In vivo antimalarial property of *Markhamia lutea* (Benth.) K. Schum leaf and stem-bark crude extracts and spectrophotometric evaluation of alkaloids, flavonoids, and phenolic contents

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Abstract

Malaria is endemic to developing countries despite several efforts from the World Health Organization (WHO) and other organizations, and this has been attributed to many factors, including resistance to first-line antimalarial drugs. This study, therefore, evaluated phytochemical levels and antimalarial properties of *Markhamia lutea* (Benth.) K. Schum leaf and stem-bark extracted with various solvents as potential future alternative antimalarial drug development. Aqueous, ethanol, ethyl acetate, and methanol extracts from *M. lutea* leaf and stem-bark were evaluated for alkaloid, flavonoids, and phenolic levels using an ultraviolet-visible spectrophotometer. The extracts were further evaluated for antimalarial properties at 100-400 mg/kg on *Plasmodium berghei* NK65-induced mice using a 4-day test. The ethyl acetate leaf extract (50.8 µg/mg) gave the highest Quercetin Equivalent of Flavonoids (QEF), followed by ethanol and methanol leaf extracts with 46.2 and 47.5 µg/mg QEF, respectively. Methanol exhibited the highest level of Gallic Acid Equivalent of Phenolics (GAEP) with 213.4 µg/mg, while aqueous leaf extract was the lowest with 96.1 µg/mg GAEP. The results showed that aqueous and methanol leaf extract, ethanol, and methanol stem-bark extract possessed antimalarial activity with the lowest ED_{50} of 237.5 and 240.6, 233.8, and 236.6 mg/kg, respectively. The extracts of *Markhamia lutea* leaf and stem-bark demonstrated antimalarial properties with high contents of phenolic and flavonoid components, while the extracts showed no acute toxicity at the tested doses in the animals studied.

Introduction

The burden of malaria is still immense in Africa despite the World Health Organization's (WHO) efforts, which registered 619,000 mortalities in the World Malaria Report recently. The African region is the most affected, with 234 million morbidities and 593,000 deaths in 2021 alone. According to the WHO, malaria control efforts are still facing many challenges, including a rise in resistance to insecticide-treated mosquito nets, resistance to antimalarial drug remedies, and drops in the primary malaria-fighting tools effectiveness in Africa.[1] A recent report on malaria-endemic countries showed that Uganda was among the four countries that accounted for almost half of all morbidities, with 1.7 million morbidities between 2019 and 2021.[1] Recent reports showed a significant increase in malaria morbidities and mortalities despite steady declines after some decades. This has been attributed to the malaria mosquito and parasite adaptation to drugs, mak-

ing them resistant to treatments.[1] With a recent increase in the malaria resistance to first-line drugs as reported by the WHO, with a greater than 10% failure rate in the activities of Artemisinin-based combination therapies in Uganda, Angola, Burkina Faso, the Democratic Republic of the Congo, there is an urgent need to search for alternative drugs that are safe, less toxic, cheap and available. Many plants are reportedly used in Uganda for malaria, among which *Markhamia lutea* (Benth.) K. Schum leaf and stem-bark are included, but there is no information on the antimalarial efficacy of this plant whatsoever.

Markhamia species roots, barks, stems, and leaves are being used in folklore in the treatment of different ailments, including anemia, parasitic diseases, backache, diarrhea, intercostal pain, sore eyes, external skin diseases, rheumatoid arthritis, pulmonary troubles, scrotal elephantiasis.[2-4] *M. lutea* (Bignionaceae) is among the listed plants commonly used for different treatments, including malaria in Uganda.[2,4]

It is reported to have antiplasmodial, antiviral, anti-leishmanial, antimalarial, antimicrobial antiprotozoal, antiviral, anticancer, anti-trypanosomal, and antioxidant properties.[5-7] The plant was reported to contain alkaloids, saponins, tannins, terpenes, carbohydrates, quinones, and phenols.[6-9] *M. lutea* leaf ethyl acetate crude extract was reported to have significant anti-parasitic *in vitro* activity and low cytotoxicity on MRC5 and KB cells with identified components including musambins A-C, musambiosides A-C, and cycloartane triterpenoids.[10]

Despite its general use for different ailments and its use for malaria, there has been no study on its *in vivo* antimalarial activities and appropriate solvent with optimum activity based on the extracted constituents. Therefore, this study has presented the antimalarial effects *M. lutea* leaf and stem-bark extracted with ethanol, ethyl acetate, methanol, and water to establish the best solvent-bearing the most active antimalarial properties for future drug development and also the alkaloidal, flavonoids, and phenolic levels of each extract.

Materials and Methods

Quercetin was purchased from Targetmol (Boston, USA) while gallic acid (CAS No. 5996-86-8), atropine sulphate anhydrous, anhydrous sodium carbonate (CAS No. 497-19-8), vanillin (CAS No. 121-33-5) Folin–Ciocalteu reagent, aluminium chloride (CAS No. 7784-13-6), sodium acetate, sodium hydroxide (CAS No. 1310-73-2), bromocresol green (CAS No. 76-60-8), methanol AR, methanol HPLC grade, disodium hydrogen phosphate (CAS No. 7558-79-4), acetic acid, citric acid (CAS No. 77-92-9), and Whatman paper No. 1 were purchased from Sigma Aldrich (Steinheim, Germany).

