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in vitro FRACTIONATION OF *Vernonia amygdalina's* ETHYLACETATE LEAF EXTRACTGUIDEDBY ANTIPLASMODIAL ACTIVITY

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Abstract: The traditional use of *Vernonia amygdalina* (VA) to control various diseases have been the world's cultures for centuries. Many research have been done on antiplasmodial activity of this plant, although relatively few have been done on fractionating the active components responsible for its antiplasmodial activity. The subject of fractionation of compounds from plants is highly relevant to the identification of lead molecules for drugs. The aim of this research is to fractionate the ethylacetate leaf extract of VA using *in vitro* antiplasmodial activity-guided studies. Solvent partitioning was used to obtain hexane, chloroform, ethylacetate and methanol extracts of VA. These were examined *in vitro* for antiplasmodial activity at different concentrations. The chloroform, ethylacetate and methanol extracts showed an appreciable antimalarial activity with the ethylacetate extract showing the highest activity after 24 hours of incubation. This was used to guide the selection of the solvent extract for fractionation. Column chromatography was used to separate the ethylacetate extract into forty-two fractions. Fractions that shared similar R*f* values were combined together after developing spotted plates in tank containing hexane-ethyl acetate (1:4), thus reducing the number to five. The total recovery of the VA extract was 39.56%. These pooled fractions were tested *in vitro* for antimalarial activity. Preparatory TLC of VA-4 revealed 4 compounds. *In vitro* antiplasmodial activity showed sub-fraction VA-4A to have the highest% inhibition. TLC of VA-4A revealed two eluting compounds. These fractions may contain compounds that can be used in pharmaceutical industry for development of novel lead drugs for management of malaria.

Keywords: *Antiplasmodial activity, Fractionation, Vernonia amygdalina*

INTRODUCTION

Malaria is an infectious disease that can be fatal and is caused by *Plasmodium* parasites which are spread from person to person through bites from female anopheles mosquitoes carrying the infection (WHO 2019). Malaria-related deaths and morbidities are major global health concerns, particularly in tropical and subtropical areas. Five species of *Plasmodium* parasite can infect human (Hardman and Limbird 2001). These are; *Plasmodium falcifarum*, *Plasmodium vivax*, *Plasmodium ovale, Plasmodium malariae* (which cause the most serious form of malaria) and *Plasmodium knowlesi* (a zoonosis that caused malaria in Macaque but can also be transmitted to humans) (Singh *et al*. 2004). Human malaria induced by *Plasmodium falciparum* is known to pose a serious risk to life, therefore *P. falciparum* infection is a serious medical emergency.

Conventional antimicrobial drugs are used to treat this life-threatening disease and over the years, various antimalarial drugs have been produced. The problem, however, is that there is a varying degree of response by the individual parasites to the drug used. Strains resistance to most antimalarial drugs makes the parasites to be able to multiply in the presence of the drugs. This therefore causes a major drawback in the control of malaria. The emergence of *Plasmodium falciparum* resistant strains to numerous antimalarial medications has sparked researchinto the discovery of novel anti malarial drugs (Bickii*et al*. 2000).

Medicinal plants are various plants in which some of their parts contain substances that can be used therapeutically or are used as building blocks to produce effective drugs (Sofowora 2008). In addition to their therapeutic benefits, many plant products are eaten in reasonable amount as food. However, some plant products are only used for medicinal purposes, while others are more potent (e.g. containing cardioactive glycosides), and should only be used in small doses that are suitable for treating specific diseases (Mohammed 2008). Plants have a vast array of bioactive phytochemicals and are diverse, making them potential sources of therapeutic agents against a variety of ailments (Farombi 2003). One key source of information used to control the spread of malaria is the use of natural products as many plant extracts and essential oils exhibit larvicidal activities and adult mosquito repellency properties. These make it imperative to search for novel antimalarial drugs from natural products (with higher efficacy and fewer side effects). *Vernonia amygdalina* is one of the plants commonly used for malaria treatment locally. However, there is little or no information in the literature on the isolation of the active components of the plant responsible for this property. Therefore, there is need to have scientific evidence of the antimalarial property of the plant, test for the solvent extract with highest activity as well as fractionate/isolate compounds responsible for the antimalarial activity of the plant.

