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Cultivation, phytochemical and *in vitro* anti-plasmodium activity of *Artemisia annua* L. (Asteraceae)

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Abstract
The chemical synthesis of Artemisinin for the production of Artemisinin-based Combination Therapies (ACTs) is complex, uneconomical and produces low yields, thus, the main source of Artemisinin is the Chinese plant, *Artemisia annua*. The present study was aimed to determine the adaptability, phytochemical constituents and *in vitro* anti-plasmodium activity of hexane and aqueous leaf extracts of *A. annua* cultivated in Kano State, Nigeria. The phytochemical analysis revealed the presence of cardiac glycosides, steroids, terpenoids, alkaloids and flavonoids in both extracts, while saponins, tannins, phenolic compounds and anthraquinones were only detected in the aqueous extract. The *in vitro* anti-plasmodium screening showed that both extracts have anti-plasmodium activity; with aqueous extract having the highest activity at the concentration of 10, 5, 2.5 and 1.25 mg/ml which was statistically significant (P≤0.05) when compared to the positive control. The study has shown that Kano soils may be suitable for the commercial cultivation of this important medicinal plant.

Keywords: Artemisinin, *Artemisia annua*, anti-plasmodium, phytochemical constituents and commercial cultivation

Introduction

Malaria is one of the major diseases in developing nations particularly in Africa, where about 2 million deaths are reported annually [1, 2]. This occurrence is mainly in areas where the malaria causing parasite *Plasmodium falciparum* has developed resistance to the conventional treatments of malaria such as Chloroquine, sulphur-pyrimethamine combinations and to some extent quinine which previously has been effective in the treatment [3]. Malaria has enormous economic costs and was reported to accounts for 40 % of public health costs in Africa, it costs Africa $12 Billion of it GDP annually. The World Health Organization's estimate has put the mortality rate of malaria for children under five in Africa at 729 per 100, 000 [4]. Historically, *A. annua* has been traditionally grown in China, and was been found to be a component of the plant flora in Asia with notable presence in countries like Vietnam and India [5, 6]. It was cited in the earliest Chinese medical prescriptions related to the Mawangdui tomb dating back to 168 BC. In the 1960s, an initiative by the Chinese government under President Mao led to the first isolation and chemical characterization of the active chemical compound in *A. annua* responsible for its potency in killing *Plasmodium* malaria parasite [7]. The World Health Organization recommends artemisinin-based combination therapy (ACT) in regions where multi drug resistance is prevalent [4]. ACTs contain two or more drugs one of which is artemisinin, an effective antimalarial drug effective against *Plasmodium falciparum, P. vivax, P. malariae* and *P. ovale* [8]. Artemisinin for ACTs is obtained by chemical synthesis or extraction from *A. annua*, a short day plant possessing a shoot and root system and could attain a height of around 2 metres [9]. However, the process of chemically synthesizing artemisinin is complex, uneconomical and produces low yields [10]. Therefore, the main source of artemisinin is the plant, *A. annua* [11]. Derivatives of artemisinin found in *A. annua* that also have antimalarial effects include artemisinic acid, artemether, arteether, artesunate, Dihydroartemisinin and hydro-artemisinic acid [12, 13].Clones of *A. annua* have been developed and introduced into African continent since 1990 (EABL, 2005) [14]. Apart from being the main source of artemisinin, *A. annua* has been used as a source of essential oils, crafting of aromatic wreaths and as a natural herbicide [15-17]. The commercial cultivation *A. annua* has been embraced in some East African countries like Kenya, Tanzania, Uganda and Madagascar.

These countries currently export *A. annua* to Pharmaceutical companies in India and other European countries where the final products are made. It has been reported by many researchers that the quantity of artemisinin depend on some factors such as soil, growing conditions, seasons, geographic locations, harvesting period and post-harvest processing [18]. Other factors that have an effect on artemisinin content include age of growth, species and ratooning [19, 20]

The main objective of this work was to determine the adaptability, phytochemical constituents and *in vitro* anti-plasmodium activity of hexane and aqueous leaf extracts of *A. annua* cultivated in Kano State, Nigeria. The findings of this study would give an insight into the suitability of Kano soils for commercial cultivation of *A. annua* and also for local production of ACTs.