Collection of plant material

The leaf and stem-bark of *Markhamia lutea* (Benth.) K. Schum were collected from Rukararwe, Bushenyi district, Southwestern Uganda, with coordinates of $0^0$31.47889'S 30^0 12.99765'E as shown in Figure 1, generated from ArcMap 10.8, before their identification and authentication was done by Dr. Eunice A. Olet of the

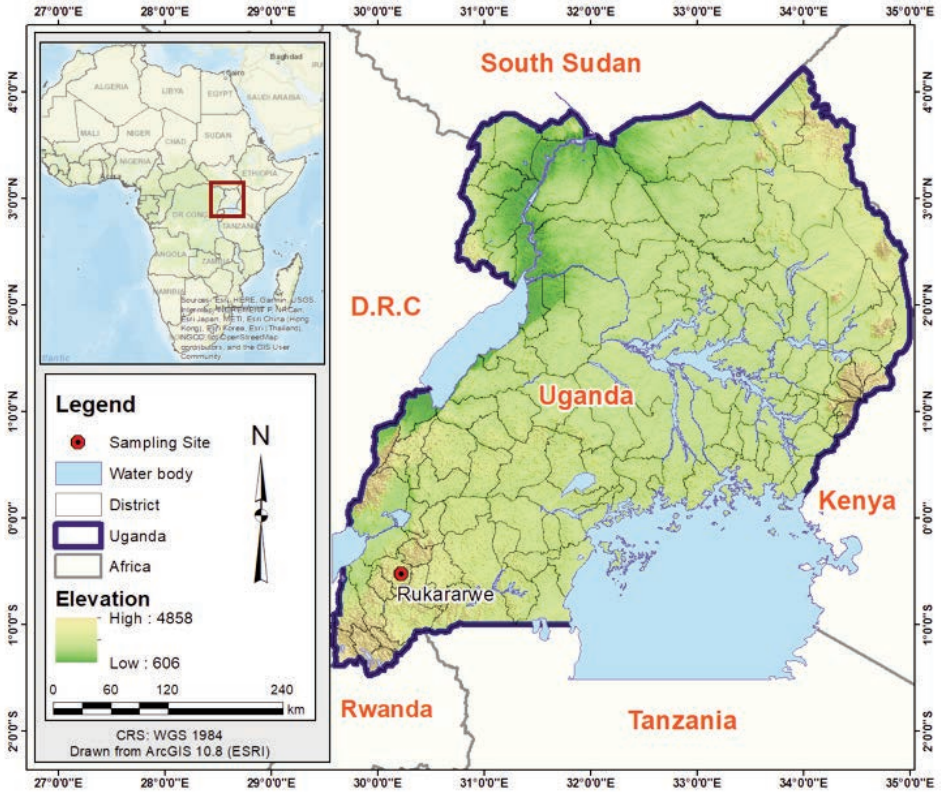

Figure 1. Map indicating the sample site of *Markhamia lutea* (Benth.) K.Schum at Rukararwe, Bushenyi district, Uganda (ArcMap 10.8 generated).

Department of Biology, Mbarara University of Science and Technology (MUST), Mbarara, Uganda before their voucher specimens were deposited at the Makerere University Herbarium, Kampala with assigned voucher number: 51269. The plant was further subjected to confirmation on the Plants of the World Online (POWO) website (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:110020-1). The *M. lutea* stem-bark and leaves were oven-dried at 50 and 40°C, respectively, for 48 h and thereafter pulverized mechanically using an electric grinder, and separately stored in amber bottles.

Extraction process

Aqueous extract of powdered stem-bark and leaves of 50 g each were separately prepared in 500 mL using decoction and infusion methods, respectively, while ethyl acetate, methanol, and 70% ethanol of both parts were separately prepared using the maceration method. The ethanol and methanol extractions were achieved in 72 h, while ethyl acetate was done within 3 h at room temperature. The extracts were all separately filtered using Whatman no. 1 filter paper and thereafter concentrated *in vacuo* at 45°C except for the aqueous that was lyophilized and were coded as AQL and AQSB for aqueous leaf and stem-bark, EtOHL and EtOHSB for ethanol leaf and stem-bark, EtOAcL and EtOAcSB for ethyl acetate leaf and stem-bark, MeOHL and MeOHSB for methanol leaf and stem-bark, respectively. The percentage yield was determined using the formula below, and the yields for AQL, AQSB, EtOHL, EtOHSB, EtOAcL, EtOAcSB, MeOHL, and MeOHSB were 21.9, 24.1, 19.9, 15.5, 2.9, 9.5, 0.2 and 0.9%, respectively. Thereafter, the samples were screened for the presence of phytochemicals using the method of Balamurugan *et al*.[11]

Determination of total alkaloid levels in *M. lutea* leaf and stem-bark extracts

Total alkaloidal content was determined by using the modified methods of John *et al*.[12] and Patel *et al*.[13] To prepare bromocresol green (BCG) solution, 6.98 mg BCG was heated with 0.3 ml of 2N NaOH and 0.5 ml distilled water at 50°C for 15 min to attain dissolution of the components which was further made up to 1 L with distilled water in a measuring cylinder. A phosphate buffer solution of pH 4.7 was prepared from 2 M Na_2HPO_4 and 0.2 M citric acid.[12]

Preparation of atropine standard curve

Atropine standard concentration (1 mg/mL) was prepared using methanol, from which the working solution of 10-100 µg/mL was obtained. One milliliter (1 mL) of the solution was measured into a separating funnel before 5 mL each of phosphate buffer (pH 4.7) and bromocresol green were separately added and shaken vigorously. The complex formed was extracted serially into 1, 2, 3, and 4 mL of $CHCl_3$ in a 10 mL measuring cylinder that was adjusted to volume with $CHCl_3$. Thereafter, the absorbance of the chloroform containing alkaloidal component was taken at 415 nm using a Jenway 6705 UV-VIS spectrophotometer against the blank prepared, and the linear regression equation of y=0.0092x-0.003; r^2=0.9929 generated was used to determine the content in the samples. The total alkaloidal content was expressed as microgram Atropine Equivalence of Alkaloids (AEA)/mg crude extract.

