



Characterization of Malaria Preventive Extracts from *Myrsine africana* Seeds

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Authors' contributions

This work was carried out in collaboration among all authors. Authors RS, OA and BC designed the study. Author BC performed the statistical analysis. Author OA wrote the protocol and author RS wrote the first draft of the manuscript. Authors OA and BC managed the analyses of the study. Author RS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: The use of pharmaceutical anti-malaria drugs in many rural areas is not common. Various plant extracts have been used as anti-plasmodial agents. *Myrsine africana* seed extracts are common anti-malaria agents amongst the Maasai community of Kenya.

Aims: This study aimed at characterizing the chemical constituents of methanolic, aqua and n-hexane extracts of *Myrsine africana* seeds.

Study Design: An independent measures design was used.

Methodology: The extracts were obtained by maceration of the seeds before subjecting to physical-chemical analysis, functional groups, bio-metal concentrations and phytochemicals screening. Antibacterial studies were conducted using *E. coli* and *S. aureus*. The extracts were thereafter screened for presence of quinine and chloroquine by UV VIS spectroscopy.

Results: The results indicated the extracts were weakly acidic with moderate solid content. The FT-IR peaks of the extracts indicated abundance of carboxylic acids and benzylic groups. The extracts

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had a moderate iron concentration with mild copper, cobalt and zinc concentrations. The extracts were also rich in tannins, phenols, saponins, alkaloids and steroids. The antibacterial proficiency of both stains used increased with concentration of extracts and were highest at 50.0 mg/mL. Methanolic and water extracts of the seeds also showed appreciable quinines and chloroquinines concentrations.

Conclusions: *M. africana* seed methanolic and water extracts can be used as anti-plasmodial drugs to help curb malaria in rural tropical regions.

Keywords: *M. africana*; seed extracts; characterization; anti-plasmodial.

1. INTRODUCTION

One of the most dangerous diseases worldwide is malaria. The disease is quite viral especially in Africa and other tropical regions. It is the leading in causes of deaths in tropical countries [1]. Over 106 malaria endemic countries were reported in 2010 [2]. There were 216 million cases of malaria in 2010, 81% of these were in the World Health Organization (WHO) Africa Region [2]. An estimated 655,000 persons died of malaria in 2010 and 86% of the population was children under the age of 5 years and 91% of the deaths occur in the region of Africa [3].

Malaria is an infection caused by a parasite known as *plasmodium* [4]. Four *plasmodium* species causes malaria in human beings, these are: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovare* and *Plasmodium malarae*. *Plasmodium falciparum* is the most dangerous since it causes most of the infections and has more severe symptoms [5]. These parasites are transmitted by blood sucking vectors between humans. The female anopheles' mosquito. This mosquito is commonly referred to as 'a vector' because it only spreads the disease but cannot cause it. The vector breeds well in stagnant water bodies.

Malaria can be curbed by prevention or treating the disease. Prevention measures are propagated as they are cheaper and reduce chances of death. There are several preventive measures taken to curb the effects of this disease. Physical preventive measures include clearing bushes, draining stagnant water bodies and other breeding sites near homesteads. Other methods involve use of drugs and vaccines to prepare the human body for malaria attack.

Most of the common malaria preventive compounds are alkaloidal in nature [6]. Chloroquinines and quinines are two of these compounds which have extensively been used to prevent plasmodium species in humans [7].

Quinine is an alkyl amino alcohol usually presented as a salt in most formulations [8]. It can be taken orally or parenterally and work within 3 hours once in the human body [9]. Chloroquine has a faster speed of action once in the human body. It is used for both prevention and treatment of malaria [10]. Both compounds have been in use for quite a long duration dating back to the early 21st century. These anti-plasmodial products are quite abundant in malaria preventive and curative plants such as cinchona and other plants.

Myrsine africana is a tropical plant of medium size. The plant is common amongst the Maasai community of Kenya in prevention of malaria. The common name of the plant is 'Oseketeki'. The plant is also used in treatment of blood pressure, kidney diseases, stomach ache and cleansing of the uterus. The anti-plasmodial concoctions are prepared from seeds of these plant. The seeds are then boiled in water for 30 to 40 minutes. Alternatively, they can be soaked though this will take longer (4 to 5 hours). The liquid is taken either hot or cold.

