THE QUANTITATIVE PHYTOCHEMICALS CONTENT OF THE ROOT BARK OF GREWIA MOLLIS, THE ANTIOXIDANT AND CYTOTOXICITY ACTIVITIES OF ITS EXTRACTS

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ABSTRACT

Grewia mollis plant is medicinally important plant from North Eastern part of Nigeria. The quantitative phytochemical content of the root bark of the plant were determined with the antioxidant and cytotoxic activities of its extracts obtained by serial extraction with solvents of increasing polarity using soxhlet apparatus. The quantitative phytochemical analysis showed that flavonoid was the phytochemical with the highest concentration while alkaloid had the lowest concentration in the root bark. The cytotoxicity activity determined by the brine shrimp lethality test showed that the water extract was the most active with LC_{50} of 3.50µg/ml, followed by the methanol extract with LC_{50} value of 11.61 µg/ml, while the remaining extracts had moderate activity with LC_{50} values of acetone (120.77 µg/ml,),ethyl acetate (426.20 µg/ml,) and hexane the least with (730.76 µg/ml,).The root bark extracts showed good antioxidant activity with three extracts while two of the extracts were not active. The standard ascorbic acid showed IC_{50} value of 50.00 µg/ml, while the active extracts has the IC_{50} values for water 108.00 µg/ml, ethyl acetate 112.00 µg/ml, and acetone 127.00 µg/ml. The non active extracts were the hexane extract with LC_{50} value of 1350.00 µg/ml and methanol 1957.00µg/ml.

Key words: Grewia mollis, quantitative phytochemical, cytotoxicity and antioxidant activity.

INTRODUCTION

Grewia mollis is a shrub or small tree up to 20 ft tall, belonging to Tiliacaea family widely distributed within the Northern, middle belt of Nigeria and some African countries. Various parts of the plant are used in food and medicine. The stem bark powder or mucilage is used as a thickener in local cakes made from beans or corn flour commonly called "Kosai" and "Punkasau" in Hausa respectively. The dried stem bark is ground and the powder mixed with beans or corn flour thereby enhancing the texture of the food product (Dalziel, 1937).
The flowers and young shoots are sometimes used as a soup or sauce vegetable. The infusion of the bark obtained by cold or hot maceration in water is used in beating mud floors, or mixed with the mud or the walls of huts to give a smooth surface and prevent termite. The mucilaginous property of the bark or leaf is used in application to cuts and sores to prevent breeding. It is known to be a strong fire resistant. The plant is use medicinally at times of child birth to ease the coming out of the child (Dalziel 1937).

Some findings demonstrated that the mucilage obtained from the stem bark can serve as a good binder in paracetamol formulations. Also the recent reports suggest that high concentration of stem bark in dietary exposure may cause some adverse effects, especially liver injury. Phytochemical studies of G. mollis indicated the presence of tannins, saponins,

flavonoids, glycosides, phenols, steroids and the absence of alkaloids in the leaves and stem bark while their presence was revealed in the roots (Martins *et al.,* 2008; Wilson, 2010).

METHODOLGY

Collection of Plant Material: The plant material 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.

Quantitative Phytochemical Determination

Determination of Alkaloids: This was done by the alkaline precipitation gravimetric method described by Harborne, (1973). A measured weight of the sample was dispersed in 10% acetic acid solution in ethanol to form a ratio of 1:10 (10%). The mixture was allowed to stand for 4h at 28^{o}C. It was later filtered via what man No 42 grade of filter paper. The filtrate was concentrated to one quarter of its original volume by evaporation and treated with drop wise addition of conc. aqueous NH_4OH until the alkaloid was precipitated. The alkaloid precipitated was received in a weighed filter paper, washed with 1% ammonia solution dried in the oven at 80^{0}C. Alkaloid content was calculated and expressed as a percentage of the weight of sample analyzed.

Determination of Flavonoids: This was determined according to the method of Harborne (1973). 5gram of the sample was boiled in50mlof 2M HCl solution for 30min under reflux. It was allowed to cool and then filtered through whatman No 42 filter paper. A measured volume of the extract was treated with equal volume of ethyl acetate starting with drop. The flavonoid precipitated was recovered by filtration using weighed filter paper. The resulting weight difference gave the weight of flavonoid in the sample.