Total flavonoid content determination of *M. lutea* leaf and stem-bark extracts

The total flavonoid content of the sample was determined using a modified $AlCl_3$ colorimetric method as described by Baba and Malik[14] and Wangalwa *et al*.[15] Three milliliters (3 mL) of methanol was added to 1 mL each of 1 mg/mL concentration of *M. lutea* leaf and stem-back extracts in a 10 mL volumetric flask, agitated before the addition of 0.2 mL of 10% $AlCl_3$ solution and 0.2 mL of 1M sodium acetate. The solution was made to the mark with methanol before the incubation. Thereafter, the incubation of the solution was done in the dark at room temperature for 30 minutes. The absorbance of the resulting solution was then taken at 420 nm using a Jenway 6705 UV-VIS spectrophotometer. Working solutions of 10-100 µg/mL of quercetin standard were prepared from the standard concentration of 1 mg/mL and were used to prepare a calibration curve with methanol substituting for the sample in the blank and the linear regression equation of y=0.0092x-0.0083; r^2=0.9982 generated was used to determine the content in the samples. The total flavonoid content was expressed as microgram Quercetin Equivalence of Flavonoids (QEF)/mg crude extract.

Total phenolic content determination of *M. lutea* leaf and stem-bark extracts

A modified Folin-Ciocalteau method described by Baba and Malik [14] and Wangalwa *et al*.[15] was used to determine the total phenolic content of the sample. One milliliter (1 mL) concentration of 0.5 mg/mL *M. lutea* leaf and stem-bark extracts was pipetted into a measuring cylinder, and 2 mL of 10% (v/v) Folin-Ciocalteu reagent was added together with 2 mL of 7.5% (w/v) Na_2CO_3 solution with incubation of the solution done in 30 minutes at 40ºC. The absorbance of the solution was taken at 760 nm using a Jenway 6705 UV-VIS spectrophotometer. Also, working solutions of 1-100 µg/mL gallic acid were prepared and used to develop a calibration curve for the standard and the linear equation of y=0.0137x+0.0545; r^2=0.972 generated was used to determine the gallic acid concentration in the samples. The total phenolic content in *M. lutea* leaf and stem-bark was expressed as microgram Gallic Acid Equivalence/mg (GAE).

High-Performance Liquid Chromatography analysis of *M. lutea* leaf and stem-bark extracts

High-Performance Liquid Chromatography conditions

The High-Performance Liquid Chromatography (HPLC) analysis was performed on a UFLC Prominence Shimadzu chromatograph (Japan) at the Analytical and Pharmaceutical Laboratory, MUST, Uganda. The HPLC machine comprised SIL-20AC HT autosampler, column oven (CTO-20AC), UV-visible detector (SPD-20A), LC 20 AD pumps, and an online degassing unit (DGU-20A). For each *M. lutea* leaf and stem-bark extract (aqueous, ethanol, ethyl acetate, methanol), 1 mg/mL concentration was prepared and filtered with 0.22 µm. A reversed-phase HPLC assay was carried out using isocratic elution with a flow rate of 0.8 mL/min at a column temperature of 35°C, injections volume of 20 µL, a mobile phase of 1% acetic acid/methanol in ratio 7:3 delivered by pump A and B, respectively and detected under the wavelength of 230 and 254 nm. The acquisition time for each injection was 40 minutes.

Solvents and deionized water were prior filtered through a 0.45 µm nylon membrane with the aid of Buckner, enhanced by ILMVAC GmbH vacuum pump. All solvents were of HPLC grades. Data were processed with LC-Solution Software.

Gas Chromatography-Mass Spectrometric of *M. lutea* leaf and stem-bark extracts

Gas Chromatography-Mass Spectrometric (GC-MS) analysis was carried out in a Shimadzu GCMS-QP2020 NX with an RXI-

Sil MS capillary column measuring 30 meters in length, 0.25 millimeters in internal diameter, and possessing a 0.25- µm film thickness, with cross bonds similar to 5% diphenyl/95% dimethyl siloxane was utilized. Helium was used as the carrier gas at a flow rate of 1.60 milliliters per minute, and the injector temperature was set at 250°C. Column interfaced with 5675C Inert MSD with Triple-Axis detector. Helium gas was used as carrier gas and was adjusted to a column velocity flow of 1.0 mL/min.

Other GC-MS conditions are ion-source temperature, 230°C; interface temperature, 250°C; pressure, 100:1 kPa; Solvent Cut Time: 3 minutes; and Injection Mode: Spitless with injection temperature of 250°C. The column temperature started at 50°C for 5 min and changed to 150 V at the rate of 4°C/min. The temperature was raised to 250°C at the rate of 20°C/min and held for 5 min. The total elution time was 30 min. The relative percent amount of each component was calculated by comparing its average peak area to total areas. One milligram of each extract was dissolved in 1 mL of hexane and filtered with 0.22 µm before the sample was loaded for injection into the machine.

Acute oral toxicity of *M. lutea* leaf and stem-bark extracts

The median lethal dose (LD_{50}) of each sample was determined *in vivo* using the 'up-and-down' method of the Organisation for Economic Co-operation and Development.[16] This method is guided by the 'aot 425 software'. Five healthy female Swiss albino mice (*Mus musculus* L.) weighing 20-22 g were used for each extract (8 extracts totaling 40 animals). The mice were fasted for 3-4 hours before dosing, while the food and water were withheld for 1 hour after treatment administration. In this method, the 1st animal was administered with the herbal supplement at 175 mg/kg as predicted by the software and observed for any sign of toxicity for 48 h (short-term outcome), during which the animals were observed for loss of appetite, reduced mobility, ruffled fur, signs of dizziness or mortality before the next predicted dose of 550 mg/kg was administered to the 2nd animal. This was also observed for 48 h before the 3rd animal was dosed with aliquot at 2000 mg/kg, and this same dose was administered to the 4th and 5th animals before the software indicated 'stop dosing'. Thereafter, the dosed animals were subjected to normal lives for an additional 12 days (long-term outcome) to make 14 days for each animal. The LD_{50} was automatically generated by the software on day 14, while the animals that reached day 14 post-administration were all humanely euthanized. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All euthanasia was performed under halothane anesthesia, and all efforts were made to minimize suffering.

