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The GC-MS Analyses of Ethanol Extract of the Root Bark of Grewia mollis with the TLC Analysis, Antimicrobial, Antioxidant and Cytotoxic Activities of its Solvent - Solvent Fractions

[1] Mshelia E. H.*, [2] Adamu H. M., [3] Abayeh, O.J., [4] Umar H.Y., [5]Ali L & [6]Mohammed A.H.

*Corresponding author: subwang10@yahoo.com (08035941403)

[1.4,6] Department of Chemistry, School Science, Federal College of Education (Tech.) Gombe

[2] Department of Chemistry, Abubakar Tafawa Balewa University, Bauchi

[3]Department Chemistry, University of Port Harcourt, Rivers state

[5]Department of Integrated science federal College of Education (Tech.) Gombe

Abstract: *Grewia mollis is a plant growing in the wild in North Eastern Nigeria and have been used by the local people in preparation of their food and in the treatment of infections and other diseases. The GC-MS of the ethanol extract of the root bark of Grewia mollis with the TLC analysis, antimicrobial, antioxidant and cytotoxic activities of its solvent - solvent fractions were determined. The ethanol extract contain fourteen different compounds which can be separated by solvent extraction followed by TLC with a polar solvent system to elute it. Butanol fraction is the fraction with highest activity followed by the chloroform fraction while the hexane fraction showed least bioactivities. Based on the finding of this work the use of the ethanol extract by the folklore of the North East Nigeria for the treatment of infections and other diseases is justified.*

Keywords: Grewia mollis, GC-MS, *antimicrobial, antioxidant and cytotoxic activities*

1. Introduction

The importance of plant product is well-recognized since the beginning of human civilization. Plants occupy an important position in the socio-cultural and health systems of many countries and there is a growing interest in exploring their therapeutic and nutritional properties. Historically, plants have provided a good source of anti –infective agents; emetine, quinine and berberine remain highly effective instruments in the fight against microbial infections. Plants containing protoberbrines and related alkaloids, picratima – type indole alkaloids, and Garcinia biflavonones used in traditional African system of medicine have been found to be active against a wide variety of microorganisms. Seeking remedies for human ailment from the environment has formed the basis for therapeutics (Oluwole *et al* 2007).

Herbal medicine involves the use of plants for medicinal purposes. The term "herb" includes leaves, stems, flowers' fruits, seeds, roots, rhizomes and barks (Gordon & David 2001*)*. The uses of plants for healing purposes have been the most ancient form of medicine known.

The quest for plants with medicinal properties continues to receive attention as scientists are in need of plants, particularly of ethno botanical significance for a complete range of biological activities, which ranges from antibiotic to anti cancerous (Gandhiraja *et al,* 2009). Today, there are a lot of scientific interests for the development of plant products as dietary supplements

Therapeutic plants have always been valued as a mode of treatment of variety of ailments in folk cultures and have played a very important role in discovering the modern day medicines with novel chemical constituents (Chan, 2003; Haider *et al*., 2004; Devi *et al*., 2008; Shirin *et al*., 2010). The efficacy of medicinal plants for curative purposes is often accounted for in terms of their organic constituents like essential oils, vitamins, glycolsides, etc Mshelia *et al,* 2016b). Antioxidants present in plants are claimed to be helpful against cancer, cardiovascular and various chronic diseases. The presence of various biofunctional and chemo-preventive compounds in plants, believed to have health-boosting properties, are a major reason for their increased consumption Mshelia *et al,* 2016a).

For the majority of the world population medicinal plants represent the primary source of the health care. As it was reported by the World Health organization (WHO), about 80% of people in peripheral communities use only medicinal herbs for the treatment of many diseases (Sahito *et al* 2003).

Several plants and herbs species used traditionally have potential antibacterial, antifungal and antiviral properties (Mshelia *et al* 2008; Akinniyi *et al* 2007; Zaria *et al,* 1995; Shelef, 1983; Zaika, 1988) and this has raised the optimism of scientists about the future of phyto antimicrobial agents (Das *et al*, 1999; Lozoya and Lozaya 1989*)*.

Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava, 1990). The beneficial medicinal effect of plant materials typically results from the combinations of secondary products present in the plant. The medicinal actions of plants are unique to a particular plant species or group and are consistent to the concept of the combination of secondary products, which are taxonomically distinct (*Wink, 1999)*. The ethno pharmacology provides an alternative approach for the discovery of bioactive active agent that is the study of medicinal plants with a history of traditional use as a potential source of substances with significant pharmacological and biological activities (Ambasta, 1992*)*.

This work will determine the compounds present in the ethanol extract obtained by soxhlet extraction of the root bark of Grewia mollis using GC-MS spectroscopy. The solvent extraction of the ethanol extract will be carried out using different solvents the TLC of the different fractions will also be determine. The antimicrobial, antioxidant and cytotoxic activities of the different fractions will be determined.

Grewia mollis belong to the family Tiliceae and occurs widely in Tropical Africa, from Northern Nigeria, Senegal and Gambia Eastward to Somalia and Southward to Angola, Zambia and Zimbabwe (Burkill, 2000; Katende, 1995; Kokwaro, 1993*).* Grewia mollis is a shrub or small tree that grows up to 10.5m tall (Sharma, 2002). The fruit is used for the treatment of malaria fever (Fowler, 2006*)* while a medicinal salt is obtained from the ash of the stem and leaves which has been used for treatment of stomach ache (Persson, 1982).

The water extract of the stem bark is used as anti rat and ant termite. The mucilaginous bark and leaves are applied to ulcers, cuts, sores and snake bites while the bark and root

preparation are taken to treat cough (Lockett, 2000; Brink, 2007*.)*. In East Africa, the leaves are pounded and mixed with water and taken against stomach problem and also given to constipated domestic animals (Ruffo, 2002).

The decoction of the leaves is used in baths and drinks against rickets in children and difficult birth ((Lockett, 2000). Grawia mollis is frequently used in traditional ritual in Sudan and Ethiopia (Persson, 1982*).* In recent time some findings demonstrated that the mucilage obtained from the stem bark can serve as a good binder in paracetamol formulations (Martins *et al* 2008. Muazu, *et al* 2009). A reports suggest that high concentration of stem bark in dietary may cause some adverse effects, especially liver injury (Wilson, 2010).

2. Material and Methods

Collection of plant material

The root bark of *Grewia mollis* was collected in Hawul local Government Area of Borno State. The collection was done in September when the leaves were green. The infected parts were removed and the healthy fresh bark was air dried under a shade and pulverized using motorized miller.

Extraction of plant material

The powdered root bark of Grewia mollis were serially extracted with hexane, ethyl acetate, acetone, ethanol and distilled water using soxhlet extractor apparatus for 8 hours each (Vogel, 1979). The extracts were evaporated to dryness on rotary evaporator, the percentage yield of the extracts were then determined.

GC-MS Instrumentation For The acetone extract

The Samples chemical compositions were analyzed on Shimadzu GC-MS-QP 2010 plus instrument (Shimadzu, Japan) equipped with GC-MS flame ionization detector (FID) carrying Agilent DB-5 ms column (30 m long x 0.25 mm internal diameter x 0.25 µm film thickness), was used. Samples were prepared by dissolving aliquot amount in 2 ml solvent chloroform. About 1.5 ml of this solution was with-drawn using a borosilicate glass syringe and filtered into an amber GC-MS vial (VZS-0209A-100; Cronus, UK) using 0.02 µm disposable PTFE filter cartridge (Sartorius Stedim, Germany) (Gumel *et al*, 2012a).

The GC-MS method was observed according to Gumel *et al*, (2012) with slight modification. A sample (1 µl) was automatically injected into the GC-MS at a split ratio of 1:50. The injection temperature was 280°C. The GC oven temperature ramping was set as follows: 40°C for 1 min then increased to 120°C at 15°C per- minutes; held at 120°C for 2 min then increased to 250°Cat 10°C per-minutes; then held at 250°C for 15 minutes. Helium (0.41 bar) was used as the carrier gas at a flow rate of 48.3 ml per-minute. Mass spectra were acquired at 1250 scan speed, 50-600 m/z scan mass using electron impact energy of 70 eV at 200°C ion-source temperature and 280°C interface temperature (Gumel *et al,* 2012; Gumel *et al,* 2012b).

