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Effect of *Alstonia congensis* Engl. extract on early and established plasmodial infection

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Abstract
This study was conducted to investigate the effect of leaf, bark and root of *Alstonia congensis* on early and established infections of *Plasmodium berghei* in animal models. The study was carried out between March 2015 to April 2017. *A. congensis* was collected from Emekuku area of Imo State, while *P. berghei* was obtained from Institute of Advanced Medical Research and Training, University College hospital, Ibadan. The albino mice and rat were purchased from Animal Science and Production Department of Michael Okpara University of Agriculture, Umudike, Umuahia. Acute toxicity studies of the crude extracts were carried out in albino mice. The antiplasmodial activities during early and established infections were evaluated in the albino mice by examining the blood smear under a microscope. The extract of *A. congensis*thus possesses antiplasmodial activity and showed a dose-dependant chemosuppression and competed favourably with artesunate which was the control drug. There was no evidence of drug-induced symptoms at all the doses of the extract administered in acute study. The finding may support the traditional use of the plant to treat plasmodial infection (malaria). It would therefore, be worthwhile to purify active components by a bioassay guided isolation in order to assess the parasite life phase on which the plant extracts are most active.

Keywords: toxicity, anti-*Plasmodium*, *Alstonia congensis,* chemosuppression

Introduction

The importance of plants in medicine remain even of greater relevance with the current global shift to obtain drugs from plant sources, as a result of which attention has been given to the medicinal value of herbal remedies for safety, efficacy and economy (WHO, 2003) [53]. Plants in general constitute a wide array of phytochemicals and when used in high dosages can elicit harmful effect on the body (Wang *et al.,* 2007) [51]. Phytotherapeutic agents or phytomedicines are standardized herbal preparations consisting of complex mixture of one or more plants which are used in most countries for the management of various diseases. Usually, the active principles responsible for their pharmacological action are unknown. One basic characteristic of phytotherapeutic agents is the fact that they normally do not possess an immediate or strong pharmacological action (Akerele, 1991) [4]. For this reason, phytotherapeutic agents are not used for emergency treatment. Other characteristics of herbal medicine are their wide therapeutic use and great acceptance by the population. In contrast to modern medicines herbal medicines are frequently used to treat chronic diseases.

Ethnobotanical survey have shown that these traditional medicines have been found to be effective especially in the treatment of malaria which is of great concern to any African nation (WHO, 2002) [52]. There is an urgent need to explore and utilize the naturally endowed rich biodiversity of indigenous communities through research that could translate to benefits for mankind.

According to Dike *et al*, (2012) [15], *A congensis* ranked as 3rd preferred herbal remedy for malaria treatment.Owing to increased resistance of malaria to orthodox antimalarial drugs, adverse side effect and the renewed interest in plant drugs, this study investigated the effect of leaf, bark and root of *Alstonia congensis* on early and established infections of *Plasmodium berghei* in animal models.

Materials and Methods

Fifty grams (50 g) of the pounded dried plant materials (leaf, bark and root powder) were weighed and extracted with 400 ml of aqueous (distilled water) and 400 ml of ethanol using Tedong extraction method. The processes were run for 2 hours each after which the samples were evaporated to dryness using water bath. The dried extracts were weighed and kept in a well labeled sterile specimen bottles and stored in a refrigerator at 4degree celsius until is required.

Preparation of *A. congensis* for experiment

One gram (1g) of each of the dried extract of leaf, bark and root were weighed and dissolved in 10ml distilled water. The volume used was calculated according to the body weight of the animals and was calculated using Tedong *et al* (2007) equation,

$$Vml = \frac{D \times P}{C}$$

D = dose used
P = body weight
C = concentration

Acute Toxicity/Lethal dose (LD_{50}) test

The medium lethal dose of the crude extracts of leaf, bark and root of *A. congensis* were determined by Lorke’s (1983) [30] method using the oral routes with the assistance of Pharmacist Solomon Nwafuru of Federal Medical Centre,

Owerri. The acute oral toxicity study was conducted in compliance with OECD guideline 425, which stipulate the use of only three animals (Jonsson *et al*., 2013) [28]. The test was divided into two stages.

