Herbal Medicine in the Treatment of Malaria: Vernonia amygdalina: An Overview of Evidence and Pharmacology

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1. Introduction

Traditional medicines occupy a central place among rural communities of developing countries for the provision of health care in the absence of an efficient public health care system (WHO, 2003).

The use of traditional remedies is common in sub-Saharan Africa, and visits to traditional healers remain a mainstay of care for many people because of preference, affordability, and limited access to hospitals and modern health practitioners (Homsy et al., 1999).

It is an important part of medical care in Uganda and throughout Africa, representing first line therapy for 70% of the population, (Homsy et al., 2004). For many, traditional herbal medicines may be the only source of treatment available. The main reasons to explain this are: traditional medicines are often more accessible compared with licensed drugs; there are no records attesting the resistance to whole-plant extracts possibly due to the synergistic action of their constituents; phytotherapy, possibly produces fewer adverse effect than chemotherapy (Willcox and Bodeker, 2000).

In Africa more than 2,000 plants have been identified and use as herbal medicines. However, very few of these plants have been screened for safety in resource-constrained countries including Uganda. It is time to ask in a systematic and scientific manner how these local treatments work, what are the best means to establish their safety and can they be used as traditionally prepared? The source of antimalarial drugs such as artemisinin derivatives and quinolines currently in use today were isolated from medicinal plants. Renewed interest in traditional pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for plants usage, but also with the discovering of novel compounds of pharmaceutical value for the treatment of malaria.

Herbals are as old as human civilization and they have provided a complete storehouse of remedies to cure acute and chronic diseases. Numerous nutraceuticals are present in medicinal herbs as key components. Scientific evaluation of herbal products has been limited, yet herbal products are the most commonly consumed health care products. Because of known pharmacological effects and potential interaction of many of these compounds with therapeutic drugs, a history of herbal intake should be considered as part of routine medical history and should be evaluated before any change in prescription drugs and before medical procedures (Schwartz et al 2000).
At present, work conducted on traditional medicine in Africa has mainly concentrated on the collection, identification, and classification of herbal products for treatment of different ailments. However, research in the areas of safety and toxicology is lacking. Traditional medicines, like modern pharmaceuticals can do harm, but because humans have been using herbal drugs for long time, they are considered safe and non-toxic so the toxicological actions of these agents have been mostly ignored, even while the effectiveness is either already known or under study (O'Hara et al., 1998). Willcox (1999), carried out a clinical study on ‘AM’ (coded to protect the intellectual property right of the traditional healers), a popular antimalarial herb that has a long history of use among the people of south-western Uganda. In her result, 'AM' significantly reduced parasite count between day 1 and day 7, patients showed symptomatic improvement, but 50% of them experienced some side effects including vomiting, nausea and stomach upset. These were partly attributed to malaria itself as well as to 'AM' ingestion (Willcox, 1999).

If the origin of herbs’ toxicity is not identified, the adverse effects may be wrongly associated with other environmental exposures or some traditional belief. Failure to establish the true cause of exposure also means that the patient continues taking the toxic herb. Thus, the screening of traditional remedies for safety and toxicity is recommended to protect public health. On the other hand, several plants used in Uganda traditional medicine can cause damage to genetic material and therefore have potential to cause long-term damage in patients when administered as medicinal preparations (Steenkamp, 2005).

1.1 The main groups of active principle or constituents obtained from medicinal herbs
The therapeutic effects of plant species are determined by their constituents. These affect the condition and function of the various human body organs, clear up residual symptoms or destroy the cause of the disease in most cases infectious micro-organisms. They help increase the body’s resistance to disease, retard or delay the processes of natural aging or facilitate the adaptation of the organism to certain conditions (Forantisek, 2001). Over the centuries, man used medicinal plants even though he was unable to find a rational explanation for their effects. It was not until the 19th century and after the rapid development of organic chemistry and pharmacology, that man determined which active or group of principles are responsible for a given therapeutic effect. Knowledge of these substances frequently served as a model for the synthetic preparation of new medicines, enabling the drug to be modified and made more effective. It was soon discovered that a better therapeutic effect was often obtained by the particular combination of active principle naturally present in each plant that by a single, isolated substance. The most important constituents are the secondary metabolism in plants, which includes alkaloids, glycosides, essential oils, tannins and the bitter principles. Products of secondary metabolism of plants are responsible for the plants’ therapeutics effects. Of greater importance for the plants themselves of course are the products of primary metabolism which are necessary for the proper function of the basic life processes in plants. Primary metabolism products are also used by man. This group includes sugars, fatty oils, organic acids, vitamins and protein. These products of primary metabolism themselves may have no therapeutic effect but may possibly increase the efficiency of the therapeutically important principles (70)

1.2 Vernonia amygdalina
(Aseraceae), also called bitter leaf is a popular African vegetable that grows as a shrub or small tree indigenous to Central and East Africa including Uganda (Huffman et al, 1996). It
produces large mass of forage and is drought tolerant (Hutchison and Dalziel, 1963), it is 2 – 5 m with petiolate leaf of about 6 mm diameter and elliptic shape. The leaves are green with a characteristic odour and a bitter taste. No seeds are produced and the tree has therefore to be distributed through cutting. It is known locally as Omubirizi in south-western Uganda and used traditionally for pain relief and malaria attack. Patients are instructed to soak the plant leave in hot water about (80°C). They should then drink half a glass (about 0.25l) two times daily for 4-7 days. Smaller doses are prescribed for children according to their weight. They are used as vegetable and stimulate the digestive system in some other countries in the continent.

