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PHYTOCHEMICAL, CYTOTOXICITY AND MICROBIAL SCREENING OF EUPHORBIA HIRTA LINN.

A O. OYEWALE, AMINA MIKA and F. A. PETERS

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ABSTRACT

Phytochemical screening of the successive solvent extracts of *E. hirta* showed, that the ether extract contains cardiac glycosides; the ethyl acetate extract contains tannins and cardiac glycosides; while the water extract contains saponins, alkaloids, tannins and cardiac glycosides. Based on the LC_{50} values calculated, the ether ($LC_{50} = 162.18 \pm 63.1\mu g/ml$) extract is highly toxic, while the ethyl acetate ($LC_{50} = 1513.56 \pm 16.3\mu g/ml$) and water ($LC_{50} = 1412.5 \pm 4.7\mu g/ml$) extracts are within safety limit on the Brine Shrimp cytotoxicity scale.

The crude water extract showed appreciable inhibitory effect on *S. aureus* and *Ps. aeruginosae*, but minimal inhibition on the growth of *E. coli* and no effect on *S. typhi* and *K. pneumoniae*. Ethyl acetate crude extract was active on *E. coli, S. aureus* and *Ps. aeruginosae*, but showed minimum inhibition on *S. typhi*. The petroleum ether crude extract showed appreciable activity on *S. aureus,* average activity on *Ps. aeruginosae,* and *K. pneumoniae*, but minimum activity on *E. coli*. *S. typhi* was resistant to water and petroleum ether extracts. *K. pneumoniae* was resistant to water and ethyl acetate extracts.

Key Words: *Euphorbia hirta* , Phytochemisty, Cytotoxicity, Microbial,

INTRODUCTION

Euphorbia hirta L, synonymous to *Euphorbia pilulifera Linn* (Sofowora, 1982), is a member of the *Euphorbiaceae* family, which is occasionally characterised by production of milky juice (Hazleton and Hellerman, 1984; El-Naggar *et al*, 1978). *E. hirta Linn* is a common weed in the tropics and is widespread in Northern Nigeria (Hutchinson and Dalziel, 1954).

The plant is reported to be effective in the treatment of asthma and hence, the name 'Australian Asthma Herb' and other respiratory tract inflammations, coughs, chronic bronchitis and other pulmonary disorders; and for relieving hay-fever and catarrh of the head (Sofowora, 1982). In East Africa and Ghana an infusion of the leaves is given to sheep, goat, cattle and breast-feeding women to increase lactation. *E. hirta* is also reported to be used in sore, wound and boil treatment; and as prescription against dysentery and scorpion venom in Nigeria.

Preliminary screening of *E. hirta L,* revealed the presence of a gum resin, calcium oxalate crystals, sugars, mucilage, volatile substances, mesityl, palmitic, oleic and linoleic acids (Kerharo and Adams, 1974). The plant is reported to be rich in flavonoids, present as the 3-rhamnoside of which the derivatives leucocyanidol, quercitol, camphor and aquercitol has been isolated (Estrada, 1959; and Blanc and Sacqui-Sannes, 1972). Baslas and Agarwal (1980) also reported the presence of Taraxerone, campesterol, stigmasterol, 1-inositol, cycloartenol, ellagic acid and myricyl alcohol. El-Naggar *et al* (1978) and Sofowora (1982) reported the isolation of shikimic acid, l-inositol, glucose, fructose, and sucrose. Other constituents reported include jambulol, euphosterol, a phenolic substance ($C_{28}H_{18}O_{15}$), β-amyrin acetate, 1-hexacosanol, ingenotriacetate, tinyatoxin, 12-deoxy-4β-hydroxylphorbol-13-dodecanoate-20-acetate, and 12-deoxy-4β-hydroxylphorbol-13-phenyl-20-acetate (Dhar *et al*, 1968).

Yoshida (1988, 1990, 1991) reported the isolation of isomeric ellagitannins, with dibenzofuranoid structure, from *E. hirta* whole plant. Similar compounds isolated from the leaves of the plant are the polyphenols, gallic acid, quercitrin, myricitriu, 3,4-di-O-galloylquinic

A. O. OYEWALE, Department of Chemistry, Ahmadu Bello University, Zaria.
AMINA MIKA, Department of Chemistry, Ahmadu Bello University, Zaria.
F. A. PETERS, Department of Chemistry, Ahmadu Bello University, Zaria.

acid, 2,4,6-tri-O-galloyl-D-glucose and 1,2,3,4,6-penta-O-galloyl-beta-D-glucose (Chen, 1991).