Antimalarial study on *M. lutea* leaf and stem-bark extracts

In vivo antimalarial activity of *M. lutea* leaf and stem-bark extracts was assessed through a 4-day suppressive test as described in a previous study by Ajayi *et al.*[17] A total of 90 healthy Swiss albino mice of both sexes (18-22 g) were obtained from the MUST Animal Research Facility, Uganda. They were kept separately in different cages under a 12-hour light/dark cycle and were fed with commercial food pellets with free access to water. This was maintained for two weeks for acclimatization before the assay.

Preparation of inoculum and antimalarial assessment

Plasmodium berghei NK65 (Chloroquine-sensitive, CQ-sensitive) strain was obtained through the United States BEI Resources, NIAID, NIH, as contributed by Thomas F. McCutchan before it was activated in mice at the Animal Facility Laboratory, MUST, Uganda.[17] The mice were infected with standard inoculum prepared from donor mice 2 h before the drug administration (post-infection), and the mice were randomly assigned to 26 groups of 3 mice per group (2 females and 1 male). The administration of aliquot to animals was conducted daily for 4 days as follows: Groups I, II, and III received AQL extract at 100, 200, and 400 mg/kg. Groups IV, V, and VI received AQSB extract at the same doses; likewise, groups VII, VIII, and IX received EtOHL extract, while groups X, XI, and XII received EtOHSB extract. Groups XIII, XIV, and XV received EtOAcL; groups XVI, XVII, and XVIII received EtOAcSB; groups XIX, XX, and XXI received MeOHL; and groups XXII, XXIII, and XXIV received MeOHSB extract. Also, groups XXV and XXVI were administered with artemether-lumefantrine at 4 mg/kg (positive control) and water at 10 mL/kg (negative control). On day five, the smears were prepared by collecting blood from the tail of each animal, fixed with methanol, and stained with 10% Giemsa-stain before the parasitemia levels were examined by counting both the parasitized and non-parasitized erythrocytes in eight random views under a light microscope at 100× oil immersion objective lens. From this count, the percentage parasitemia levels and chemosuppression were determined as follows;

$$\text{Percentage parasitemia} = \{Na/Nb\}100 \quad (1)$$

where "Na" is the total number of parasitized red blood cells, while "" is the total parasitized and non-parasitized red blood cells.

$$\text{Percentage chemosuppression} = \{A\text{-}B/A\}100 \quad (2)$$

where "A" is the negative control group percentage parasitaemia level, and "B" is the test group percentage parasitaemia levels.

Thereafter, the animals were observed for 28 days post-inoculation to monitor their survival. The animals that showed signs of loss of appetite, reduced mobility, ruffled fur, or signs of dizziness during this period were removed and euthanized with halothane and those that reached day 28 were all euthanized using halothane. The carcasses of the animals were appropriately disposed of by incineration.

Data management and analysis

All quantitative data were expressed as mean ± Standard Deviation (SD). The effective doses were determined using Microsoft Excel 2016, while the variation in the data set was analyzed through one-way analysis of variance. The means variation was considered at a 95% confidence level using Tukey's Multiple Comparison post-hoc Test through Graph Pad Prism10 software 2023 version.

Results and Discussion

Phytochemicals in *M. lutea* leaf and stem-bark

The extracts of *M. lutea* (Benth.) K. Schum showed the presence of various phytochemicals in different solvents, with saponin absent in ethyl acetate extract. Also, anthraquinone was absent in all the extracts tested. Alkaloids were present in all the extracts aside from ethyl acetate stem-bark extract, which was absent, while a traceable amount was observed in ethanol and ethyl acetate

leaf extracts. Steroids were present in aqueous ethanol, methanol stem-bark extracts, and aqueous leaf extract with traceable amounts in ethyl acetate and methanol leaf extract, whereas it was absent in ethanol leaf extract.

Alkaloids, flavonoids, and phenolic contents in *M. lutea* leaf and stem-bark

Chemical contents in *M. lutea* leaf and stem-bark showed different yields. Flavonoid content in the leaf extracted with ethyl acetate was the highest at 50.8 µg/mg, which was significant compared to the rest; in addition, ethanol and methanol extracts gave 46.2 and 47.5 µg/mg, respectively, in the leaf, which showed that the leaf is rich in flavonoids than the stem-bark (Table 1). The aqueous extract of the stem-bark was the lowest, with a significant content of 4.3 µg/mg. The ethanol and methanol extracts showed quantifiable amounts of alkaloids, with ethanol having the highest content of 21.6 µg/mg in the leaf, whereas methanol extracts had 13.5 µg/mg in the same part, as shown in Table 1.

Though the stem-bark extracted with ethanol and methanol gave comparable amounts of respective 0.8 and 0.6 µg/mg atropine equivalence of alkaloidal content, the amount is significantly different from that of the leaf part ($p<0.0001$). The extracts of all the solvents gave a considerable amount of phenolic contents, with stem-bark extracted with methanol having the highest content of 213.4 µg/mg crude extract, while the lowest was recorded in ethanol leaf extract, as shown in Table 1. Generally, phenolic content was high in the stem-bark compared to the leaf part (Table 1).