This plant has ethnomedical use in treating veneral diseases, gastrointestinal problems and malaria (Kambizi et al, 2001; Huffman et al, 2003; Hamill et al, 1992). Furthermore, they are used as local medicine against leech, which are transmitting bilharzias. Free living chimpanzees eat the leaves, if they have attacked by parasites (Huffman, M.A. 2003). There are reports concerning the hypoglycaemic, antineoplastic antibacterial and antioxidant properties of the plant. (Akah et al, 1992; Izevbige et al, 2004; Taiwo et al, 1999; Iwalewa et al, 2005). Despite the varied uses of the plant, there are no information on its analgesic properties and exact toxicology on sub-chronic exposure, although some reports describe its antiplasmodial effects (Abosi et al 2005; Masaba et al 2000; Tona et al, 2004; Wilcox et al, 1999). Previous phytochemical reports have shown the presence of steroid, saponins, flavonoids.

2. Methods

2.1 Research design

Ninety traditional healers were identified through community and healers association leaders. Once identified, study staff members approached the individuals to determine eligibility. Eligibility criteria included 30 years of age and older, recognition as a traditional healer by the local community council, and having established an active practice in the community. Three districts (Kanungu/Bwindi area, Bushenyi and Mbarara) south-western Uganda were identified. With consent a taxonomist samples of antimalarial herb were obtained for this research study.

To evaluate consistency, interviews were conducted by a person specially trained in interview administration and who is fluent in the language of the participants. The ethnographic interview included questions about common plant names, sources of products, method of preparation, purpose of use, quantity of herbs use and perceived benefit of herbs in ameliorating malaria symptoms and improving overall health. Ethical forms were used in order to assure them of the defense of their knowledge and intellectual property right was applied. Traditional healer’s name, age, gender, and ethnicity tribe, were asked. We relied on the knowledge of healers and the taxonomist to select the products of greatest importance. This enabled us target products that have a high likelihood of possessing significant pharmacological activity.

A strategy was developed that respected the healers’ rights to maintain propriety of unique blends of herbal medicine. Also, a memorandum of understanding was developed that disclosed our study objective, which is to characterize the pharmacologic activity and to elucidate the toxicity of these remedies in order to determine any potential adverse effect. We emphasized that we were interested in general knowledge about the remedy and not in specific formulation, and that it was not our intention to use the knowledge gained from this research.
study for commercial profit. Rather we would report back to them in a workshop the remedy’s indication and contraindication after completion of the study. We requested that all parties sign the agreement and copies were kept in a secure file. The result of the study was used to determine the most common botanical/herbal products used by the healers to treat malaria. Among the herbal products, *Vernonia amygdalina* which appeared in 80% in the interview was chosen for studies.

**Fidelity level:** The fidelity level (Fl) (Alexiades et al., 2000) among the healers from the same district was calculated according to the following formula:

\[
\text{Fl (\%)} = \left( \frac{N_p}{N} \right) \times 100
\]

Np is the number of healers from one given district that claim a use of a plant species to treat a particular disease, and N is the number of healers from the same district that use the plants as a medicine to treat any given disease. The formula was applied in order to compare data from different district where the survey was performed.

### 2.2 Extract preparation

Leaves of *V. amygdalina* were collected from the botanical garden of the Rukararwe Traditional Medicine Health Center, a division of Rukararwe Partnership Workshop for Rural Development (RPWRD) in Bushenyi district. The plant was authenticated by Dominic Byarugaba, a botanist with the Department of Plant Biology, Mbarara University of Science and Technology (MUST), Uganda. A voucher specimen is kept in the department. The plant material was air-dried and grounded into a coarse powder. About (350g) of this powder were macerated in 2 L of distilled water for 24 h with occasional shaking (GFL 3017 Germany) and then extracted using a soxhlet extractor (Gallemkamp, England). The resultant extract was evaporated in a water bath, under controlled temperature not exceeding the one used by the healers in their plant preparation (80°C) to yield a 32.3 g of semi solid residue.

### 2.3 Animals

Adult Wistar rats (130 – 150g) and Swiss albino mice (18 – 26g) of either sex, maintained at Animal Facility Centre were used for the acute toxicity and 14 days sub-chronic experiments. The animals were kept in plastic cages at room temperature and moisture, under a naturally illuminated environment of 12:12 h dark/light cycle. Animals were fed the standard diet and had access to tap water *ad libitum.* Male Wistar rats were used for the 6 week exposure studies. At dosing, animals were 8 to 12 weeks old. All animals were clinically monitored at the time of delivery and during acclimation period, and were maintained at the Animal Facility Centre. Animals found unsuitable were excluded from the experiment. Animals were housed in plastic cages under same conditions described above; they were fed with standard diet (Mice pallet), and had access to tap water *ad libitum.* The animal experiments were conducted according to the NIH Guide on Laboratory Animals for Biomedical Research (NIH, 1978) and ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann et al, 1983).

### 2.4 Antiplasmodial activity

The test was conducted according to the curative procedure described earlier (Saidu et al, 2000; Adzu et al, 2003). A donor mouse infected with rodent malaria (*Plasmodium berghei*)
was anaesthetized with chloroform and the abdomen opened. Blood was collected through
cardiac puncture with a sterile needle and syringes in such a way that 0.2 ml of the blood
containing about 1 x 10^7 infected red cells. Twenty-five mice were inoculated i.p. with 0.2
mL each. The mice were then randomized and grouped into five (n = 5) and treated as
follows on day 3 (D3). Group 1 received normal saline, group 2 received chloroquine (CQ,
5mg/kg, i.p.); while groups 3-5 received (50, 100, and 200 mg/kg, i.p.). The treatment
continued daily until day 7. Thick and thin blood smear were collected daily from tail blood,
fixed with methanol, stained with 4% Giemsa at pH 7.2 for 45 min and examined
microscopically (Nikon YS2-H Japan). The increase/decrease in parasitaemia, defined as the
number of infected and uninfected red blood cell RBCs, were counted on five different
fields, and mean survival time (within 30 days) was recorded.

