1-amine	97	7.417	$C_6H_{11}N$	4.34
3	Cyclobutanone,2,3,3,4-tetramethyl	126	10.692	$C_8H_{14}O$	27.40
4	Hygrine/2-prpanone,1-(methyl-2-pyrrolidinyl)-,	141	13.783	$C_8H_{15}NO$	0.46
5	1-(3-amino propyl)piperidine	142	14.342	$C_8H_{18}N2$	3.39
6	3-Pyridinecarboxylic acid,1,2,5,6-tetrahydro-1-methyl,methyl ester	155	14.817	$C_8H_{13}NO_2$	0.76
7	Morpholine,4-(2-methyl-1propenyl	141	15.308	$C_8H_{15}NO$	22.55
8	6-methyl-6-azabicyclo(3,2,1)octane	125	17.717	$C_8H_{15}N$	2.86
9	1-Methyl-pyrrolidine-2-caroxylic acid	129	18.075	$C_6H_{11}NO_2$	1.12
10	5-hydroxy piperidine carboxylic acid	145	18.217	$C_6H_{11}N_3O$	9.65
11	1,4-cis-Cyclohexanedicarboxylic acid,dimethyl ester	200	18.392	$C_{10}H_{16}O_4$	2.21
12	Boranamine,N-ethyl-1,1-dipropyl	141	18.625	$C_8H_{20}BN$	0.92
13	2-(E)-Hexenoic acid,(4S)-amino-5-methyl	143	18.742	$C_7H_{13}NO_2$	6.14
14	1-Piperidinepropanenitrile	138	31.667	$C_8H_{14}N_2$	1.39
15	2-Pyrolidine methanol,1-methyl(1-methyl-2pyrrolidinyl)-methanol	115	31.217	$C_6H_{13}NO$	1.57

Mw. = Molecular weight; Rt. Time = retention time.

Table 5. Molecular weights, retention times, formulae and peak areas of compounds present in F_{12} as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt. time	Formula	Peak area (%)
1	(trans/cis)-3-(Dimethylamino)-4,5-dicyanocyclohex-1-ene	175	6.742	$C_6H_{13}N_3$	2.19
2	Methanamine,N-(3-methyl-2-butenyliden	97	7.300	$C_6H_{11}N$	19.13
3	N,N-Dimethyl-1,3-butadien-1-amine	97	7.433	$C_6H_{11}N$	28.05
4	Cyclobutanone,2,3,3,4-tetramethyl	126	10.700	$C_8H_{14}O$	1.71
5	Hygrine/2-propanone,1-(methyl-2-pyrrolidinyl)-,(R)	141	13.808	$C_8H_{15}NO_2$	11.31
6	N-(6-chloro-2-pyrazinyl)-2-(1-piperidinyl)acetamide	245	14.367	$C_{11}H_{15}CIN_4O$	2.49
7	Morpholine,4-(2-methyl-1-propenyl)	141	15.317	$C_8H_{15}NO$	7.35
8	Proline, N-methyl-butyl ester	185	17.925	$C_{10}H_{19}NO_2$	2.01
9	Cyclohexanol,2-amino-1-methyl-4-(1-methylethyl)	155	18.625	$C_{10}H_{21}N$	2.28

Mw. = Molecular weight; Rt. Time = retention time.

compound might be considered as the cause of the high toxicity of the fraction; since free proline was found to inhibit the growth of mammary tumors induced by N-methyl-N-Nitrosourea in rats as reported by Kalinovsky et al. (2005). They suggested its use as an inhibitor of breast cancer development.

Surprisingly, *M. pseudopetalosa* tubers were used in the folkloric medicine by the natives of the South Blue Nile State for the treatment of breast cancer growth without any knowledge of its chemical constituents. Furthermore, acetamide which is present in F_{12} was found to be cytotoxic at high concentrations in maternal animals (Kennedy, 1986) and may consequently add to the increased cytotoxicity of the fraction at the appropriate concentration.