Determination of Tannin: Tannin content was determined by the Folis-Denis colorimetric method described by Kirk and Sawyer (1998). 5g sample was dispersed in 50mls of distilled water and shaken. The mixture was allowed to stand for 30min at 28^{o}C before it was filtered through what man No. 42 grade of filter paper. 2mls of the extract was dispersed into a 50ml volumetric flask. Similarly 2ml standard tannin solution (tannic acid) and 2ml of distilled water were put in separate volumetric flasks to serve as standard and reagent was added to each of the flask and the 2.5ml of saturated Na_2C0_3 solution added. The content of each flask was made up to 50mls with distilled water and allowed to incubate at 28^{o}C for 90 min. Their respective absorbance was measured in a spectrophotometer at 260nm using the reagent blank to calibrate the instrument at zero.

Saponin determination: The method used was that of Obadoni and Ochuko (2001). The samples were ground and 20 g of each were put into a conical flask and 100 cm^3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of

diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage.

Determination of Anthraquinone contents: The method described by Soladoye and Chukwuma, (2012) is used for the determination of the anthraquinone. 50 mg of the fine powder sample was soaked in 50 ml of distilled water for 16 hours. This suspension was heated in water bath at 70^{o}C for one hour. After the suspension was cooled, 50ml of 50% methanol was added to it and then filtered. The clear solution was measured by spectrophotometer at a wavelength of 450nm and compared with a standard solution containing 1mg/100ml alizarin with the absorption-maximum 450nm

Determination of Cardiac glycosides: Cardiac glycoside content in the sample was evaluated using Buljet's reagent as described by El-Olemy et al, 1994. 1g of the fine powder of root bark of Grewia mollis was soaked in 10ml of 70% alcohol for 2hrs and then filtered. The extract obtained was then purified using lead acetate and Na_2HPO_4 solution before the addition of freshly prepared Buljet's reagent (containing 95ml aqueous picric acid + 5ml 10% aqueous NaOH). The difference between the intensity of colours of the experimental and blank (distilled water and Buljet's reagent) samples gives the absorbance and is proportional to the concentration of the glycosides.

Determination of total phenols by spectrophotometric method: The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amylalcohol were also added. The samples were made up to mark and left to react for 30 min for colour development. This was measured at 505 nm.

Determination of total terpenoids About 2 g of the plant powder was weighed and soaked in 50 ml of 95% ethanol for 24 h in a conical flask. The extract was filtered and the filtrate extracted with petroleum ether (60-80^{0}C) and concentrated to dryness. The dried ether extract was treated as total terpenoids (Kwaji et al, 2015; Ladan et al., 2014).

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^{0}C**.**

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 Labs, 2015).

HATCHING 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^{0}C 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 Labs, 2015).

Preparation of Test Sample: Samples were prepared by dissolving 20mg of the plant extracts 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.

Cytotoxicity Test (Bioassay): Brine shrimp lethality bioassay was carried out using brine shrimp larvae (Artemia salina) to determine the Cytotoxicity of the plant extracts. 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 6, 12 and 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%.

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

LETHAL CONCENTRATION (LC50) DETERMINATION: The lethal concentrations of plant extract resulting in 50% mortality of the brine shrimp (LC50) 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 LC50 was derived from the best-fit line obtained. LC_{50} values were obtained from the best-fit line, plotted of concentration against Percentage mortality.

Antioxidant Activity of Plant Extracts Antioxidant activity (DPPH free radical scavenging activity) of the extracts was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical as described by Brand-Williams *et al.*, 1995 (Rajani et al 2013). The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid was used as standard. 2ml of 0.002% of DPPH solution in methanol was mixed with 2ml of plant extract solution of varying concentrations (100, 125, 250, 500, and 1000 µg /ml). Corresponding blank sample were prepared and L-Ascorbic in (100-1000 µg /ml) was used as reference standard. Mixture of 2ml methanol and 2ml DPPH solution was used as control. These solutions were kept in dark for 30min, and optical density measured at 517nm using UV-Vis spectrometer, LT-290 Labtronics model**.** The reaction was carried out in duplicate and the decreased in absorbance was measured at 517nm. The DPPH radical scavenging activity (S %) or inhibition % was calculated using the equation.

$$S\% = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of the blank control (containing all reagent except the solution extract) and, A_{sample} is the absorbance of the sample.