2.5 Evaluate the antinociceptive activity of the selected antimalarial
2.5.1 Acetic acid-induced writhing in mice
Analgesia was assessed according to the method of Siegmund et al. (1957), as was modified
by Koster et al, 1959). The mice were divided into different groups (of five mice each). They
were differently pre-treated with the extract (50, 100, 200mg/kg i.p), aspirin (100mg/kg i.p)
and normal saline (10ml/kg i.p). 30, 60, 90 and 120min after the treatment, 0.7%
acetylsalicylic acid (ASA) (Sigma Chemicals Co) 10ml/kg i.p was administered to the mice.
They were placed in a transparent cage, 5mins after administration of acetic acid, the
number of abdominal constrictions (writhes) made within 10min of every mouse was
recorded. The results of the treatment groups were compared with those of normal saline
pre-treated control. The percentage of the writhes was calculated as (test mean/control
mean) x 100.

2.5.2 Formalin test
For the formalin studies, rats were injected with 0.05 ml of formalin (2.5% formaldehyde)
into the sub-plantar surface of the left hind paw 30min after treatment with saline, extract or
ASA. Severity of pain (for both control and test groups (n = 5)) were simultaneously
observed and rated as scores using (Dubuisson et al, 1977) pain measurements. This was
rated as follows: (0) rat can bear weight on injected paw; (1) light resting of the paw on floor;
(2) partial elevation of the injected paw, and (3) total elevation, licking and biting of paw.
These observation were recorded every minute for the first 10 min (early phase) and at
every 5 min for the period between 15 and 60min (late phase).

2.5.3 Tail-flick test
This test as first described [28], and subsequently modified (Janssen et al, 1969; Asongalem
et al 2004a) was used. Briefly, before treatment, the terminal (2 cm) of each rat tail was
immersed in hot water contained in a 500ml beaker and maintained at 55 ± 1°C using a
thermo-regulated hot plate (Ugo Basile, Socrel DS-35) and the time (in seconds) between the
onset of stimulation and tail withdrawal was measured as the tail-flick latency. Twenty five
rats that shows response within 0 – 4 s were selected and grouped into five (n = 5) for the
study. Immediately after basal latency assessment, normal saline, reference drug (Pethidine
hydrochloride; Bayer, England) or the plant extract were administered and the reaction time
again recorded at 30 and 60min. after administration the extract. (50 – 200 mg/kg p.o)
(Sanchez-Mateo et al, 2006).
2.6 Acute and sub-chronic toxicity of the herbs

2.6.1 Acute – toxicity test

The intraperitoneal (i.p) acute toxicity of the extract was evaluated in Swiss albino mice using a slightly modified Lorke’s method (Lorke, 1998). Briefly, this method involved the determination of LD$_{50}$ value in biphasic manner. The animals were starved of feed but allowed access to water 24 h prior to the study. In the initial investigatory step (phase 1), a range of doses of the extract producing the toxic effects was established. This was done by intraperitoneal administration of geometric doses of the extract (10, 100, 1000, 1500 mg/kg) to four groups of mice (n = 4). Based on the results obtained, a phase 2 investigatory step was done by giving more specific doses (200, 400, 600, 800 mg/kg i.p) to four other groups of mice.

The mice were observed for 24 h for such behavioral signs as, excitement, dullness, ataxia or death. The LD$_{50}$ was estimated from the geometric mean of the dose that caused 100% mortality and the dose which caused no lethality.

The same procedure was used in rats which received (1000, 2000, 3000, 5000 mg/kg oral) in phase 1 and (1500, 3000, 4000, 5000 mg/kg oral) in the second phase.

2.6.2 Two weeks sub-chronic toxicity test

Twenty eight Wistar rats divided into four weight-matched groups, of seven rats each, (both sexes) were used for the study. Three test groups received 500, 1000, 2000 mg/kg $V. amygdalina$ by gavage with biomedical needle (G 16, Length 76.2mm, diameter 3mm, Straight Harvard Apparatus) for 14 days. The control group received normal saline. Food and water intake were measured daily while the animal’s body weights were taken every other day. All animals were observed at least once daily for clinical signs (behavior such as lethargy, hyperactivity, depression and diarrhea). On day 14, immediately prior to euthanasia, all animals were anesthetized with chloroform and bled via the descending aorta for hematology and clinical chemistry determination. Organs were dissected and weighed to determine absolute and relative weight. The blood for clinical chemistry was allowed to clot in microtainer separator tubes, centrifuged and sera collected and stored at -20°C till ready for biochemical analyses. Commercial kits for Biosystem BTS-310, (Biosystem S.A Costa Brava 30, Barcelona, Spain) and Vitrous DT systems, Orthoclinical Diagnostics Johnson Company (US17) were used to analyze liver function, renal function and the electrolyte test.

The hematological tests were carried out in an ethylene diamine tetra-acetic acid (EDTA) – anticoagulated blood. Hemoglobin (Hb) concentration was analysed by the cyanmethaemoglobin method, packed cell volume (PCV) by the micro-method, and white blood cell (WBC, total and differential) and platelet counts by visual methods Dacia et al, 1991). The mean cell hemoglobin concentration (MCHC) was calculated by dividing Hb by PCV ( Dioka et al, 2002).

