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FLAVONOIDS AND ANTIMALARIAL ACTIVITY OF THE METHANOL LEAF EXTRACT OF *Spondias mombin* LINN (Anacardiaceae)

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ABSTRACT

Two flavonoids were isolated as yellow crystals by column chromatography and gel filtration of acetone soluble portion of n-butanol fraction of the methanol leaves extract of Spondias mombin using silica gel (60-120 mesh size) and sephadex (LH20). The structure elucidation of the isolates was achieved by UV, IR, 1D and 2D NMR spectral data. The oral median lethal dose of the extract was studied using Lorke's method. The anti-malarial activity of the extract was scientifically studied using suppressive and curative tests against chloroquine sensitive Plasmodium berghei (NK65) infected mice. The LD_{50} of the extract was found to be greater than 5000 mg/kg. The two experiments have shown dose-dependent chemo-suppressive effect at tested doses of 250, 500 and 1000 mg/kg body weight which compared with the standard chloroquine at 5 mg/kg and equal volume of normal saline. The difference in means was analyzed using statistical tool. This study afforded hyperin [quercetin-3-O-β-D-galactopyranoside] (I) and quercetin (II). The extract was found to be practically non-toxic in mice and it was found to have significant anti-malarial activity.

Keywords: Spondias mombin, Anti-malaria, Oral median lethal dose, Hyperin and Quercetin.

INTRODUCTION

Spondias mombin is a fruitiferous tree that grows in tropical rainforest and coastal areas of the world (Tingshuang *et al.,* 2008). It is distributed in tropical America, Brazil and Nigeria among others (Bicas *et al*., 2011; Mattietto and Matta, 2011). *S. mombin,* is locally called *"tsadarmasar"* in Hausa, *akika-etikan* in Yoruba*, ijikara* in Igbo and *chabulli* in Fulani. The leaves, fruits or barks juice have been widely used for various folk medicinal purposes (Akubue *et al*., 1983). The fruit decoction is used as a diuretic and febrifuge and the barks and leaves mixture is used as an emetic and for the treatment of hemorrhoids, gonorrhea and leucorrhea (Ayoka *et al*., 2005). Several biological activities of the plant have been reported which include; antiviral (Corthout *et al.,* 1994), antibacterial and mollus-cicidal (Abo *et al*., 1999), β-lactamase inhibitory (Coates *et al.,* 1994), anti-infammatory (Abad *et al*., 1996), wound healing (Villegas *et al.,* 1997), antipsychotic, anticonvulsant and sedative(Ayoka *et al*., 2005). A previous phytochemical screening of the plant revealed the presence of alkaloids, anthraquinones, flavonoids, phenols, saponins, steroids and tannins (Njoku and Akumefula, 2007). The leaves and stems of the plant contained geraniin (Corthout *et al*., 1994).

Malaria is a serious parasitic diseases transmitted through the bite of an infected female anopheles mosquito. There are five species of parasites which belong to *plasmodium* genus namely; *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* but the most deadly is *P. falciparum* (WHO, 2014). *P. falciparum* is the most virulent which is responsible for severe clinical malaria (Sanni *et al*., 2002). Malaria causes fever, fatigue, vomiting, headaches, yellow skin, seizures and death in severe situations (Caraballo, 2014). There were 198 million cases of malaria worldwide, where Africa took the highest percentage (WHO, 2014). The continuous claiming of lives by malaria is increasing which necessitate search for new antimalarial agents. However, many herbal preparations have been used as remedy against malaria for thousands of years all over the world (Saxena *et al*., 2003). *S. mombin* is one of the plants that are ethno-medicinally used for the treatment of malaria in Nigeria. Therefore, it is very important to scientifically validate its claimed anti-malarial effect.

On continuous search of phytochemical constituents from the plant, we report the isolation of two flavonoids which identified as hyperin and quercetin from the acetone-soluble portion of n-butanol of the methanol leaves extract of *S. mombin*.

MATERIALS AND METHODS

Experimental Animals

Swiss mice (72) of either sex weighing 18-30 g body weight were obtained from animal house facilities of the Department of Zoology, Ahmadu Bello University, Zaria. All the mice were fed with laboratory diet and water and they were maintained under standard conditions (12 hours light and 12 hours dark cycle) in a propylene cages at room temperature.

Malaria Parasite

Swiss mice infected with chloroquine-sensitive *Plasmodium berghei* (NK-65) was obtained from National Institute of Medical Research, Lagos, and the parasite was kept alive by continuous intra-peritoneal passage in fresh mice at the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria.

