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Anti-plasmodial and Radical Scavenging Activities of *Croton megalobotrys*

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Research Article

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ABSTRACT

The root, stem bark and leaves of *Croton megalobotrys* were extracted and tested for their anti-plasmodial activity. In *in vivo* screening test against *P. berghei* (ANKA) infection in NMRI albino mice, the stem bark extract produced a statistically significant suppressive effect (74.5%; $p<0.05$) in early infection and a residual inhibitory effect of 86.9%. In an established infection, a mean survival time (MST) of 16.2 days was achieved although parasitaemia was not completely eliminated. Impressive anti-plasmodial activity was observed in *in vitro* test with IC_{50} values of 1.74 ± .47 µg/mL and 3.78 ± 1.03 µg/mL for the hexane fraction of the stem bark extract against D_6 and W_2 strains of *P. falciparum* respectively. The chloroform fraction of the stem bark extract yielded a cinnamate derivative (*E*)-tetratriacontyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenate, while the fourth semi-purified fraction of the chloroform fraction (AA-CC4) in qualitative DPPH assay showed activity at a loading dose of 0.05 µg, thus exhibiting radical scavenging activity comparable to that of ascorbic acid.

INTRODUCTION

Medicinal plants are used for treatment of a variety of diseases. They are sources of modern medicine and a major source of remedy in developing countries. Even when some knowledge of traditional use of plants in Africa was lost due to lack of documentation [1], active experimentation on medicinal plants by local population continued [2]. The emergence of new diseases such as HIV/AIDS and resurgence and development of resistance of others such as malaria have encouraged medicinal plant use. Scientific research is sometimes carried out to support or refute claims of medicinal value of the plants used in traditional medicine. Some medicinal plants of Botswana have been evaluated for their antimicrobial [3,4], antimalarial [5,6] and radical scavenging [7] activities. Most of them, collected from medicinal plant vendors, have been shown to contain useful compounds with known biological activities [8].

In the present work the leaf, stem bark and root extracts of *C. megalobotrys* (Euphorbiaceae) were evaluated for antimalarial activity *in vivo* against *P. berghei* in mice. The crude stem bark extract (AA-CCR) which produced more than 70% parasite suppression was partitioned by liquid–liquid extraction into the *n*-hexane (AA-CHE), chloroform (AA-CCE), *n*-butanol (AA-CBE) and residual aqueous (AA-CWE) fractions. These fractions, together with the crude extract, and six semi-purified fractions (AA-CC1 to AA-CC6) from the chloroform fraction were evaluated *in vitro* against two strains of *P. falciparum.* The essence was to determine the effect of the extract on chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* as well as the fraction likely to contain the antimalarial activity. Radical scavenging activity of the extracts was also determined.

MATERIAL AND METHODS

Plant material

Croton megalobotrys, leaves, and roots (voucher code: A2003/3) were collected from Maun in Ngami District of Botswana in

July 2003. The stem bark (voucher code: A2003/4) was collected from Mapoka, the North East District, Botswana in June 2003. They were authenticated in the herbarium of Biological Sciences Department, University of Botswana.

General methodology

The 1D [^{1}H (300 MHz), ^{13}C (75.4 MHz), DEPT] and 2D [COSY, HMQC, HMBC) spectra were acquired on Bruker Avance DPX 300 and referenced to residual solvent signals. Low-resolution mass spectra were obtained on Finnigan MAT LCQ^{DECA} instrument. The ultraviolet and visible (UV-VIS) spectra were taken on Shimadzu UV-2101PC UV-Vis Scanning Spectrophotometer. Infrared (IR) spectrum was measured on a Perkins Elmer System 2000 FT-IR Spectrophotometer using KBr pellets. Melting points were recorded using Stuart Scientific melting point apparatus. Analytical thin layer chromatograms were run on readymade 0.25 mm thick layer of Merck silica gel 60 $F_{254+366}$ coated aluminium foil. Spots on the chromatograms were detected by observing in UV light (254 or 366 nm) and/or sprayed with vanillin-sulphuric acid spray. Preparative thin layer chromatograms were run on 0.5 mm thick layer Merck silica gel 60 $HF_{254+366}$ containing $CaSO_4$ (binder) coated on 20×20 cm glass plates. Normal chromatography was conducted using different sizes of columns packed with Merck silica gel 60, particle size 0.0400-0.0630 mm and Sephadex LH-20.