Stage One

Determination of the toxic range of the leaf, bark and root extracts of *Alstonia congensis.* Mice were divided into 9 groups of 3 animals in each group. Each group received a dose (10, 100, 1000mg/kg) of the ethanolic extracts of leaf, bark and root suspended in distilled water respectively. The doses were administered orally and the treated animals observed for 72hrs for number of deaths.

Stage Two: Determination of lethality of leaf, bark and root extracts.

The doses used in this stage were determined from the number of deaths per dose recorded in the stage one test. Since no death occurred in the stage one test, three different higher doses: 1600mg/kg, 2900mg/kg and 5000mg/kg were administered to another group of animals at one dose per animal. The treated animals were monitored for number of deaths for 24hrs and continued to 72hrs. The LD_{50} in this test is determined by calculating the geometric mean of the test and most toxic doses.

$$LD_{50} = \sqrt{\text{minimum toxic dose} \times \text{maximum tolerated dose}}$$

Experimental animals and parasite inoculation

Parasite strain and inoculation

Albino mice and rats weighing between 15 to 27gm were used for this study. The study was carried out at the laboratory of Animal Science and Production Department, Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State. Animals were caged in groups for one week prior to initiation of the experiment for acclimatization to laboratory conditions. Albino mice previously infected with *P. berghei* were used as donor animals. The parasite strain was obtained from Institute of Advanced Medical Research and Training (IAMRAT) University College Hospital, Ibadan. The infected mice were brought to Umuahia in a plastic cage.

Two of the infected mice from IAMRAT were anaesthesized using cotton wool dipped in chloroform. The abdomen were opened using scissors. Using 2ml syringe, blood were withdrawn from the heart and put in a sterilized bottle. The dilution was made based on the parasitemia of the donor mice and the RBC count of normal mice in such a way that 1ml blood contains 5×10^7 infected erythrocytes (Animut, 2002) [7]. Each mouse was inoculated by intraperitoneal injection with a blood suspension (0.2ml) containing about 1×10^7 parasitized erythrocytes.

***In vivo* Antimalarial Screening**

Test on Early Malaria Infection (4-day suppressive test)

This test was a modified Makinde *et al,* (1989) [32] and Peters and Robinson, (1992) [47] methods. One hundred and forty mice were divided into 14 groups of 10 mice each. All mice were infected with *Plasmodiumberghei* at the commencement of the experiment (day 0). Treatment commenced 3hours after infection on day 0 and continued daily for four days (from day 0 to day 3). Groups 1-12 received 200, 400, 600, 800mg doses of leaf, bark and root extracts/kg body weight and was given to them orally using gavage to ensure safe ingestion (Bello *et al.,* 2016) [11]. Group 13 which serve as the control 1 (positive) received 20mg/kg of artesunate/kg. Mice in 14th group received 0.2ml distilled water and serve as control 2 (negative). On the 5th day (i.e. day 4) blood samples were collected from the animals tail snip of each of the mouse (Innocent *et al.,* 2009) [23] and thin smears were prepared and stained with 10% Giemsa solution, then each stained slides were examined under the microscope with an oil immersion objective of 100x magnification power to determine parasitemia by counting minimum of five fields per slide. The percentage suppression and parasitemia were calculated using the formula indicated:

$$\text{Percentage parasitemia} = \frac{\text{Number of infected RBC} \times \text{Number of field} \times 100}{\text{Total RBC} \times \text{Number of fields}}$$

$$\text{Av \%Suppre} = \frac{\text{Av Parasitaemia in neg. Control} - \text{Av. Parasit in drug treated} \times 100}{\text{Av. Parasitaemia in neg. Control}}$$

Test on Established Infection (Curative or Rane test)

The method was a modified on Ryley *et al*, (1970) [48]. Another one hundred and forty mice were divided into fourteen groups of ten mice, all were infected with *P. berghei* on the first day of the experiment (day 0). The mice were not treated until the parasitaemia was established i.e. after 10 days of infection with *P. berghei*. Groups 1-12 received 200, 400, 600 and 800mg/kg of leaf, bark and root extracts respectively for 3 days morning and evening. Group 13 received 20mg/kg artesunate as control 1 and 14th group received 0.2ml distilled water and serve as control 2 for the same period. On the 3rd day, blood samples were collected from tail snip of each mouse and thin smears were prepared and stained with Giemsa solution and examined under the microscope so that the average percentage (%) parasitaemia could be evaluated for each of the doses. After the fifth day, the animals were fed and observed for 28 days. Any death that occurs during this period was noted and used to determine the mean survival time.