General Experimental Procedure

Column chromatography was carried out using chromatographic column (75 by 3.5 cm) on silica gel 60-120 mesh size (Merk KGaA, Germany) and sephadex LH-20 (Sigma). Cary 60 UV spectrophotometer, Cary 630 FTIR machine and 400 MHz Advance Bruker NMR Spectrophotometer were used for the structural elucidation of the isolates.

The leaves of *S. mombin* were collected on 10th January, 2018 from Makarfi Local Government Area of Kaduna state-Nigeria. The identification and authentication was achieved at the Herbarium Unit, Department of Botany, Faculty of Life Science, Ahmadu Bello University, Zaria. The herbarium specimen with a voucher number (2384) was kept for future reference. The fresh leaves of the plant were air-dried under the shade and pulverized to fine powder using mortar and pestle.

Extraction and Isolation

The powdered leaf material (1800 g) was exhaustively extracted by maceration method with 75% hydro-methanol (5 L) for 7 days with frequent shaking every day. The combined extract was concentrated by evaporation into an open air which yielded a dark solid residue (180 g) coded methanol leaf extract (MLE). The MLE (170g) was successively fractionated using n-hexane (2 L), chloroform (2 L), ethylacetate (2 L) and n-butanol (1.5 L) which yielded four (4) different fractions and thin layer chromatographic profile was established. The n-butanol fraction (20 g) was further fractionated with acetone (200 mL) which afforded acetone-soluble portion and acetone-insoluble portion. The acetone-soluble portion (5 g) was subjected to column chromatography on silica gel (60-120 mesh size) using 75cm by 3.5cm glass column packed using wet slurry method. The prepared sample paste was loaded on to the packed column and eluted gradiently with ethylacetate (100%) followed by different ratios of ethylacetate : methanol and washed with methanol (100%). The progress of separation was monitored by thin layer chromatography using solvent system I: ethylacetate : chloroform : methanol : water (15:8:4:1) and solvent system II: ethylacetate : methanol (7:3). Fraction eluted with ethylacetate : methanol (49:1) afforded flavonoid mixture (45mg) which was subjected to gel filtration chromatography on sephadex (LH-20), eluted slowly with methanol to afford two flavonoids. The isolates were subjected to spectral analysis using UV, IR, 1D and 2D- NMR spectroscopy.

PHARMACOLOGICAL STUDIES

Acute Toxicity Study

The study involve two phases, in the first phase, 9 Swiss mice were randomly grouped into 3 groups, each containing 3 mice. Varying doses of the MLE (10, 100 and 1000 mg/kg body weight) were administered orally to group 1, 2 and 3 respectively and observed for sign of toxicity and mortality within 24 hours. Based on the results of the first phase, the second phase was carried out by an oral administration of varying doses of MLE (1,600, 2,900 and 5000 mg/kg body weight) to 3 fresh groups containing one mouse each and they were observed for sign of toxicity or mortality within 24 hours (Lorke, 1983).

Antimalarial Studies

Four-day suppressive test in mice

The 4-day suppressive test was adopted using chloroquine-sensitive *P. berghei* (NK65) infected mice. The blood of the donor mouse with 36% parasitaemia level was collected by eye puncture. 0.2 mL of the blood was diluted with 12 mL normal saline which produced the standard inoculum containing approximately 1 x 10^7 parasitized erythrocytes. Thirty (30) Swiss mice of either sex, weighing 18-30 g were inoculated intra-peritoneally with the *p. berghei* infected erythrocytes solution (0.2 mL/kg).

Three (3) hours post inoculation, the parasitaemia was confirmed and they were randomly divided into five groups of six mice each. Group 1 was subjected to normal saline, group 2, 3 and 4 were administered orally with the MLE at varying doses of 250, 500 and 1000 mg/kg body weight respectively, while group 5 was administered orally with the standard

chloroquine at a dose of 5 mg/kg body weight. The treatment was only once in a day for 4 consecutive days (day 0 to day 3). A day after the fourth treatment, thin blood smears were made from the tail of each mouse onto slides. The blood films on the slides were fixed using methanol and stained with 10% Giemsa for 15 minutes. The numbers of parasites were counted under the microscope (X 100 objective lens) (Peters, 1967).

