Antiplasmodial Activity of Extracts and Fractions of Casuarina Equistifolia against Plasmodium Berghei in Mice

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Abstract
The antiplasmodial activity of the extracts and fractions of *Casuarina equistifolia* was carried out using both the suppressive and established infection (curative test) procedure in laboratory Swiss albino mice. The results of the suppressive test showed that the dichloromethane extract, ethyl acetate fraction, n-butanolic fraction and residual water soluble fraction of *C. equistefolia* reduced parasitaemia by >50% when tested at an oral dose of 200mg/kg/day indicating that the median effective dose (ED50) of is <200mg/kg/day. In the curative test procedure the 80% methanolic extract exhibited a very good activity (70.59%) against *Plasmodium berghei*. Other extracts and fractions like dichloromethane, petroleum ether, chloroformic, ethyl acetate, n-butanolic and the residual aqueous all showed antiplasmodial activity with a statistically significant difference ($P<0.05$, 0.01) when compared to the control. Although all extracts, fractions and the chloroquine phosphate standard drug employed in this study reduced the parasitaemia, but could not clear all the parasitaemia in animals after termination of treatment. The survival time was significantly different ($P<0.05$) in all extracts and fractions employed in this study when compared to the control group. The chloroquine phosphate standard drug group recorded a low survival time of 14.0±0.81days. This could probably be as a result of some side effects of the drug mode of administration (intraperionneally) employed in this study.

KEYWORDS: *Casuarina equistifolia*, *Plasmodium berghei*, Extract, Fractions, Suppression.

INTRODUCTION
Plasmodium berghei is a rodent equivalent of *P. falciparum* which causes cerebral malaria in humans. However, *P. berghei* does not cause cerebral malaria in an albino immunodeficient inbred strain (BALB/C) mice (Albay *et al*., 1999). *P. berghei* belongs to a group of four *Plasmodium* species that infect murine rodents from Central Africa. These are *P. vinckei*, *P. chabaudi*, *P. yoelii* and *P. berghei* (Killick- Kendrick, 1978). The use of *P. berghei* for the study is because, the genome of *Plasmodium berghei* has been sequence and it shows a high similarity both in structure and gene content, with the genome of the human malaria parasite *Plasmodium falciparum* (Janse *et al*., 2006).

Casuarina is a shrub native to Australia and Island of the pacific. They are common in tropical and subtropical areas. Common names include Coast she-oak, Beach *Casuarina*, beach oak, Beach she-oak, whistling tree, Coastal She-oak, Horse tail she oak and Coast she-oak. The specific name equisetifolia is derived from the Latin word 'equisetum' meaning "horse hair" referring to the resemblance of the dropping branch lets to horse tail (Boland *et al*., 2006). The tree has delicate, slender ultimate branches and leaves that are no more than scales, making the tree look more like a wispy conifer. Australian pine grows to a height of 12m and width of 7m. The plant does especially well in windswept locations and is widely planted as wind – breaks. In Panama the fruit is mixed with powdered nutmeg used in relieving toothache (Walter and Memory, 1977). Recently Onwuliri and Umezurumba (2003) reported its efficacy against *Salmonella typhi*.

Traditional medicines are the primary sources of healthcare and have been in use since time immemorial. Their conservation is vital to people that do not have access to adequate medical resources. There are several plants that have long been utilized by traditional practitioners for curing diseases: Awe and Makinde (1997a), reported

that the leaves of *Carica papaya* is usually used in combination with other plants like *Alstonia boonei*, *Cymbopogon citratus* and *Azadirachta indica* as remedies against fevers in tropical countries. Several researchers have evaluated the claim of anti-malarial efficacies of plants in laboratory rodents, *Morinda lucida* (Awe and Makinde, 1997b), *Erythrina senegalensis* (Saidu *et al*., 2000). *Zizypus spina–christi* (Adzu *et al*., 2007), others are *Croton mubango*, *Nauclea pobeguinii* and *Pyrenacantha staudii* used as anti-malarial remedies in the Democratic Republic of Congo (Mesia *et al*., 2005). Olliaro and Trigg, (1995) opined the need for drug development with priority given to research on drugs and drug combinations to treat multi drug resistant *P. falciparum* which remains a major operational problem in many countries especially Africa.