2.6.3 Six weeks sub-chronic toxicity test

For the six-week exposure studies, twenty eight male Wistar rats were divided into four weight-matched groups of seven rats each. Three of the four test groups received 750 1500, 3000 mg/kg $V. amygdalina$ by gavage with biomedical needle (G 16, Length 76.2mm, diameter 3mm, Straight Harvard Apparatus) consecutively for 43 days. The control group received distilled water vehicle only, via gavage.
Food and water intake were measured daily while the animal’s body weights were taken preexposure and weekly during exposure. All animals were observed at least once daily for clinical signs (behavior such as lethargy, hyperactivity, depression and diarrhea), and once/week clinical observations were performed on each rat by removing it from its cage and examining it for changes in general health. On day 44, immediately prior to euthanasia, all animals were anesthetized with chloroform and bled via the descending aorta for hematology and clinical chemistry determination. Organs were dissected and weighed to determine absolute and relative weight. The blood for clinical chemistry was allowed to clot in microtainer separator tubes, centrifuged and sera collected and stored at -70 °C until performing the biochemical analyses. The biochemical and hematological parameter were analyzed in the (New Italian Laboratory). Mbarara University of Science and Technology (MUST).

The collected plasma samples were analyzed using a (HumanStar 180 and Humalyzer 2000, Germany) autoanalyzer. Sixteen biochemical parameters were studied; plasma sodium, potassium and chloride were also assayed with (Humalyzer 17410, Germany) autoanalyzer with appropriate Human Kits.

Hematological parameters were analyzed using (Beckman and Coulter, USA) with the appropriate kit (Coulter ACT 5 diff Diluent’s).

3. Results

In the study, a total of 90 healers from - sub-county from the three districts were interviewed. Most of them were members of traditional healers association in their district. Men dominate the practices of traditional medicine.

Most of the traditional healers interviewed (81 out of 90), indicated to have passed through a several routes to become healers. All of the 81 were raised in families with traditional healers, who had involved them in the healing process and they were familiar with the profession. After some time they started to practice on their own. All reported on the importance of the family environment of a traditional healer in the context of acquiring knowledge and experience by members of the family. Furthermore it was reported that, the entrance into practice through these routes is facilitated through training by an experienced healer or a family member, who decides when the apprentice is ready to become an independent healer or to take up the practice. Eight out of 81 of the traditional healers indicated to have been instructed through dreams by their ancestral spirits to take up the traditional healing practice. They were required to learn and observe traditional healing procedures as dictated by the spirits. Indeed, the ancestral spirits are considered to be supernaturally powerful and ignoring them is to invite punishment to an individual and or her family. Of the 90 healers interviewed, two indicated to be self-taught healers.

The leaves are the most frequently used plant part (56.3%), the root and fruits are used about 30% and 8.5% respectively, and the less used plant part is the bark (5.3%). The majority of the remedies are prepared in the form of decoction of fresh leaves. In our study area people do usually not store remedies for prolonged period of time. When needed they go out and collect the plant and prepare the remedy from fresh or sun dried material. Powders are prepared by pounding the fresh plant part or the crushed plant material after sun drying.

Decoction is the most frequent method way of remedy preparation (65%) followed by infusion (13%), which is used for the powders; the maceration (11%) is mostly used for the
root preparation. Some remedies are prepared from a single plant species; however, in a few cases mixtures of plants or other substances are added as noted in Table 2.

Most of the remedies are taken orally and by external application as body bath, steam bath, and as ointment. Fumigation is mainly used in the treatment of headache and chest pain. For most of the remedies, the administered dose depends on the patient’s age, physical and health condition, and the duration of the illness. The doses vary from a teacup (70 ml) for adults to a handful (25 ml) for a child; a lack of agreement among the healers on doses of remedies was sometimes noted. The variation of the doses from one healer to another may show that the plants have a low degree of toxicity. For pharmacological investigation the active doses of these plants may not be high since they appear to treat the patients with low doses. The duration of treatment is not given for all remedies. According to the healers duration of treatment is difficult to determine and depends on how long the patient has been ill. The patient is supposed to take the remedy until healed. The only person able to determine the end of a treatment is the patient himself since the remedy is taken at home in the absence of the healers.

The reported adverse effects for the use of these medicinal plants are vomiting and dizziness. According to the healers these effects are generally due to an overdose of the remedy. The adverse effects are generally moderate, and disappear at the end of the treatment. Also, patients are advice to drink a lot of milk, meat soup or porridge made from sorghum to help alleviate serious side effects.

| Districts | Sub-County             | Numbers of healers | Sex | Age range |
|-----------|------------------------|--------------------|-----|-----------|
|           |                        |                    | Males | Females |         |
| Kanungu   |                        | 5                  | 4    | 1        | 28–76   |
|           |                        | 5                  | 3    | 2        | 30–79   |
|           |                        | 5                  | 5    | 0        | 42–85   |
|           |                        | 5                  | 5    | 0        | 35–55   |
|           |                        | 5                  | 4    | 1        | 65–85   |
| Bushenyi  | kyangyenyi             | 5                  | 5    | 0        | 47–70   |
|           |                        | 5                  | 5    | 0        | 38–80   |
|           |                        | 5                  | 5    | 1        | 27–70   |
|           |                        | 5                  | 5    | 0        | 49–104  |
|           |                        | 5                  | 5    | 0        | 56–80   |
| Mbarara   | Nyakajojo              | 10                 | 8    | 2        | 45–73   |
|           | Kinoni                 | 10                 | 10   | 0        | 55–62   |
|           | Rugando                | 10                 | 9    | 1        | 50–61   |
|           | Bugamba                | 10                 | 9    | 1        | 50–59   |

Table 1. An overview of the traditional healers interviewed.
3.1 Acute toxicity test
The median lethal dose LD\textsubscript{50} was established to be 560 ± 1.21 mg/kg i.p. in mice and 3.32 ± 0.15 g/kg oral in rats. Adverse signs of gaiting, reduction in stereotypic activities and deaths were however seen in high doses.