Results

Table 1: acute toxicity (LD_{50}) test of the crude leaf extract of *A. congensis*

Stages	Doses mg/kg	Mortality
Stages 1	10	0/3
	100	0/3
	1000	0/3
Stages 2	1600	0/1
	2900	0/1
	5000	0/1

Table 2: acute toxicity (LD_{50}) test of the crude bark extract of *A. congensis*

Stages	Doses mg/kg	Mortality
Stages 1	10	0/3
	100	0/3
	1000	0/3
Stages 2	1600	0/1
	2900	0/1
	5000	0/1

Table 3: acute toxicity (LD_{50}) test of the crude root extract of *A. congensis*

Stages	Doses mg/kg	Mortality
Stages 1	10	0/3
	100	0/3
	1000	0/3
Stages 2	1600	0/1
	2900	0/1
	5000	0/1

At respective doses of 10, 100 and 1000mg/kg, all the three animals given the extracts of leaf, bark and root survived beyond the two weeks of observation without any sign of illness. When the extract was increased to 1600, 2900 and 5000 all the animals equally survived. All the mice that received the doses (10, 100, 1000, 1600, 2900 and 5000mg/kg) of the extract survived beyond the 2weeks of observation.

The medium lethal dose toxicity value (LD50) of the extract must be above 5000mg/kg. There was no gross physical and behavioral changes including rigidity, sleep, diarrhea, depression, abnormal secretion and hair erection within the observation period.

Table 4: Result of effect of *A. congensis* extracts (leaf, bark and root) on early plasmodial infection (4- day Suppression)

Sample	Dosage	% Parasitemia	% Suppression	Significance
Leaf	0.2ml Distilled water (Control 2)	53.0 ± 4.8	0.00	-
	200mg/kg	15.0 ± 5.3*	71.13	P < 0.05
	400mg/kg	10.0 ± 0.0*	81.13	P < 0.05
	600mg/kg	7.0 ± 4.8*	86.79	P < 0.05
	800mg/kg	3.0 ± 4.8*	94.34	P < 0.05
	20mg/kg Artesunate	0.0 ± 0.0*	100.00	-
Bark	0.2ml Distilled water (Control 2)	53.0 ± 4.8	0.00	-
	200mg/kg	14.0 ± 5.2*	73.58	P < 0.05
	400mg/kg	12.0 ± 4.2*	77.36	P < 0.05
	600mg/kg	8.0 ± 4.2*	84.91	P < 0.05
	800mg/kg	0.0 ± 0.0*	100.00	P < 0.05
	20mg/kg Artesunate	0.0 ± 0.0*	100.00	-
Root	0.2ml Distilled water (Control 2)	53.0 ± 4.3	0.00	-
	200mg/kg	13.0 ± 4.8*	75.47	P < 0.05
	400mg/kg	12.0 ± 4.2*	77.36	P < 0.05
	600mg/kg	7.0 ± 4.8*	86.79	P < 0.05
	800mg/kg	1.0 ± 3.2*	98.11	P < 0.05
	20mg/kg Artesunate	0.0 ± 0.0*	100.00	-

The results are expressed as mean ± SEM, P < 0.05 is significant, N = 10 * = significant