Extraction and isolation

The air dried and powdered leaf (15 g) and root (17.9 g) of *C. megalobotrys* were extracted in methanol by Soxhlet extraction method [9] to yield upon solvent evaporation, the crude leaf (CML,1.6 g) and crude root (CMR, 1.8 g) extracts respectively. The stem bark was scraped off the stem wood, dried in air and powdered. The dried and pulverized material (622.4 g) was extracted in *n*-hexane/chloroform/methanol/water (1:14:4:1). Removal of the solvent from the extract gave a brown crude extract (AA-CCR, 54.6 g), part of which (50.0 g) was dissolved in minimal amount of water and subjected to liquid–liquid partitioning sequentially with hexane, chloroform and butanol to yield the *n*-hexane (AA-CHE, 0.9 g), chloroform (AA-CCE, 4.5 g), *n*-butanol (AA-CBE, 11.6 g) and the residual aqueous (AA-CWE, 10.6 g) fractions. Since the n-hexane fraction was rather small, no further fractionation work could be done on it. The chloroform fraction was divided into two portions. The first portion (1.5 g) was adsorbed in 1.5 g of silica gel and loaded on a silica gel column (150 g) packed and eluted with $CHCl_3$/EtOAc (8:2) to yield (*E*)-tetratriacontyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (10 mg). The second portion (3.0 g) was subjected to vacuum liquid chromatography and eluted with 100% hexane, Hex/$CHCl_3$ (1:1), $CHCl_3$ (100%), $CHCl_3$/MeOH (8:2), $CHCl_3$/MeOH (1:1) and MeOH (100%) to give six fractions; AA-CC-1, AA-CC-2, AA-CC-3, AA-CC-4, AA-CC-5 and AA-CC-6. These fractions were kept for the anti-plasmodial and DPPH free radical scavenging activity tests.

In vivo anti-plasmodial activity

The dried leaf (CML,1.6 g), root (CMR, 1.8 g) and stem bark (AA-CCR, 3.0 g) crude extracts of *C. megalobotrys* were evaluated for anti-plasmodial activity in NMRI white albino mice infected with 1 x 10^7 *P. berghei* (ANKA) parasitized erythrocytes. The effects of the extracts were assessed on early infection and established infection as described in Abosi and Raseroka[5]. The residual effect of the extracts was also assessed using the Repository or Prophylactic test.

Evaluation of the repository activity or prophylactic test

This method is a modification of that of Peters [10], used to assess any possible repository activity of the extract. Twenty-five NMRI mice weighing 18 ± 2 g, housed in groups of fives in plastic cages at room temperature (20°C) and kept in constant experimental conditions were used in the experiment. They had constant supply of dog feed and water and allowed free movement. Each group of mice was given a different plant extract for three consecutive days. A dose of 1.2 mg/kg per day of pyrimethamine, a prophylactic drug, was given to a standard control group. Sterile distilled water was given a placebo. An inoculum size of 1 x 10^7 *P. berghei* parasitized erythrocytes obtained from a donor mouse previously infected with *P. berghei* parasites was then passage into each mouse on the fourth day of treatment. Seventy-two hours later, tail blood smears were made, stained by Giemsa and percentage parasitaemia assessed. Mean percentage suppression of parasitaemia was calculated using the formula [11]:

$$a=\frac{b-c}{b} \; x \; 100$$

Where:

a=Mean percentage suppression of parasitaemia

b=Mean% parasitaemia in placebo group

c=Mean% parasitaemia in extracts tested group.

In vitro anti-plasmodial activity

The anti-plasmodial activity test was based on the method of Desjardin., *et al* [12]. Chloroquine-resistant (W_2) and chloroquine-sensitive (D_6) isolates of *P. falciparum* continuously maintained in culture by standard methods [13,14] were used in the bioassay.

For the test, two fold dilutions of test samples were prepared over a 64-fold concentration range. 25 µL aliquots of each dilution were added to each well of a 96-well flat bottom microculture plate. 200 µL aliquots of a 0.9% parasitized erythrocytes in culture medium were added to all the test wells. Parasitized and non-parasitized erythrocytes and solvent controls were incorporated in all tests. The plates were incubated at 37 °C in a gas mixture of 3% O_2, 6% CO_2 and 91% N_2 and pulsed with 25 µL of culture medium containing 0.5 µCi of [G^3H]-hypoxanthine after 24 hr incubation. It was further incubated for 18 hours and harvested on to glass fiber filters (Packard Filtermate Harvester Unifilter-96), washed thoroughly with distilled water and radioactivity measured by liquid scintillation. Raw data representing the parasite counts was generated and directly imported to the data analysis software (Oracle), which gave the results as IC_{50} values.