3.2 Acetic acid-induced writhing in mice
The aqueous extract of \textit{V. amygdalina} (50, 100, 200mg/kg i.p) exhibited a significant (P<0.05) antinociceptive activity against acetic acid-induced writhing in mice. 50 and 100 mg/kg oral doses exhibited a dose-dependent anti-nociception that progressively reduced over a period of 90 min post-treatment. However, at 120min the reduced anti-nociceptive activity increases again at these doses. The dose of 200 mg/kg on the other hand caused a total anti-nociception up to 120min. These results compared favorably with those of aspirin (100mg/kg i.p; Fig 1).

![Fig. 1. Effect of aqueous extract of \textit{V.amygdalina} leaves on acetic acid-induced writhing in mice for 5 min (NS, normal saline; Va, \textit{V. Amygdalina}; ASA acetysalicylic acid). All data are presented as means ± S.E.M., n=5. The asterisk (*) denotes, significance (p<0.05) between treated group and NS control](image)

3.3 Formalin and tail-flick tests
The extract at doses of 100 and 200 mg/kg induced significant (p<0.05) reduction in pain response in both phases (aphasic and tonic) of pain induced by formalin in comparison with control (Fig 2).
In all cases ASA, a positive analgesic agent demonstrated significant anti-nociceptive action with a slightly stronger pharmacological intensity than \textit{V. amygdalina} at 200 mg/kg in the late phase. The extract exerted no significant effect on nociception in tail-flick as values...
obtained correspond with those with saline. However pethidine the reference agent markedly prolonged the tail-flick reaction time in rats (Fig 3).

Fig. 2. Effect of aqueous extract of *V. amygdalina* leaves on formalin test in rats (NT, non treated animals; Va, *V. Amygdalina*; ASA acetyl salicilic acid). All data are presented as means ± S.E.M., n=5. The asterisk (*) denotes significance (p<0.05) between treated group and NT control.

Fig. 3. Effect of aqueous extract of *V. amygdalina* leaves on thermal stimulus-induced tail-flick test in rats (NS, normal saline; Va, *V. Amygdalina*; PH, penthidine hydrochloride). All data are presented as means ± S.E.M., n=5. The asterisk (*) denotes significance difference (p<0.05).
3.4 Antiplasmodial activity

The extract caused a significant (P < 0.05) and dose-dependent reduction in mean parasitaemia in mice infected with Plasmodium berghei in comparison to CQ (5 mg/kg). The extract caused a parasitaemia reduction of 52% in 50 mg/kg, 64% and 73% in 100 and 200 mg/kg respectively (Table 2). One animal death was recorded in the 200 mg/kg extract groups throughout the 30 days observation period of the experiment while the remaining mice recovered fully. All mice in the saline group were lost within 15 days of the study.

| Treatment       | Dose (mg/kg, p.o.) | Mean parasitaemia (D₀–D₈) | Inhibition (%) |
|-----------------|--------------------|---------------------------|---------------|
| NS (10ml/kg)    | -                  | 14.2 ± 0.25               | -             |
| V. amygdalina   | 50                 | 6.7 ± 0.17*               | 52.8          |
|                 | 100                | 5.0 ± 0.22*               | 64.8          |
|                 | 200                | 3.7 ± 0.17*               | 73.9          |
| CQ              | 5                  | 3.1 ± 0.25*               | 78.2          |

Values are mean count S.E.M. (for n = 5)
*Significantly (p<0.05) different from saline control group
N.S., Normal saline

Table 2. Curative activity of the aqueous extract of V. amygdalina and CQ against Plasmodium berghei in mice.

3.5 Sub-chronic toxicity (14 days exposure)

No treatment deaths occurred and no treatment related clinical signs were noted during the study. The extract did not exert significant changes on mean body and organ weight, fluid and food intake (Table 3). All animals demonstrated a progressive increase in body weight during the exposure. The organ weights were expressed as a percentage of the body weight (% relative organ weight), rather than as absolute weights, so as to take into consideration differences in the organ weight that may solely be attributable to differences in the body weights of the respective rats. The hematology result showed a significant decrease (p<0.05) in red blood count at the dose of 2000mg/kg compared to control (Table 4). The result of the clinical chemistry parameter showed a dose-dependent increase in direct and total bilirubin, there was also an increase in uric acid at the doses of 500 and 1000mg/kg compared to control (Table 5).

3.6 Six weeks exposure

Clinical observation, Body and organ weight

At the end of the 43-day-period of drug administration, No overt signs of toxicity were seen in any of the animals during the course of the study. No statistical difference was observed between the body and organ weight of the control group and the assay group in the male rats receiving the three doses, all animals exhibited a gain in body weight. Organ weights (% relative organ weights) were similar to those of the corresponding organs from the control. (Fig 5 and 6).
Table 3. Effect of *V. Amygdalina* on daily food and fluid intake, body weights and organs weight of rats (14 days exposure).

| Treatment (mg/kg) | Food Intake (g SEM) | Fluid Intake (mL SEM) | Initial Body Weight (g) | Final Body Weight (g) | Liver (Absol. Relat.) | Heart (Absol. Relat.) | Kidney (Absol. Relat.) | Lungs (Absol. Relat.) | Spleen (Absol. Relat.) |
|------------------|---------------------|----------------------|-------------------------|-----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| 0 (Control)      | 131.82 7.04         | 155.63 11.64         | 147.92 7.35             | 178.92 6.51           | 7.03 0.18 0.19       | 7.96 0.65 0.35       | 1.43 0.49 0.79        | 1.42 0.12 0.80       | 0.07 0.84 0.05        |
| 500              | 131.82 10.52        | 148.33 19.22         | 161.38 14.77            | 181.11 14.11          | 6.78 0.42 0.47       | 6.72 0.83 0.46       | 1.34 0.09 0.76        | 1.66 0.23 0.97       | 0.20 0.76 0.62        |
| 1000             | 113.49 10.14        | 120.16 6.51          | 137.62 14.32            | 158.17 17.93          | 5.37 0.58 0.52       | 5.64 0.64 0.44       | 1.26 0.10 0.85        | 1.50 0.10 0.99       | 0.11 0.63 0.46        |
| 2000             | 129.82 12.20        | 147.16 12.18         | 134.58 9.72             | 156.94 6.85           | 6.43 0.41 0.41       | 6.74 0.74 0.48       | 1.36 0.09 0.88        | 1.33 0.09 0.87       | 0.07 0.74 0.42        |

Values are expressed as mean ± S.E.M. for n = 7.