Solvent- Solvent Separation

The separation technique used for the solvent-solvent separation was the one used by USA National Cancer institute described by Stuffiness and Douro's (1979). The ethanol extract obtain

from soxhlet extraction was subjected to solvent-solvent separation with different solvents. The fractions were allowed to dry under stream of air over night or water bath.

The extract was dissolved in equal volume of chloroform and water. The water fraction was mixed with an equal volume of n- butanol in a seperatory funnel to yield the water and butanol fractions. The chloroform fraction was dried in a stream of air and extracted with equal volume of hexane and 10% water in methanol mixture. This yielded the hexane fraction and the 10% water in methanol mixture. The 10% water in methanol mixture was then diluted to 20% water in methanol by adding water. This was then mixed with carbon tetrachloride in a seperatory funnel giving the carbon tetrachloride fraction. The 20% water in methanol fraction was further diluted to 35% water in methanol and mixed with chloroform to yield the chloroform and 35% water in methanol fractions

Thin layer chromatography (TLC)

The numbers of compounds present in each fraction namely n-butanol, water, n-hexane, carbon tetra chloride, chloroform and 35% water in methanol fractions were determined by TLC. 10µl of a 10mg/ml concentration were spotted on Merck silica gel F_{254} plates. Three eluting system selected to separate high, intermediate and low polarities (ethyl acetate/methanol/water (40:5.4:4) EMW polar, chloroform/ethyl acetate/formic acid 5:4:1 CEF intermediate polarity/acidic and Benzene/ethanol/ammonium 90:10:1 BEA non-polar/basic) were used. Separated compounds were examined under visible light marked and sprays with anseldehye/ sulphuric acid spray reagent.

Brine Shrimp Lethality Assay

Brine shrimp eggs were commercially available. For this experiment, brine shrimp egg without shells “Artemia Revolution” 120g were obtained from NT labs (Fry care) laboratories LTD UK, Serial No. 7//3380900038///3.Made in England. Eggs were stored in a refrigerator at 5^0C (NT laboratoryLTD UK 2015)**.**

Preparation of Artificial Sea water

Artificial sea water was prepared by dissolving 35g of sea salt in1litre of distilled water for hatching the brine shrimp eggs (NT laboratoryLTD UK 2015).

Hacthing of brine shrimp

An artificial seawater was Prepared at full strength. To obtain an optimum result a solution of specific gravity of 1.022 at 24^0C was prepared by dissolving 35g sea salt sodium chloride NaCl pre- liter of water. The seawater was added to the brine shrimp Hatcher in a heated aquarium aerate from bottom of the unit so that all eggs are kept in suspension and moving. The brine shrimp bottle was shaken before dispensing into the aquarium (each drop gives from 1500 to 2000 nauplii, three drops (5000 nauplii) and are hatched in approximately 250ml sea water.

The hatcher is illuminated very well for a minimum of three hours preferably for 12hours. The hatching time depend on temperature at 24^0C (which is average tropical aquarium

temperature) hatching take place between 24-48 hours (maximum hatch 44-48hours). The Nauplii is then used directly for the cytotoxicity test (NT laboratoryLTD UK, 2015);

Preparation of Test Sample

Samples were prepared by dissolving 20mg of the fractions in 10ml of suitable solvent (stock solution # 1). Solution of varying concentrations (1000, 500, 250,125,100 µg /ml) were obtained by the serial dilution technique of the stock solution.

Cytotoxicity Test (Bioassay): Brine shrimp lethality bioassay was carried out using brine shrimp larvae (Artemia salina revolution) to determine the cytotoxicity of the fractions (Ghosh and Chatterjee, 2013). To each sample vial corresponding to 1000,500,250,125, and 100µg/ml, 4ml of artificial seawater was added and 10 brine shrimps were introduced into the tubes using pipette, and the final volume in each vial was adjusted with artificial seawater to make a total volume of 5ml. The test tubes were left uncovered in the light, the nauplii were counted against a lighted background using magnifying hand lens and the number of the surviving shrimps were counted and recorded after 24hours. Control test was also carried out using artificial seawater only. Nauplii were considered dead if they were lying immobile at the bottom of the vial.