TABLE 1: Phytochemicals constituents of the root bark of Grewia mollis on dry weight basis expressed as $mg100^{-1}g$

Phytochemical	1st	2nd	3rd	Sum	Mean.
Tannin	0.1450	0.1300	0.1502	0.4252	0.1417
Saponnin	0.0518	0.0531	0.0501	0.1550	0.0517
Alkaloid	0.0031	0.0032	0.0031	0.0094	0.0031
Anthaquinone	0.0450	0.0425	0.0475	0.1350	0.0450
Flavonoid	0.8280	0.8422	0.8139	2.4841	0.8280
Cardiac glycoside	0.0190	0.0530	0.0142	0.0862	0.0287
Terpenoid	0.3451	0.3344	0.3411	1.0206	0.3402
Phenol	0.0014	0.0021	0.0019	0.0054	0.0018

TABLE 2: Brine shrimp lethality test for cytotoxicity of the root bark extracts of Grewia mollis

EXTRACTS	Concentration. (PPM)	% Motality	LC_{50}(µg/ml
Hexane	10	10.00	730.76
	100	33.33	
	200	30.00	
	500	50.00	
	1000	56.25	
Ethyl acetate	10	27.78	426.20
	100	29.41	
	200	44.44	
	500	55.56	
	1000	77.78	
Acetone	10	38.89	120.77
	100	60.00	
	200	83.33	
	500	94.44	
	1000	100.00	
Methanol	10	22.22	11.61
	100	61.11	
	200	83.33	
	500	100.00	
	1000	100.00	
Water	10	33.33	3.50
	100	88.89	
	200	84.21	
	500	100.00	
	1000	100.00	

The result is the mean of three readings

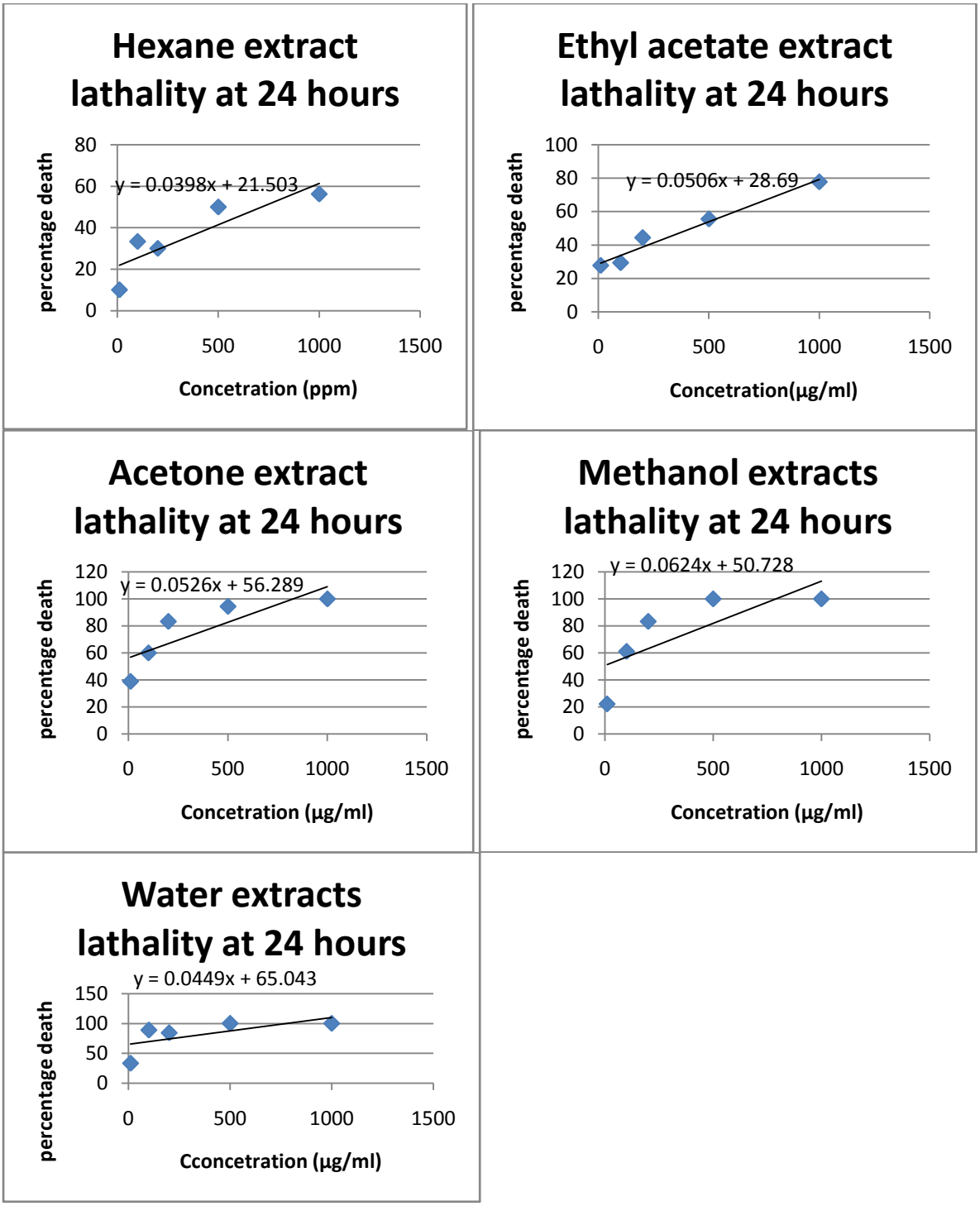

Figure 1; Graphs for brine shrimp lethality test of root extracts of Grewia mollis