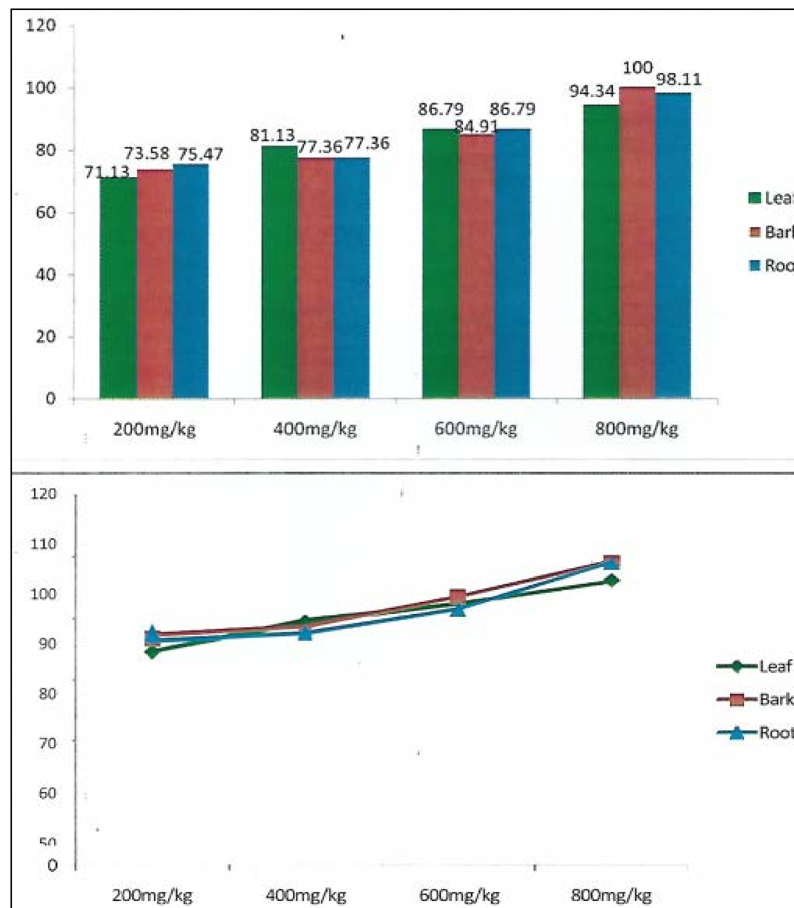

Fig 1&2: Showing suppressive effect of extract on early plasmodial infection

The percentage parasitemia before the treatment was 53.0±4.8 and suppression was 0.00. When 200mg/kg of leaf, bark and root were given, parasitemia became (15.0±5.3, 14.0±5.2, 13.0±4.8), respectively and suppression were (71.13, 73.58, 75.47) respectively. When 400mg/kg of leaf, bark and root were given, parasitemia were (10.0±0.0, 12.0±4.2, 12.0±4.2 respectively and suppression were 81.13, 77.38, 77.36 respectively. For 600mg/kg of leaf, bark and root, Parasitemia were (7.0±4.8, 8.0±4.2, 7.0±4.8) and suppression were (86.79, 84.91, 86.79) respectively. For 800mg/kg of leaf, bark and root parasitemia were 3.0±4.8, 0.0±0.0, 1.6±3.2 and suppression were 94.34, 100, 98.11 respectively.

All the extracts at the doses used showed significant change in parasitemia. There was statistically significant ($p<0.05$) suppression of parasitemia. The antiplasmodial activity produced by the extracts were statistically significant ($P<0.05$) and competed favourably with artesunate.

Table 5: Effect of different parts of *A. congensis* on *P. berghei* in mice (Before treatment and 4 days after treatment)

Treatment	Samples	% Parasitemia before treatment	% Parasitemia 4 days after treatment	% Suppression
0.2ml distilled water (Control 2)		37.0 ± 9.5	49.0 ± 15.2	-32.43
200mg/kg	Leaf	37.0 ± 6.8	15.0 ± 5.3	59.46
	Bark	38.0 ± 6.3	14.0 ± 5.2	63.16
	Root	38.0 ± 9.2	13.0 ± 4.8	65.79
400mg/kg	Leaf	37.0 ± 7.3	10.0 ± 0.0	72.97
	Bark	38.0 ± 6.3	12.0 ± 4.2	68.42
	Root	38.0 ± 9.2	12.0 ± 4.2	68.42
600mg/kg	Leaf	37.0 ± 7.3	7.0 ± 4.8	78.95
	Bark	38.0 ± 6.3	8.0 ± 4.2	78.95
	Root	38.0 ± 9.2	7.0 ± 4.8	81.58
800mg/kg	Leaf	37.0 ± 7.3	3.0 ± 4.8	91.89
	Bark	38.0 ± 6.3	0.0 ± 0.0	100
	Root	38.0 ± 9.2	1.0 ± 3.2	97.37
20mg/kg Aretesunate		37.0 ± 9.5	0.0 ± 0.0	100

The results are expressed as mean ± SEM
P < 0.05 is significant, n = 10, SEM = Standard error of mean

The percentage parasitemia with extracts from different parts of the plant ranged from 37.00 ± 6.80 to 38.00 ± 9.20. After 4 days of treatment, the lowest doses of the plant extracts (200mg/kg), reduced parasitemia to 13.00 ± 4.80 to 15.00 ±5.30 with activity of 59.46% to 72.97% respectively. While the highest doses of the plant extracts (800mg/kg), reduced parasitemia from 0.00 ± 0.00 to 3.0 ± 4.80 with activity of 91.89% to 100%.