There are several reports on the pharmacological activities of *E. hirta* whole plant extract e.g. anti-ulcer activity (Lin and Hsu, 1988) and the sedative and anxiolytic activities (Lanhers *et al*, 1991a). The use of Amoeba proteus as a low-cost multipurpose biochemical model for cytotoxic screening of plant extracts was employed for the extraction of the active constituents of the aqueous extract of the aerial parts of *E. hirta* (Duez *et al*, 1991). Other reported activities of the plant, *E. hirta* include the analgesic, antipyretic and anti-inflammatory activities of the whole plant (Lanhers *et al*, 1991b); anti-diarrhoeic (Pousset, 1981; Ajao *et* al, 1985; Galvez, 1993; Tona *et al*, 1999a), the anti-malarial (Tona *et al*, 1999b), anti-amoebic and anti-spasmodic (Ridet and Chartol, 1964; Tona *et al*, 2000) activities of various parts of the plant.

The aim of this study is to carry out phytochemical screening on the plant *Euphorbia hirta;* and to determine the cytotoxic level on the brine-shrimp test and activity on some microorganisms, of the crude and purified solvent-extracts of the plant.

EXPERIMENTAL

Sample Collection, Preparation and Extraction

The sample of *Euphorbia hirta*, (whole plant except the floral part) was collected within the premises of Ahmadu Bello University, Zaria, Nigeria, in August and September. The plant was cleaned, air-dried, pulverized and the powdered sample extracted successively with a Soxhlet extractor. The solvents used were petroleum ether (60-80°)→ethyl acetate→ chloroform → ethanol → distilled water. The solvents used were Analar* grades and purified by distillation.

Phytochemical Screening

Preliminary phytochemical screening was performed on each of the petroleum ether, ethyl acetate, chloroform, ethanol and water extracts of the plant, *E. hirta*.

Test for Saponins

(a) The Frothing Test:- For the frothing test, a small quantity of each extract was shaken with distilled water in a test tube. Frothing which persisted on warming was taken as evidence for the presence of saponins. In order to remove 'false-positive' results, the blood haemolysis test was performed on extracts that frothed in water.

(b) The Blood Haemolysis Test:- Small portions of the plant extracts were diluted with 5ml water. To 1ml of this solution was added 1ml of rat blood sample in normal saline and set aside for 3 hours. The solution was then observed for haemolysis. A positive control test was carried out using 1% solution of saponin. A negative control test was also carried out with 1ml of water.

Test for Alkaloid

The extracts were cleaned (to remove non-alkaloidal compounds capable of giving 'false positive' results) (Trease and Evans, 1989); and then divided into two equal portions and each was treated with few drops of Mayer's, and a drop of Wagner's reagents. A creamy white (Mayer) and reddish brown (Wagner) precipitate was taken as a preliminary evidence for the presence of alkaloids.

Test for Tannins

A small quantity of each extract in water (10ml) was boiled and filtered. To the filtrate was added few drops of 1% $FeCl_3$ solution, a blue-green precipitate was taken as evidence for the presence of tannins. The formation of a red precipitate when an aqueous extract of the plant extract was boiled with 1% HCl was taken as evidence for the presence of phlobatinnins (Trease and Evans, 1989).

Test for Anthraquinones

Borntrager's test:- A small portion of each extract was shaken with benzene (10ml) and filtered. 10% ammonia solution (10ml) was added to the filtrate and shaken. The presence of pink colour formed in the lower ammoniacal phase showed the presence of free anthraquinones.

Test for Cardiac Glycosides

Standard test procedures (Trease and Evans, 1989) were used for cardiac glycoside identification.

(i) *Legal Test*: A deep red colour that faded to a brownish yellow showed the presence of Cardenolides in the extracts.

(ii) *Keller-Kiliani Test*: A brown ring obtained at the interface indicated the presence of a de-oxy sugar characteristic of cardenolides.

(iii) *Salkowski Test*: A reddish-brown colour at the interface showed the presence of a steroidal ring as the aglycone portion of the cardiac glycoside.

Thin Layer Chromatography (T. L. C.)

Thin layer chromatography was used to clean up the extracts. The chromatograms were developed using: - Water extract - Petroleum ether (60-80°): ethyl acetate (1:1); and Ethyl acetate extract - petroleum ether (60-80°): ethyl acetate (3:2). The spots were viewed under UV fluorescent lamp.