TABLE 3: DPPH free radical scavenging activity of Grewia mollis root bark extracts

Extracts	Concentration (µg/ml)	Absorbance (Mean) n=3	Percentage (Mean) n=3	IC50 (µg/ml)
n-hexane	100	0.667	7.87	1350.00
	125	0.626	13.58	
	250	0.602	16.90	
	500	0.533	26.34	
	1000	0.437	39.64	
Ethyl acetate	100	0.426	41.16	112.00
	125	0.200	72.33	
	250	0.193	73.39	
	500	0.186	74.26	
	1000	0.180	75.09	
Acetone	100	0.453	37.38	127.00
	125	0.362	49.95	
	250	0.274	62.11	
	500	0.225	68.92	
	1000	0.195	73.11	
Methanol	100	0.699	3.50	1957.00
	125	0.658	9.16	
	250	0.674	6.91	
	500	0.590	18.46	
	1000	0.521	27.99	
Water	100	0.679	6.17	108.00
	125	0.320	55.85	
	250	0.250	65.42	
	500	0.202	72.10	
	1000	0.178	75.46	
Ascorbic acid	100	0.097	86.65	50.00
	125	0.084	88.44	
	250	0.082	88.72	
	500	0.062	91.48	
	1000	0.059	91.85	

The mean absorbance is the mean of three different readings

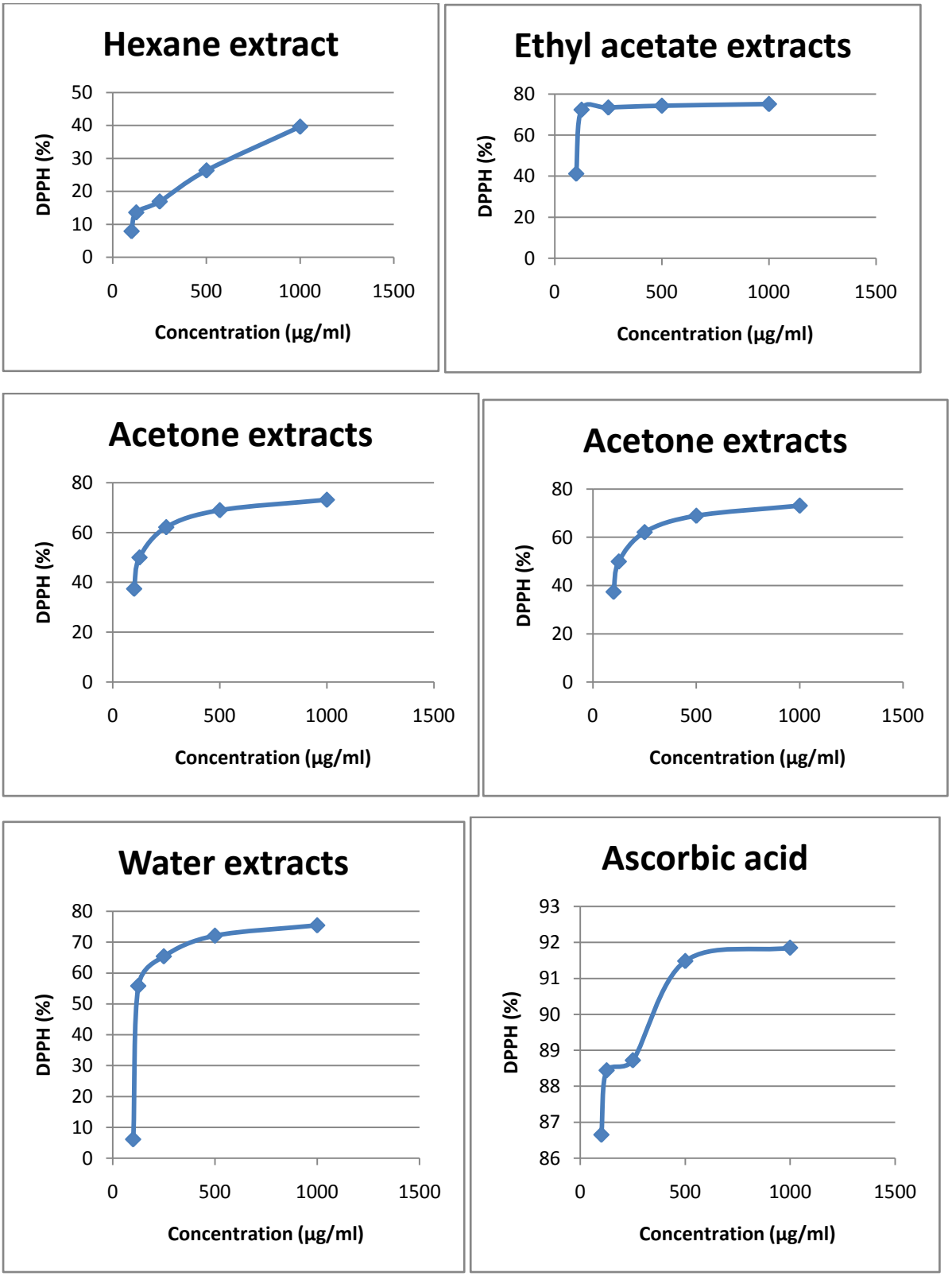

Figure 2: DPPH free radical scavenging activity of Grewia mollis root bark extracts

DISCUSSION

Table 1 shows the results for the quantitative phytochemical content of the root bark of Grewia mollis. The flavonoid (0.8280mg100^{-}1g) was the highest phytochemical detected in the plant while the lowest was alkaloid (0.0018 mg100^{-1}g). Tannin and terpenoid was also seen in moderate quantity compared to the other phytochemicals in the root bark of the Grewia mollis.

The toxicity of herbal extracts expressed as LC_{50} values is commonly valorized either by comparison to Meyer's or to Clarkson's toxicity index. According to Meyer's toxicity index, extracts with LC_{50} < 1000 µg/ml are considered as toxic, while extracts with LC_{50} > 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 LC_{50} above 1000µg/ml are non-toxic, LC_{50} of 500 - 1000 µg/ml are low toxic, extracts with LC_{50} of 100 - 500µg/ml are medium toxic, while extracts with LC_{50} of 0 - 100 µg/ml are highly toxic (Clarkson *et al.*, 2004).

The brine shrimp lethality test (BSLT) is considered a rapid, cheap and simple test to evaluate the lethality of medicinal plant extracts which acts as a preliminary indicator to the cytotoxic and antitumor potential (Ghareeb *et al.*, (2015). Bioactive compounds are almost always toxic at higher dose. In vivo lethality in a simple zoological organisms can be used as a marker for screening in the discovery of new bioactive naturals as an antitumor agent according to Ghosh and Chatterjee, (2013).