Relative organs weight = Absolute Organ Weight / Final Organ Weight × 100.
Table 4. Effect of *V. amygdalina* on selected hematological parameter in rats (14days Exposure)

| Parameter            | Control       | 500mg/kg     | 1000mg/kg    | 2000mg/kg    |
|----------------------|---------------|--------------|--------------|--------------|
| Hb (g/dl)            | 12.17±0.27    | 9.97±0.73    | 11.88±0.78   | 10.54±0.47   |
| PCV (%)              | 36.14±0.79    | 29.71±2.20   | 35.42±2.34   | 31.42±1.41   |
| MCHC (g/dl)          | 32.14±0.34    | 30.28±0.60   | 32.00±0.65   | 30.85±0.40   |
| RBC (x10\(^12\)/L)  | 4.64±0.13     | 3.84±0.25#   | 4.30±0.35#   | 3.58±0.14#   |
| Platelet (x10\(^9\)) | 156.42±3.77   | 139.85±4.38  | 153.28±6.95  | 136.71±3.82* |
| WBS (x10\(^9\))     | 6.57±0.43     | 5.57±0.87    | 6.68±0.51    | 4.74±0.89#   |
| Neutrophil (%)       | 20.57±2.42    | 25.57±1.95*  | 24.00±1.77*  | 21.00±1.67   |
| Lymphocyte (%)       | 79.28±2.53    | 74.42±1.95   | 77.4±2.47    | 83.28±3.48*  |

* Values are expressed as means ± S.E.M for n = 7
* Significantly increased (P ≤ 0.05) compared to control.
# Significantly reduced (P ≤ 0.05) compared to control.

Table 5. Effects of *V. Amygdalina* on Clinical Chemistry Parameter in rats. (14days exposure)

| Parameters            | Control       | 500 mg/kg    | 1000 mg/kg   | 2000 mg/kg   |
|-----------------------|---------------|--------------|--------------|--------------|
| AKL. Phos (iu/L)      | 212.1±2.21    | 215.5±3.77   | 218.8±8.11   | 218.7±3.45   |
| Total Protein (g/L)   | 71.5±1.11     | 74.3±2.69    | 77.0±0.54    | 73.7±2.21    |
| Albumin (g/L)         | 38.3±1.25     | 39.7±1.04    | 40.0±0.94    | 39.6±0.76    |
| Direct Biliru (µMol/L)| 1.8±0.82      | 2.5±1.01*    | 3.2±1.51*    | 4.0±1.78*    |
| Total Biliru (µMol/L) | 4.2±2.65      | 6.28±2.63*   | 5.71±2.53*   | 6.57±3.19*   |
| ALT (iu/L)            | 32.7±5.80     | 34.60±10.18  | 35.10±11.04  | 33.70±6.23   |
| AST (iu/L)            | 42.28±10.27   | 40.43±10.68  | 41.29±12.32  | 41.14±5.52   |
| Chol (µMol/L)         | 1.23±0.32     | 0.92±0.33    | 1.25±0.34    | 1.10±0.28    |
| Trig (µMol/L)         | 0.51±0.04     | 0.86±0.18    | 0.60±0.60    | 0.47±0.06    |
| HDL (iu/L)            | 0.44±0.39     | 0.44±0.31    | 0.36±0.07    | 0.42±0.44    |
| LDL (µMol/L)          | 0.37±0.20     | 0.53±0.19    | 0.69±0.20    | 0.64±0.16    |
| VLDL (µMol/L)         | 0.23±0.02     | 0.39±0.08    | 0.27±0.02    | 0.21±0.03    |
| CHO/HDL (µMol/L)      | 2.80±0.84     | 1.95±0.73    | 2.60±0.69    | 2.72±0.80    |
| K+ (mMol/L)           | 6.92±0.28     | 8.04±0.50    | 7.02±0.52    | 7.35±0.48    |
| Na+ (mMol/L)          | 139.14±3.34   | 140.71±5.84  | 147.43±2.79* | 145.57±1.06* |
| Cl (mMol/L)           | 107.57±2.72   | 110.70±5.32  | 117.40±4.37* | 114.00±1.46* |
| HCO3⁻ (mMol/L)        | 23.71±0.91    | 22.88±1.32   | 21.14±0.39   | 21.14±0.40   |
| Uric Acid (µMol/L)    | 106.6±6.74    | 164.6±19.18  | 168.6±8.21   | 134.8±9.50   |
| Urea (mMol/L)         | 6.80±0.44     | 7.31±0.39    | 6.82±0.72    | 4.60±0.41#   |
| Creat (mMol/L)        | 76.57±2.80    | 73.71±8.4    | 70.14±2.52   | 76.85±4.06   |

Values are expressed as means ± S.E.M. for n = 7
* Significantly increased (P ≤ 0.05) compared to control.
# Significantly reduced (P ≤ 0.05) compared to control.

ALT, Alanin Amino transferase
AST, Aspartate amino transferase
ALK. Phos, Alkaline Phosphatase.
Fig. 5. Evolution of the mean ±SE of rat body weight during the subchronic toxicity of Vernonia amygdalina extract.