The control 2 (0.2ml distilled water) showed a negative activity, parasitemia was increased. The extract showed a significant reduction ($P<0.05$) of parasitemia. The mean treatment of all the parts showed a significant different from the control 2.

Table 6: Effect of different parts of *A. congensis* on *P. berghei* in mice (Established infection)

Sample	Dosage	%Parasitemia	%Activity	Significance
Leaf	0.2ml Distilled water (Control 2)	66.0 ± 5.2	0.00	-
	200mg/kg	14.0 ± 5.2*	78.79	P < 0.05
	400mg/kg	13.0 ± 4.8*	80.30	P < 0.05
	600mg/kg	10.0 ± 4.7*	84.85	P < 0.05
	800mg/kg	8.0 ± 4.2*	87.88	P < 0.05
	20mg/kg Artesunate	0.0 ± 0.0*	100.00	P < 0.05
Bark	0.2ml Distilled water (Control 2)	69.0 ± 3.2	0.00	
	200mg/kg	5.0 ± 5.3*	92.75	P < 0.05
	400mg/kg	6.0 ± 5.2*	91.30	P < 0.05
	600mg/kg	2.0 ± 4.2*	97.10	P < 0.05
	800mg/kg	0.0 ± 0.0*	100.00	P < 0.05
	20mg/kg Artesunate	0.0 ± 0.0*	100.00	P < 0.05
Root	0.2mlDistilledwater (Control 2)	66.0 ± 5.2	0.00	-
	200mg/kg	6.0 ± 5.2*	90.91	P < 0.05
	400mg/kg	5.0 ± 5.3*	92.42	P < 0.05
	600mg/kg	3.0 ± 4.8*	95.45	P < 0.05
	800mg/kg	0.0 ± 0.0*	100.00	P < 0.05
	20mg/kgArtesunate (control 1)	0.0 ± 0.0*	100.00	P < 0.05

