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ANTIMALARIA ACTIVITY OF ETHANOLIC EXTRACTS OF LEAVES OF *TERMINALIA CATAPPA*. L. COMBRETACEAE (INDIAN ALMOND)

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ABSTRACT
The leaves of Terminalia catappa were air dried, grounded and soaked with ethanol. The extracts obtained (47.23g, TC1) was fractionated sequentially using aqueous methanol with petroleum ether, chloroform and ethyl acetate respectively. The residue of ethanol extract (marc) was extracted with 5M HCl, basified and extracted with chloroform. These were labeled as TC1-01–TC1-05 for the plant. Each of these fractions was phytochemically screened to detect the class of secondary metabolite present. The fractions obtained from the plant were found to be selectively active against brine shrimp larvae. These fractions were also subjected to antimalaria parasites bioassay. Fractions TC1, TC1-02 and TC1-05 were found to be active against tested organisms, with TC1-02 being the most active. TC1-02 was further subjected to activity guided column chromatography that led to the isolation of two pure compounds TC1-02-1 and TC1-02-64. Compound TC1-02-64 was found to be active against the malaria parasite. This was further purified and subjected to qualitative and quantitative analysis.

Key Words: Fractionation, Antimalaria, Ethanol, Column Chromatography, Terminalia catappa.

INTRODUCTION

Malaria is a vector-borne infectious disease caused by protozoan parasites. It is wide spread in tropical and sub-tropical regions including part of the American, Asia and Africa (Snow *et al*., 2005). Each year, it causes diseases in approximately 515 million people and kills between one and three million people, majority of whom young children in sub-Saharan Africa (Snow *et al*., 2005). Adults are less likely to die from malaria, but still suffer from the sickness (Adams, 2008). Infection often peaks during the rainy season, and higher rates of bedridden workers affect the agricultural productivity of families, communities, and nations (Newman,*et al*., 2008). This made the disease to be associated with poverty and regarded as a major hindrance to economic development (Joy *et al.,* 2003). Experts estimate that the annual costs of malaria treatment and lost in productivity due to malaria total between $2 billion and $3 billion in Africa (Newman,*et al*., 2008).

Malaria parasites are transmitted by female anopheles mosquitoes bites. The parasites multiply within red blood cells (RBC) causing symptoms that include anaemia as well as other general symptoms such as fever, chills, nausea, and flu-like illness and in severe cases coma and death (Joy *et al.,* 2003).

Malaria transmission can be reduced by preventing mosquito's bites with mosquito nets and insect repellents on by mosquito control measures such as spraying insecticides inside houses and draining standing water where mosquitoes lay their eggs. Taking preventive drugs (both traditional and orthodox drugs) can reduce risk of infection (Mary *et al*, 2000). Resistance acquired by the mosquito to insecticides, and the parasite to drugs, has led to new therapeutic challenges, particularly in the treatment of *P. falciparum*.

The plants have shown significant killing capacity against plasmodium parasites, these includes; Cinchona tree, Artemisia tree, *Artabotrys uncinatus* (China: arteflene), *Azadirachta indica* (Asia : nimbolide) and *Brucea javanica* (China: terpenoids). Many more plants might have antimalarial potential. (Ying and Yu-Lin, 1998). The research study reports on the active compounds present in *Terminalia catappa*. L. responsible for its activity against malaria parasite.

MATERIALS AND METHODS

Sample Collection

Leaves of *Terminalia catappa* were collected at Sharada Phase I, Kano Municipal Local Government Area on 11th August, 2007. The leaves were identified at the Department of Biological Science, Bayero University, Kano.

Extraction and Fractionation

The air-dried sample (200g) was percolated using Ethanol (1.3L) for a period of 10 days. The extracts were drained and concentrated under reduced pressure using Rotavapor (R110 at 40^{0}C).

The ethanol extract was allowed to dry and its weight was recorded. This was labeled as TC1 as Ethanol extracts *T. Catappa.*

Ethanol extract (10g) was dissolved in 60% aqueous methanol (150ml, Aq. MeOH) and partitioned with petroleum ether (150ml), chloroform (150ml) Ethyl acetate (150ml) sequentially. All fractions obtained were collected in weighted beakers and were labeled as TC1-1 to TC1-5 (Sofowora, 1984).