Statistical Analysis The percentage of deaths and (LC_{50}) were determined using statistical analysis. Percentage mortality (M %) was calculated by dividing the number of dead nauplii by the total number, and then multiply by 100%.

$$\text{Percentage of Death (\%M)} = \frac{Total\ number\ of\ nauplii - number of\ nauplii\ alive}{total\ number\ of\ nauplii} \text{X100}$$

Lethal Concentration (LC_{50}) Determination: The lethal concentrations of fractions resulting in 50% mortality of the brine shrimp (LC_{50}) was determined from the 24 h counts and the dose-response data were transformed into a straight line by means of a trendline fit linear regression analysis, the LC_{50} was derived from the best-fit line obtained. LC_{50} values were obtained from the best-fit line, plotted of concentration against Percentage mortality (Rajan *et al,* 2013).

Qualitative antioxidant determination

Procedures in brief, the fractions were re-dissolved in appropriate solvent and spotted on the silica-gel $60F_{254}$ plates and develop the chromatogram in appropriate solvent systems. The plates are sprayed with a methanolic solution of DPPH (2mg/ml). Thus, antioxidants activity appears as yellow bands on a light purple background and the intensity of the yellow colour shows the degree of antioxidant activity. After spotting the fractions on the TLC plates, even uneluted plates also can be used to determine the qualitative antioxidant analysis. The uneluted plates also can be sprayed with 0.2% DPPH methanol solution and sample spots will be evaluated for radical scavenging activity (Brand-Williams *et al,* 1995).

Source and Maintenance of Organism

Gram negative and Gram positive were obtained and confirmed at the laboratory of the Department of medical microbiology and parasitology, Federal Teaching Hospital, Gombe. They were maintained on Muller-Hinton agar (MHA) (Oxoid, UK) to obtain isolated colonies.

Disc-Agar Diffusion Method

Fractions were tested for antibacterial activity by the disc diffusion method. A single colony was aseptically transferred with an inoculating loop to about 20 ml of the prepared nutrient agar. Filter papers are cut out with a diameter of 1cm. the filter paper is then transferred to the oven and sterilized for one hour. Using sterilized forceps, the filter papers are then transferred to the various fractions. The filter paper is left in the extracts for about 20 minutes so as to soak the fractions very well. The filter papers were then transferred to a cultured agar plates, the plates are then incubated at 37ºC for 24 hours in the incubator. Negative controls were performed using paper discs loaded with acetone and standard loaded with gentmicin and ampiclox. The zone of inhibition was determined after the 24hours.