MATERIALS AND METHOD

Collection and preparation of the Plant Material

Fresh leaves of *Casuarina equistifolia* (*Casuarinaceae*) whistling pine leaves were collected from the Federal College of Forestry, Jos, Plateau State, Nigeria. The plant was authenticated by a Botanist Mr Segun Olayenju of Biotechnology Garden Department of Biological Sciences, University of Abuja.

The leaves collected were spread thinly on a flat clean tray and allowed to air dry at room temperature for seven days to prevent spoilage due to moisture condensation and then reduced to coarse powder using a wooden pestle and mortar (Sofowora, 1982).

Preparation of Extracts and Fractionation.

The method described by Mesia *et al*. (2005) was modified putting into consideration Silver *et al.* (1998). The dried and powdered leaves of *C. equistifolia* (300g) was separately and exhaustively macerated first with 4500mls of 80% methanol and then with 250mls of dichloromethane for three consecutive days each. The maceration was exhaustive by occasional shaking using a mechanical or magnetic stirrer for homogenization of the final solution and saturation of the solvent. Each macerate was filtered using Whatman filter paper (cat no. 1001 090, 99mm diameter, Whatman International Limited England). An 8g subsample of each dried 80% methanolic extract was suspended in 200mls of distilled water. The solution was filtered using a Whatman filter paper to obtain a filtrate for partitioning. The filtrate was partitioned successively with 150mls each of the following solvents; petroleum ether, ethyl acetate, chloroform and n-butanol obtaining their corresponding fractions designated as FR1-5 respectively.

Experimental Animals

Swiss albino mice of both sexes weighing between 15 – 22g were obtained from the Animal House of the Department of Pharmacology, University of Jos. The mice were fed using standard rodent diet and allowed free access to water. The animals were kept in plastic cages measuring 25cm x 10cm with a metal mesh cover under normal room temperature. All the procedures in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and Guidmated (1989).

Parasite Species

The parasite species used, *Plasmodium berghei* was obtained from the Nigeria Institute for Medical Research (NIMR), Lagos and was maintained in the Animal house of the Department of Pharmacology, University of Jos through weekly passage in laboratory mice. A donor mouse with parasitaemia level of (++) =11-100 parasite per 100 thick film field was used to infect the experimental animals intra-peritoneally (Adzu, *et al*., 2007).

4 – Day Suppressive (Peters) Test

Thirty five (35) mice of either sexes were each inoculated with 0.2ml of the diluted inoculums intra–peritoneal (i.p), then grouped into seven groups (n =5) as described by Adzu, *et al*., (2007). Treatment of animals commenced immediately (Peters *et al*., 1993; Saidu *et al*., 2000; Dikasso *et al*., 2006; Adzu *et al*., 2007) and then daily day one today three (D1 – D3) until day four (D4) when blood was collected from the tail of each mouse after each mice was restrained in a mouse restrainer and the tail cut with a pair of scissors and the blood was quizzed out and smeared unto a microscopic slide to make a thick film (Saidu *et al*., 2000). Percentage

suppression was determined as follows:

% suppression = parasitaemia in control – parasitaemia in treated group x 100 parasitaemia in control
(Mesia *et al*., 2005; Adzu *et al*., 2007).

Curative Test (Rane Procedure)

The procedure described by Adzu, *et al*., (2007), Saidu, *et al*., (2000) was adopted which is similar to the suppressive test, except that treatment started on day three (D3) after infection was established by a pre-treatment blood smear of each mouse. Treatment was then continued daily (D4-D6) until D7 when another smear was collected (post treatment smear) and examined for parasitaemia suppression. The mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice in each group over a period of thirty (30) days of post inoculation with *Plasmodium berghei.*

Staining of the films

Dried blood smears of each mouse was stained using 3% Giemsa stain at pH 7.2 for 30 minutes on the slide rack and after that, each slide was washed off the stain using distilled water and allowed to dry. Percentage suppression was estimated as stated above.

Statistical Analysis

Results were expressed as mean $\pm$ standard error of mean (SEM). The student t-Test was used to compare results among groups for any significant difference in parasitaemia of mice treated with leaf extracts and fractions (x) and the control group (μ) by the formular:

$$t = \frac{x - \mu}{S/\sqrt{n}}$$

RESULTS

Table 1, shows the extract and fraction yield of *C. equistifolia* leaf, each macerate and corresponding percolate gave a dried methanolic extract of 3.54% by weight and a dried dichloromethane extract of 0.71% respectively. The 8g subsample extracted and partitioned, exhaustively and successively, yielded the corresponding dried fractions; petroleum-ether (2.38%), chloroform (4.13%), ethyl acetate (4.25%), n-butanol (4.38%) and residual aqueous fraction (21.63%) by weight respectively.

