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ANTI-PLASMODIAL EFFECT OF LUPEOL ISOLATED FROM THE STEM BARK OF DIOSPYROS MESPILIFORMIS (HOSTCH.)

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ABSTRACT
The stem bark of *Diospyros mespiliformis* was air dried, ground and soaked in ethanol. The ethanol extract was macerated sequentially using n-Hexane, chloroform, ethyl acetate, methanol and water. The resulting fractions were assayed against *Plasmodium falciparum* using thin smear method. All the fractions of *D. mespiliformis* were found active against the malaria parasite at 5000, 2000, 1000, and 500µg/ml with percentage elimination of 91.7, 84.7, 81.9 and 73.6 respectively. The bioactivity-guided column fractionation of the ethanol extract of stem- bark of *Diospyros mespiliformis* led to the isolation of compound Dp-01-1A, a white solid with solvent system CHCl3/EtAc (7:3) and Rf value 0.91. The spectral data, IR; ^{1}H, 13C and DEPT NMR revealed the compound as Lupeol, a pentacyclic triterpenoid which shows high anti-plasmodial activity (84.4%, 78.1% and 68.8%) at 1000, 100 and 10µg/ml respectively. The results support the popular use of the plant in treating malaria fever in Kano State of Nigeria.

KEYWORDS: Anti-plasmodial, Lupeol, Stem-bark, *D. mespiliformis.*

INTRODUTION
Diospyros mespiliformis(*Ebenaceae*), known as African Ebony, jackalberry, and "*Kanya*" in Hausa. It is a large deciduous tree found mostly in the savannah of Africa. It is a tall upright tree that can reach a height of 25m, with a trunk circumference of more than 5m and dense evergreen canopy. The bark is black to grey with a rough texture. The inner skin of the bark is reddish; leaves are simple, alternate leathery and dark green. The fruit is a fleshy berry with an enlarged calyx, yellow to orange when ripe (Palmer and Pitman, 1972).

The plant is widely used for the treatment of malaria fever in northern Nigeria (Etkin, 1997). The leaf and bark extract of *Diospyros mespiliformis* showed significant antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Microsporum gypseum* (Sadiq*et al.*, 2013). Antimicorbial studies on the leaves, stem bark and root of *D. mespiliformis* showed broad spectrum activities against 9 gram-positive bacteria, 8 gram negative bacteria, and 6 fungal strains (Adeniyi*et al*, 1996). Neuropharmacological activities of the aqueous extract of stem bark of *Diospyros mespiliformis*in mice has been reported (Adzu*et al*, 2002). Previous research work on the aqueous extract of the stem bark of the plant exhibited remarkable *in-vivo* anti-plasmodial potency in mice (Adzu and Salawu, 2009). However, this paper reports the isolation and *in-vitro* anti-plasmodial effect of lupeol, a pentacyclic triterpenoid obtained from the stem bark of *D. mespiliformis* (Ebenaceae).

MATERIALS AND METHOD
General Procedure
The solvents used in the research work are of analytical grade. Glass wares were properly washed. Column chromatography was done using silica gel (50-200 mesh). Thin layer chromatography (TLC) was performed on 20 × 20cm precoated aluminium TLC plates. TLC bands were visualized under ultraviolet (UV) lamp (at 254nm and 356nm) or by exposure to iodine. *Plasmodium* species were obtained from Malam Aminu Kano Teaching Hospital (AKTH) and transported to the Department of Microbiology, Bayero University, Kano. ^{1}H and 13C NMR spectrum were recorded on Varian Mercury 600MHz and 151MHz respectively. IR spectrum was recorded on Nicolet Magna 550FT Spectrophotometer.

Collection of Plant Material
The stem bark of *Diospyros mespiliformis*(Hostch.) was collected on 22nd July, 2013 from Dan Sarai Nassarawa Local Government Area of Kano State of Nigeria. The

stem bark and leaves were authenticated by Prof. Bala Sidi Aliyu of Plant Biology Department, Bayero University, Kano. The plant material was air dried and ground into fine powder.