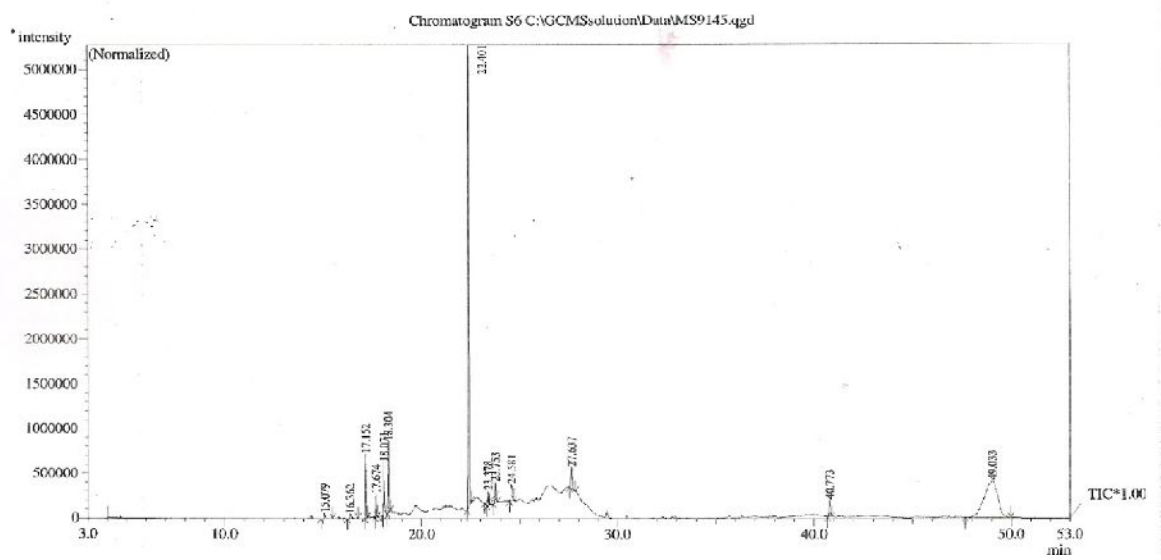


Figure 1: GC-MS spectrum of ethanol extract of Grewia mollis root bark

Table 1: Compounds identify by the GC-MS spectroscopy present in the Ethanol extracts of root bark of Grewia mollis

Peak	Retention time (min)	% composition of compounds	Name of compounds	Formula
1	15.079	0.41	2-methyl glutaconic acid diethyl ester	$C_{10}H_{16}O_4$
2	16.362	0.66	Benzenedicarboxylic acid dimethyl ether	$C_{10}H_{10}O_4$
3	17.152	3.10	Ethyl-2-(1-ethoxycarbonyl-2-oxocyclopentyl) propionate	$C_{13}H_{20}O_5$
4	17.674	1.14	2H-Pyran-5-carboxylic acid, 4,6-dimethyl-2-oxo ethyl ester	$C_{10}H_{12}O_4$
5	18.071	3.75	2H-Pyran-5-carboxylic acid, 2,6-dimethyl-2-oxo ethyl ester	$C_{10}H_{12}O_4$
6	18.304	4.53	Diethylphthalate	$C_{12}H_{14}O_4$
7	22.401	38.12	1,2-Benzenedicarboxylic acid bis(2-methypropyl ester	$C_{16}H_{22}O_4$
8	23.378	1.16	Dibutyl phthalate	$C_{16}H_{22}O_4$
9	23.753	1.78	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$
10	23.753	1.46	Pentadecanoic acid methyl ester	$C_{16}H_{32}O_2$
11	24.581	2.16	Phthalic acid butyl undecylester	$C_{23}H_{36}O_4$
12	27.637	2.73	1,2,3-Propanetricarboxylic acid,2-hydroxy-tributy ester	$C_{18}H_{32}O_7$
13	40.773	1.72	1,2-Benzenedicarboxylic acid bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$
14	49.033	40.20	Lup-20(29)-ene-3,28-diol	$C_{30}H_{50}O_2$

Table 2: Percentage of recovery, colour and texture of solvent-solvent extraction of the ethanol extract of the root bark of Grewia mollis

S.No	Solvent	% Recovery	Colour of	texture

		of fraction	fraction	fraction
1	n-butanol	28.3086	Light brown	Powder
2	Water	13.3900	Light brown	Powder
3	n-hexane	3.1936	Green	Crystalline
4	Carbon tetrachloride	3.0629	Yellow	Crystalline
5	Chloroform	7.5050	Yellow	Crystalline
6	35% water in methanol	32.3086	Brown	Powder

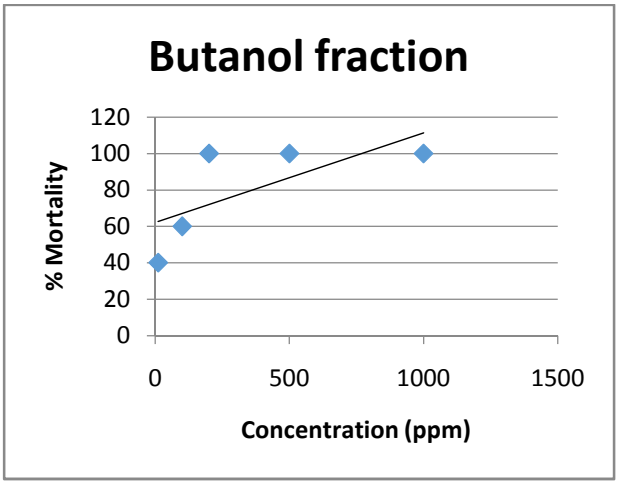
Table 3: Number of spots visualized on TLC plates of the fractions from solvent extraction of the ethanol extract of the root bark of Grewia mollis and viewed under visible light and sprayed with anisaldehyde/sulphuric acid mixture