The results are expressed as mean ± SEM,
P< 0.05 is significant, n=10

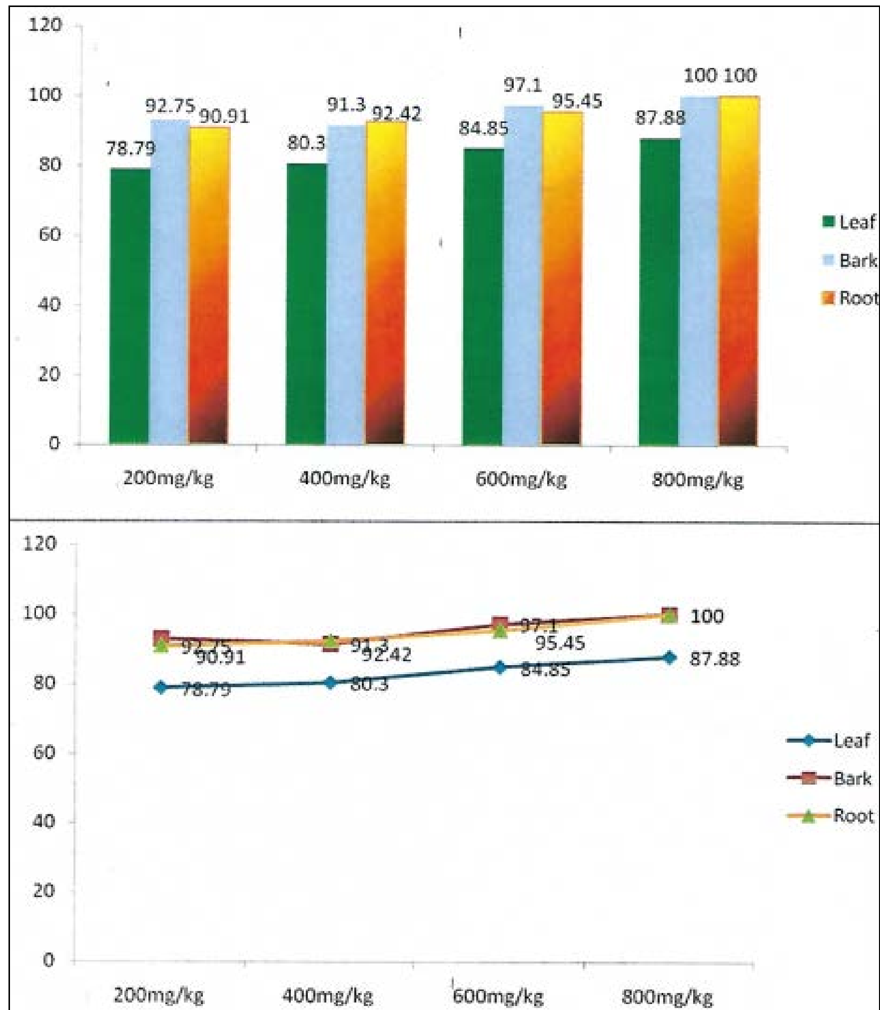

Fig 3&4: Showing effect of extract on established infection

The percentage parasitemia before the treatment was 66.0±5.2 and activity was 0.00. When 200mg/kg of leaf, bark and root were given, parasitemia became (14.0±5.2, 5.0±5.3, and 6.0±5.2), respectively and activity were (78.79, 92.75, and 90.91) respectively. When 400mg/kg of leaf, bark and root were given, parasitemia were (13.0±4.8, 6.0±5.2, and 5.0±5.3 respectively and activity were 80.30, 91.30, and 92.42 resepctively. For 600mg/kg of leaf, bark and root, parasitemia were (10.0±4.7, 2.0±4.2, and 3.0±4.8) and activity were (84.85, 97.10, and 95.45) respectively. For 800mg/kg of leaf, bark and root parasitemia were 8.0±4.2, 0.0±0.0, and 0.0±0.0 and activity were 87.88, 100, and 100 respectively. All the extracts at the doses used showed significant ($P<0.05$) change in parasitemia.

There was clear reduction of parasitemia. The activity was pronounced in all the extracts. There was statistically significant ($P<0.05$) change when compared with the control 2.

Table 7: Comparative effect of different concentrations of extracts on *P. berghei* in mice.

Extract Treatment	Dose (mg/kg)	% parasitemia mean ± SEM	% Activity	Significance
Leaf	200	14.0 ± 5.2	78.79	P < 0.05
Bark	200	5.0 ± 5.3	92.75	P < 0.05
Root	200	6.0± 5.2	90.91	P < 0.05
Leaf	400	13.0 ± 4.8	80.30	P < 0.05
Bark	400	6.0 ± 5.2	91.30	P < 0.05
Root	400	5.0 ± 5.3	92.42	P < 0.05
Leaf	600	10.0 ± 4.7	84.85	P < 0.05
Bark	600	2.0 ± 4.2	97.10	P < 0.05
Root	600	3.0 ± 4.8	95.45	P < 0.05
Leaf	800	8.0 ± 4.2	87.88	P < 0.05
Bark	800	0.00 ± 0.00	100.00	P < 0.05
Root	800	0.00 ± 0.00	100.00	P < 0.05
Artesunate	20	0.00 ± 0.00	100.00	-
Distilled water	0.2ml	66.0 ± 0.52	-32.43	-