MATERIALS AND METHODS

Collection and Identification of Plant Material

Fresh leaves of *V. amygdalina* (bitter leaf) were collected from Tukuntawa ward in Municipal Local Government Council of Kano State, and identified at Plant Biology Department, Bayero University, Kano.

Extraction of the powdered leaf of the plant (Abosi and Raseroka 2003)

The leaves of *V. amygdalina* were plucked from the stem, cleaned, air-dried under shade and ground into powder. The powder was stored in an airtight container and kept in a cool, dry place. Extraction was carried out by dispersing 2.17 kg of the powdered

leaves in 5.0L of 99% ethanol (Qualinkems) in an air-tight plastic bucket. The bucket containing the powdered leaves wastightly closed, shaken and left to stand at room temperature. Every day for four days, the soaked leaves were filtered, the extracts were then collected and concentrated by evaporation using a rotary evaporator at temperature of 37°C to give 240 g. Reclaimed solvents were put reused for soaking (Abosi and Raseroka 2003). The extract was exhaustively and successively partitioned with n-hexane, chloroform, ethyl acetate and aqueous methanol to afford the hexane, chloroform, ethyl acetate and methanol residue fractions.

In Vitro Antiplasmodial Assay

Sourcing of malaria parasite (*Plasmodium falciparum*)

Human blood samples infected with malaria parasites (*Plasmodium falciparum*) were sourced from Bayero University Kano Clinic. Venous blood was collected from patients diagnosed with malaria using 5cm^3 disposable syringes. The samples were immediately transferred into EDTA coated bottles with tightly fitted plastic corks and mixed thoroughly, then transported to the microbiology laboratory, Bayero University Kano.

Screening of blood samples for *Plasmodium falciparum* using thin smear method (Tankeshwar 2010).

After thorough mixing, a small drop of each blood sample was placed at one end of a clean grease-free glass slide using a clean capillary tube. Using a clean cover slip as spreader, the drop was allowed to extend along it. Holding the slide and spreader at an angle of about 30^o, the spreader was pushed along the slide, drawing the blood behind it, spreading the drop of the blood to make a film about 40-50mm in length (two thirds of the slide). The film was air dried by waving the slide. When completely dried, the film was placed on staining rack and 1-2 drops of absolute methanol was added and allowed to dry on the film, then covered with several drops of Giemsa stain for 15 minutes. The stain was then washed with distilled water, drained and dried in the air at room temperature. The dried films were observed under microscope using an oil immersion at ×100 magnification. An average was obtained from the reading of 3 microscopic fields using the relation

$$\% \text{ parasitaemia} = \frac{\text{Number of infected cells} \times 100}{\text{Total number of cells}}$$

***In vitro*antiplasmodialactivity of plant extracts (Trager and Jensen 1976; Srivastava *et al*. 2007)**

Plasmodium falcifarum positive blood obtained from Bayero University Kano Clinic, was maintained at not more than 5% parasitemia in complete RPMI 1640 medium (SIGMA ALDRICH) supplemented with 25 mM HEPES, L-glutamine and 40 µg/cm3 gentamycin by modified candle jar method (Trager and Jensen 1976; Srivastava *et al*. 2007). The culture was routinely monitored through Geimsa staining of the thin smears. Standard drug (Combisunate 20/120 (Ajanta Pharma Limited)) and extracts (stock solutions) were prepared in distilled water and dimethylsulfoxide (DMSO) respectively and then diluted to achieve the required concentrations of 500, 1000, 2000 and 4000µg/cm^3 using the dilution formula:

$C_1V_1=C_2V_2$

Where C_1= Initial concentration

V_1= Initial volume

C_2=Final concentration

V_2= Final volume

Into a glass vial containing 1cm^3 of 5% parasitemia erythrocytes, 1cm^3 of the test solution and 2cm^3 of the culture medium were added, mixed thoroughly and incubated for 24 hrs at 37°C in candle jar.