Radical scavenging activity

The preliminary screening method

12 mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was dissolved in 50 ml of methanol (0.24 µg/ml) and placed in a spray bottle. The crude extracts and fractions obtained from stem bark of *C. megalobotrys* were spotted on TLC plates and developed appropriately. These were done in duplicates. The TLC plates were then dried using hair dryer and visualized using UV 254 nm. One of the dried plates was sprayed with the DPPH reagent and the other with the vanillin-sulphuric acid spray. The zone of inhibition which appeared as a yellow spot on a purple background due to the disappearance of the purple color of DPPH indicated the radical scavenging properties. The DPPH sprayed plates were compared with the vanillin-sulphuric acid sprayed plate to locate the probable position of compounds with radical scavenging properties.

The semi-quantitative method

The crude extracts and fractions obtained from the stem bark of *C. megalobotrys* were spotted on TLC plates in amounts ranging from 0.05 µg to 100 µg, dried and sprayed with DPPH reagent. Ascorbic acid was tested in the same way and it served as a standard. The zone of yellow colour in a purple background due to the disappearance of the purple colour of DPPH was a sign of activity and this was compared with that of the standard. The minimum inhibitory loading dose is the minimum amount of sample applied to the TLC that caused the inhibition.

RESULTS

Isolated compound from the chloroform fraction (AA-CCE) of the stem bark:

The chloroform fraction (AA-CCE) of *C. megalobotrys* was subjected to silica gel chromatography to afford white amorphous powder. Its EI-MS spectrum showed a molecular ion peak at *m/z* 670 $[M]^+$ consistent with a molecular formula $C_{44}H_{78}O_4$. The IR spectrum showed absorption bands at 3500, 1700, 1670 and 1260 cm^{-1} suggesting the presence of free -OH, -C=O, -C=C- and -C-O respectively. The ^{1}H NMR spectrum **(Table 1)** and ^{1}H-^{1}H COSY spectra showed the presence of aromatic ABX proton spin system signals [δ_H 6.92 (1H, *d*, *J*=8.1), δ_H 7.09 (1H, *dd*, *J*=8.2, 1.8 Hz) & δ_H 7.05 (1H, *d*, *J*=1.8 Hz)], olefinic *trans* coupled proton signals [δ_H 7.62 (1H, *d*, *J*=15.9 Hz) & δ_H 6.30 (1H, *d*, *J*=15.9 Hz)] and signals due to the protons attached to a long chain carbon atoms [δ_H 4.20 (2H, *t*, *J*=6.7 Hz), δ_H 1.71 (2H, *m*), δ_H 1.27 (42H, *m*) and δ_H 0.89 (3H, *t*, *J*=6.9 Hz)]. The presence of the methoxy group was evident from the sharp singlet signal at δ_H 3.94 (δ_C 56.3). The ^{13}C NMR spectrum **(Table 1)** showed nine aromatic carbons signals [δ_C 167.7, 148.2, 147.1, 144.9, 127.4, 123.4, 116.1, 115.0 and 109.7]. The DEPT and HMQC spectra indicated that five of these carbons are protonated [δ_C 144.9, 127.4, 123.4, 116.1, 115.0, 109.7] which suggested a phenyl propanoid skeleton. An olefinic proton resonating at δ_H 7.62 (δ_C 145.0) showed HMBC correlation with a conjugated carbonyl (δ_C 167.7), olefinic carbon (δ_C 116.1) and aromatic carbons [C-1 (δ_C 127.4), C-6 (δ_C 123.4), C-2 (δ_C 109.7)] deducing the assignment of carbons resonating at δ_C 116.1 & 145.0 to C-1' and C-2' respectively. The methoxy protons (δ_H 3.94) showed HMBC correlation to the carbon resonating at δ_C 147.1 indicating its attachment on the aromatic ring. Based on the HMQC and HMBC spectral data analysis protons at δ_H 4.21, 1.71 and 0.89 were assigned to C-1″ (δ_C 65.0), C-2″ (δ_C 29.1) and a terminal C-28″ (δ_C 14.4) of the long chain respectively. The ESI-MS spectral fragmentation ions by McLafferty rearrangement resulted into fragments at *m/z* 194 [ferulic acid] indicating a loss of 476 mass unit [-CH_2=CH $(CH_2)_{31}CH_3$]. This compound was therefore identified as (*E*)-tetratriacontyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate

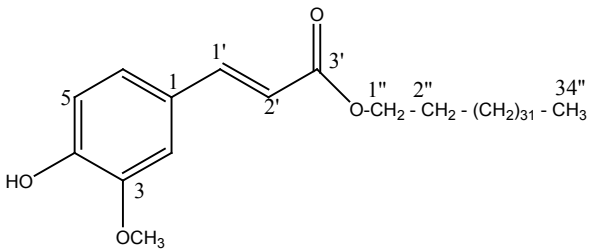

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Table 1. ^{1}H (300 MHz) and ^{13}C (75.4 MHz) of (E)-tetratriacontyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate in $CDCl_3$.

Position	δ^{1}_{H}	δ^{13}_{C}
1		127.4
2	7.05, d (1.8)	109.7
3		147.1
4		148.2
5	6.92, d, (8.1)	115.0
6	7.09, dd, (8.2, 1.8)	123.4
1′	7.62, d (15.9)	144.9
2′	6.30, d (15.9)	116.1
3′		167.7
1″	4.20, t (6.7)	65.0
2″	1.71 m	29.1
3″-27″	1.27, s	23.0-32.3
28″	0.89, t (6.3)	14.4
OCH_3	3.94, s	56.3

In vivo anti-plasmodial activity

The results of the *in vivo* anti-plasmodial activity of the extracts are shown in **Table 2.** In the established infection; the parasitaemia initiated by the standard inoculum of 1 x 10^7 increased gradually with time in all groups. A decrease in parasite count was observed in the group treated with root, stem bark and the leaf extracts when compared to the placebo. The stem bark extract demonstrated a strong anti-plasmodial activity at highest dose employed as evidenced by the mean survival time of 16.2 ± 1.3 days it produced at 500 mg/kg per day indicating a dose dependent effect. It also exhibited good mean parasite suppression in both early infection (74.5%) and the repository state (86.9%) at the same concentration.

Table 2. Antimalarial activity of C. megalobotrys extracts against P. berghei in mice.

Plant part	Dose [a]	Early infection		Repository infection		MST[f]
		Count[b]	Suppression[c]	Count[d]	Suppression[e]	
Leaf (CML)	500	23.4 ± 2.5	41.5	NT	NT	10.2
Root (CMR)	500	20.2 ± 1.6	49.5	NT	NT	13
Stem -AACCR	500	10.2 ± 1.8	74.5	3.0 ± 0.9	86.9	16.2
Stem-AACCR	250	20.0 ± 3.5		7.8 ± 2.3	66.1	11.6
Stem-AACCR	125	23.0 ± 2.5		22.4 ± 2.6	2.6	11.2
Chloroquine	5	5.4 ± 1.4	86.5	NT	NT	30
Placebo	0	40.0 ± 0.9	0	23.0 ± 2.3	0	6.8
Pyrimethamine	1.2	NT		2.2 ± 0.2	90.4	NT

[a] Dose of extract in mg/kg per day
[b] Mean parasite count ± standard error in early infection
[c] Mean percent suppression in early infection
[d] Mean parasite count ± standard error in repository state of extract
[e] Mean percent suppression in repository state
[f] Mean survival times obtained in established infection

In vitro anti-plasmodial activity

The results obtained from the *in-vitro* anti-plasmodial activity assessment of *C. megalobotrys* stem bark extract against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* are shown in **Table 3.** This result showed a very good anti-plasmodial activity for a crude extract. On partitioning of the crude extract into the *n*-hexane, chloroform, *n*-butanol and aqueous fractions, it was possible to assess the nature of the compounds causing the anti-plasmodial activity. The *n*-hexane fraction representing the least polar group of compounds produced an IC_{50} of 1.74 ± 0.47 µg/ml and 3.78 ± 1.03 µg/ml for D_6 and W_2 isolates respectively. The chloroform fraction produced an IC_{50} of 8.34 ± 1.66 µg/ml for D_6 isolates and 10.28 ± 3.68 µg/ml for W_2 isolates. The *n*-butanol fraction and the aqueous extract were inactive at the highest concentration (50 µg/ml) tested.