Materials and Methods

Cultivation of *A. annua*

The seeds of *A. annua* were obtained from Bioresources Development Centre, Odi, Bayelsa State, Nigeria. The methods described by [21, 22] were adopted for the cultivation of the plant in Kano State, Nigeria.

Nursery Bed

A well-drained light loamy soil was used for the cultivation. The nursery bed was tilled and watered evenly. The seeds (2 g) were mixed with sand, spread uniformly over the nursery bed and covered with a thin layer of soil. The bed was kept moist by frequent watering, and germination was observed on the fifth day.

Transplanting

The land was ploughed twice and laid into bed of convenient size after the application of organic fertilizer. The land was watered for two days before the seedlings were transplanted at a spacing of 60 cm between rows and 45 cm between the plants (Figure 1)

Fertilizer Application and Weeding

Organic manure was applied to the growing *A. annua* about 6 weeks after transplanting. Weeding was conducted manually (i.e using hand and hoe).

Plant Collection, Identification and Preparation

The plant was collected at its full flowering stage [23], it was then identified and authenticated by a Taxonomist at the Ethnobotany Unit of Bioresources Development Centre, Kano, a reference sample number, BDCKN /EB/1896 has been deposited in the Herbarium. The leaves were dried and powdered using pestle and mortar.

Extraction of the Powdered Plant Material

The powdered plant material (100 g) was successively macerated with hexane and distilled water (500 ml each) for 48 hours, the mixture was shaken occasionally. The filtrate obtained was evaporated to dryness at 40 ^{0}C using a rotary evaporator and a water bath.

Preliminary Phytochemical Screening of Aqueous and Hexane Extracts of *A. annua*

The Preliminary Phytochemical screening of the aqueous and hexane extracts was conducted using the standard laboratory procedures [24-28]

Anti-plasmodium Activity of Aqueous and Hexane Extracts of *A. annua*

Sourcing of Malaria Parasites for the Assay

Malaria parasites infected blood samples were obtained from Nana Diagnostic Laboratory and Clinic of Bayero University, Kano, Nigeria, consent of the management allowed the collection of the samples. Venous blood from patients recommended for malaria parasites test (MP test) was obtained using 5 ml disposable plastic syringes and needles. The samples were immediately transferred into K_3-EDTA disposable plastic sample bottles with tightly fitted plastic corks.

Confirmation of *Plasmodium falciparum* positive blood samples using thin smear

After thorough mixing, a small drop of each blood sample was placed at the centre of a clean grease free glass slide, at least 2 mm from the edge using a clean capillary tube. A clean cover slip was placed in front of each drop at an angle about 45° and then drawn backward to be in contact with the drop of blood, the drop was then allowed to run along the full length of the edge of the cover slip. With a fast and smooth movement, the cover slip was pushed forward to form even thin smear on each glass slide. The smear was left to dried and stained using the Leishman's stain. The stained slide was then observed under a high power objective (×100) using oil immersion. The smears were screened thoroughly for *Plasmodium falciparum* infected RBC's. An average parasitemia was obtained from the reading of 3 microscopic fields. Blood samples with 5 % parasitemia were used for the research [29].

Separation of the Erythrocytes

About 5 ml of blood sample with 5% parasitemia was centrifuged at 2500 rpm for 15 minutes. After centrifugation, the supernatant (plasma) was discarded while the sediments (erythrocytes) were further centrifuged with normal saline at 2500 rpm for 5 minutes. The supernatant was then discarded and the erythrocytes were suspended in normal saline.