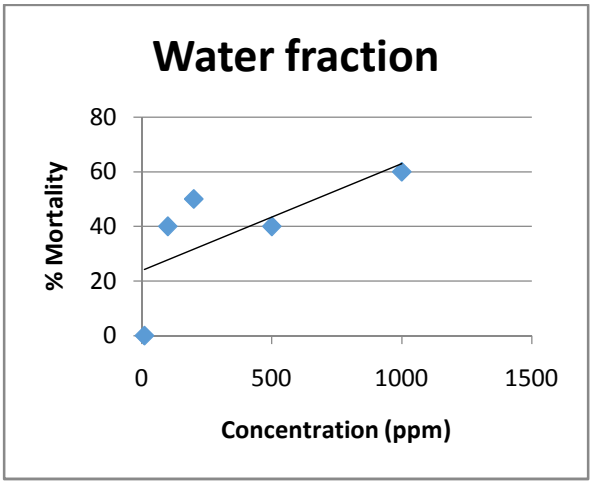
S.No.	Fraction	Visible light			Sprayed with anisaldehyde		
		BEA	EMW	CEF	BEA	EMW	CEF
1	n-butanol	2	3	0	4	4	0
2	Water	0	2	0	0	3	0
3	Hexane	0	1	1	0	1	2
4	Carbon tetrachloride	0	1	0	3	1	1
5	Chloroform	2	3	0	2	3	2
6	35% water in methanol	0	0	0	2	3	0

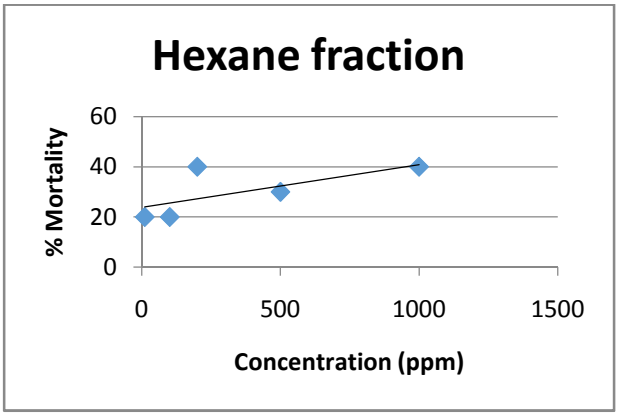
Table 4: Brine Shrimp Lethality Test for the Solvent-solvent fraction of the ethanol Extracts of the root bark of Grewia mollis

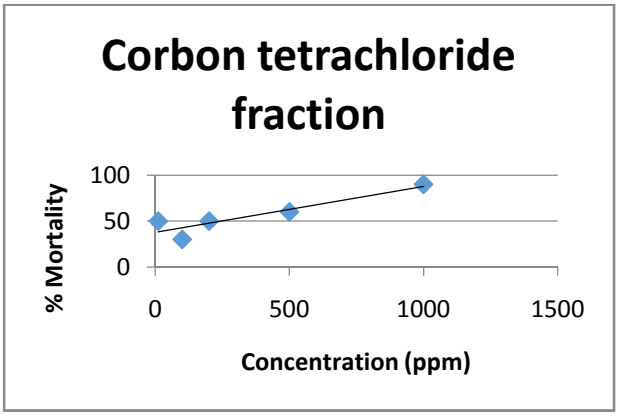
Fraction	Concentration (ppm)	% Mortality of naupiles	LC_{50}- (µg/ml)
Butanol	10	40	25.12
	100	60	
	200	100	
	500	100	
	1000	100	
Water	10	0	619.49
	100	40	
	200	50	
	500	40	
	1000	60	
Hexane	10	20	1632.50
	100	20	
	200	40	
	500	30	
	1000	40	
Carbon tetrachloride	10	50	245.91
	100	30	
	200	50	
	500	60	
	1000	90	