The success rate of *Myrsine africana* in prevention of malaria amongst the Maasai people was quite high. Actually, very few if any cases of Maasai people deaths could be traced down to malaria. This study aimed at screening the extracts of *Myrsine africana* plant for chemical constituents and presence of quinine and chloroquinine compounds.

2. MATERIALS AND METHODS

2.1 Design of Experiment

M. africana seeds were collected from Narok county (co-ordinates, 1.1041°S, 36.0893°E). The seeds were shade-dried before boiling in water for 40 minutes (10%^{w/v}). A similar concentration (10%^{w/v}) was soaked in n-hexane and methanol solution for 5 hours. The extracts obtained were subjected to physical-chemical, phytochemical

and antibacterial analysis by disc fusion method [11]. The extracts were also concentrated to obtain residues which were subjected to functional group analysis by FT-IR. The residues were also digested in aqua regia acid and subjected to bio-metal analysis. Four metals (zinc, iron, cobalt and copper) were analyzed using AAS spectroscopy. The extracts were screened for presence of quinines and chloroquinines using UV-VIS spectroscopy.

Extraction and analysis of physical chemicals, functional groups, antibacterial property, bio-metal concentration and phytochemicals was done at Maasai mara university, Kenya. UV VIS screening of quinines and chloroquinines was done at Multimedia University, Kenya.

2.2 Requirements

All chemicals and reagents used were analytical grade and were obtained from Sigma-Aldrich Co. (Germany). The chemicals and reagents were in Maasai Mara University chemistry laboratory, Kenya.

For antimicrobial analysis; Muller-Hinton agar (Sigma-Aldrich), nutrient agar, potato dextrose agar, sterile distilled water, ethanol.

Access to; Atomic Absorption Spectrometer (PG-990), FT-IR (Shimadzu-119) UV-VIS (Shimadzu-1800), pH metre (Hanna G114).

2.3 Methods

2.3.1 Characterization of extracts

The extracts were characteriseed for the pH, conductivity, total solids, volatile solids and solubility using conventional methods. A pH meter and conductivity meter were used for pH and conductivity respectively.

For bio-metal analysis, the digested extracts were serially diluted using tripple-distilled water and filtering using Whatman #42 filter paper at each dilution stage. The bio-metals were analyzed after formulation of calibration curve using standard salts prepared for each of the bio-

metal analyzed. (Table 1) summarizes the conditions used during the bio-metal analysis.

For functional group analysis, the extracts were gradually concentrated until all the water was dried. The samples were then cast into pellets using potassium bromide pellet before analyzing for functional groups using IR Spectrometer.

2.3.2 Phytochemical screening of the extracts

2.3.2.1 Test for phenols [12]

3 ml of aqueous ferric chloride solution was added to 10 ml of the sample solutions, shaken and observations made. Formation of green coloration indicated presence of phenols.

2.3.2.2 Test for flavonoids [13]

Onto the test samples, 2 g of vanillin powder was added and the mixture agitated in an acidic medium.

The procedure was confirmed by adding 3 ml of dilute ammonia solution to 2 ml of aqueous filtrate followed by 1 ml of concentrated sulfuric acid. Formation of yellow deposits confirmed presence of flavonoids.

2.3.2.3 Test for tannins [13]

About 0.1 g of the dry samples were boiled in 4ml distilled water in a boiling tube then filtered. A few drops of 0.1% ferric chloride solution were then added and observations of change in color to brownish-green made.

2.3.2.4 Test for saponins [13]

The sample was added to 3 ml distilled water and vigorously agitated until a stable, persistent froth formed. 3 drops of olive oil were then added and shaken vigorously. Presence of emulsion indicated positive results.

2.3.2.5 Test for terpenoids (Salkowski's test) [13]

About 3 ml of the samples were mixed with 1 ml of chloroform and 1 ml of concentrated sulfuric acid. Formation of intense red-brown color indicated presence of terpenoids.