Test for Functionality: Confirmatory Spray tests were carried out for alkaloids, cardiac glycosides and phenolic Compounds. The samples were spotted on TLC plates coated with silica gel and sprayed with the appropriate reagents using a manual spray-pump.

For alkaloids, iodine-potassium iodide reagent was used. It was prepared using a mixture of 5% iodine in KI solution:water:2M acetic acid (2:3:5). A brown colour was taken as a positive result for alkaloids (Kirchner, 1967).

For cardiac glycosides, 1,3- Dinitrobenzene reagent was used. It was made up of solution A (10% 1,3-dinitrobenzene in benzene) and solution B (6g NaOH in 25ml H_2O into which 45ml CH_3OH had been added). The spotted sample was first sprayed with solution A, heated to 60 °C and then sprayed with solution B. A purple colour which changed to blue and which faded rapidly was taken as positive (Touchstone, 1992).

For phenolic compounds (tannins), ferric sulphate-potassium ferricyanide reagent was used. It consists of solutions, (a) 0.5% ferric sulphate in 0.5M H_2SO_4, and (b) 0.2% potassium ferricyanide. Equal volumes of solutions (a) and (b) were mixed and sprayed on the sample spots. Observation was made for colour change before 10mins and after heating to110°C (Kirchner, 1967). The fact that hydrolysable tannin solutions turn blue with iron salts and condensed tannins give brownish-green was used for assessment (Trease and Evans, 1989).

Colour reaction test with ammonia gas for flavonoids (condensed tannins) was carried out. The spots were exposed to ammonia gas and viewed with ultraviolet (UV) light. Assessment was done based on the colour observations (Haslam, 1966).

Cytotoxicity Test

The Brine Shrimp (*Artemia salina*) Lethality (Meyer *et al*, 1982) method was used for the cytotoxicity test. A stock solution of each of the extracts was prepared to a concentration of 10mg/ml in Dimethyl Sulphoxide (DMSO). For each extract, concentrations of 1000, 500, 250, 125, and 62.5 µg/ml were made by serial dilution from the stock. A test tube containing only DMSO was used as the control. Triplicate test determinations were made.

Ten shrimps, initially hatched from its eggs in sea water, were collected with teat pipettes and placed in each test tube and made up to 5.0ml with sea water, giving a total of thirty shrimps per dilution. The test tubes were maintained under illumination for twenty-four hours and the survivor shrimp larvae were counted macroscopically in the tubes against lighted background. From the survivors the number of deaths at each dose and control were recorded. The LC_{50}, at 95% confidence interval, were determined for the extracts based on the recorded percentage death using the method of Linear regression analysis (Saunders and Fleming, 1971).

Antimicrobial Test Using the Agar Diffusion Method

This test is the anti-bacterial test and the method used is the Plate Dilution Method (Collins and Lyne, 1976). Mueller-Hinton agar (38g MHA in 1litre of distilled water), which is the recommended medium for most commonly encountered bacteria, was used for this test.

Solutions of the extracts were prepared (1000µg/ml) in appropriate solvents – distilled water for water extract, DMSO for petroleum ether and ethyl acetate extracts, and then diluted to give concentrations of 1000, 500, 250, 125, and 62.5 µg/ml for each extract. The activity of the extracts was then tested against microorganisms.

The microorganisms: *Staphylococcus aureus, Pseudomonas aeruginosae, Klebsiella pneumoniae, Salmonella typhi and Escherichia coli* were sub-cultured to nutrient Agar Slants, and incubated for 24hours at a temperature of 37°C. Before innoculating the plates, the broth culture was further diluted (one drop of broth culture to four drops of normal saline- 9% NaCl solution). A control containing only normal saline was also prepared.

RESULTS AND DISCUSSION

Phytochemical Test

The results of the phytochemical screening (Table 1) on *E. hirta* showed that the ether and chloroform extracts contain cardiac glycosides; the ethyl acetate extract contains

Table 1: Results of Phytochemical Screening of *Euphorbia hirta L.*

Extract	Saponin		Alkaloid		Tannin	Phloba-tannin	Anthra-quinone	Cardiac glycoside		
	Fr.	B.H	M.	W.				Legal	K/K	Sal
Water	+	+	+	+	+	-	-	+	-	-
Ethanol	+	+	-	-	+	-	-	-	+	+
Ethyl acetate	-	-	-	-	+	-	-	+	+	-
Pet. ether	-	-	-	-	-	-	-	-	+	+
Chloroform	-	-	-	-	-	-	-	-	+	+

NOTE: A positive result is indicated by (+), while a negative result is indicated by (-).
Fr. = Frothing Test; B.H. = Blood Haemolysis Test; K/K = Keller-killiani Test;
Sal = Salkowski Test, Lieb. = Libermamm's Test, M = Mayer's Test, W = Wagner's Test.