Preparation of *Plasmodium falciparum* Culture Medium

The medium was prepared by dissolving 10.4 g of the powdered material into one liter of distilled water and then autoclaved at 121 °C for 15 minutes as instructed by the manufacturers. Venous blood (2 ml) from the main vein of white healthy Rabbit's pinnae was withdrawn using a disposable 5 ml syringe (BD 205 WG). This was defibrinated by allowing it to settle for at least one hour. The defibrinated blood was centrifuged at 1500 rpm using spectre merlin centrifuge for 10 minutes and the supernatant layer was collected in a sterilized tube. The sediment was further centrifuged at 1500 rpm for 5 minutes and the supernatant layer was added to the first test tube. The sediments were discarded and the serum collected was supplemented with the salt of RPMI 1640 medium (KCl 5.37mM, NaCl 10.27mM, $MgS0_4$ mM, $NaHPO_4$ 17.73mM, $Ca(NO_3)_2$ 0.42 mM, $NaHCO_3$ 2.5mM, and glucose 11.0 mM. (BDH ltd, UK). The medium was sterilized by 40 µg/ml gentamicin sulphate [30].

Preparation of the Test Concentrations

Exactly 20 mg of each of extract and the positive control drug (Artemether-lumefantrine) were dissolved in 1ml of dimethyl sulfoxide (DMSO) in separate vials (stock solution). Using serial doubling dilution, four different concentrations (10, 5, 2.5 and 1.25 mg/ml) of each extract were prepared.

In vitro Assay of the Extracts on *Plasmodium falciparum* Culture
Exactly 0.1 ml of test solution and 0.2 ml of the culture medium were added into a tube containing 0.1 ml of 5 % parasitemia erythrocytes and mixed thoroughly. The sensitivity of the parasites to the tested fractions was determined microscopically after incubation for 24, 48 and 72 hours at 37 °C. The incubation was undertaken in a bell jar glass containing a lighted candle to ensure the supply of required quantity of Carbon dioxide (about 5% Oxygen gas, 2% and about 93% nitrogen gas as demonstrated by [31].

Determination of Activity
At the end of each incubation period, a drop of a thoroughly mixed aliquot of the culture medium was smeared on microscopic slides and stained by Leishman's staining techniques. The mean number of erythrocytes appearing as blue discoid cells containing life rings of the parasite (that appeared red pink) was estimated and the percentage elimination by the samples was determined [31].
The activity of the tested samples was calculated as the percentage elimination of the parasites after each incubation period, using the formula below;

$$\% = \frac{N}{Nx} \times 100$$

Where;
% = Percentage activity of the extracts
N = Total number of cleared RBC
Nx = Total number of parasitized RBC
Note: RBC = Red Blood Cells

Statistical Analysis
The level of significance was tested using One-way ANOVA followed by Duncan Multiple Range Test (DMRT). Results were regarded as significant when $P \leq 0.05$.

Results and Discussion
The physical properties of hexane and aqueous extracts of *A. annua* were summarized in the table 1;

Table 1: Physical Properties of Hexane and Aqueous Extracts of *A. annua*

Property	Hexane Extract	Aqueous Extract
Colour	Dark green	Dark brown
Texture	Gummy	Gummy
Percentage yield	4. 92%	30.5%

Preliminary Phytochemical Screening of Hexane and Aqueous Extracts of *A. annua*
The preliminary phytochemical screening of aqueous and hexane extracts of *A. annua* revealed the presence of cardiac glycosides, steroids, terpenoids, alkaloids and flavonoids in both extract. In addition, saponins, tannins, phenolic compounds and anthraquinones were only detected in the aqueous extract (Table 2).