The Cytotoxicity activity in the brine shrimp lethality bioassay, indicate that the extracts are biologically active. The test extracts showed different mortality rate at different concentrations. The mortality rate of the brine shrimp was found to be increased with increase of the concentration for each extracts, a plot of concentration of the extracts verse percentage mortality showed an linear correlation between them. From the graphs in figure 1, the LC50 values of the different extracts were evaluated.

Table 2 and figure 1 shows the result for the brine shrimp lethality test for the extracts of the root bark of Grewia mollis. The lethality test at 24 hours shows that the water extract has LC50 of 3.50µg/ml, followed by the methanol extract with LC50 of 11.61 µg/ml. The hexane has LC50 of 730.76 µg/ml and ethyl acetate 426.20 µg/ml while acetone 120.77 µg/ml after 24hours.

The result showed that the water and methanol extracts were active where as the extracts of acetone and ethyl acetate are moderately active while that of hexane is weakly active. The activity of the extracts may be attributed to the presence of phytochemicals like flavonoid (0.8280), tannin (0.1417), terpenoid (0.3402), saponnin 0.0517 and anthraquinone (0.0450) which were detected in the root.

Free radicals and reactive oxygen species are generated in living cells as a result of physiological and biochemical processes and these are causative agents for many chronic diseases such as cancer, diabetes, aging and other degenerative diseases in humans, usually due to oxidative damage of proteins, lipids and DNA (Ghosh *et al.*, 2008 and Ahmed *et al.*, 2016). Harmful effects of disturbed antioxidant can be largely prevented by intake of antioxidant substances (Mishra and Singh, 2012) Table 3 and figure 2 showed the DPPH free radical scavenging activity of the root bark of Grewia mollis extracts. The standard ascorbic

acid showed IC_{50} value of 50µg/ml while that of water extract showed IC_{50} value of 10 µg/ml. The ethyl acetate extracts and acetone extracts also showed good scavenging activities of IC_{50} of 112µg/ml and 127µg/ml respectively. The least scavenging activity was seen in methanol extract of IC_{50} value of 1957µg/ml.

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