Herbal Medicines Journal **2018; Vol. 3, No. 3:92-100**
DOI: 10.22087/herb%20med%20j.v3i3.742 **ISSN:** 2538-2144

Original Article

Antiplasmodial and Cytotoxic Properties of the Ethanolic Extract of the Leaves of *Sarcocephalous Latifolius*

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Received: 07.05.2019; Accepted: 25.05.2019

Abstract

Background and Aim: *Sarcocephalus latifolius* serves multiple purposes in the treatment of different ailments in Nigeria. In this study, antiplasmodial and cytological activities of the ethanolic extract of leaves of *S. latifolius* were assessed using albino mice and *Allium cepa* models.

Materials and Methods: For the antiplasmodial test, a total of 25 mice were divided into five groups of five mice. The groups were administered plant extract (25, 50 and 100mg/kg), chloroquine (10mg/kg) and distilled water respectively. This test involved two phases. In the suppressive phase, the extract was orally administered for four days after inoculation, and blood smear was prepared on the fifth day. In the curative phase, the mice were inoculated with parasites three days before the administration of the extract to ensure the full development of parasites, and the extract was administered for five days. Blood smears were prepared along the periods of administration and five days after the administration. In cytotoxic study, onion bulbs were exposed to 25, 50 and 100 mg/ ml concentrations of the extract for 24, 48 and 72 hours. Subsequently, the macroscopic and microscopic investigations were evaluated.

Results: All doses exhibited significant antiplasmodial activity dose-dependently. Root length significantly decreased in all concentrations compared to control. After 72 hours, the percentage root length and root inhibition at all concentrations were decreased. *S. latifolius*, therefore, demonstrated significant antiplasmodial activities. Availability of metabolites, such as alkaloids and flavonoids, may elicit endoperoxidation thereby causing death of parasites.

Conclusion: This finding supports the traditional use of *S. latifolius* against malaria. Cytoarchitecture revealed aberrations like sticky telophase, vagrant metaphase and telophase which may indicate the signs of toxicity that could cause cell death.

Keywords: Antimalarial, *S. latifolius***,** Chromosomal Aberrations, Toxicity.

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Please cite this article as: Oloyede A M, Larinde S O. Antiplasmodial and Cytotoxic Properties of the Ethanolic Extract of the Leaves of *Sarcocephalous Latifolius*. Herb. Med. J. 2018;3(3):92-100.

Introduction

Malaria is a prominent public health problem endemic in countries of Sub-Saharan Africa (1). It has been reported that malaria causes at least 300 million infections and also results in one million deaths

annually (2). The infection has caused unparalleled sufferings and gross economic loss in most African countries including Nigeria. In the face of expensive orthodox drugs, local medicinal plants have continued to play a vital role in primary health care as they are readily affordable and easily accessible in the community. The antimalarial potential of plants have been proven in the Amazon since 1770 (3).

Sarcocephalus latifolius (African peach) is one of the numerous plant species strongly reported to have medicinal value belonging to the family Rubiaceae. It is locally referred to as Egbesi (Yoruba), *Tafashiya* or *tuwon biri* (Hausa), *Ubuluinu* in (Igbo), *mahyann* are all found in Nigeria (4). Extracts from various parts of *S. latifolius* are reported to have a wide range of medicinal properties, and are commonly used locally in the treatment of malaria (5) hypertension, dental problems, diarrhea and dysentery (6). *S. latifolius* is also antipyretic and antinociceptive (7). This study was conducted to confirm the veracity of the traditional claim of *S. latifolius* being antimalarial, and also to investigate its cytotoxicity on the chromosomes.

Materials and Methods

Plant Collection

As the plant material used for this study, *Sarcocephalus latifolius* was purchased from Shomolu market, Lagos state, Nigeria. The plant was identified and authenticated by Mr Oyebanji, a taxonomist from the Department of Botany, University of Lagos, where a voucher specimen with herbarium number LUH7352 was deposited.

Preparation of Plant Extract

Applying the methodology already used by Oloyede *et al*. (8), though with modifications, the leaves of the plants were air dried for 14 days, and then were homogenized using a miller to convert it into powdery form. 482.1g of the powder was allowed to stand in 1120ml of 99.7% absolute ethanol for 72 h. Hence, it was decanted and filtered using a muslin cloth. The extract was later evaporated to dryness in a hotplate subjected to 40° C. Finally, the dried extract weighed 33.9g, giving a percentage yield of 7.03%. The dry ethanolic extract was dissolved in distilled water in order to make the stock solution from which the various doses administered were prepared to be used by serial dilution.

Animal Stock

Swiss albino mice weighing 15- 27g were bred in the Animal House of University of Lagos, Lagos State, and were maintained in the University of Lagos Animal House. They were fed with growers' pellet and were given water ad libitum. They were kept in plastic cages with saw-dust to be provided with warmth and to prevent cages from being messy. The animals were acclimatized for 10 days. Four mice were used for each experiment, and the experiments were repeated where necessary.

Micro-Organisms

A chloroquine-sensitive strain of *Plasmodium berghei* was purchased from the National Institute of Medical Research (NIMR) in Yaba, Lagos State, and was maintained by sub-passages in mice.

Parasite's Inoculation

As demonstrated by Oloyede *et al*. (8), the inoculum consisted of 5 x 10^7 *P. berghei* parasitized red blood cells per ml. It was through counting the number of parasitized red blood cells against the total number of red blood cells that the parasitaemia was determined. The desired volume of blood gained from the donor mouse was then suitably diluted with Phosphate buffer of pH 7.2 so that the final inoculum (0.2 ml) for each mouse contained the required number of parasitized red blood cells (that is 1.0×10^7 parasitized red blood cells). Consequently, each mouse was inoculated on the first day intra-peritoneally with 0.2ml of the final inoculum containing 1×10^7 parasitized red blood cells which is the standard inoculum for the infection of a single mouse.