The results are expressed as mean ± SEM
$P<0.05$ is significant, n = 10

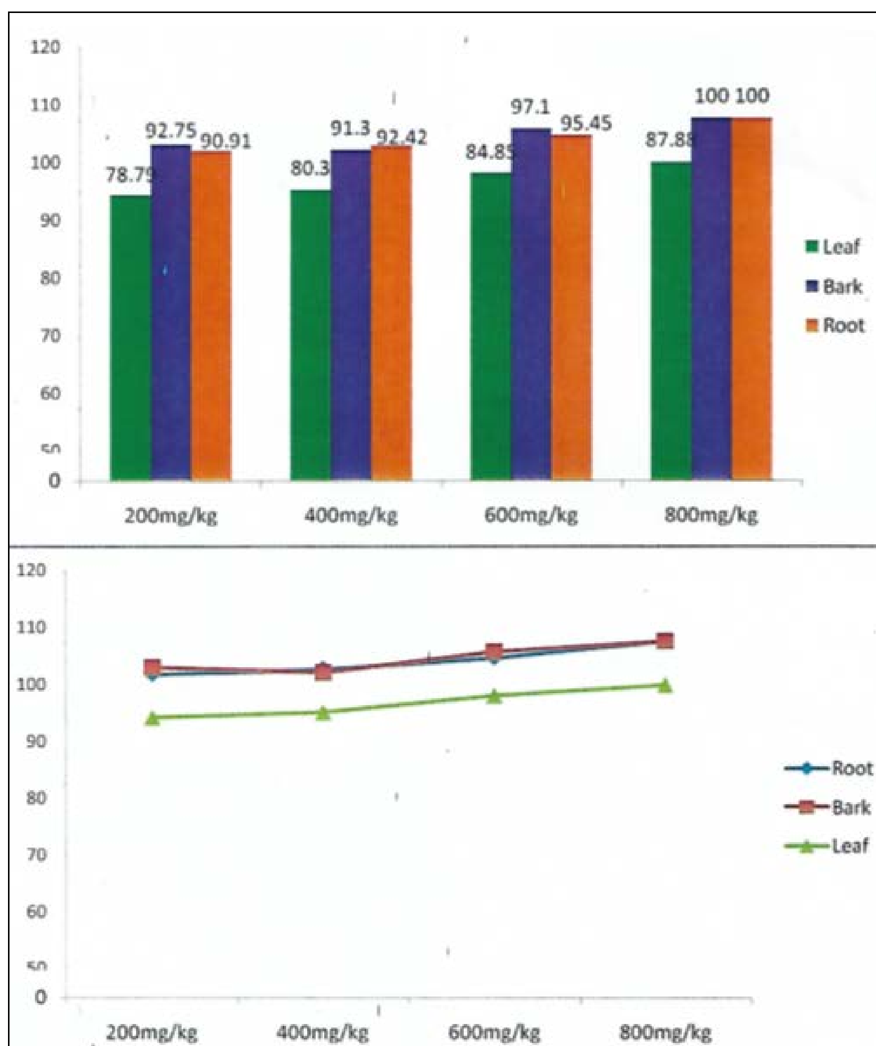

Fig 5&6: Showing comparative suppression of leaf, bark and root extract on plasmodial infection.

The highest activity was observed in the bark and root extracts at dose of 800mg/kg (100%). For the lowest dose (200mg/kg) the bark showed highest activity (92.75%), root (90.91) and leaf (78.79) with parasitemia 5.0±5.3, 6.0±5.2 and 14.0±5.2 respectively. For 400mg/kg, the root showed activity of 92.42% followed by bark, 91.3% and then the leaf 80.3% with parasitemia 5.0±5.3, 6.0±5.2 and 13.0±4.8 respectively.. But for 600mg/kg the bark was highest 97.1% followed by the root (90.91%) and finally the leaf (78.79%) with parasitemia 2.0±4.2, 3.0±4.8 and 10.0±4.7 respectively. For 800mg/kg of leaf, bark and root parasitemia were 8.0±4.2, 0.0±0.0, and 0.0±0.0, and activity were 87.88, 100 and 100 respectively. The bark was the preffered part as it had the highest activity at lowest dose. The bark and the root competed perfectly with the control 1 (artesunate). The comparative analysis of the plant parts in different concentrations showed a progressive suppression as the concentration increased.

Discussion

Plants have undoubtedly been a rich source of new drugs in use today. It is estimated that over 1200 plant species from 160 families are used to treat malaria and fever, (Wilcox & Gerald, 2004) [54]. A number of studies have been carried out to evaluate the inhibitory effect of various plant extracts on *P. falciparum,* (Milijaona *et al.*, 2003). Similarly, the *in vivo* anti plasmodial properties of several plant extracts have been studied in mice, (Awe *et al.*, 1990; Ajaiyeoba *et al.*, 2006) [8, 2]. Following the trend, this study presents the results from the evaluation of the *in vivo* anti plasmodial activity of *Alstonia congensis* commonly used in the Eastern and Western part of Nigeria. *Alstonia congensis* (umbrella tree, shade plant) is commonly used in the Eastern part of Nigeria to treat malaria and other plasmodial infections, (Choudhury *et al.,* 2010) [13].