Extraction and Fractionation

The air dried sample (500g) was percolated using ethanol (1L) for a period of two weeks. The crude ethanol extract was drained and concentrated at 40°C on a rotary evaporator. The extract was weighed (26.4g).

The crude ethanol extracts (11.8g) was macerated sequentially using n-haxane, chloroform, ethyl acetate, methanol and water. The fractions were dried by exposing to air at room temperature (Sofowora, 1984).

MALARIA PARASITE ASSAY

Preparation of Test solution

A stock solution of 10000µg/ml was prepared by dissolving 20mg of each test fraction in 2ml of Dimethyl sulphoxide (DMSO). Sample solutions of 500, 1000, 2000, 5000µg/ml were prepared from the stock solution by serial dilution.

Sourcing of Malaria Parasite for Assay Malaria parasites of infected blood samples containing a parasiteamia of *Plasmodium falciparum* were collected from the Department of Haematology, Aminu Kano Teaching Hospital (AKTH). The samples were received in K3-EDTA coated disposable plastic sample bottles with tightly fitted plastic corks, and transported to the Microbiology Laboratory of Bayero University Kano.

Separation of Erythrocytes (5% Parasitaemia) From Serum of Blood Samples

In this method (Dacie and Lewis, 1968), 50% dextrose solution (0.5ml) was added to each blood sample (5ml) which was defibrinated and then centrifuged at 2500rpm for 15minutes in a spectra merlin centrifugation machine. Blood samples with higher parasitaemia (>5%) were diluted with fresh malaria parasite negative erythrocytes as described by Hanne *et al.* (2002).

Preparation of *Plasmodium falciparum* Culture Medium

Venus blood (2ml) from the main vein of white healthy rabbit pinnae was collected using a syringe (BD 205WG). This was defibrinated and allowed to settle for at least 45minutes (Lewis, 1968). The blood was then centrifuged. The sediments were discarded and the serum collected was supplemented with RPMI 1640 salt medium (KCl 5.37mM, NaCl 10.27mM, MgSO4 2.56mM, NaHPO4 17.73mM, $Ca(NO_3)$. 0.42mM, NaHCO3 2.5mM and glucose 11.0mM (BDH Ltd, UK) as demonstrated by Devo et al. (1985). The medium was sterilized by 50 gentamicin sulphate (Trager, 1982).

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

The assay was performed using RPMI 1640 as the culture medium used for cultivation of *P. falciparum* as demonstrated by Devo *et al.*, 1985. Controls were prepared without the plant extracts. Each test solution (0.1ml) and culture medium (0.2ml) were added into test tubes containing 5% parasitaemia erythrocytes and mixed thoroughly. The sensitivity of the parasites to each tested fraction at concentration of 500, 1000, 2000, and 5000µg/ml was determined microscopically at 37°C after 24 and 48 hours of incubation. The incubation was carried out under a bell jar system with a lighted candle that ensured the condition being atmospherically inert which contain CO2 (about 5%), O2 gas 2% and about 93% nitrogen gas (Muktar*et al.*, 2006).

Determination of the Activity

After 24 and 48 hours of incubation, an aliquot of the culture medium was dropped on a microscopic slide and stained using Giemsa's staining techniques. The average percentage elimination of the erythrocytes that appeared as blue discoid cells was determined using the formula.

$$\% = \frac{N}{N_x} \times 100$$

Where % = percentage activity of the extracts
N = Total number of cleared Red Blood Cells (RBC)
N *x* = Total number of parasitized RBC