Table 1. AAS conditions used to analyze the bio-metals

Bio-metal	Wavelength	Bandwidth	Lamp current	Flame	Sensitivity
Co	240.7 nm	0.4 nm	5.0 ma	Air/Acetylene	0.05 mg/L
Cu	324.7 nm	0.4 nm	5.0 ma	Air/Acetylene	0.03 mg/L
Fe	248.3 nm	0.2 nm	5.0 ma	Air/Acetylene	0.05 mg/L
Zn	213.9 nm	0.4 nm	4.0 ma	Air/Acetylene	0.01 mg/L

2.3.2.6 Test for alkaloids (Mayer's test) [13]

About 3 ml of ammonia solution was added onto the sample followed by 10 ml of chloroform. The mixture was shaken well then filtered. The chloroform layer was then evaporated off and 3 ml of Mayer's solution added to the remaining solution. Formation of a cream precipitate indicated positive test for alkaloids.

2.3.2.7 Test for steroids [14]

The sample solution was dissolved in 10 ml of chloroform followed by 3 ml of concentrated sulfuric acid. Formation of red precipitates indicated presence of steroids.

2.3.3 Antimicrobial analysis of the extracts

Antimicrobial studies were conducted for both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*). All aseptic techniques were considered to minimize the contamination rates.

2.3.3.1 Media preparation

28.0 g of Muller-Hinton's agar media was dissolved into 600 ml of sterile distilled water in a media dispensing bottle. The mixture was gradually boiled to completely dissolve the media. Caution was taken not to break the media bottle by loosening the bottle stopper occasionally to avoid pressure build up. The media was then sterilized by autoclaving along with petri-dishes and all apparatus to be used at 121°C and 15 psi pressure for 15 minutes. The bacterial suspension was further diluted 1:100 to achieve a working suspension of 10^6 CFU/mL. The extracts were dissolved in 5.0% dimethyl sulfoxide (DMSO) to achieve varying concentrations (10.0 to 50.0 mg/mL). The media was allowed to cool to 45°C before dispensing in sterile petri dishes. The media plates were allowed to cool, inverted and stored in the refrigerator at 4°C for 24 hours.

2.3.3.2 Antimicrobial sensitivity tests

Sterile media plates were sub-divided into six equal parts using a marker pen and labelled accordingly. The test microbes were then spread aseptically on different media plates to prevent cross-contamination. Sterile octodiscs impregnated with different bio-slurry samples were then placed on the surface of the plates. The plates were then inverted and incubated at 37°C for 24 hours. The inhibition zones were noted and recorded in millimeters.

2.3.4 Screening of quinines and chloroquinines by UV-VIS spectroscopy

The *M. africana* seed extracts were screened for presence of quinine and chloroquine by checking their absorbance at 236 nm and 343 nm respectively. Distilled water, methanol and n-hexane were used as the blanks in the analyses.

2.4 Data Analysis

Data was reported as mean \pm standard deviation. The degree of freedom value was maintained at 8 with 95% confidence level being used for f-test statistics done. All the data obtained was analysed using Ms Excel and Originlab (version 6.5) statistical packages.