High-Performance Liquid Chromatography fingerprint of *M. lutea* leaf and stem-bark

HPLC fingerprint is one of the most sensitive, reliable, and reproducible means of identification of component(s) or extracts. In this study, the HPLC fingerprint of *M. lutea* leaf fingerprint at 230 nm showed characteristic peaks at 4.9 and 13.8 mins that are peculiar to aqueous, ethanol, and methanol extracts (Figure 2a). Also, *M. lutea* stem-bark at 230 nm wavelength showed character-

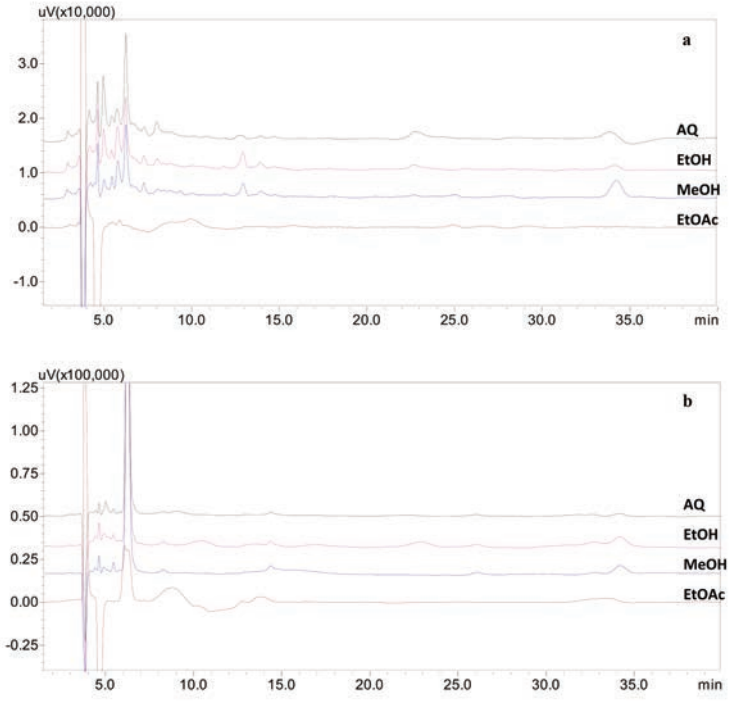

Figure 2. HPLC fingerprints of *M. lutea* leaf (**a**) and stem-bark (**b**) @ 230 nm wavelengths AQ, Aqueous; EtOH,Ethanol; MeOH, Methanol; EtOAc,Ethyl Acetate

Table 1. Phytochemical contents of *M. lutea* leaf and stem-bark.

Phytochemicals	QE flavonoids (µg/mg)	AE alkaloids (µg/mg)	GAE phenolics (µg/mg)
EtOH leaf	46.19±0.33	21.60±0.19	86.25±0.084
EtOH sb	9.09±0.063	0.80±0.5[a]	137.40±0.00
AQ leaf	5.69±0.00	0.00	96.08±0.30
AQ sb	4.34±0.063	0.00	135.10±0.29
MeOH leaf	47.50±0.063	13.48±0.063	103.00±0.084
MeOH sb	7.68±0.063	0.62±0.11[a]	213.40±0.59
EtOAc leaf	50.79±0.20	0.00	107.70±0.84
EtOAc sb	8.37±0.16	0.00	197.50±0.69

Data are expressed as mean ± Standard Deviation, SD; same superscripted letter within the columns are comparable ($p=0.05$). QE, Quercetin Equivalent; AE, Atropine Equivalent; GAE, Gallic Acids Equivalent.

istic peaks at 6.2 and 14.4 mins in all the extracts. In addition, aqueous, ethanol, and methanol extracts showed peaks at 22.02, 26.02, and 34.2 mins, which are diagnostic, as shown in Figure 2b.

At 254 nm wavelength, *M. lutea* leaf showed a unique peak at 4.9 min, which was prominent in aqueous extracts but also observed in ethanol and methanol, as shown in Figure 3a. There was another characteristic peak at 34.5 min, which was prominent in ethanol and methanol extracts and diagnostic (Figure 3a). The fingerprint of *M. lutea* stem-bark showed characteristic peaks at 4.6 and 25.9 min, which were peculiar to aqueous, ethanol, and methanol extracts but missing in ethyl acetate extract (Figure 3b). There was a peak at 22.9 min, which was peculiar to ethanol extract alone and could be used for identification of the extract, as shown in Figure 3b.

Gas Chromatography-Mass Spectrometric analysis of the active *M. lutea* extracts

The extracts of ethanol stem-bark, methanol leaf, and stem-bark were analysed using Gas Column Chromatography to identify the components, and 35 compounds from each of the three extracts were identified. Ethyl alpha-d-glucopyranoside (19.4%) appearing at a retention time of 10.43 min (Figure 4a) was the most abundant in the ethanol stem-bark extract followed by E,E,Z-1,3,12-nonadecatriene-5, 14-diol with 18.8% at 15.16 min and ethyl 13-methyl-tetradecanoate with 12.1% at 15.39 min while ketone, methyl 2-methyl-1,3-oxothiolan-2-yl with 0.14% at 7.40 min was the least abundant as shown in *Supplementary Table 1*.

In methanol leaf extract, phytol was the most abundance (13.92%), followed by vitamin E (11.89%), squalene (10.52%), and 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (9.07%) appearing at retention times of 14.63, 22.66, 20.32 and 5.95 mins, respectively as shown in Figure 4b. Also, in methanol stem-bark extract, sitosterol was the most abundant (Figure 4c) with 12.75% followed by pentadecanoic acid with 11.67% and 2-(Isobutoxymethyl)oxirane with 7.73% appearing at retention times of 24.61, 13.75 and 8.83 minutes, respectively as shown in *Supplementary Table 1*.