Table 2: Functionality Spray Test Result for Extract Fractions of *E. hirta*.

Fraction	I_2-KI Spray (Alkaloid)		Dinitrobenzene (Cardiac glycoside)		$Fe_2(SO_4)_3$-$K_3Fe(CN)_6$ (Tannin)		Ammonia (Flavonoid)	
	EA	W	EA	W	EA	W	EA	W
1	-	+	-	-	+	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	+	+	+	+	-
4	-	NA	-	NA	+	NA	+	NA
5	-	NA	-	NA	+	NA	+	NA
6	-	NA	-	NA	-	NA	-	NA

Note: EA= Ethyl acetate extract W= Water extract NA= Not applicable
(+) = positive response (-) = negative response.

tannins and cardiac glycosides; the ethanol extract contains saponins, tannins and cardiac glycosides; while the water extract contains saponins, alkaloids, tannins and cardiac glycosides. Earlier report on the plant by Ridet and Chartol (1964) indicated the presence of saponin whose glycone is rhamnose with a triterpenoid aglycone; tannin and resins in the fresh plant.

These results showed that the ethanol and water extracts on one hand and the petroleum ether and chloroform extracts on the other, extracted virtually the same classes of compounds. Subsequent investigation on the plant was therefore centered on the petroleum ether, ethyl acetate and water extracts. The water extract was of particular interest because it is the conventional medium of administering the plant's extract for the reported medicinal uses. The ethyl acetate and water extracts were purified and fractionated by TLC; six spots and three spots were obtained for the extracts respectively. The fractions were then screened (Table 2).

Brine-Shrimps (Toxicity)

The results of the toxicity test are shown in was Table 3. The death responses for the three extracts reduce with decrease in the concentrations of the extracts. Only the ether extract gave a 100% death response at the highest concentration (1000 µg/ml) used, while for the other extracts the death response of the Brine-shrimp reduces gradually with decrease in concentration.

The Brine-Shrimp lethality test has been used to estimate LC_{50} for comparison of potencies of drugs (Meyer *et al*, 1982). The probits of the quantal responses were extracted from the probit table (Saunders and Fleming, 1971). From the probit values, the LC_{50} of each extract was calculated by the method of linear regression (Saunders and Fleming, 1971).

The LC_{50} values showed that the ether (162.18 ± 63.1µg/ml) extract is highly toxic, while the ethyl acetate (1513.56 ± 16.3µg/ml) and water (1412.5 ± 4.7µg/ml) extracts are within safety limit on the cytotoxicity scale. Extracts are said to display toxicity in the brine shrimp assay when $LC_{50} < 1000\mu g/ml$ (Meyer *et al*, 1982). The high toxicity of petroleum ether extract compared to the others could be attributed to the fact that, the result of the phytochemical screening showed that it contained mostly cardiac glycosides, which are known to be poisonous.

Table 3: Dose, Log_{10}Dose, % Death Response, Probits* and LC_{50} for the Extracts

DOSE (µg/ml)	Log_{10}DOSE (X*)	Water		Ethyl acetate		Petroleum ether	
		% Death	Probit	% Death	Probit	% Death	Probit
1000	3.000	43.33	4.82	40	4.75	100	8.09
500	2.699	23.33	4.26	30	4.48	80	5.84
250	2.398	13.33	3.87	20	4.16	60	5.25
125	2.092	10.00	3.72	6.7	3.45	30	4.48
62.5	1.796	3.33	3.12	6.7	3.45	20	4.16
LC_{50} (µg /ml)		1412.54± 4.7		1513.56±16.3		162.18 ± 63.1	

Table 4: Result of Antimicrobial Test for the Crude ether, Water and Ethyl Acetate Extracts

Extract	Microorganism	Zones of Inhibition (mm) for Various Concentration (µg/ml)					Standard
		1000	500	250	125	62.5	
Water	*E. coli*	M	-	-	-	-	-
	S. aureus	17	12	-	-	-	-
	Ps. aeruginosae	10	8	-	-	-	-
	S. typhi	-	-	-	-	-	-
	K. pneumoniae	-	-	-	-	-	-
Ethyl acetate	*E. coli*	10	M	-	-	-	-
	S. aureus	10	M	-	-	-	-
	Ps. aeruginosae	12	7	-	-	-	-
	S. typhi	M	-	-	-	-	-
	K. pneumoniae	-	-	-	-	-	-
Petroleum ether	*E. coli*	M	-	-	-	-	-
	S. aureus	17	11	10	-	-	-
	Ps. aeruginosae	10	-	-	-	-	-
	S. typhi	-	-	-	-	-	-
	K. pneumoniae	10	-	-	-	-	-