Table 2: Phytochemical Constituents of Aqueous and Hexane Extract of *A. annua*

Phytochemicals	Extracts	
	Hexane	Aqueous
Tannins	-	+
Anthraquinones	-	+
Cardiac glycosides	+	+
Saponins	-	+
Phenolic compounds	-	+
Flavonoids	+	+
Alkaloids	+	+
Terpenoids	+	+
Steroids	+	+

Key
+ = Present
- = Absent

In vitro Anti-plasmodium Activity of Hexane and Aqueous Extracts of *A. annua*
The *in vitro* anti-plasmodium screening of hexane and aqueous and extracts of *A. annua* showed that both extracts have anti-plasmodium activity. The highest activity was observed in the aqueous extract at the tested concentrations, 10, 5, 2.5 and 1.25 mg/ml ($P \leq 0.05$) when compared to the positive control, Artemether-lumefantrine (Table 3).

Table 3: *In vitro* Anti-plasmodium Activity of Hexane and Aqueous Extracts of *A. annua*

Concentration (mg/ml)	Percentage Elimination		
	Positive Control	Aqueous Extract	Hexane Extract
10	100^a	100^a	84^c
5	100^a	94^b	82^c
2.5	94^b	82^c	74^d
1.25	82^c	73^d	68^e

Values in the same column with different superscript differs significantly ($P \leq 0.05$)

Fig 1: Transplanted Seedlings of *Artemisia annua*, Kano State, Nigeria

Fig 2: Growing *Artemisia annua*, Kano State, Nigeria

Discussion

The plant was collected at full flowering stage; this is due to the fact that the quantity of the active component, artemisinin is highest at this stage [21, 22]. Although, the artemisinin content has not yet been quantified, the study has shown that *A. annua* has well adapted to the climatic conditions of Kano State [Figure 1 and 2]. Thus, Kano State may soon join the league of *A. annua* producers in Africa. The world market for products including artemisinin derivatives is now growing rapidly and the demand for artemisinin is increasing, finding suitable geographic regions for *A. annua* is a critical research area [4].

The phytochemical screening showed that cardiac glycosides, steroids, terpenoids, alkaloids and flavonoids were present in both extract, while saponins, tannins, phenolic compounds and anthraquinones were only detected in the aqueous extract. These variations could be attributed to the differences in the polarity index of hexane and water [32]. The absence of saponins and tannins in the hexane extract supported the finding of [33].

The *in vitro* anti-plasmodium screening showed that both hexane and aqueous extracts of *A. annua* have anti-plasmodium activity. The aqueous extract had the highest activity at 10, 5, 2.5 and 1.25 mg/ml ($P \leq 0.05$) when compared to the positive control drug, Artemether-lumefantrine. The anti-plasmodium activity observed could be attributed to the presence of Artemisinin and other antimalarial compounds in the plant. Also, the presence of a complex matrix of phytoconstituents in the aqueous extract might enhance the bioavailability and thus the anti-plasmodium activity of the extract [34].

Artemisinin and its derivatives exert their anti-plasmodium properties by interfering with the plasmodial hemoglobin catabolic pathway and inhibition of heme polymerization. Also, the flavonoids present in *A. annua* are linked to suppression of CYP450 enzymes responsible for altering the absorption and metabolism of artemisinin in the body [35, 36]. Recently, the antiplasmodial activities of artemetin, casticin, chrysoplenetin, chrysosplenol-D, cirsilineol and eupatorin were reported to be the possible synergistic compounds found in the antimalarial tea, these flavonoids have been extracted from *A. annua* and their IC_{50} were much higher than that of artemisinin [37, 38].

The anti-plasmodium activity reported in this study is comparable to those reported for some indigenous plants like *Cassia nigricans, Jatropha curcas, Mangifera indica, Vernonia amygdalina* [32, 39-41].

Although, *A. annua* may not cause haemato toxicity or hyperlipidemia, it should be used with caution during pregnancy due to a potential risk of embryo toxicity at higher dose [42]

Conclusions

To the best of our knowledge, this study has reported for the first time the cultivation of *A. annua* in Kano State, Nigeria, and it can be concluded that Kano soils may be suitable for the commercial cultivation of this important medicinal plant. A study is ongoing to isolate, identify and quantify the Artemisinin content.

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