Acute toxicity of *M. lutea* leaf and stem-bark

The acute toxicity study on all the extracts (aqueous, ethanol ethyl acetate, and methanol) of *M. lutea* leaf and stem-bark showed no mortality at all the doses tested up to 2000 mg/kg, and there was no sign of distress aside from the calmness at the highest dose of 2000 mg/kg. Hence, the LD_{50} generated by the 'aot software' gave above 2000 mg/kg.

Antimalarial efficacy of *M. lutea* leaf and stem-bark

There was a reduction in the parasitaemia levels of all the tested extracts at 100 to 400 mg/kg from 17.1 to 5.1%. At 100 mg/kg, aqueous leaf extract showed a significant reduction in parasitaemia level of 7.7% which was comparable to that of ethyl acetate leaf with 8.6% parasitaemia level (p=0.26), methanol leaf (8.5% parasitaemia level) (p=0.48) and stem-bark (8.1% parasitaemia level) (p=0.97) extracts while ethyl acetate stem-bark was the least with 11.3% parasitaemia though the result was significant compared to the negative control (p<0.0001) as shown in Table 2. At 200 mg/kg, ethanol stem-bark extract gave the highest reduction of 5.6% parasitaemia level, which was comparable to that of the aqueous leaf (6.2% parasitaemia level) (p=0.79), and methanol stem-bark (6.4% parasitaemia level) [extracts while ethanol leaf extract was the gave the lowest parasitaemia level of 8.9% that was significant (p<0.0001) compared to the negative control (Table 2). At 400 mg/kg, there was a further reduction in the parasitaemia level of animals dosed with ethanol stem-bark (5.1%) extract, a

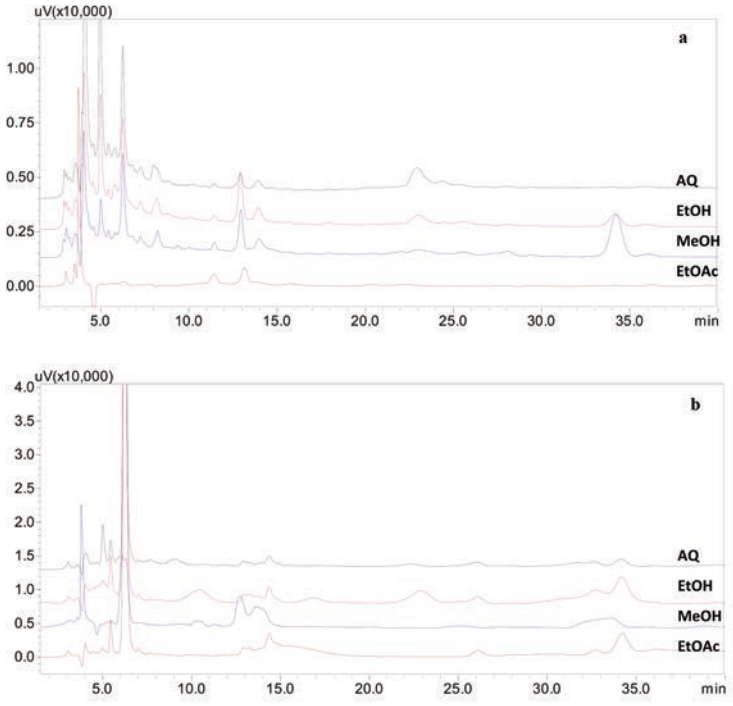

Figure 3. HPLC fingerprints of leaf (**a**) and stem-bark (**b**) @ 254 nm wavelengths. AQ, Aqueous; EtOH,Ethanol; MeOH, Methanol; EtOAc, Ethyl Acetate.

reduction that was comparable to that of the aqueous leaf (6.0% parasitaemia level) (p=0.099), methanol leaf (5.6%) (p=0.75) and stem-bark (5.7%) (p=0.58) extracts whereas ethyl acetate leaf extract exhibited the lowest parasitaemia reduction of 7.8% as shown in Table 2. Percentage suppression established that showed aqueous leaf has the highest at 100 mg/kg with 54.8% which was comparable to ethyl acetate leaf (p=0.13), methanol leaf (p=0.29) and stem-bark (p=0.92) extracts with 49.6, 50.4 and 52.5% chemosuppression, respectively, though the activity was significantly different from that of positive control with 91.6% chemosuppresion (p=0.0001) while ethyl acetate (33.9%) and ethanol (35.6%) stem-bark gave the lowest chemosuppression as shown in Table 2. The