Chloroform	10	20	42.30
	100	100	
	200	100	
	500	100	
	1000	100	
35% water in methanol	10	50	233.57
	100	50	
	200	40	
	500	60	
	1000	60	









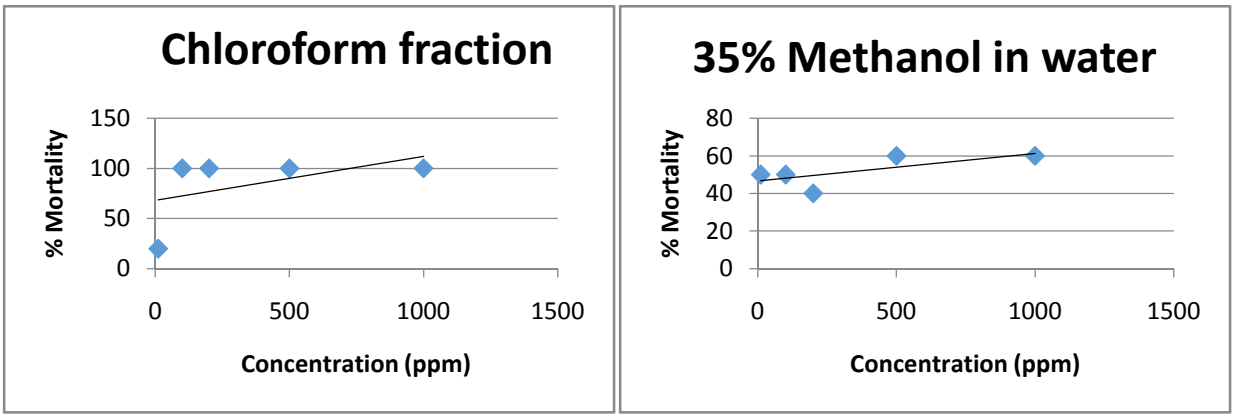


Figure 2: Cytotoxic effects of the fractions of solvent extraction of ethanol extract of the root bark of Grewia mollis on brine shrimp

TABLE 5: Antimicrobial and antioxidant activities with the LC_{50} of fractions of solvent/solvent extraction of ethanol extract of root bark of Grewia mollis

Fraction	Salmonella typhi	Staphylococcus aureus	Escherichia coli	Shigella dysentariae	LC_{50}	Antioxidant activity
n-butanol	23	18	7	11	25'12	+++
Water	3	2	0	0	619.49	+
n-hexane	0	0	0	0	1632.50	-
Carbon tetrachloride	5	0	0	0	245.91	++
Chloroform	15	8	0	0	42.30	++
35% water in methanol	12	20	0	4	233.57	-
GENTIMICIN	27	18	16	14	ND	ND
Ampicilin	32	22	22	21	ND	ND
Water	0	0	0	0	-	ND

3. Results and Discussion

Figure 1 showed the GC-MS spectrum of the ethanol extract of the root bark of Grewia mollis, while table 1 showed the detailed compounds present in the extract as shown in the spectrum of figure 1. The table showed that there are fourteen different compounds belonging to different classes of organic compounds. The types of compounds present in the extract include organic acids, esters, ethers, phthalates and terpenes. The compounds extracted by the ethanol as shown above are mostly polar to certain degree which might be because the solvent ethanol is polar in nature, therefore the polar compounds are soluble in it.

Table 2 shows the percentage recovery, colour and texture of solvent-solvent extraction of methanol extracts from serial extraction of the root bark of Grewia mollis. The lowest percentage yield was obtained with carbon tetrachloride while the highest was 35% water in methanol followed by the n-butanol fractions. The n-hexane, carbon tetrachloride and chloroform were crystalline in nature, while all the remaining were amorphous powder after drying. The high percentage of recovery of the 35% methanol in water may be because it is the most polar solvent and were able to extract most of the polar compounds from the ethanol extract which is also polar. The same reason may be the one responsible for high percentage recovery of the n- butanol compared to the remaining solvents. The least percentage recovery from the carbon tetrachloride and the hexane fraction may be because they are non polar and the ethanol extract contain less amount of non polar compounds.