3. RESULTS AND DISCUSSION

3.1 Physical-chemical Analysis

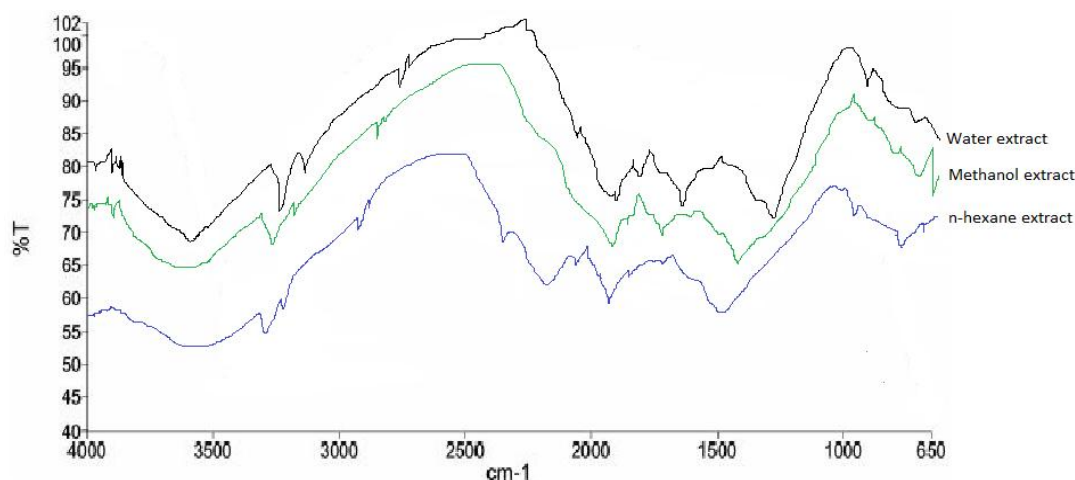
The pH of the extracts was found to be slightly acidic. Water and n-hexane extracts were found to have relatively similar pH values while the pH value of methanol extracts was more acidic ($P \geq 0.05$, $n = 8$). The pH values obtained were quite conducive to allow for proper drug delivery and assimilation into the blood system [15]. The pH of blood plasma ranges between 6.5-7.2 [16] and more deviation from this range would cause

Table 2. Physical-chemical properties of *M. africana* extracts

Parameters	Sample extract		
	Water	Methanol	n-hexane
pH	5.29 \pm 0.04	4.25 \pm 0.89	5.32 \pm 0.02
E. Conductivity (mS)	0.68 \pm 0.10	0.41 \pm 0.19	0.01 \pm 0.11
Total Solids (%)	18.23 \pm 0.24	13.55 \pm 0.15	13.17 \pm 2.13
Volatile Solids (%)	7.14 \pm 0.98	6.86 \pm 1.23	5.88 \pm 0.14
Solubility (g/100ml water at 37°C)	0.68 \pm 0.01	0.21 \pm 0.02	0.01 \pm 0.01

Table 3. Bio-metal concentrations of *M. africana* extracts

Bio-metal	Sample extract concentration (mg/Kg)		
	Water	Methanol	n-hexane
Copper	4.80±0.00	2.40±0.00	7.15±0.00
Iron	10.20±0.00	24.50±0.00	22.15±0.00
Cobalt	7.10±0.00	6.38±0.00	6.65±0.00
Zinc	1.60±0.00	4.55±0.00	2.35±0.00

Fig. 1. FT-IR spectra of *Myrsine africana* seed extractsTable 4. Phytochemicals screened in *M. africana* extracts

Phytochemicals	Samples		
	Water	Methanol	n-hexane
Tannins	++	++	++
Alkaloids	+	++	-
Steroids	++	++	++
Phenols	++	++	++
Saponins	++	++	++
Flavonoids	+	-	-
Terpenoids	-	+	-

Table 5. Zonal inhibition of *M. africana* extracts against bacteria

Samples	Test bacteria	% zonal inhibition (mm) at different concentration of extracts		
		10.0 mg/mL	25.0 mg/mL	50.0 mg/mL
Water	<i>S. aureus</i>	9.0	11.0	17.5
	<i>E. coli</i>	7.0	10.5	16.0
Methanol	<i>S. aureus</i>	6.5	11.5	18.5
	<i>E. coli</i>	6.0	12.5	19.0
n-hexane	<i>S. aureus</i>	6.0	8.5	12.5
	<i>E. coli</i>	6.0	9.0	13.0

undesired reactions. However, the blood has a buffering capacity and is able to regulate slightly more acidic or alkaline solutions from the specified pH range. Tse et al. [17] reports the pH

value of several malaria preventive drugs to range between 5.0 to 6.0. At lower pH values, protons present in the extracts react with hydroxyl groups in quinine to form salts [18].

Table 2 illustrates the physical-chemical parameters of the antimalaria extracts obtained.