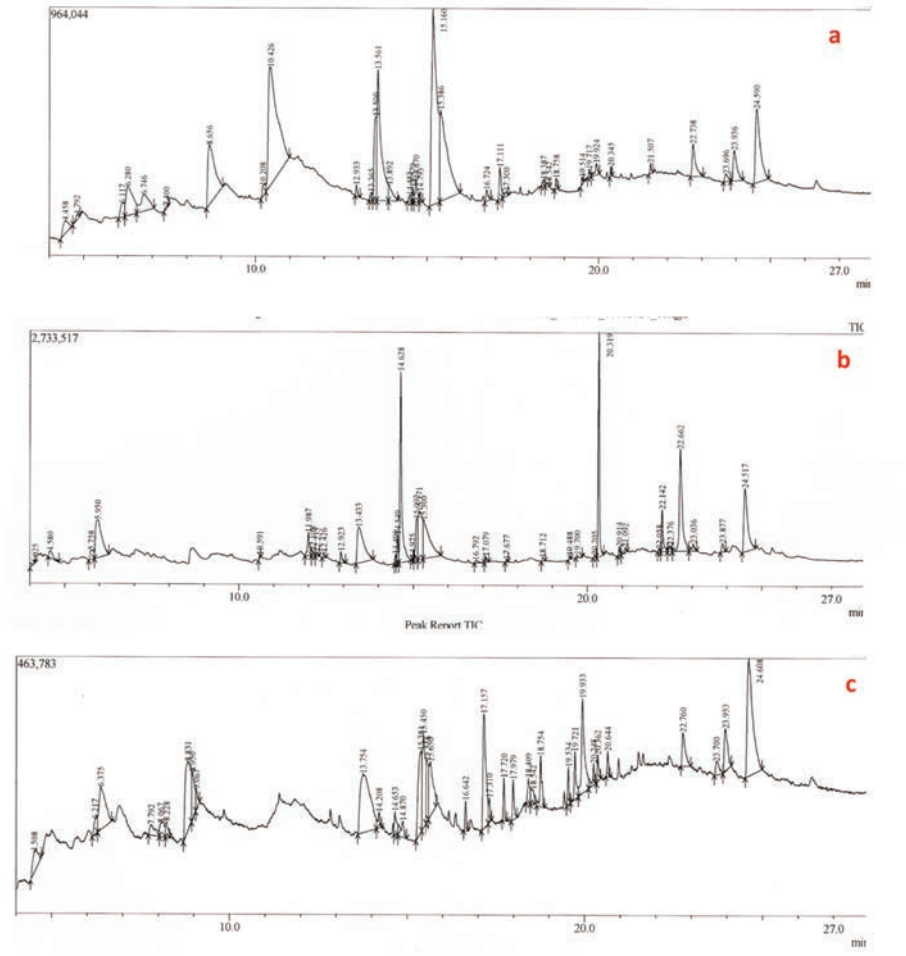

Figure 4. Gas Chromatography-Mass Spectrometric (GC-MS) chromatograms of *M. lutea* ethanol stem-bark (**a**), methanol leaf (**b**) and methanol stem-bark (**c**) extracts.

Table 2. Antimalarial properties of M. lutea leaf and stem-bark extracts on P. berghei-infected mice at 100-400 mg/kg.

Extracts	100 mg/kg		200 mg/kg		400 mg/kg	
	% Parasitaemia	% Chemosuppression	% Parasitaemia	% Chemosuppression	% Parasitaemia	% Chemosuppression
Negative control (10 mL/kg)	17.12±0.72	0.00	17.12±0.72	0.00	17.12±0.72	0.00
AQ leaf	7.74±0.090^{a,c}	54.81±0.53^{n}	6.22±0.15^{e}	63.69±0.88^{r,s}	5.99±0.060^{i,j}	64.99±0.35^{v}
AQ SB	9.04±0.20^{b}	47.20±1.18^{m}	8.19±0.25^{f,f}	52.16±1.48^{o,p,q}	7.30±0.066^{k}	57.37±0.38^{t,u}
EtOAc leaf	8.64±0.083^{a,b}	49.55±0.48^{m,n}	7.72±0.35^{f}	54.89±2.02^{o,p,q}	6.81±0.16^{j,k}	60.20±0.93^{u}
EtOAc SB	11.32±0.29^{d}	33.89±1.66^{l}	8.37±0.20^{f}	51.09±1.15^{o,p}	7.71±0.24^{k}	54.94±1.43^{t}
EtOH leaf	9.39±0.43^{b}	45.17±2.51^{m}	8.86±0.074^{f}	48.23±0.43^{o}	7.38±0.028^{k}	56.90±0.16^{t,u}
EtOH SB	11.03±0.27^{d}	35.59±1.59^{l}	5.59±0.48^{e}	67.33±2.83^{r}	5.14±0.37^{i}	69.96±2.15^{w}
MeOH leaf	8.49±0.60^{b,c}	50.41±3.48^{m,n}	7.09±0.94^{f,g,h}	58.58±5.49^{q,s}	5.61±0.27^{i}	67.23±1.60^{v,w}
MeOH SB	8.13±0.67^{b,c}	52.52±3.93^{m,n}	6.40±0.35^{e,h}	62.60±2.05^{r,s}	5.69±0.48^{i}	66.78±2.83^{v,w}
ACT (4 mg/kg)	1.43±0.069	91.63±0.40	1.43±0.069	91.63±0.40	1.43±0.069	91.63±0.40

Data are expressed as mean ± Standard Deviation, SD; the same superscripted letter within the column means p=0.05; SB, Stem-Bark; AQ, Aqueous; Etoac, Ethyl Acetate, Etoh, Ethanol; Meoh, Methanol; ACT, Artemisinin-Based Combination Therapy.

medium dose of 200 mg/kg showed an increase in the activity of ethanol stem-bark with the highest chemosuppression of 67.3%, which was comparable to the activities of aqueous leaf (p=0.64) and methanol stem-bark (p=0.33) extracts with 63.7 and 62.6%, respectively. At 400 mg/kg, ethanol stem-bark extract gave the best activity with 70.0% reduction, which was comparable to those of aqueous leaf, methanol leaf, and stem-bark with 65.0, 67.2 and 66.8%, respectively, but the activity was significantly different from that of positive control with 91.6% chemosuppression (Table 2). The effective doses with 50 and 90% activity (ED_{50} and ED_{90}) results showed that aqueous and methanol leaf, ethanol, and methanol stem-bark gave the lowest ED_{50} of 237.5 and 240.6, 233.8, and 236.6 mg/kg, likewise gave lowest ED_{90} of 427.6 and 433.0, and 420.9 and 425.9 mg/kg, respectively. Meanwhile, ethyl acetate stem-bark gave the lowest ED_{50} and ED_{90} of 291.5 and 531.2 mg/kg, respectively, as shown in Figure 5.