Blood smears from each vial were fixed in methanol, stained with Giemsa's stain and the numbers of infected RBCs per field were counted. The antimalarial activity of the test extracts was expressed as 50% inhibitory concentration (IC_{50}). All experiments were performed in triplicates and the results were expressed as percentage of growth inhibition. Extract with highest % inhibition was chosen for the study.

Fractionation of the Active Extracts (Triadisti and Zamzani 2023)

The ethylacetate extract showed the highest antimalarial activity *in vitro.* Five (5) of the ethylacetate extract was loaded on a column (pre-packed with silica gel) to separate it into its component fractions. Silica gel (50-200 mesh, Park scientific limited Northampton, UK) was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase. The slurry was prepared by mixing150g of silica gel and 750cm^3 of hexane and poured down the glass column (3 × 50cm**)** carefully. The tap of the column was left open to allow free flow of solvent into a conical flask below to ascertain whether the set-up is in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. At the end of the packing process, the tap was locked. The column was allowed 24h to stabilize, after which, the clear solvent on top of the silica gel was allowed to drain down to the silica gel meniscus. The silica gel was washed twice by pouring n-hexane and draining. The hexane was then added onto the column (nearly full). The sample was prepared by mixing 5gof the extract and 10g of silica gel, solubilized with methanol and allowed to dry. The dried sample was carefully added onto the column. The set-up was allowed until when the sample settled completely, and then silica gel was added on top of the sample in an amount near the size of the sample. The column tap was opened to allow the eluent to flow at the rate of 0.2cm^3 per second. The extract was eluted using solvent combinations of gradually increasing polarity using hexane, chloroform, ethyl acetate and methanol (selected based on analytical thin layer chromatography of the extracts). The solvent systems used were; n-hexane 100%; n-hexane: chloroform 80:20, 60:40, 50:50, 40:60 and 20:80; chloroform 100%; chloroform: ethylacetate 60:40 and 40:60; ethylacetate 100%; ethylacetate: methanol 80:20, 60:40 and 50:50, then lastly methanol 100%. A reagent bottle was used to add each solvent combination carefully by the sides of the glass into the column each time. This helps to prevent solvent droplets from falling directly and disturbing the sample layer in the column which may lead to non-uniform draining of the fractions. The eluted fractions were collected in aliquots of 50cm^3 in collecting bottles.

The fractions were concentrated using rotary evaporator and then separated based on their relative mobility in solvent systems and colour reactions with ultra-violet (UV) light using pre-coated silica gel (Xtra SIL G/UV_{254}, Macherey-Nagel Germany) aluminium plates. A strip of the pre-coated silica gel was cut out. Using a micropipette, a spot of the sample was applied on the plate about 1.0 cm from the edge. The drop was dried using hot air dryer. The strip was lowered into the chromatographic tank containing and saturated with the solvent system. The tank was covered with a glass lid. The solvent was allowed to ascend until the solvent front reach about ¾ of the length of the strip. The strip was then removed and dried by a hot air dryer and viewed under UV lamp at 356 and 254nm (in a dark room) to identify the fluorescing spot, which was measured with a ruler and the relative retention factor (Rf) value calculated based

on the formula:
Rf = Distance travelled by the spot from the origin / Distance travelled by the solvent from the origin to the solvent front.
Forty-two fractions were collected from the column. Fractions with near/the same Rf values were pooled together after developing spotted plates in tank containing hexane-ethyl acetate (1:4), thus reducing the number of fractions to five. These pooled fractions were tested *in vitro* for antimalarial activity as earlier described. The fraction with highest activity was subjected to preparative thin-layer chromatography for further fractionation (purification) into individual components.