The conductivities of the samples were high as expected in the polar solvents (water and methanol). The ions in n-hexane could not ionize in the non-polar solvent limiting their detection and thus an almost negligible conductivity value. The relatively higher conductivities in the water extract can be attributed to more solid content in the extract. The water extracts also had a higher pH value. Conductivity is a function of pH and soluble ions in solution [19]. High conductivity of the extracts implies better conveyance within the blood [20]. This theory is further supported by the relatively high solubility values (0.68 ± 0.01 g/100 ml water at 37°C) of the water extracts. The water extracts had higher solid content compared to the other extracts acidic ($P \geq 0.05$, $n = 8$). This is partially attributed to the method of extraction used. Most of the samples in the anti-malaria compounds might also be quite polar and thus enjoy better detection in water. About 39.16%, 50.62% and 44.65% of the solid matter in water, methanol and n-hexane extracts respectively was volatile. The volatility of medicinal extracts is an indicator of organic residues present. When abundant in medicinal extracts, these residues affect the stability of the main medicinal components present [21]. Volatile matter also increases the emulsion of medicinal extracts prompting for less external emulsifiers. The water extracts had a relatively higher solubility at normal human temperature (37°C). High solubility implies more miscibility of the drugs and thus better conveyance in watery solutions.

3.2 Bio-metal Concentrations of the Anti-malaria Extracts

The solvents used did not have any particular effect on the concentration of bio-metals in the samples. Iron was the most abundant bio-metal in the extracts. The concentration of copper in the n-hexane extracts (7.15 ± 0.00 mg/Kg) were significantly higher than those of water and methanol extracts ($P \geq 0.05$, $n = 8$). According to Gabrić et al. [22], n-hexane is a suitable solvent for extracting copper ions in organic compounds. Copper is an important component of several metalloenzymes significant in coordinating human body immune functions [23]. Copper ions have also been reported as important co-factors in malaria preventive extracts [24]. Table 3 illustrates the bio-metal concentrations in the malaria preventive extracts.

The methanol extracts had the highest iron abundance (24.50 ± 0.00 mg/Kg) while water extracts had the lowest iron abundance (10.20 ± 0.00 mg/Kg). Apart from blood formation, iron plays a key role in immunity of the body [25]. Iron metalloenzymes work in coordination with platelets and are key in inhibiting entry and spread of plasmodium species in the blood [26]. During the intraerythrocytic stage of plasmodium maturity, influence of high iron concentration cause the parasite degrade hemoglobin and in the process release ferriprotoporphyrin IX (FePPIX) complex [27]. This complex cause reactive oxygen species (ROS) which induce oxidative stress leading to parasitic death [27]. These extracts are rich in malaria preventive and curative compounds [28]. All the three extracts had a significantly similar cobalt concentration ($P \geq 0.05$, $n = 8$). The choice of extraction solvent did not thus influence the availability of cobalt ions in the extracts. The case was different in zinc abundance where the methanolic extracts were significantly more than the other extracts ($P \geq 0.05$, $n = 8$). Both cobalt and zinc are crucial co-factors used in synthesis of heme and other immune cells involved in anti-plasmodial activities [29]. β -heme can induce heme toxicity leading to death of plasmodium species. On contrary, immune cells such as macrophages, monocytes and neutrophils induce chemical signals (cytokines and chemokines) that fight the plasmodium species [30]. Abundance of these bio-metals is thus vital in formation of anti-plasmodial compounds.

3.3 Functional Groups of the Anti-malarial Extracts

The FT-IR spectra of the three extracts were quite similar with a lot of resemblance in peak positions and intensities. Water and methanolic extracts exhibited presence of adsorbed moisture at around 3850 cm^{-1} . The two extracts also had alcoholic OH peaks at around 3300 cm^{-1} resulting from presence of alcohol in these extracts. There was also a wide peak between $3000\text{--}3400\text{ cm}^{-1}$ in all the extracts indicating presence of carboxylic OH groups in the *M. africana* seeds. These peaks, coupled with the carboxylic C=O peaks at $1770\text{--}1810\text{ cm}^{-1}$ (in the water and methanolic extracts) indicate presence of carboxylic acids in the extracts. All the extracts had considerably long carbon chains with regard to the pronounced sp^3 C-H peaks between $2800\text{--}2900\text{ cm}^{-1}$. Similar peaks were observed in the

extracts of *Persea americana*, *Jatropha podagrica* and *Picralima nitida* plants with anti-plasmodial potential [31]. The three FT-IR spectra of *M. africana* seeds are illustrated in Fig. 1.