Curative test in mice (Rane test)

In the curative test, thirty (30) Swiss mice of either sex, weighing 18-30 g were inoculated intra-peritoneally with standard inoculum containing approximately 1 x 10^7 *P. berghei* (NK65) infected erythrocytes, seventy two (72) hours post inoculation, the parasitaemia level of all the mice was confirmed and they were randomly divided into five groups of six mice each. Group 1 was subjected to normal saline, group 2, 3 and 4 were administered orally with the MLE at varying doses of 250, 500 and 1000 mg/kg of body weight respectively while group 5 was administered orally with the standard chloroquine at a dose of 5 mg/kg body weight. The treatment lasted for four (4) consecutive days at a single dose per day. A day after the fourth treatment, thin blood smears were made from the tail of each mouse onto the slides. The slides were fixed using methanol, stained with 10% Giemsa solution for 15 minutes and examined for the parasite count under the microscope (Ryley and Peters, 1970).

RESULTS

Table I: Comparison of ^{1}H and ^{13}C-NMR spectrum of compound I with the literature

Position	^{1}H-NMR δ compound	^{13}C-NMR δ II	Guvenal and Demrezer, 2005 ^{13}*C*NMR	δ^{1}H-NMR
2	-------	158.80	158.7	------
3	-------	135.80	135.2	-------
4	-------	179.50	178.9	---------
5	------	163.20	163.0	------
6	6.20[d,J= 2Hz]	100.30	101.3	6.12[d,J=2.0Hz]
7	------	163.50	167.2	------
8	6.40[d,J= 2.0 4Hz]	95.00	95.7	6.30[d,J=2.0Hz]
9	-----	158.60	158.5	-----
10	------	105.60	105.0	-----
1`	-----	123.00	122.2	------
2`	7.85[d,J= 2.2Hz]	117.80	116.0	7.82[d,J=2.0Hz[
3`	-----	145.90	146.2	------
4`	-----	150.10	148.5	-----
5`	6.88[d,J= 8.5Hz]	116.20	117.6	6.85[d,8.0Hz]
6`	7.60[dd,J =2.04Hz,8 .5Hz]	126.20	122.9	7.57[dd,J=2.0 Hz,7.5Hz]
1``	5.16[d,J= 7.8Hz]	105.60	105.8	5.04[d,J=7.6Hz]
2``	3.83[m]	73.30	73.2	3.82[m]
3``	3.56[m]	75.20	75.2	3.54[m]
4``	3.83[m]	70.10	70.0	3.85[m]
5``	3.49[m]	77.30	77.1	3.45[m]
	3.66 [m]	62.00	61.9	3.65[m]
6``a	3.56 [m]			3.58[m]
6``b				

Table II: Comparison of 1H and 13C-NMR spectrum of compound II with the literature

Position	^{1}H-NMR δ compound	^{13}CNMR δ II	Ahmadu *et al,* ^{13}*C*-NMR	2007, δ^{1}H- NMR
2	-------	156.85	158.7	------
3	-------	135.83	135.7	-------
4	-------	175.95	179.5	---------
5	------	161.12	163.2	------
6	6.17(d,J= 2.04Hz)	97.85	100.1	6.21(d,J=2.0Hz)
7	------	164.19	166.8	------
8	6.38(d,J= 2.12Hz)	93.03	94.90	6.41(d,J=2.0Hz)
9	-----	147.38	158.60	-----
10	------	103.13	105.7	-----
1`	------	122.76	123.0	------
2`	7.73(d,J= 2.04Hz)	120.23	117.6	7.76(d,J=2.0Hz)
3`	-----	144.83	146.1	------
4`	-----	146.63	150.1	-----
5`	6.88(d,J= 8.4Hz)	114.61	116.3	6.87(d,J=8.5Hz)
6`	7.63(dd,J =2.2Hz8.9 Hz)	124.84	123.10	7.58(dd,J=2.0 Hz, 8.0Hz)

Table III: Anti-plasmodial activity of methanol leaf extract of plant in early infection (suppressive test)

Treatment (mg/kg)	Average parasitaemia ± SEM	Percentage suppression
N/S	32.60±1.97	00%
MLE 250	17.07±2.73*	48%
MLE 500	11.03±1.23*	66.2%
MLE 1000	10.27±0.29*	68.5%
CQ 5	1.47±0.40*	95.5%

Table IV: Anti-plasmodial activity of methanol leaf extracts of plant in established infection (curative test)