Preparative Thin Layer Chromatography of the Most Active Fraction

The thin-layer plate consists of a layer of the adsorbent (Silica gel G for TLC- lobachemie laboratory reagent and fine chemicals, Mumbai India) spread over an inert, flat glass support. The layer was stabilized by incorporating a binding agent (plaster of Paris, 100%) in the mixture. The plate was prepared by shaking a mixture of the adsorbent and the binding agent with an appropriate volume of water. A uniform layer of this slurry was spread over clean plate and allowed to set. The plate was then dried in an oven at 110^0Cfor two and half hours and stored in a dessicator.
The most active fraction of the plant was dissolved in ethyleacetate and applied on the plate as streak with capillary tube. After the sample had been applied and dried, the plate was placed in a tank containing and saturated with hexane: ethyleacetate: methanol (3:2:2) 1cm deep. When the solvent has moved to a line that has been scored across the plate about 1cm from the top edge the plate was removed and dried quickly. The separated bands were visualised by viewing under ultraviolet light and their positions marked. The bands were then scraped, washed with ethylacetate and centrifuged at 4000 revolutions per minute for 10 minutes.The eluents were subjected to further antiplasmodial activity *in vitro*. Eluent that exhibited maximal activity was then labelled as the most active sub-fraction.

Statistical Analysis
IC_{50} calculations were performed using Microsoft Excel.

RESULT AND DISCUSSION

Solvent partitioning was used to obtain hexane, chloroform, ethylacetate and methanol extracts of *Vernoniaamygdalina*. Figure 1 showed the result of *in vitro* Antiplasmodial Activity at different concentrations from these four extracts of *Vernoniaamygdalina*. Chloroform, ethylacetate and methanol extracts demonstrated appreciable antimalarial activity with the ethylacetate crude extract showing the highest activity after 24 hours of incubation.This may be due to the fact that ethylacetate is semi-polar and is capable of extracting both some portion of polar as well as portion of non-polar unlike hexane which have more affinity for non-polar compounds (Alkasim, 2016). Ethylacetate is semi-polar solvent effective in extracting alkaloids, aglycon and glycoside compounds (Houghton and Raman, 1998), sterol, terpenoids and flavonoids (Cowan 1999). This result of preliminary *in vitro* antiplasmodial activity was used to guide the selection of the solvent extract for fractionation. Approximately five (5) grams of the extract was repeatedly loaded into the column and separated into its respective fractions. Forty-two fractions were collected from the column (Table 1). Fractions with near/the same R*f* values were pooled together after developing spotted plates in tank containing hexane-ethyl acetate (1:4),

thus reducing the number of fractions to five (Table 2). The total recovery (i.e., eluted amount) extract was 9.89g (i.e., 39.56%). These pooled fractions were tested *in vitro* for antimalarial activity as earlier described (Figure 2). Fraction four (ethylacetate: methanol-80:20) showed the highest antimalarial activity *in vitro* after 48 hours. However, the IC_{50} of the respective fractions were depicted in Table 3. Preparatory thin layer chromatography (TLC) of VA-4 revealed 4 compounds (plate 1). *In vitro* antiplasmodial activity showed sub-fraction VA-4A to have the highest percentage inhibition (Figure 3). Thin layer chromatogram of VA-4A (plate 2) revealed two eluting compounds.