The result in table 3 showed the amount of compounds present in each fraction from the solvent extraction. The ethyl acetate: methanol: water (EMW) solvent system was the best eluting solvent system compared to the other two solvent systems because it was able to separate more compounds. The result also showed that n-butanol contain higher number of compounds since it has higher number spots.

Table 5 showed the antimicrobial activity of fractions obtained from solvent-solvent separation of ethanol extract of Grewia mollis root bark. The results showed that five of the fractions have showed some degree of inhibition on the test organisms. The n-butanol fraction showed the highest activity, by inhibiting all the four test organisms. The hexane extract did not show activity on any of the test organism while the chloroform inhibited the growth of two organisms and carbon tetra chloride inhibited the growth of only one organism. The highest zoned of inhibition was shown by n-butanol against salmonella typhi.

The high activity of the n-butanol fraction can be attributed to the high number of compounds as shown by the TLC in table 3. The possible compounds that can show this activity from the GC-MS can be due to organic carboxylic acid, some esters and the phthalates (Arun et al, 2013).

The toxicity of herbal extracts expressed as LC_{50} values is based on comparison to Meyer's or to Clarkson's toxicity index. According to Meyer's *et al (*1982), toxicity index, extracts with LC50 < 1000 µg/ml are considered as toxic, while extracts with LC50 > 1000 µg/ml are considered as non-toxic (Meyer *et al*., 1982). Clarkson's toxicity criterion for the toxicity assessment of plant extracts classifies extracts in the following order: extracts with LC50 above 1000 µg/ml are non-toxic, LC50 of 500 - 1000 µg/ml are low toxic, extracts with LC50 of 100 -

500 µg/ml are medium toxic, while extracts with LC50 of 0 - 100 µg/ml are highly toxic (Clarkson *et al*., 2004).

Table 5 also showed the LC_{50} values for the fractions, two of the fractions n-butanol with LC_{50} value of 24.12µg/ml and chloroform with LC_{50} value of 42.57 µg/ml are highly toxic which may be due to the ability of this solvents to extract the active components. Two of the fractions 35% methanol in water with LC_{50} value of 233.57 µg/ml and carbon tetrachloride fraction with LC_{50} vale of 245.91 µg/ml are moderately active while the water fraction is weakly active with LC_{50} value of 619.49 µg/ml and the hexane is non-active with LC_{50} value of above 1000 µg/ml. The in activity of the hexane fraction is due to its inability to extract any active compounds from the ethanol extract which is more polar while the activity may be due to the presences of the compounds in the ethanol extract as shown by the GC/MS which include terpenoids, phthalate, carboxylic acids and esters (Ahmed et al*,* 2016; Arun *et al*, 2013; Basri *et al*, 2014; Hanan *et al,* 2012; Ghias *et al,* 2011). The antioxidant activity is in good correlation to the LC_{50} values of the different fractions which may be possible that the same type of compound is causing both bioactivities (Ghosh *et al,* 2008; Mishra and Singh, 2012). The antioxidant and cytotoxic activity may be due to the presence of terpenes, compounds with free hydroxyl group and benzene carboxylic compounds from the ethanol extract as seen in the GC-MS which are good scavenger of free oxygen or oxygen ions Reddy and Jayaveera, 2014).

4. Conclusion

It is concluded from this study that there are fourteen different compounds from the ethanol extract of the root bark of Grewia mollis, which can be fractionated using different solvents in solvent extraction. Ethyl acetate/Methanol/water is the best solvent system to be used for the isolation of the individual compounds from the different fractions using column or preparative TLC chromatography. The present study have revealed that the fractions from the solvent extraction had good antimicrobial, antioxidant and cytotoxic activity, which proved that the use of the ethanol extract of the root bark of Grewia mollis by the traditional people in the treatment of different infections and diseases have been justified.

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