M = Minimum inhibition, - = No inhibition

Antimicrobial Tests

Dilution susceptibility testing methods are used to determine the minimal concentration (µg/ml) of an antimicrobial agent inhibiting or killing a microorganism. Results of the microbial test are shown in Table 4. The water extract, at concentrations greater than 500µg/ml, showed appreciable inhibitory effect on *S. aureus* and *Ps. aeruginosae*, but minimal inhibition on the growth of *E. coli* and no effect (at least at the given concentrations) on *S. typhi* and *K. pneumoniae*. {Any extract with zone of inhibition, i.e. diameter, 6mm or more was considered active (Boda, 1997)}. Ethyl acetate extract, at concentrations greater than 500µg/ml, was active on *E. coli, S. aureus* and *Ps. aeruginosae*, but showed minimum inhibition on *S. typhi*. The petroleum ether extract showed appreciable activity on *S. aureus*, even at concentrations as low as 250µg/ml, but only active on *Ps. aeruginosae*, and *K. pneumoniae*, at a concentration of 1000µg/ml, but minimum activity on *E. Coli*. *S. typhi* was resistant to water and petroleum ether extracts. *K. pneumoniae* was resistant to water and ethyl acetate extracts.

The fact that the plant extracts were found to inhibit the growth of these bacteria could account for their reported medicinal uses. *S. aureus* is known to be involved in wound infection, diarrhea, conjunctivities and vomiting caused by food poisoning. *E. coli* also causes wound infection (especially abdominal wound infection), urinary tract infection and diarrhea. *Ps. aeruginosae* infects the respiratory tracts of cystic fibrosis patients, causes burns and wounds infection. *S. typhi* causes gastro entritis and typhoid fever; and *kleb. pneumoniae* causes urinary tract diseases and acute diarrhea in children (Falkow *et al*, 1987; Bailey, 1987).

When the antimicrobial tests were repeated on the fractions from the water and ethyl acetate extracts, the activity by the ethyl acetate extract was reproducible on the four isolates on which it was initially active, i.e *E. coli, Ps. aeruginosae, S. aureus* and *S. typhi*. For the water extract fractions the activity on *Ps. auruginosae* and *S. aureus* were reproducible, while that on *S. typhi* was not reproducible.

Table 5: Antimicrobial Test of the TLC fractions of Water and Ethyl Acetate Extracts

Fraction (R_F Value)	Zones of Inhibition (mm) of Various			
	E. coli	*Ps. aeruginosae*	*S. aureus*	*S. typhi*
W_1 (0.06)	-	10	M	NA
W_2 (0.50)	-	-	-	NA
W_3 (0.79)	-	-	-	NA
EA_1 (0.23)	10	15	17	10
EA_2 (0.46)	12	10	14	9
EA_3 (0.75)	M	17	12	M
EA_4 (0.85)	M	12	12	10
EA_5 (0.96)	11	13	18	M
EA_6 (0.99)	M	15	-	M

(-) = 'No inhibition' M = 'Minimum inhibition' NA = 'Not available'

Functionality and microbial test performed on the TLC fractions (Tables 2 and 5) of water extract revealed that the W_1 extract fraction is saponin, which was active on *Ps. aeruginosae* and *S. aureus*. The W_2 fraction did not respond to any of the functionality test and was not active on any of the microorganisms. The W_3 fraction is an hydrolysable tannin which was not active on any of the microbes. The EA_1, EA_3, EA_4 and EA_5 ethyl acetate fractions were condensed tannins (the EA_3, EA_4 and EA_5 being flavonoids) and were active on *E. coli, Ps. aeruginosae, S. aureus,* and *S. typhi*. The EA_6 fraction did not respond to any of the functionality tests, but was active on *Ps. aeruginosae, E. coli* and *S. typhi*. This comparative study on the activities of *E. coli, Ps. aeruginosae, S. aureus,* and *S. typhi* with different solvent extracts of *E. hirta* complements earlier reports on the antimicrobial activity of the plant by Collier (1949); Pousset (1981); Laurens *et al*, (1985); and Ajao *et al* (1985).

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