Table 1: Extracts and Fractionation Yield of *C. Equistifolia*

Extracts and Fractions	Yield (%)
80% methanolic extract	3.54
Dichloromethane extract	0.71
Petroleum-ether fraction	2.38
Chloroformic fraction	4.13
Ethyl acetate fraction	4.45
n-Butanolic fraction	4.38
Residual water-soluble fraction	21.63

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Anti-malarial activities of extracts and fractions.

In the 4 day suppressive tests, infected mice were administered 200mg/kg extracts/ fractions a day (Table, 2). The 80% methanolic extracts produced the least (40.66%) reduction in parasitaemia whereas the residual fraction gave 61.11% reduction. However the dichloromethane extract produced the highest reduction of 66.78% while the petroleum ether, chroloformic, ethyl acetate and n-butanolic fraction yielded 43.29%, 43.89%, 55.95% and 51.19% reduction in parasitaemia respectively.

Table 2: Antiplasmodial activities of plant extracts and fractions *Casuarina equistifolia* against *Plasmodium berghei* in mice (suppressive test).

Treatment	Dose mg/kg/day	Parasitaemia count	Suppression (%)
80% methanolic extract	200	58.60±17.91	40.66*
Dichloromethane extract	200	32.80±7.97	66.78**
Petroleum-ether fraction	200	56.00±4.92	43.29**
Chloroformic fraction	200	55.40±17.29	43.89*
Ethyl acetate fraction	200	43.50±15.85	55.95*
n-Butanolic fraction	200	48.20±11.13	51.19**
Residualwater-soluble fraction	200	38.40±11.28	61.11**
Normal saline (-ve control)	0.2ml	98.75±26.32	_
Chloroquine phosphate (5mg/kg)	5	11.20±3.16	88.66**

(* $p < 0.05$, ** $p < 0.01$)

The curative test revealed that the extracts and fractions were able to reduce the parasitaemia observed in the pre-treatment data, although could not totally clear the parasitaemia including the chloroquine phosphate standard drug after termination of treatment (Table, 3). The 80% methanolic extract reduced the parasitaemia level from10.2±0.97 on day three (D3) to 3.0±0.75 after termination of treatment (D7) compared to the 0.2ml normal saline (control group) 12.0±0.49 on day three (D3) to 13.6±0.75 in day seven (D7) after termination of treatment.

Table 3: Antiplasmodial activities of plant extracts and fractions *Casuarina equistifolia* against *Plasmodium berghei* in mice (curative test).

Test substance	Dose (mg/kg/day)	Pre-treatment (D_3)	Post-treatment (D_7)	Percentage Reduction (%)
80%Methanolic extract	200	10.2±0.97	3.0±0.32	70.59**
Dichloromethane extract	200	11.2±0.74	3.6±0.51	67.86**
Petroleum-ether fraction	200	6.6±0.68	4.4±0.51	33.33**
Chloroformic fraction	200	9.4±1.33	3.6±0.25	63.83**
Ethyl acetate fraction	200	7.8±1.40	5.2±0.58	33.33**
n-Butanolic fraction	200	6.0±0.70	3.8±0.58	36.67**
Residual aqueous fraction	200	8.8±1.07	4.4±0.25	50.00**
Chloromethane Phosphate	5	7.8±0.86	2.0±1.23	74.39**
Normal Saline	0.2ml	12.0±0.49	13.6±0.75	-

** Indicates significant difference ($p< 0.05,0.01$)
D_3 = day 3, D_7 = day 7after infection was initiated Each result is with a mean of 5 mice

The result of mean survival time (mst) of the extracts and fractions in the established infection (curative) is shown on table 3. The group administered 200mg/kg/day of ethyl acetate fraction survived for a longer days (26.0±1.52), other extracts and fractions gave above average of survival in days. The chloroquine group survived for 14.0±0.81while the 0.2ml normal saline treated group (control group) survived for 9.6±0.81 days.

Table 3: Mean survival time (mst) in days following established infection (Curative test).