Isolation and Purification of Compound Dp-01-1A

The crude extract (13.8g) of *D. mespiliformis* was mixed with silica gel until a homogenous solid mixture was formed. The sample mixture was then carefully applied onto a column that was packed with silica gel (264g). An additional portion of silica gel (9.02g) was added to form a protective layer on top of the loaded sample. The column was eluted in this order with n-hexane (2 litres), n-hexane: chloroform (1:1, 2 litres), chloroform (2 litres), chloroform: ethyl acetate (1:1, 2 litres), ethyl acetate (2 litres), ethyl acetate: methanol (1:1, 2 litres) and methanol (2 litres). The eluants were collected in portions of about 75ml per beaker providing 163 fractions. Each fraction was concentrated and analysed on TLC. Similar fractions were combined on the basis of their TLC profile. From the TLC, Dp-01-1A gave a spot of a single compound with solvent system CHCl3/EtAc (7:3) and Rf value 0.91. The Compound was obtained from the pooled fraction of Dp-01-1A by filtration as a white solid (12mg) and was subjected to spectral analysis. Malaria parasite assay were conducted on some selected fractions, including Dp-01-1A (Table 2):

Spectral Data of Dp-01-1A

The Compound is a white solid. IR(cm^{-1}):
3358, 2926, 1640, 1452, 1380, 1035, 883; ^{1}H NMR(MeOD, 600MHz):δ 4.55, 4.69(2H, s, H-29a, 29b), 3.2 (^{1}H, dd, J=9.6, 6.3Hz, H-3), 2.38(^{1}H, m, H-19), 1.70, 1.02, 0.99, 0.97, 0.88, 0.84, 0.77, (each ^{3}H, s, Me -30, Me - 26, Me -27, Me -23, Me -28, Me -25, Me -24); 13C NMR(MeOD, 151MHz): δ 150.51(C-20), 109.5(C-29), 78.4(C-3), 55.42(C-5), 50.46(C-9), 48.13(C-18), 47.99(C-19), 42.69(C-17), 42.55(C-14), 40.74(C-22), 39.64(C-8), 38.66(C-13), 38.11(C-1), 37.29(C-10), 36.89(C-4), 34.07(C-7), 33.69(C-16), 28.96(C-23), 26.63(C-2), 25.21(C-21), 22.32(C-30), 20.58(C-28), 20.56(C-11), 18.04(C-6), 15.14(C-15), 17.02(C-12), 14.72(C-25), 14.71(C-26), 13.62(C-24), 13.04(C-27).

RESULTS AND DISCUSSION

From this study all the fractions obtained from the stem bark of *Diospyros mespilifomis* showed some degree of antiplasmodial activity as shown in Table 1. At the concentration of 2000 and 5000µg/ml, the ethanol extract of the stem back (Dp1) of *Diospyros mespliformis* exhibited a very high parasite elimination of 84.7% and 91.7% respectively (Table 1). The ethyl acetate soluble fraction (Dp4) has also showed a very good activity at the said concentrations (80% and 84%, respectively), while the chloroform soluble fraction (Dp3) proved to be less active than any other fraction (Table 1). From the results, all other fractions showed some degree of anti-plasmodial activity, but their activities were less than that of ethanol extract (Dp1).

The anti-plasmodial effect of compound Dp-01-1A (lupeol) was high with percentage elimination of the parasite (84.4%, 78.1% and 68.8%) at 1000, 100 and 10µg/ml respectively after 48 hours of incubation (Table2), this supports the use of *D. mespiliformis*in folklore remedies for the treatment of malaria fever in Hausa land.

Table 1: Anti-plasmodial Activity Results of *Diospyros mespiliformis* Fractions.

Fractions	Conc. µg/ml	Average no. of parasites per field before incubation	Day 1	Day 2	Overall average no. of parasites after 48 hours	Percentage elimination at the end of incubation
Control	36	36	36	36	0%	Control
Dp1	5,000	36	4	2	3	91.7%
	2,000		7	4	5.5	84.7%
	1,000		8	5	6.5	81.9%
	500		10	9	9.5	73.6%
Control		25	25	25	25	0%
Dp2	5,000	25	4	3	3.5	86%
	2,000		8	4	6	76%
	1,000		6	11	8.5	66%
	500		12	12	12	52%
Dp3	5,000	25	7	5	6	76%
	2,000		10	5	7.5	70%
	1,000		14	9	11.5	54%
	500		13	10	11.5	54%
Dp4	5,000	25	5	3	4	84%
	2,000		6	4	5	80%
	1,000		7	4	5.5	78%
	500		10	8	9	64%
Dp5	5,000	25	6	4	5	80%
	2,000		7	5	6	76%
	1,000		10	6	8	68%
	500		14	12	13	48%
Dp6	5,000	25	4	3	3.5	86%
	2,000		6	2	4	84%
	1,000		12	11	10.5	58%
	500		13	9	11	56%