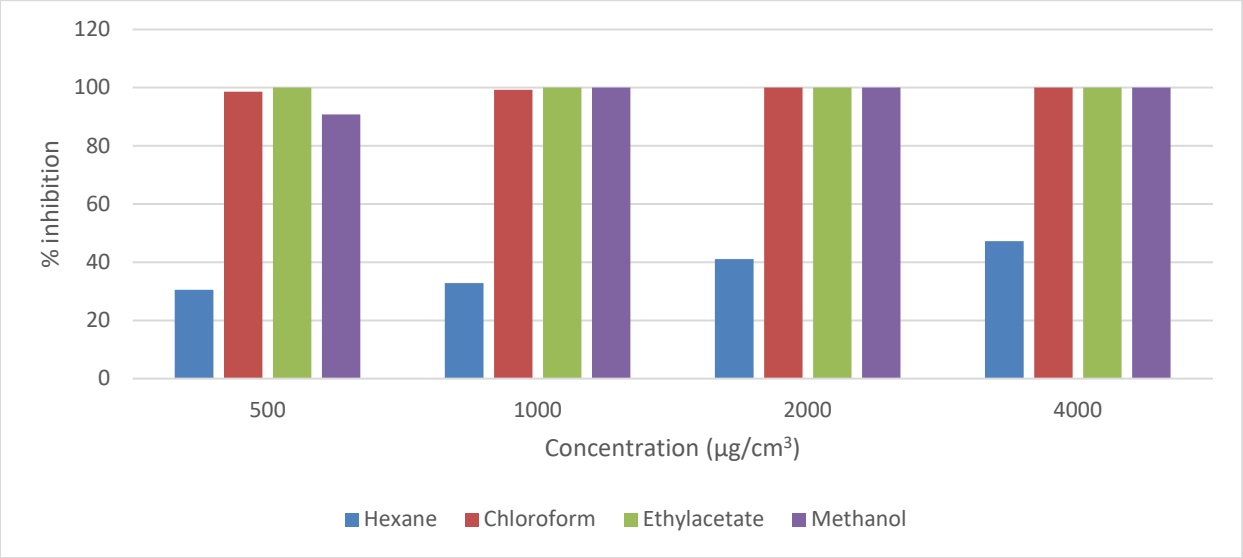

Figure 1: *In vitro* anti plasmodial activity of *Vernonia amygdalina* crude extracts after 24 hours of incubation

Table 1: Fractionation of V*ernonia amygdalina* Ethylacetate Leaf Extract

S/N	Fraction Label	Fractions (column chromatography)	Solvent System (column chromatography)
1	I	01-02	N-hexane (100%)
2	II	03-05	N-hexane: Chloroform (80:20)
3	III	06-08	N-hexane: Chloroform (60:40)
4	IV	09-12	N-hexane: Chloroform (50:50)
5	V	13-15	N-hexane: Chloroform (40:60)
6	VI	16-17	N-hexane: Chloroform (20:80)
7	VII	18-20	Chloroform (100%)
8	VIII	21-24	Chloroform: Ethylacetate (60:40)
9	IX	25-26	Chloroform: Ethylacetate (40:60)
10	X	27-29	Ethylacetate (100%)
11	XI	30-34	Ethylacetate: Methanol (80:20)
12	XII	35-37	Ethylacetate: Methanol (60:40)
13	XIII	38-39	Ethylacetate: Methanol (50:50)

14	XIV	40-42	Methanol (100%)

Table 2: Pooling of V*ernonia amygdalina* Ethylacetate Leaf Extract

S/N	Fraction Label	Pooled Fractions (column chromatography)	Amount Recovered (mg)	Rf Values (TLC)	Appearance
1	A	1-2	1050	0.56	Orange
2	B	3-14	2020	0.65	Yellowish green
3	C	15-19	1200	0.78	Yellowish green
4	D	20-25	1030	0.92	Dark brown
5	E	26-42	4590	0.74	Dark brown

Fractions from repeated column chromatography were separated based on their relative mobility in solvent systems and colour reactions with ultra-violet (UV) light (at 254nm and 356nm wavelengths) of the TLC chromatograms. The fluorescing spot was identified and measured with a ruler and relative retention factor (Rf) value calculated. Forty-two fractions were collected from the column, and fractions with near/the same Rf values were pooled together after developing spotted plates in tank containing hexane-ethylacetate (1:4), thus reducing the number of fractions to five.