The Residue obtained (marc) after extraction with ethanol was repercolated with 5M Hydrochloric acid (500ml) for the period of 3 days. Aqueous acid fraction obtained was basified with solution of sodium hydroxide and partitioned with chloroform. The chloroform fraction was collected in a weighted beaker (After evaporating excess chloroform using Rotavapor) (Djilani *et al,* 2006).

Chemical Analysis

Plants extracts were phytochemically screened using standard techniques for the qualitative detection of Alkaloid, Flavanoids, Resins, Steroids, Sugars, Tannins and Saponins (El-olemy *et al*., 1994; Sofowora, 1984; Evans, 1995).

Brine Shrimp Lethality Test (BST) Meyer, *et al*., 1982.

The fractions obtained from the two plants were subjected to the test for their activity against Brine Shrimp Larvae (*Artemia Salina*).

Artemia salina (leach) eggs (50mg) were added in a hatching chamber containing ocean sea salt water. The hatching chamber was kept under on inflorescent bulb for 48 hours for the eggs to hatch into shrimp larvae.

The fractions TC1-1 to TC1-5 (20mg) were separately dissolved in methanol (2ml) from which 500, 50, and 5ml of each solution was transferred into vials corresponding to 1000, 100 and 10 mg/ml respectively. Each dosage was tested in triplicate. The vials (9 per test fraction) and one control contain 500ml of solvent were allowed to evaporate to dryness in about 48 hours at room temperature 4.5ml of ocean sea salt water was added to each vial. The final volume of solution in each vial was adjusted to 5ml with sea salt water immediately after adding the shrimps 24 hours later, the number of surviving shrimps at each dosage was counted and recorded.

Malaria Parasite Assay

Sourcing of Malaria Parasites for the Assay

Malaria parasites infected blood samples from Haematology Department, Muhammad Abdullahi Wase Specialist Hospital, Kano provide clinical blood samples containing heavy parasitaemia of *Plasmodium falciparum*.

Venous blood from patients recommended for malaria parasites test (MP test) using 5ml disposable plastic syringes and needles (BD and 20 SWG). The samples were immediately transferred into K_3-EDTA disposable plastic sample bottles with tightly fitted plastic corks and mixed thoroughly and then transported to the Microbiology laboratory at Bayero University in a thermoflask containing water maintained at 4^0C as demonstrated by Dacie (1968).

Preparation of *Plasmodium falciparum* Culture Medium

Venous blood (2ml) from the main vein of white healthy rabbits pinnae was withdrawn using a disposable 5ml syringe (BD 205 WG). This was defibrinated by allowing it to settle for at least one hour (Dacie and Lewis, 1968). The defribranated blood was centrifuged at 1500rpm using spectre merlin centrifuge for 10minutes and the supernatant layer was collected in a sterilized tube. The sediment was further centrifuged at 1500rpm for fine minutes, and the supernatant layer was added to the first test tube. The sediments were discarded and the serum collected was supplemented with the salt of RPMI 1600 medium (KCl 5.37mM, NaCl 10.27mM, $MgS0_4$ mM, $NaHPO_4$ 17.73mM, $Ca(NO_3)_2$ 0.42mM, $NaHCO_3$ 2.5mM, and glucose 11.0 mM. (BDH ltd, UK) as demonstrate by Devo *et al* (1985). The medium was sterilized by 40µg/ml gentamicin sulphate (Trager, 1982).

***In-Vitro* Assay Of the Activity of the Extracts on *Plasmodium falciparum* Culture**

A 0.1ml of tested solution as 0.2ml of the culture medium were added into a tube containing 0.1ml of .5% parasitaemia erythrocytes and mixed thoroughly. The sensitivity of the parasites to the tested fractions was determined microscopically after incubation for 24 and 48 hours at 37°C.The incubation was undertaken in glass bell jar containing a lighted candle to ensure the supply of required quantity of CO_2 (about 5% 0_2 gas, 2% and about 93% nitrogen gas as demonstrated by Muktar *et al*, (2006).