Fig. 6. Plots of the mean SE. Of the harvested organ weights during the sub-chronic toxicity study of V.amygdalina extract.
3.7 Hematological analysis

The mean values of the nine haematological parameters are reported in (Table 6).

| Dosage (mg/kg) | WBC (10⁹/l) | RBC (10¹²/l) | HGB (g/dl) | HCT (%) | MCV (fL) | MCH (pg) | MCHC (g/dl) | PLT (10⁹/l) | MPV (fL) | NE (%) | LY (%) |
|---------------|-------------|--------------|------------|---------|---------|---------|-----------|-----------|---------|--------|--------|
| Control       | 4.8 ± 0.8   | 8.4 ± 1.1    | 16.9 ± 2   | 44 ± 1  | 55 ± 1 | 38 ± 4 | 436 ± 161 | 43 ± 1   | 6.7 ± 0.1| 5.4 ± 0.8 |
| 750mg/kg      | 6.6 ± 1.0   | 8.7 ± 1.1    | 17.2 ± 3   | 45 ± 1  | 53 ± 2 | 38 ± 4 | 429 ± 161 | 42 ± 2   | 6.5 ± 0.3| 1.0 ± 0.4 |
| 1500mg/kg     | 8.0 ± 0.7*  | 8.2 ± 0.6    | 16.7 ± 1   | 43 ± 1  | 53 ± 2 | 38 ± 4 | 555 ± 161 | 38 ± 4   | 6.9 ± 0.6*| 6.7 ± 0.1 |
| 3000mg/kg     | 8.5 ± 0.3*  | 8.4 ± 0.2    | 17.1 ± 0.2 | 44 ± 0.8| 52 ± 2 | 38 ± 4 | 436 ± 161 | 43 ± 1   | 6.7 ± 0.3| 7.3 ± 0.3*|

Values presented as mean ± standard deviation.

WBC = White blood cell; RBC = Red blood cell; HGB = Hemoglobin; HCT = Hematocrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular hemoglobin; MCHC = Mean corpuscular hemoglobin concentration; PLT = Platelets; NE = Neutrophil; LY = Lymphocyte

Statistical analysis: ANOVA, then Dunnett’s test. *Mean value of group is significantly different from control at p<0.05.
There was also a significant increase in the values of Neutrophil count (1.08 ± 0.7 in 750 mg/kg/d) versus control (0.53 ± 0.02). However, no major difference was noted between the control and assayed groups.

### 3.8 Biochemical analysis

Clinical chemistry analysis of the rats indicate increased ASAT and ALAT ratios in the 1500 mg/kg/d dose, compared to control values (Table 7). In addition, significant increased in HDL levels were noted in the same dose level when compared to controls.

Table 7. Mean clinical chemistru values of rats after repeated (6 weeks) orad dosing with aqueous extract leave of *V. amygulina*.

| Dose (Mg/kg/d) | ASAT (U/L) | ALAT (U/L) | ALB (g/dl) | TRIG (mg/dl) | UREA (mg/dl) | CALC (mg/dl) | T.PROT (g/L) | PHOS (mg/dl) | CREAT (mg/dl) | T.PROT (g/L) | T. BILI (mg/dl) | URICAC (mol/l) | D.BILI (mg/dl) | T.CHO (mg/dl) | HDL (mg/dl) | LDL (mg/dl) | K (mmol/l) | NA (mmol/l) | AMY (U/L) |
|---------------|------------|------------|------------|--------------|--------------|--------------|--------------|-------------|---------------|--------------|----------------|----------------|---------------|--------------|--------------|------------|-----------|---------|----------|--------|
| Control       | 132 ± 26   | 93 ± 8     | 3.4 ± 2     | 120 ± 4      | 52 ± 1.5     | 12 ± 0.5     | 33 ± 1.0     | 24 ± 0.3    | 0.5 ± 0.02    | 80 ± 0.20    | 88 ± 0.19      | 4.1 ± 0.02      | 68.1 ± 0.16    | 5.9 ± 0.23   | 15 ± 0.23   |            |          |         |         |
| 750           | 202 ± 20   | 94 ± 12    | 3.5 ± 0.9   | 115 ± 9      | 56 ± 3.2     | 14 ± 0.4     | 39 ± 1.1     | 40 ± 0.6    | 0.6 ± 0.42    | 81 ± 0.12    | 69 ± 0.19      | 3.4 ± 0.2       | 55.5 ± 0.14   | 5.7 ± 0.14   | 14 ± 0.14   |            |          |         |         |
| 1500          | 373* ± 77  | 186* ± 26  | 3.7 ± 1.3   | 112 ± 3.1    | 56 ± 1.8     | 6.4 ± 0.5    | 24 ± 0.6     | 28 ± 0.6    | 0.6 ± 0.08    | 70 ± 0.18    | 83 ± 0.15      | 6.7* ± 0.2     | 59 ± 0.21     | 5.9 ± 0.14   | 14 ± 0.44   |            |          |         |         |
| 3000          | 113 ± 63   | 140 ± 23   | 3.3 ± 1.15  | 104 ± 3.7    | 57 ± 1.2     | 3.9 ± 0.3    | 24 ± 0.3     | 16 ± 0.5    | 0.5 ± 0.58    | 148 ± 0.10   | 76 ± 0.06      | 4.8 ± 0.1       | 56 ± 0.22     | 5.9 ± 0.15   | 15 ± 0.4    |            |          |         |         |

n = 7, Values presented as mean ± standard deviation

ASAT = Aspartate aminotransferase; ALAT = Alanine aminotransferase; ALB = Albumin; TRIG = Triglyceride; CAL = Calcium; T.PROT = Total protein; PHOS = Phosphate; CREAT = Creatine; T. BILI = Total bilirubin; D. BILI = Direct bilirubin; T.CHO = Total Cholesterol; HDL = High density lipoprotein; LDL = Low density lipoprotein; K = Potassium; NA = Sodium; AMY = Amylase

Statistical analysis: ANOVA, Dunnett’s test

*Mean values of groups is significantly different from control at p<0.05.*
4. Discussion

As herbal medicine become more popular especially in rural areas, pharmacological evidences to understand the action of these medicine and the underlying mechanisms, to support the proper and safe use of these medicine are indispensable. Our ethnopharmacology survey showed that medicinal plants are still widely used by the population in the area where the study was conducted. Several types of preparations of plants were used. The plants grow over an extended area and are used by healers separated by long distances.