Antimalarial activity has been related to a range of several classes of secondary plant metabolites (phytochemicals) including alkaloid, flavonoid phenol, tannin, terpenoid and quinines of which alkaloids have been the most important and have shown very interesting activities, (Tiwari *et al.,* 2011) [50]. Indeed quinine is the first antimalarial drug that belongs to the class of alkaloids, (Frederich *et al.*, 2008) [18]. Therefore, the antiplasmodial activity observed in the study may be attributed to the presence of these bioactive compounds.

The leaf, bark and root extracts in the present study was observed to show some intrinsic anti plasmodial activity. This is evident from its percentage chemosuppression both in early and established infection of *P. berghei* which is in agreement with the work carried out by Lumpu *et al,* (2014) [31], in which the leave, stem bark and root of *A. congensis,* in aqeuous and 80% MeOH extracts exhibited pronounced antiprotozoal activity against KI strains of *Plasmodium falciparum.* This result according to Lumpu *et al,* (2014) [31], can partly support and justify the traditional use of this plant parts as raw material for preparation of traditional remedies

to treat parasitic diseases such as malaria and trypanosome.
The result from this study justifies the use of *A. congensis* in combination with other plants in traditional medicine (Hilda *et al.,* 1994) [20]. All the plant parts in ethanolic extract exhibited comparable suppressive effect on *P. berghei*. However, ethanolic extract of leaf in all doses tested may be considered to have lowest (78.79%, 80.3%, 84.85% and 87.88%) suppression at 200mg/kg, 400mg/kg, 600g/kg and 800mg/kg respectively. Thus, this result may justify the traditional use of the plant for antimalarial therapy in different parts of Nigeria (Ajaiyeoba *et al.*, 2006) [2].
Artesunate used in this study suppressed parasitemia to non-detectable number which is in agreement with, Iyawe *et al*, (2009) [26], in which standard antimalarial drug cleared *P. berghei* to undetectable level. Artesunate at the doses of 20mg/kg gave a percentage suppression of 100%, a value equal to that of 800mg/kg of bark and lower than 800mg/kg of root and leaf. Mohammed *et al,* (2014) [35], used a total dose of 25mg/kg of chloroquine in his study while Awe *et al,* (1990) [8] used 5mg/kg. Both used 1.0 x 10^7 parasite whereas this present study was done with 20mg/kg artesuante and 1.0 x 10^7 parasite and all suppressed parasitemia. In untreated mice the parasite count increased as observed in previous studies (Ajaiyeoba *et al.*, 2006).
The high level of chemosuppression produced at high doses of extracts is in line with previous studies, (Ajaiyeoba *et al.*, 2006) [2]. At 200mg/kg, the bark and the root suppressed parasitemia by 73.58% and 75.47% respectively. This indicated that the minimum effective dose of the extract is approximately 200mg/kg which was in agreement with Awe *et al*, (1990) [8], findings which gave a suppression of early infection with 200mg/kg as 74.9%.

Conclusion
The present work has shown in an animal model that the extracts of leaf, bark and root of *A.congensis* was effective for chemotherapy of *Plasmodium* infections. The chemosuppression was based on the presence of phytochemicals which have been indicated as antioxidants. Hence it can be suggested as a natural medicine against plasmodial infection (malaria) which has constituted nuisance to our communities and a public health challenge in this present generation. In conclusion, the extract competed favourably with the control 1 (artesunate) in clearing *P. berghei* and so agrees with the local usage of the plant as anti plasmodial agent.

Summary
From the study, *A congensis* is a good candidate for further study. The extract of *A. congensis* can be said to be safe, as no death was observed in the animals throughout the study, even when high doses were given. The extracts of *A. congensis* leaf, bark and root were effective as antiplasmodial agent. It may be used for the treatment of malaria that results from plasmodial infectioin. The anti plasmodial (malaria) activity was based on the presence of phytochemicals which have been indicated as anti plasmodial agent. The extract may be preferable for chronic infections where orthodox medicine failed.

Ethical Approval
Ethical clearance was given by Michael Okpara University of Agriculture Umudike, Umuahia Abia State.

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