Determination of the Activity

At the end of the incubation periods 24 and 48 hours, a drop of a thoroughly mixed aliquot of the culture medium was smeared on microscopic slides and strained by Giemsa's staining techniques. The mean number of erythrocytes appearing as blue discoid cells containing life rings of the parasite (that appeared red pink) was estimated and the average percentage elimination by the samples was determined. The activity of the tested samples was calculated as the percentage elimination of the parasites after incubation period – 24 and 48 hours, using the formula below;

$$\% = \frac{N}{Nx} x100$$

Where, % = Percentage activity of the extracts

N = Total number of cleared RBC Nx = Total number of parasitized RBC

Note: RBC = Red Blood Cells (Muktar *et al.,* 2006.)

Column Chromatography of TC1-02

5g of the extract (TC1-02) was thoroughly mixed with silica gel until it changed to a non-sticky powder. It was then loaded to a silica gel column (108g; 62 x 2.5 cm).

The column was run by eluting solvents of increasing polarity as follows: petroleum ether (60-80), petroleum ether-chloroform (9:1), (8:2), (1:1), (2:8), chloroform, chloroform-ethyl acetate (1:1), (2:8), ethyl acetate, methanol. A varied quantity of the solvents was eluted and the eluant was collected in fraction of 100ml. They were allowed for complete evaporation at room temperature. Weight and Thin Layer Chromatographic analysis on pooled fraction were determined. Malaria parasite assay was reconducted on selected samples.

RESULTS AND DISCUSSION

The various extracts/fractions obtained were labeled; their weights and appearances were recorded (Table 1). Powdered plant material (200g) yielded 47.23g of the ethanol extract from the leaves of *Terminallia catappa.* From the 10g of ethanol extract; petroleum ether fraction has 5.20g (gummy, dark green), this constitute more than 50% of the extracts. While other fractions, chloroform (2.63g, Sticky, dark-green), ethyl acetate (0.68g, Crystalline, brownish) and aqueous methanol (1.03g, gummy, brownish) constitutes 43.4% of the partitioned extracts. A chloroform fraction (TC2, 0.82g) resulted from the extraction of the plant residue using mineral acid in such a way that an alkaloid is separately extracted; it is sticky and yellowish in appearance.

Table 1: Weight of Various Fractions Obtained and Their Appearances, Leaves of *Terminalia catappa*

			APPEARANCE	
FRACTIONS	**SYMBOL**	**WEIGHT**	**NATURE**	**COLOUR**
Ethanol	TC1	10.00g	Gummy	Dark-Green
Pet-Ether	TC1-01	5.20g	Gummy	Dark-Green
Chloroform	TC1-02	2.63g	Sticky	Dark-Green
Ethyl acetate	TC1-03	0.68g	Crystalline	Brownish
Aq. MeOH	TC1-04	1.03g	Gummy	Brownish
Chloroform	TC2	0.82g	Sticky	Yellow

The phytochemical screening result showed the distribution of the presence of secondary metabolites in the extracts. From Table 2, the ethanol extract (TC1) have shown the presence of alkaloid, flavonoids, resins, saponins, steroids, sugars and tannins. They were reflected in the other fractions, such that resins and saponins appeared in all the fractions, steroids were presence in TC1-02, TC1-03 and TC1-04, while sugars and tannins reflected in TC1-04. TC2 showed alkaloid present only.

The lethality test against Brine shrimp larvae (*Artemia salina*) were carried out. The Brine shrimp Test (BST) results are expressed in LC50 µg/ml values at 95% confidence interval (Table 3). From the BST results, extracts obtained from the plant exhibit activity for all the fractions tested with TC1-01 being most active among the fractions. It is also observed that, the acitivity for T. catappa decreases with increasing polarity with the exception in activity of ethanol fraction.