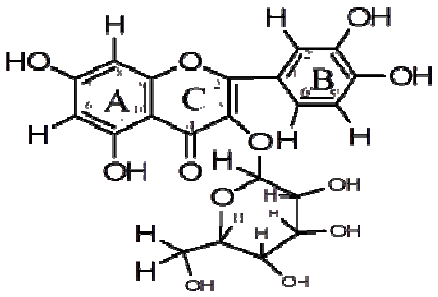
Treatment (mg/kg)	Average parasitaemia ± SEM	Percentage curation
N/S	31.80±1.88	00%
MLE 250	18.80±0.80*	42%
MLE 500	13.40±0.74*	58%
MLE 1000	10.47±0.25*	67%
CQ 5	4.97±0.68*	84%

Data were expressed as mean±SEM and the differences between the means were compared using one-way ANOVA followed by Dunnett's post-hoc test, *p*-value < 0.05 = * versus negative control; n=6, N/S-normal saline, MLE-methanol leaf extract and CQ-chloroquine.

DISCUSSION

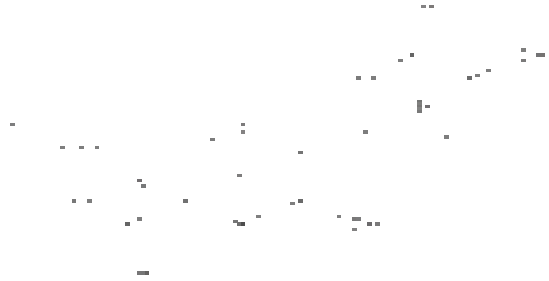
Compound I (8 mg) was isolated as brownish-yellow powder, mp 225-226^{o}C gave single spot on TLC plate using solvent system I and II with R_f –value of 0.38 and 0 .41respectively, which implied the purity of the compound and it is found to be soluble in methanol and acetone. The UV spectrum showed absorption maxima at 280 and 360 nm typical for carbonyl conjugation. The IR spectrum showed characteristic absorption frequencies at 3242.8, 2926.0, 2151, 1744, 1655, 1506 and 1088 cm^{-1} which indicated stretching vibrations for OH, aromatic C-H, -C=C-, C=O, C=C, C-C and C-O respectively. The ^{1}HNMR spectrum revealed the presence of five aromatic proton at δH 7.85 (1H, d, J=2.2 Hz), δH 7.60 (1H,dd, J= 2.04 Hz and 8.5Hz) and δH 6.88 (1H, d, J= 8.5Hz) assignable to the tri-substituted benzene ring.

The meta-coupled doublet at δH 6.40 (1H, d, J=2.04Hz) and δH 6.20 (1H, d, J=2.08Hz) assignable to the tetra substituted benzene ring. The aromatic proton signals at δH7.85, δH 7.60 and δH 6.88 were assigned to H-2′, H-6′ and H-5′ of ring B respectively, while other signals were assignable to sugar residue: δH 5.16 (1H, d J= 7.8Hz) assigned to H-1″ an anomeric-proton with a *β*-linkage as well as δH 3.83 (1H, m), δH 3.66 (1H, m), δH 3.56 (1H, m) and δH 3.49 (1H, m) which were assigned to H-2″ and H-4″, H-6″, H-3″ and H-6″ and H-5″ of the sugar moiety respectively. The ^{13}C NMR spectrum showed 21 signals, the most deshielded signal was at δC 179.59 ppm which is assigned for the carbonyl carbon (C-4), other deshielded signals at δC 163.52, 163.17, 150.11 and 145.99 were due to oxygenated aromatic carbons at C-7, C-5, C-4` and C-3` respectively while deshielding at δC 158.82 and 158.68 were due to oxygen bridge between C-2 and C-9 of the flavonoid nucleus. The HSQC spectrum gave ten methine fragments and one methylene fragment. The obtainable fragments were further joined together by COSY and HMBC spectrum. The sugar moiety was identified as galactose based on comparison of ^{1}H NMR spectrum of anomeric proton and ^{13}C NMR spectrum of C-1″ and C-6″ with the literature. The HMBC spectrum indicated that the sugar moiety is bonded to C-3 of ring c of the flavonoid nucleus through o-glycosidic linkage via β- configuration as confirmed by spin-spin coupling constant of the anomeric proton (7.8Hz). The ^{1}H and ^{13}C NMR spectrum of compound I (Table I) was thoroughly compared with the literature values of the reported compound which confirmed it as Quercetin-3-O-*β*-D-galactopyranoside (Hyperin) (Guvenalp and Demrezer, 2005).