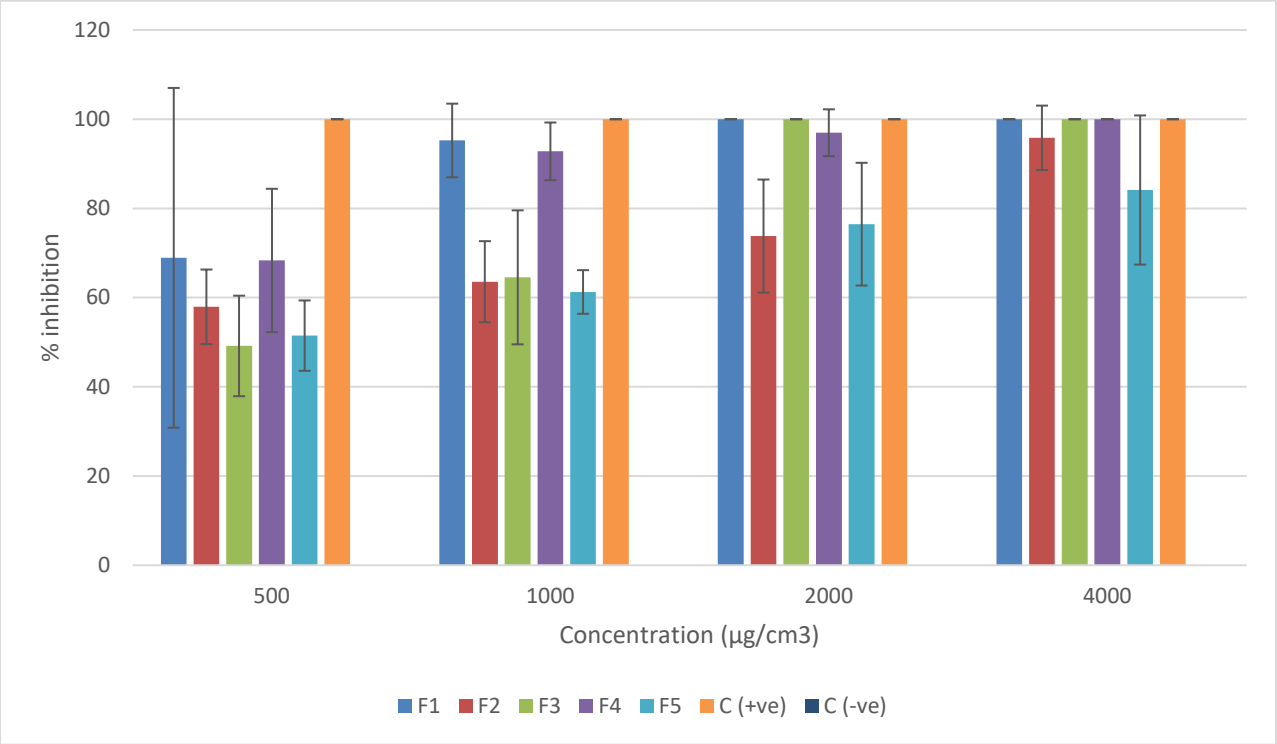

Figure 2: *In vitro* anti plasmodial activity of the five pooled fractions of *Vernonia amygdalin a*after 48 hours using *Plasmodium falcifarum* positive blood

Table 3: IC_{50} of *Vernoniaamygdalina*ethy lacetate fractions

FRACTION	IC_{50} (µg/cm3)
F1	271.45 ± 66.60
F2	985.27 ± 76.27
F3	1049.56 ± 186.91
F4	226.59 ± 25.82
F5	1329.05 ± 329.60

Results are mean ± standard deviation; method of calculation = Microsoft Excel

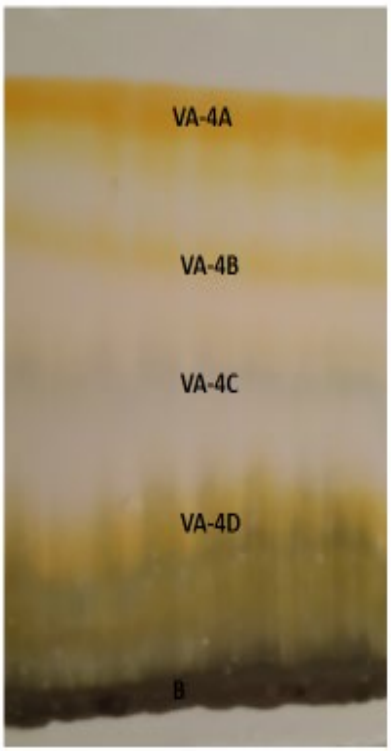

Plate 1: TLC Plate of Ethylacetate Extract of Leaves of *Vernonia amygdalina* (Fraction 4) Developed using Hexane: Ethyleacetate: Methanol (3:2:2). 4A-4D = Sub fractions of VA-4, B = Origin