Table 2: Phytochemical Result for *T. catappa.*

Fractions	**Compound Present**
TC1	Alkaloid, Flavanoids, Resins, Steroids, Sugars, Tannins, Saponins
TC1-01	Resins, Saponins
TC1-02	Resins, Saponins, steroids
TC1-03	Resins, Steroids, Saponins
TC1-04	Resins, Steroids, Sugars, Tannins, Saponins
TC2	Alkaloid

.

Table 3: Brine Shrimp Lethality Test Result:

PLANT	**FRACTIONS**	**LC_{50}**
	TC1	10.52
	TC1-O1	0.1024
	TC1-02	23.29
Terminallia catappa	TC1-03	177.56
	TC1-04	516.88
	TC2	78.34

The results of antimalaria activity of the extracts are shown in Table 4. Fractions TC1 and TC2 (alkaloid extract) have demonstrated a remarkable activity at all concentrations. The most interesting antiplasmodial activity was obtained with TC1-02, in which the microscopic examination of Giemsa's stained slides for the fraction at 5000ug/ml showed a virtual absence of the parasite after 48 hours. These observations suggest that the activity of the extract may be cytotoxic for *P. falciparum*, thereby inhibiting their development. MP test result on TC1 and TC2 has shown activity of almost 82% elimination of the parasite, whereas the rest of the fractions have about 75% elimination of the parasite (after 48 hours at 5000ug/ml).

Table 4: Antimalaria Activity of *T. catappa*

Fractions	Concentrations (µg/ml)	Average No. of parasite per field Before incubation	1st Day	2nd Day	Overall Average No. of Parasite After 48 Hours	Percentage Elimination at the End of Incubation
Control		28	28	28	28	0%
TC1	5000	28	6	5	5.5	82%
	2000		10	8	9	71%
	1000		11	11	11	61%
	500		19	13	16	54%
TC1-01	5000	28	17	6	11.5	79%
	2000		21	12	16.5	57%
	1000		23	15	19	46%
	500		26	18	22	36%
TC1-02	5000	28	2	0	1	100%
	2000		4	3	3.5	89%
	1000		9	7	8	75%
	500		13	8	10.5	71%
TC1-03	5000	28	11	7	9	75%
	2000		13	9	11	68%
	1000		17	10	13.5	64%
	500		23	14	18.5	50%
TC1-04	5000	28	10	7	8.5	75%
	2000		12	10	11	64%
	1000		15	9	12	68%
	500		19	10	14.5	64%
TC2	5000	28	11	5	8	82%
	2000		13	7	10	75%
	1000		16	8	12	71%
	500		21	8	14.5	71%

In Table 5, column fractions TC1-02-64 and TC1-02-83 have shown the highest activity; the former was obtained in an appreciable quantity for spectral analysis (TC1-02-64: a white crystalline compound; 0.14g; Rf =0.67; ^{1}HNMR = 5.4, 5.2 ppm; ^{13}CNMR = 72.05, 140.99, 121.97 ppm; IR = 3246, 1667, 2919 cm^{-1}) was found to be most active against malaria parasite, (83.65% after 48 hours at 5000 µg/ml) and it melts between 139^{o}C-141^{o}C. The qualitative and quantitative analysis of this fraction revealed the presence of carbon (36.25%), hydrogen (62.50%) and oxygen (1.25%). The percentage composition of oxygen shows that it is a mono oxygenated compound. While TC1-02-5 and TC-2-72 exhibited weak activity against the parasite; 22.69% and 38.74% after 48 hours, for the respective fractions.

Table 5: Antimalaria Activity of the Column Fractions TC1-02-1, TC1-02-5, TC1-02-33, TC1-02-54, TC1-02-64 and TC1-02-72

Fractions	PERCENTAGE ELIMINATION OF MALARIA PARASITE BY FRACTIONS AT 5,000 µg/ml
TC1-02-1	62.30%
TC1-02-5	22.69%
TC1-02-33	83.54%
TC1-02-54	55.00%
TC1-02-64	83.65%
TC1-02-72	38.74%

CONCLUSION

Conclusively, this work provides a scientific basis for using *Terminalia catappa* as antimalaria plant. The fraction TC1-02 led to isolation of active white crystalline compound.

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