Test substance	Survival (Days)
80% Methanolic extract	19.4±2.16
Dichloromethane extract	20.4±2.11
Petroleum ether fraction	18.8±2.73
Chloroformic fraction	23.6±2.99
Ethyl acetate fraction	26.0±1.52
n-Butanolic fraction	17.6±2.05
Residual aqueous fraction	15.8±1.07
Chloromethane Phosphate	14.0±0.81
Normal Saline	9.6±0.81

DISCUSSION

Malaria or *Plasmodium* species can be developmentally arrested by induced vitro culture of species in the presence of sub-lethal concentration of Artemisinin (Witkowski *et al.*, 2010). The extraction and fractionation of the powdered leaves of *C. equistifolia* indicated that the active constituents are water soluble and yielded more residual soluble fractions 21.63% by weight and the 80% methanol yielded 3.54% of the 300g each dried powdered leaves respectively. The 80% methanolic extract of *C. equistifolia* showed more solubility in chloroform 4.13% dried fraction yield by weight similar to the 73.50% fraction yield of *Croton mubango* extract partitioned in chloroform than its corresponding 3.20% and 2.12% yield of *Nauclea pobeguinii* and *Pyrenacantha staudii* respectively (Mesia *et al*., 2005). Conversely, the constituents of the leave extracts of *C. equistifolia* showed no lipophilic tendency as the dichloromethane gave the least extract yield 0.71% by weight of the dried extract. The result of the suppressive test indicated that the crude extracts (80% methanolic and dichloromethane) from the leaves of *C. equistifolia* and all the soluble fractions from the partitioning of the 80% -methanolic extracts exhibits some anti-malarial activity *in vivo.* This is similar to earlier findings by Mesia *et al.*, (2005) on the anti-malarial activities of *Croton mubango* in the Democratic Republic of Congo. The dichloromethane extract gave a comparatively higher chemo-suppression compared with the 80% methanolic extract as was the case with *Croton mubango* (Mesia *et al*., 2005). All the extracts and fractions gave significant difference ($p < 0.05$) except the 80% methanolic extract and the chloroformic fraction did not produced any significant changed at 99% level of probability ($p > 0.01$).

The 7-day curative test is a standard test which is commonly employed in screening of antimalarial activity (Peters and Ryley, 1970) and the determination of percentage suppression of parasitaemia is the most reliable parameters (Dikasso, *et al*., 2006). The extracts and fractions showed significant difference ($P<0.05$, 0.01) parasitaemia. The 80% methanolic extract gave a high percentage reduction in parasitaemia (70.59%) when compared to the negative control group (-13.33%). All the extracts and fractions employed in this study could only suppress the parasitaemia in the mice but could not clear it after termination of treatment. The chloroquine phosphate drug (positive control) suppressed parasitaemia in the test animals as previously published (Saidu *et al*., 2000; Tona, *et al*., 2001; Mesia, *et al*., 2005; Dikasso, *et al*., 2006 and Adzu, *et al*., 2007), but could not also clear all the parasites. The Petroleum ether fraction and ethyl acetate fraction showed only slight parasitaemia suppression 33.33% and 33.33% respectively.

The survival time of the Swiss albino mice treated with the extracts and fractions in this test during a period of 30 days showed increase in survival time base on extracts and fractions employed. These findings are consistent with Malann and Ajayi, (2011), Okonko, *et al.*, (2007), and Adzu, *et al*., (2007) but inconsistent with the findings of Elufioye and Agbedahunsi (2004) who recorded that survival time reduced on administration of extract of *Tithonia diversifolia* in the established infection procedure. Furthermore, the mean survival time of extracts and fractions treated groups were significantly different ($P<0.05$) compared to the control. The survival time of 200mg/kg/day of ethyl acetate fraction was high (26.0±1.52) compared to all other extracts and fractions employed in this study including the chloroquine phosphate group which is inconsistent to earlier findings of Malann and Ajayi, (2011), Elufioye and Agbedahunsi, (2004); Okonko, *et al*., (2007) on the activities of *Casuarina equisetifolia*, *Crossopteryx febrifuga*, *Setaria megaphylla*, and *Ziziphus spina-christi*. This study revealed that chloroquine phosphate standard drug group showed less survival time of 14.0±0.81 days in contrast to earlier reports of Malann and Ajayi, (2011), Elufioye and Agbedahunsi, (2004), Okonko, *et al*., (2007), and Adzu, *et al*., (2007). All mice employed in the study died within the range of 30 days of parasite innoculation.

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