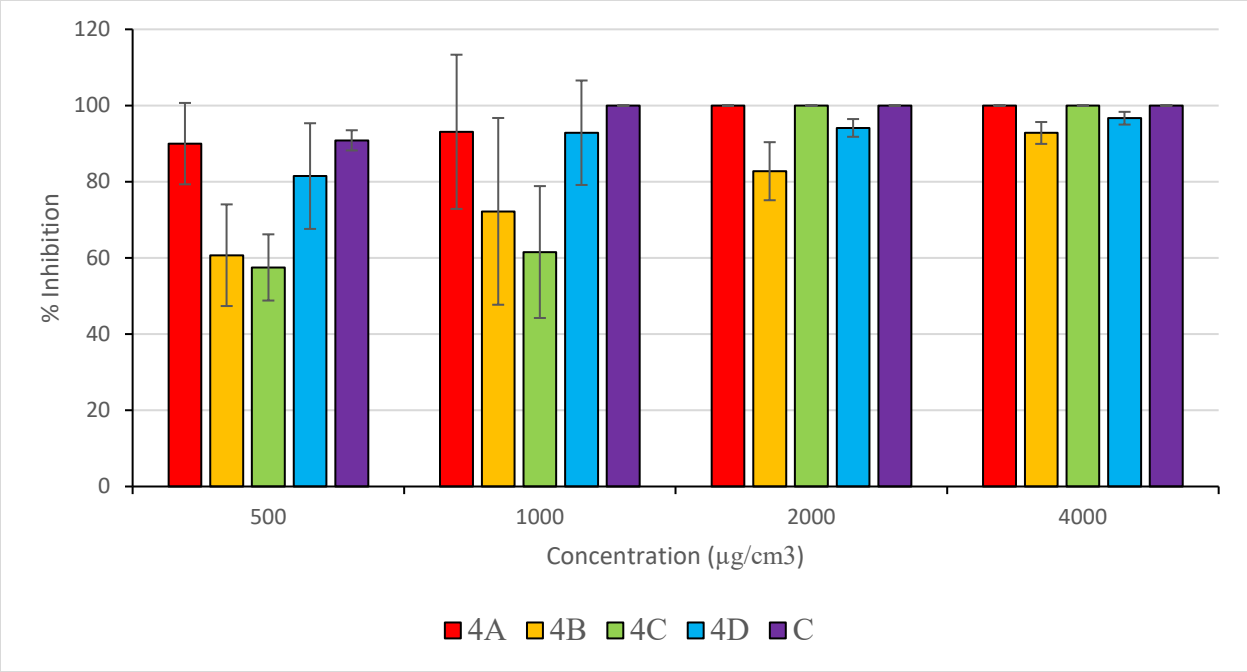

Figure 3: *In vitro* anti plasmodial activity of *Vernonia amygdalina* (fractions 4A-4D) after 48 hours using *Plasmodium falcifarum* positive blood
Note: Preparatory thin layer chromatography was used to isolate the compounds.

Plate 2: TLC Plate of Ethylacetate Extract of *Vernonia amygdalina* (Sub-Fraction 4A) Developed using Hexane: Ethyleacetate: Methanol (3:2:2) and Viewed in Daylight. 1-2 = Sub fractions of VA-4A, B = Baseline

CONCLUSION

From the results of this research, it can be concluded that *V. amygdalina* plants have an appreciable anti-malaria effect and that the ethylacetate extract possessed the highest activity over hexane, chloroform and methanol extracts. Finally, the ethylacetate extract of the plant contain some important compounds that may be responsible for the anti-malarial property of the plant.

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