In the current study, the analgesic effect of the leave extract of *Vernonia amygdalina* was assessed using three nociceptive animal models. In the writhing response model, acetic acid is injected into the peritoneal cavity of mice. The acid causes nociception in the abdomen due to the release of various substances that excite pain nerve ending (Raj, 1996). According to previous reports this assay is commonly used in mice to detect both central and peripheral analgesic efficacy of agents (Dewey, 1970; Fukawa et al 1980), *V. amygdalina* showed an ability to diminish the numbers of the writhing episode in a dose-dependent manner. The results of the writhing test alone did not ascertain whether antinociceptive effects are central or peripheral.

The formalin test is considered a model for chronic pain (Duduisson and Dennis, 1977). In this test, animals present two distinct nociceptive behavior phases, which probably involve different stimuli. The first phase initiates immediately after formalin injection and lasts 3 to 5 mins, resulting from chemical stimulations of nociceptor. The second phase initiates 15 to 20 mins after formalin injection, lasts 20 to 40 mins and seems to depend on a peripheral mechanism as well as a central one. While substance P and bradykinins are involved in the first phase, histamine, 5HT, prostaglandins and bradykinin are involved in the second phase. The effect of extract was significant in both phases. Since the mechanism of the analgesic effect of *V. amygdalina* is apparent in these two models, it can however be speculated that this effect may be linked to processes in the prevention of sensitization of the nociceptor, down-regulation of the sensitized nociceptor and/or blockade of the nociceptor at peripheral and/or central levels (Ferreira, 1990). Another possible mechanism may be that the extract blocks effect or the release of endogenous substances, including prostaglandin E2 (PG_E2) and PGF_2a, that excites pain nerve ending which is found in writhing response test model of mice (Deraedt, et al, 1980).

The extract fails to exhibit antinociceptive effect in the tail-flick test, as values obtain were not significantly different from control animals. Pethidine (50mg/kg p.o.) the reference drug used exhibited significant antinociceptive effect in rats. It is known that the tail-flick (thermal nociceptive) response appears to be a spinal reflex sensitive to opioid _µ_-agonists and non-thermal tests to opioid _κ_-agonists (Abbott, 1988; Furst et al, 1988), furthermore thermally-induced pain is also mediated by _Aδ_ and _C_ fibers. The data in the present study suggest the involvement of _κ_ opioid receptors in the analgesic activity and a decrease activity of _Aδ_ and _C_ fibers against inflammatory-induced activation but not thermally-induced activation (Puig and Sorkin, 1980).

Aqueous extracts of *V. amygdalina* were found to have *in vivo* activities against *P. berghei* in mice. At 200 mg/kg the antiplasmodial activity were comparable to CQ treated mice. Empirically, this plant is used in decoction alone, other plants may be added to reduce the side effect of nausea that result from the herb’s bitter taste (15).

The acute oral toxicity results from the *V. amygdalina* extract (3.32 ± 0.15 g/kg p.o) indicate that the extract may be safe based on the chemical labeling and classification of acute...
systemic toxicity on oral LD<sub>50</sub> values, recommended by the organization for Economic Cooperation and Development (Walum et al., 1986). It has, however, been reported, that the median lethal dose is not an absolute value but is an inherently variable biologic parameter that cannot be compared to constants such as molecular weight or melting point (Oliver, 1986). The adverse signs of gaiting, reduction in stereotypic activities and deaths were however seen in high doses.

In the sub-chronic study, the hematologic parameter shows a decrease in the RBC counts and an increased neutrophil in the treated groups. The serum chemistry parameter shows an increase in the direct and total bilirubin value. In several organs, mainly heart and liver, cell damage is followed by increased levels of a number of cytoplasmic enzymes in the blood, a phenomenon that provides the basis for clinical diagnosis of heart and liver diseases. For example, liver enzymes are usually raised in acute hepatotoxicity but tend to decreased with prolonged intoxication due to damage to the liver cells (Orisakwe et al., 2004). In this study, the extract did not exert significant effects on the serum chemistry parameters, the increased in bilirubin levels were probably due to the decrease RBC values.

Since the traditional healers reported use of the drug as prophylactics against malaria, male rats were exposed to the extract for 6 weeks. No extract-related deaths occurred, the clinical condition of the animals, body weight gain, and food consumption were unaffected. Clinical pathology parameters (hematology, serum chemistry) exhibited no treatment-related effect. Organ weight changes can be sensitive indicators of target organ toxicity, and significant changes in organ weights may occur in the absence in changes in other pathology parameters (Bailey, S.A., 2004), for example, increased liver weight associated with hepatic cytochrome P450 induction is a common finding in toxicology studies. Liver weight increases of up to 20% relative to control without microscopic evidence of hepatocellular hypertrophy or changes in serum chemistries (Amacher, et al., 2000). Similarly modest dose-related changes in kidney weight commonly occur in toxicology studies without histopathologic evidence of cellular alteration (Greaves, P., 2000).

In conclusion, the results of this study showed analgesic activity of the extract with clear and significant antiplasmodial effects in mice, no indication of toxicity in rats, incidental findings below or above standard reference levels were all within control values based on historical reference ranges. This might explain the pharmacological basis for the successes in pain and malaria treatment claimed by traditional healers who use <i>V. amygdalina</i>.

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