Conclusion

The overall results indicated that the ethanol fractions

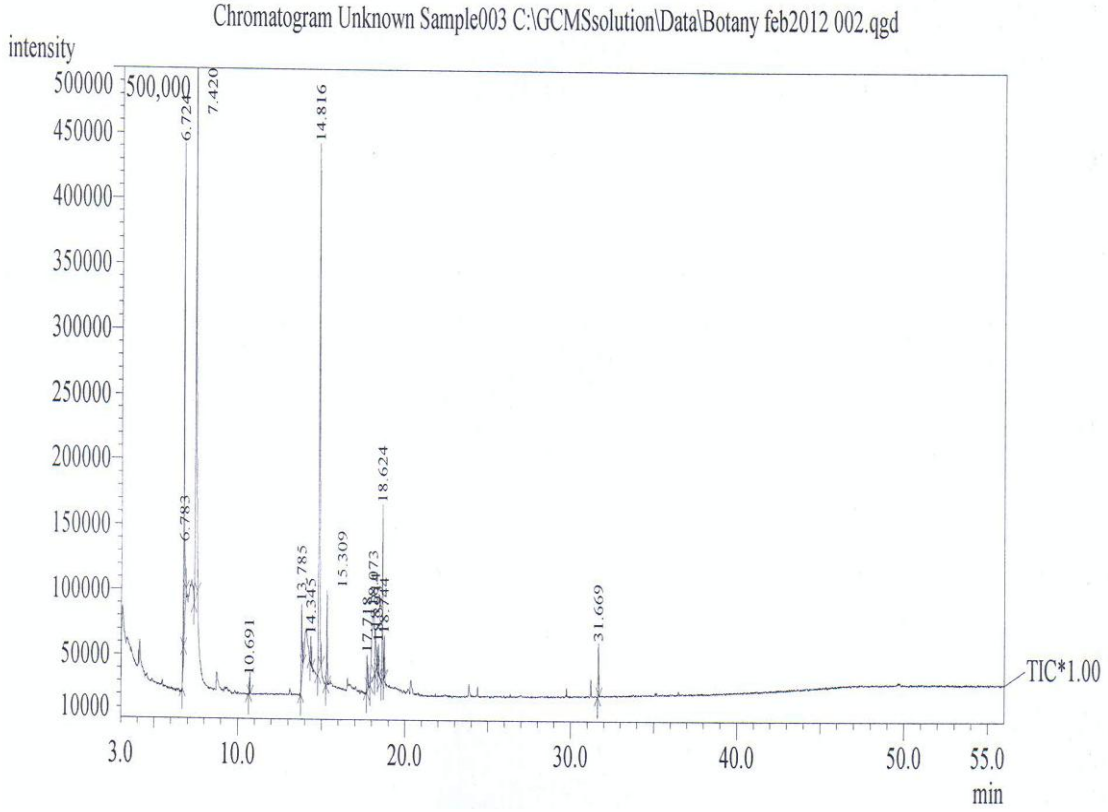

Figure 3. GC/MS total chromatogram of copounds detected in fraction (11).

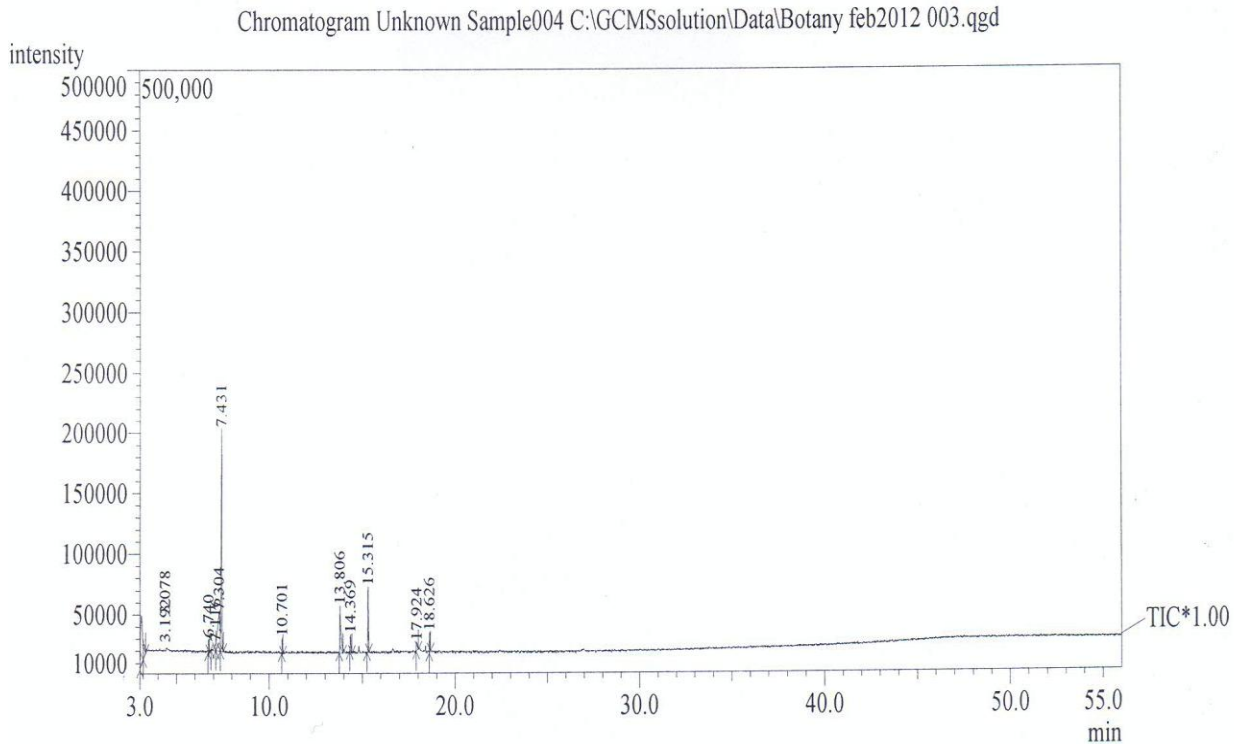

Figure 4. GC/MS total chromatogram of compounds detected in fraction (12).

exerted more cytotoxic effects than those obtained through ethyl acetate fractions.

The four ethanol fractions (F_8, F_9, F_{11} and F_{12}) with highest cytotoxicity are composed mainly of derivatives of fatty acids, derivatives of amino acids, amines, acetamide, ester cyclobutanone, methane amines and carboxylic acid derivatives.

The importance of cytotoxicity, in this study is brought about by its highly acclaimed characteristics that are closely linked to antitumor activity.

Conflict of Interest

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to the management and staff of Department of Botany, Faculty of Science, University of Khartoum; the Central Laboratory, University of Khartoum and the Institute of Medicinal and Aromatic Plants, National Council for Research, Sudan for materials and technical support.

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