This study screened the phytochemicals present in *Markhamia lutea* leaf and stem-bark extracts of aqueous, ethanol, ethyl acetate, and methanol, quantified the total flavonoid, and phenolic contents, evaluated the acute toxicity level and evaluated their antimalarial effects on *Plasmodium berghei* NK65 infected mice. The results, as indicated, presented the presence of a range of phytochemicals, including saponins, alkaloids, flavonoids, and terpenoids in most of the extracts, in particular ethanol and methanol extracts. There was an appreciable amount of alkaloids, flavonoids, and phenolic contents in the extracts, with ethanol and methanol giving the highest levels. The HPLC fingerprint of the extracts of *M. lutea* leaf and stem-bark was carried out for identification of the extracts, and the results are reproducible following the condition. There was a dose-dependent in the antimalarial activities of all the extracts with a significant reduction in the parasitaemia levels of the animal dosed with aqueous leaf, ethanol stem-bark, methanol leaf, and stem-bark extracts, and they possessed the highest percentage chemosuppression with lowest ED_{50} and ED_{90}.

The result on phytochemicals was in accordance with a review of Genus *Markhamia* phytochemicals and pharmacology by Ibrahim *et al*.,[7] who reported that the species are known for the presence of biologically active substances like flavonoids, saponins, steroids, terpenes, and terpenoids, phytosterols, tannins, phenols, coumarins, and quinones. Likewise, it was reported that the flowers, leaves, and stem-bark of *M. lutea* contain alkaloids, quinones, saponins, tannins, phenols, and terpenes.[6,8] The compounds identified by GC-MS are rich in esters and steroids, and these groups have been reported for antiplasmodial activity, for instance, a report showed glycosides of stigmasterol inhibited *Plasmodium falciparum* growth using 3D7 strain by schizont inhibition maturation assay.[18] With the presence of stigmasterol in this study and other steroids, possibly there could be the presence of sugar moiety in the extract that has contributed to the antimalarial activity in addition to the other components that the GC-MS could not identify due to the high temperature involved.

The acute toxicity result showed that all the extracts tested were acutely safe. It was reported that the extract of this plant can cause the regeneration of tissue, which shows that the plant can lead to curative properties against degeneration.[9]

Alkaloids, flavonoids, and phenolics levels reported in this study have been reported to have many therapeutic values, including antimalarial properties, and this could have contributed to the antimalarial properties exhibited in ethanol and methanol. Some of these components could have possibly exhibited the activity synergistically. Some terpenoidal and steroidal, indole, isoquinoline, benzylisoquinoline, hasubanane, naphthoisoquinoline, phenanthroindolizine, *etc*. Alkaloids have been reported for antimalarial activities, and the research continues on these promising phytochemical groups.[19-21] In addition, several flavonoids, including flavones, have been reported for either antiplasmodial or antimalarial properties.[22-24] The activity expressed in the ethyl acetate extract could have been due to the presence of flavonoids, and it was established that flavonoids are phenolic, which was reported

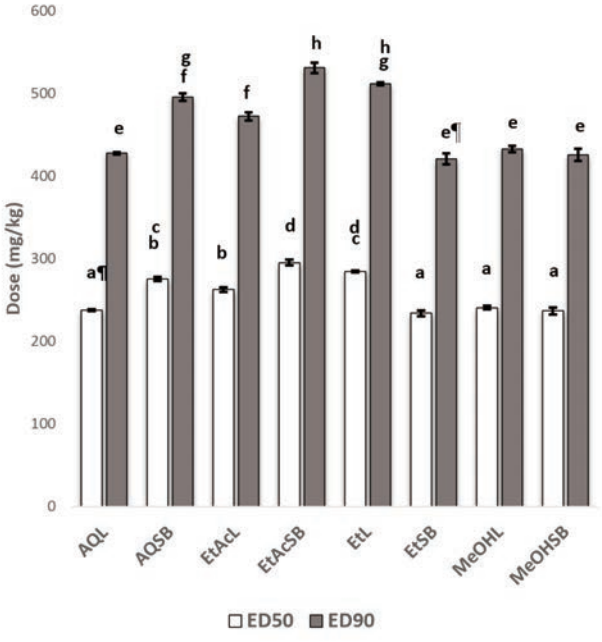

Figure 5. Effective doses (ED_{50} and ED_{90}) of *M. lutea* leaf and stem-bark extracts. Data are expressed as Mean ± Standard Deviation, SD, same superscripted letter means p=0.05; SB, Stem-Bark; AQ, Aqueous; EtAc, Ethyl Acetate, EtOH, Ethanol; MeOH, Methanol.

to possess antimalarial activity.[23] The higher antimalarial properties expressed in aqueous, ethanol, and methanol extract could have been attributed to the more polar constituents which these solvents have an affinity for. Despite the promising activities of the extracts, this study couldn't report LC-MS chemical composition due to insufficient natural product library in our laboratory; likewise, it could not report the activities of the fractions presently and also the subacute toxicity level of the active extracts, which will be reported in future studies.

Conclusions

The extracts of *M. lutea* leaf and stem-bark showed different phytochemicals and high contents of phenolic and flavonoids and the ethanol and methanol leaf showed additional appreciable amounts of alkaloids. The extract did not cause any lethality at all the dose tested. The aqueous, ethanol, ethyl acetate, and methanol extracts exhibited antimalarial properties. The study is already ongoing in our laboratories to determine the active antimalarial constituents in the extracts and also evaluate their subchronic toxicity level.

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Online supplementary material.
Supplementary Table 1. Gas Chromatography-Mass Spectrometric (GC-MS) profile of M. lutea active extracts.