The n-hexane FT-IR spectra showed presence of acetylide and nitride peaks at 2450 and 2101 cm^{-1} respectively. These peaks were suppressed in the water and methanolic extracts. The n-hexane extract spectra however lacked carboxylic C=O peak (1700-1810 cm^{-1}) and trans C=C peaks at 1330 cm^{-1} . This discrepancy is attributable to change in polarity between the n-hexane extracts and the water and methanolic extracts. The three extracts showed traces of organometallic and organohalide groups with some activity between 650-800 cm^{-1} . Organohalide groups have been proven to inhibit growth of bacteria in plant extracts [32]. In general, the above FT-IR spectra had several peaks matching those of quinine and chloroquine thus showing the potential to possess anti-plasmodial activity.

3.4 Phytochemicals Present in *M. africana* Extracts

As a rule of thumb, a good medicinal extract should be quite rich in phytochemicals. Most of the anti-plasmodial compounds are alkaloidal in nature [33]. The extracts of *M. africana* were found to be abundant in most of the phytochemicals screened (Table 4). Tannins, steroids, phenols and saponins were quite concentrated in all the three extracts screened. The choice of use of extraction solvent affected the abundance of flavonoids, alkaloids and terpenoids in the extracts. n-hexane extracts showed the least proficiency in phytochemicals screened.

The methanolic extracts exhibited more alkaloids present compared to water and n-hexane extracts. The water extract showed mild concentration of alkaloids while the n-hexane extract did not depict any trace of alkaloids. Similar findings were observed by Adia et al. [34] who carried out phytochemical analysis of anti-plasmodial extracts using methanol and water solvents. Flavonoids were the least abundant phytochemicals in the extracts and only traces of the same were observed in the water extract. Only the methanolic extracts of *M. africana* had terpenoids present.

3.5 Antibacterial Proficiency of *M. africana* Extracts

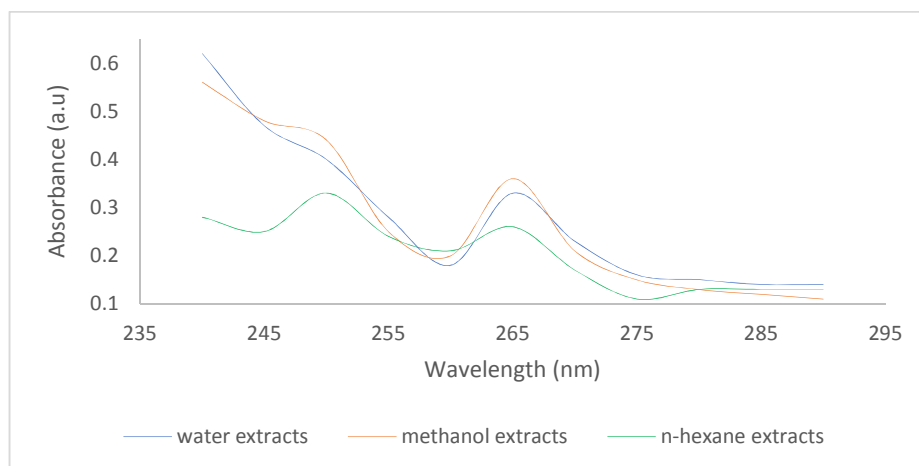
M. africana extracts were found to exhibit strong antibacterial property when exposed to both gram positive and negative bacteria. Mofenson et al. [35] reported that presence of *S. aureus* and *E. coli* in malaria-infected patients result into opportunistic infections; further weakening the patient. The antibacterial strength linearly increased with increasing extract concentration. The antibacterial effect did not differ significantly in either type of bacteria (*S. aureus* and *E. coli*) used. The highest antibacterial proficiency was seen in the water extracts (at 10.0 mg/mL) and methanolic extracts (at 25.0 and 50.0mg/mL). The antibacterial proficiency of the n-hexane extracts at 25.0 mg/L and 50.0 mg/L were found to be significantly different from those of water and methanol extracts ($P \geq 0.05$, $n = 8$). Table 5 illustrates the % zonal inhibition of different extract concentrations on *S. aureus* and *E. coli* bacteria strains.