Compound I

Compound II (7 mg) was obtained as pale yellow crystal, mp 314-316^{o}C, which gave a single spot on the TLC plate using solvent system I and II with Rf-value of 0.44 and 0.37 respectively, which indicated the purity of the compound and it was found to be soluble in methanol and acetone. The UV spectrum showed absorption maxima at 250 and 280 nm which indicated extended conjugation. The IR spectrum showed vibrational frequencies at 3228, 2920, 2176, 1715, 1651, 1506 and 1118 cm^{-1} which indicated stretching vibrations for OH, aromatic C-H, -C=C-, C=O, C=C, C-C and C-O respectively. The ^{1}HNMR spectrum revealed only five aromatic protons at δH 7.73 (1H, d, J=2.04Hz), δH 7.63 (1H, dd, J= 2.2Hz and 8.9Hz) and δH 6.88 (1H, d, J= 8.4Hz) are assignable to the tri-substituted benzene ring. The meta coupled doublets at δH 6.38 (1H, d, J=2.12Hz) and δH 6.17 (1H, d, J=2.04Hz) are assignable to tetra substituted benzene ring. The proton signals at δH7.73, δH 7.63 and δH 6.88 were assigned to H-2′, H-6′ and H-5′ of ring B respectively. The ^{13}C NMR spectrum revealed only 15 signals, the most deshielded signal was at δC 175.95 ppm which is assigned for the carbonyl carbon (C-4), other deshielded signals at δC 164.19, 161.12, 146.63 and 144.83 were due to oxygenated aromatic carbons at C-7, C-5, C-4` and C-3` respectively while deshielding at δC 156.85 and 149.38 was due to oxygen bridge between C-2 and C-9. The ^{1}H and ^{13}C NMR spectrum of compound II was thoroughly compared with the literature values of the reported compound (Table II) which confirmed it as Quercetin (Ahmdu *et al.*, 2007).



Compound II

The oral median lethal dose (LD_{50}) of the MLE in mice was found to be greater than 5000 mg/kg body weight, throughout the experiment neither mortality nor any sign of toxicity was observed which suggested that, the MLE is safe and practically non-toxic for oral administration (Lorke, 1983). Varying doses of MLE of *S. mombin* at less than 30 % of the LD_{50} value demonstrated that it is safe for ethno-pharmacological study (Vongtau *et al.,* 2004).

The in *vivo* anti-plasmodial activity of the MLE of *S. mombin* was investigated by early and established plasmodium infection tests using mice. The 4-day suppressive test is a standard test commonly used for antimalarial study in order to determine the percentage parasitaemia suppression and it is the most reliable parameter (Peter and Anatoli, 1998 and Madara *et al.,* 2010). In the 4-day suppressive test it was noticed that the percentage parasitaemia level is significantly reduced by the methanol leaf extract in the infected mice as compared with the untreated group which showed percentage parasitaemia suppression of 00%. The lowest and the highest doses of the MLE at 250 and 1000 mg/kg body weight showed percentage parasitaemia chemo-suppression of 48% and 68.5% respectively which compared with 95.5% chemo-suppression shown by standard chloroquine at a dose of 5 mg/kg body weight. Higher efficacy was observed in chloroquine treated group which was higher than the MLE treated groups which may be due to complexity and interaction of the chemical constituents within the extract which might further lead to slow absorption and poor bioavailability of the extract (Table III). The MLE of *S. mombin* has shown a dose- dependent chemosuppressive effect against *P. berghei*

In the curative test, the MLE of *S. mombin* showed dose dependent chemo-suppression in parasitaemia level of the *P. berghei* infected mice as observed in all the MLE-treated groups (group 2, 3 and 4). The lowest and the highest doses of the MLE at 250 and 1000 mg/kg body weight produced percentage parasitaemia chemo-suppression of 42% and 67% respectively as compared with 84% level of chemo-suppression produced by the standard chloroquine at the dose of 5 mg/kg body weight. The chloroquine-treated group exhibited higher efficacy than the MLE-treated groups (Table IV). The significant chemo-suppresssion noticed in the MLE-treated groups is in conformity with the traditional use of the leaves of the plant for the treatment of malaria in Makarfi Local Government Area of Kaduna State-Nigeria.

CONCLUSION

The phytochemical analysis of the methanol leaves extract of *S. mombin* afforded hyperin and quercetin. The extract was found to be orally non-toxic and it was found to have significant anti-plasmodial activity in mice.

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