Key

Dp1: Ethanol Extract, Dp2: n-Hexane extract, Dp3: Chloroform extract, Dp4: Ethyl acetate extract, Dp5: Methanol extract and Dp6: Water extract.

Table 2: Antiplasmodial Activity of Selected Column Fractions.

Pooled code	Conc. µg/m1	Percentage Elimination of plasmodium parasite at the end of incubation
Dp-01-1A	1000	84.4%
	100	78.1%
	10	68.8%
Dp-01-7	1000	72.7%
	100	63.6%
	10	54.5%
Dp-01-18	1000	87.5%
	100	84.4%
	10	71.9%
Dp-01-26	1000	72.7%
	100	71.9%
	10	65.6%
Dp-01-31	1000	81.3%
	100	78.1%
	10	65.6%

The IR spectrum of Dp-01-1A showed characteristic absorption frequencies at 3358 and 1035 cm^{-1} O-H and C-O bond vibrations respectively; the absorption at 883 cm^{-1} was due to an unsaturated C-H vibration; the C=C vibration was shown around 1640 cm^{-1}, stretching and bending vibrations due to gem-methyl groups at 1380 cm^{-1} and methine at 2926 cm^{-1}, the signal at 1452 cm^{-1} was due to methylene vibration.

The ^{1}H NMR spectrum of the compound revealed a pair of broad singlets at δ 4.55 and 4.69(^{1}H each) was an indicative of olefinic protons (H-29a&b) of the exocyclic methylene group. The signal at δ 3.2 appeared as double of doublet which could be assigned for a secondary hydroxyl group. A multiplet of one proton at δ 2.38 is attributed to 19β-H which is characteristic of lupeol, seven tertiary methyl protons were shown at δ 0.77, 0.84, 0.88, 0.97, 0.99, 1.02 and 1.70. These assignments are in good agreement for the structure of lupeol (Soumia*et al.*, 2012).

The structural assignment of Dp-01-1A was further substantiated by the 13C NMR which showed thirty signals including a secondary carbinol at C-3 position which revealed δ 78.64, the olefinic carbon of the exomethylene group appeared at δ 150.51 and 109.5, seven methyl groups appeared at [δ:28.96(C-23), 13.62(C-24), 14.72(C-25), 14.71(C26), 13.04(C-27), 20.58(C-28), and 22.32(C-30)]; ten methylene, five methine and five quaternary carbons were assigned with the aid of DEPT. The structural assignment of the compound Dp-01-1A as lupeol (Fig. 1) is in consisten with the reported values (Chaturvedula and Indra, 2012).

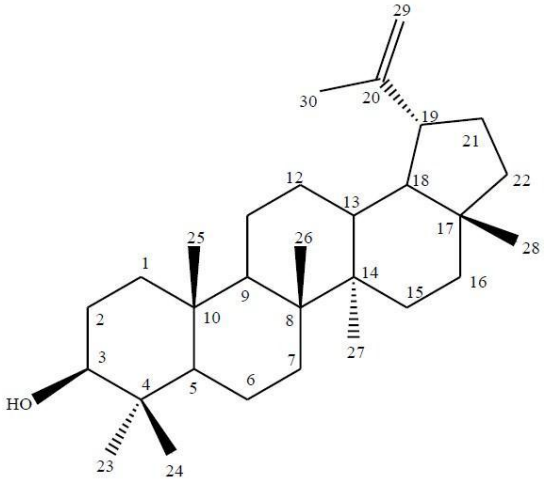

Figure 1: Proposed structure of Dp-01-1A: Lupeol.

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