Falodun et al. [36] found the methanolic extracts of *P. americana* and *Jatropha podagrica* extracts used for malaria prevention to have similar inhibitions to *S. aureus* as those in Table 5. Optimal inhibitions were observed at IC_{50} 8.695 mg/ml and 35.189 mg/ml concentrations [36]. The two extracts however showed less inhibition towards *E. coli* bacteria.

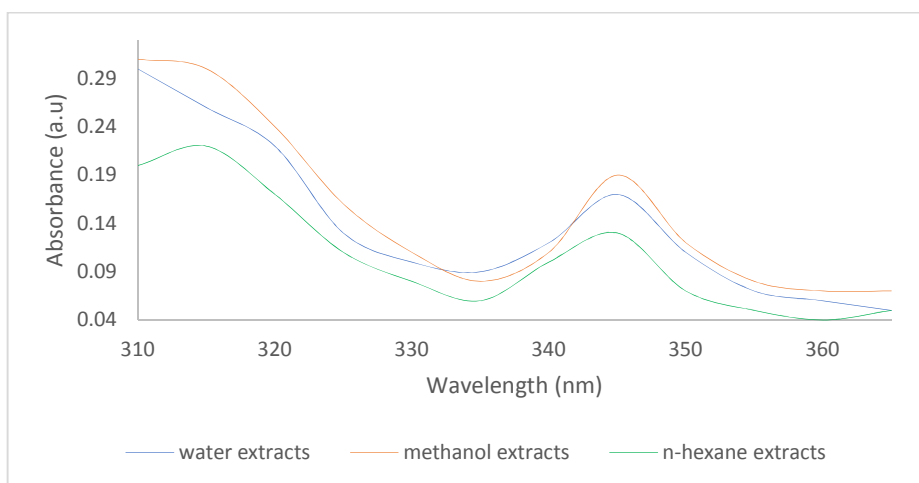
3.6 Chloroquine and Quinine Analysis in the *M. africana* Extracts

All the three extracts analyzed were found to contain some traces of the two anti-plasmodial groups (Quinine and chloroquine). The order of abundance in these groups was methanol>water>n-hexane. This indicates that methanol is the most suitable solvent for extraction of anti-plasmodial groups from *M. africana* seeds. Similar findings were observed by Falodun et al. [36] whereby the methanolic extracts gave the best anti-plasmodial activity. Alongside DMSO and chloroform solvents, methanol have over a long period been used in extraction of various anti-plasmodial compounds from plants [37]. Fig. 2 illustrates the UV-VIS spectra of these extracts when screened for presence of quinines and chloroquines.

Fig. 2 shows that the absorbance of n-hexane is quite low and thus should not be used to extract these compounds from *M. africana* seeds.



(a)



(b)

Fig. 2. UV-VIS spectra for quinine (a) and chloroquine (b) compounds in *M. africana* seed extracts

This is partially attributable to the polarity of the two anti-plasmodial compounds. Both quinine and chloroquine are quite polar unlike n-hexane.

4. CONCLUSIONS

The three *Myrsine africana* extracts (water, methanol and n-hexane) were found to be weakly acidic with low conductivity values and moderate solid content. All the extracts were rich in iron content. The extracts exhibited presence of carboxylic and benzylic groups present. The extracts were also quite rich in tannins, alkaloids, steroids, phenols and saponins. The antibacterial

proficiency of the extracts increased with the concentration of the extracts. Methanolic and water extracts showed high abundance of quinine and chloroquine anti-plasmodial compounds.

DATA AVAILABILITY

All the data used in this research is enclosed within the manuscript and any supplementary sheets attached.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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