Radical Scavenging activity of C. megalobotrys

The preliminary test showed that the AA-CC1 and AA-CC2 did not have the radical scavenging characteristics whereas all other fractions demonstrated the radical scavenging activity **(Table 4).** The semi-quantitative assay showed that the activity was dose dependent as judged by wider zones of dis colourazation observed with higher concentration. Inactive fractions did not discolor DPPH. Ascorbic acid and fractions AA-CC4 showed activity at the lowest concentration used. AAVC4, therefore, had the highest DPPH radical scavenging activity being able to decolorized DPPH at a loading of 0.05 µg.

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Table 3. In vitro anti-plasmodial activity of C. megalobotrys against two strains of P. falciparum.

Fractions	IC_{50} (µg/mL)	
	D_6	W_2
AA-CCR	3.12 ± 0.68	5.34 ± 0.78
AA-CHE	1.74 ± 0.47	3.78 ± 0.10
AA-CCE	8.34 ± 1.66	10.78 ± 0.68
AA-CWE	>50	40.28 ± 6.83
AA-CBE	>50	>50
AA-CC1	23.34 ± 0.45	23.85 ± 0.52
AA-CC2	44.24 ± 1.52	>50
AA-CC3	33.39 ± 0.91	37.38 ± 0.79
AA-CC4	7.98 ± 0.73	15.36 ± 2.63
AA-CC5	15.84 ± 1.13	20.43 ± 1.71
AA-CC6	5.64 ± 0.48	9.04 ± 0.78
Chloroquine	0.016 ± 0.003	0.029 ± 0.002

All values are the mean ± SD of four separate experiments carried out on different days.
CCR: C. megalobotrys crude extract, CHE: C. megalobotrys hexane fraction, CCE: C. megalobotrys chloroform fraction, CWE: C. megalobotrys aqueous fraction, CBE: C. megalobotrys n-butanol fraction. AA-CC1-6=six semi-purified fractions from chloroform fraction. Cut off point for activity of crude extract is IC_{50}=49.9 µg/ml.

Table 4. Radical scavenging activities of C. megalobotrys stem bark crude extract and fractions.

Fractions	Activity	Minimum loading dose (MLD)*
		(µg)
AA-CCR	yes	0.5
AA-CHE	yes	0.5
AA-CCE	yes	2.0
AA-CBE	yes	0.2
AA-CWE	yes	1.0
AA-CC1	no	>20
AA-CC2	no	>20
AA-CC3	yes	5.0
AA-CC4	yes	0.05
AA-CC5	yes	0.2
AA-CC6	yes	0.2
Ascorbic acid	yes	0.05

*MLD: Minimum Loading Dose at which activity was observed.

DISCUSSION

The Croton species are distributed throughout the tropics and are generally used as a fodder for livestock. Many of them are used in traditional medicine [15] for the treatment of a variety of ailments [16-18] including malaria [19]. *Croton megalobotrys* is one of the six croton species found in Botswana [20]. The root, seed and stem bark are used in traditional medicine to treat abdominal pains, dropsy, malaria, and to induce abortion in humans [21,22]. The stem bark and seed were well known among early pioneers in malarious areas as a cure as well as prophylactic for fevers [23] while the stem bark is known to cause paralysis in fish [24]. The oil from the seed is a very effective purgative, toxic to mice [25] and in combination with *Ricinus communis*, acts against round and tape worms [26]. Although little is known about its biological activity, our results showed that it possesses anti-plasmodial and possible antimalarial activities both *in vitro* and *in vivo*. *C. guatemalensis* [27] and *C. pseudochellus* [28] exhibited antimalarial activity hence supporting this finding. The crude extract of *C. megalobotrys* gave an IC_{50} of 3.12 ± 1.68 µg/ml for D_6 isolates and 5.34 ± 1.78 µg/ml for W_2. This activity could be attributed to the activity observed in the *n*-hexane fraction which produced IC_{50} of 1.74 ± 0.47 µg/ml for D_6 isolates and 3.78 ± 1.03 µg/ml for W_2 isolates. The fourth sub-fraction of chloroform fraction (AA-CC4) also exhibited a good radical scavenging activity. These findings probably suggest that the successful use of *C. megalobotrys* as a remedy for malaria in traditional medicine might not only be due to its schizontocidal activity. Its ability to scavenge free radical known to be generated in malaria infection [29] could have had an effect in maintaining low levels of parasitaemia that were symptom less.

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