Drug Administration

The drugs (chloroquine) and *S. latifolius* leaves ethanolic extract used in the antimalarial studies were orally administered using a stainless metallic feeding cannula.

Antimalarial Activities of the Extract

Suppressive Test

The impact of the schizontocidal activity of *S. latifolius* was experimented using the method described by Oloyede *et al*. (8). The mice were injected intraperitoneally with standard inoculum of 1x 10^7 *P. berghei* infected erythrocytes on day 0 (the first day). The mice were divided into five groups of five mice. They were orally administered shortly after

inoculation with 25, 50 and 100mg/kg day doses of *S. latifolius* extract, chloroquine 5 mg/kg and an equivalent volume of distilled water for four consecutive days. On the fifth day (day 4), thin and thick films were made from the tail blood of each mouse and stained with 10% Giemsa, which were examined microscopically with 100x magnification under oil immersion on the fifth day post-inoculation. The average percentage parasitaemia chemosuppression was calculated as follows:

100 ((A-B)/A), where A is the average percentage parasitaemia in the negative control and B is the average percentage parasitaemia in the test group (9).

Curative or Rane Test

The evaluation of the curative potential of *S. latifolius* was performed using a similar method described by Oloyede *et al*. (8). The mice were injected intraperitoneally with standard inoculum of $1x10^7$ *P. berghei* infected erythrocytes on the first day (day 0). Seventy-two hours later (72 hours), the mice were divided into five groups of four mice. The groups were orally administered with *S. latifolius* leaf extract (25, 50, 100mg/kg/day), chloroquine (5 mg/kg) was given to the positive control group and an equal volume of distilled water to the negative control group. For five days there the administration of treatment continued, and chloroquine was given daily. Thin films stained with Giemsa stain were daily obtained from the tail blood of each mouse for 5 days to check the parasitaemia level.

Calculation of Percentage Parasitaemia

The parasitaemia level was determined by counting parasites present in about five microscopic fields selected randomly. The percentage parasitaemia of each mouse was assessed, and the average percentage parasitaemia of each group was calculated as follows:

$$\%\ \text{Parasitaemia} = \frac{\text{No. of parasitized red blood cells (RBC) out of 500 erythrocytes}}{\text{Total No. of parasitized and un} - \text{parasitized RBC} \quad (10)} \times 100$$

***Allium cepa* Assay Procedure**

Allium cepa bulbs (purple onion) weighing 27-36g was used for this study. They were purchased from Oyingbo Market, Lagos, Nigeria, and were sun-dried for two weeks.

Viability Test

This test was conducted as described by Olorunfemi *et al*. (11). For de-scaling process, the older roots from the primordial root ring were carefully removed without harming the root ring. Thereafter, bulbs were suspended in distilled water (negative control) for 48hrs to determine the viable ones. The viable bulbs were subsequently chosen and used for subsequent studies. The water was changed daily throughout the period of the experiment.

Growth Root Inhibition Test

The onion bulb, exposed only to distilled water, was used as the control. The *Allium cepa* bulb root length was measured before being transferred into *S. latifolius* solution (25, 50 and 100mg kg-1). Root lengths of the *Allium cepa* bulb were measured in the intervals of 24hrs, 48hrs and 72hrs using a ruler. The result was expressed in centimeters (cm) (12).

The formulae for the calculation of both the percentage of root length and root length inhibition are as follows:

$$\text{Percentage root length} = \frac{\text{root length in test solution}}{\text{root length in control}} \times 100$$

$$\text{Percentage (\%)root length inhibition} = \frac{\text{root length in control} - \text{root length in test solution}}{\text{root length in control (water) (12)}} \times 100$$

Microscopic Examination and Determination of Mitotic Index:

Harvesting and Fixing of Root Tips

The control (water) roots were harvested after 48hours of growths while the roots treated with *S. latifolia* (25, 50 and 100mg kg-1) solution were harvested each day from hours of exposure, and were introduced into the fixative immediately (aceto-alcohol 1:3). Slide preparation was conducted in order to arrest mitosis (12).

Preparation of Slides

Root tips were gotten from the onion bulb. A clean glass slide was taken using a pair of forceps. The root tip was placed on the glass slide after which a razor blade was used to cut its tips and the remnant was discarded. The root tips were treated with a drop of 1 normal HCl for 5 minutes in order to soften the tissues. The excess 1 normal HCl was blotted out

neatly using a filter paper. The conventional feulgen-squash method (13) was used to prepare permanent slides of root meristems. Maceration was done using a dissecting needle to enhance stain uptake and to ensure the spreading of the cells in a monolayer for easy microscopic examination. A drop of lactic-acetic orcein stain was placed on the tissues and was left for 20 minutes to allow the stain penetrate the cells thoroughly (12). After staining, the slide was covered with a cover slip that would allow the stain to spread evenly over the square parts of the cover slip and the glass slide was subsequently placed between two folds of filter paper, and then pressure was applied around the square area of the cover slip for evenly squashing of the specimen. Finally, the square edges of the cover slip were sealed with the clean fingernail polish (14). This procedure aimed at preventing the drying out of the preparation by the heat of the microscope (13) with the clean fingernail polish.

Cytological Studies

The individually prepared slide was placed on the stage of the microscope and observed under the X40 objective for its mitotic stages. The total number of cells, and the total number of dividing cells were recorded. Scorings of chromosomal aberrations were taken from 5 microscopic fields for each of the different tests solutions and controlled having a total count of about 500 cells (12). Photomicrographs of normal and aberrant dividing cells were taken with National microscope (TSView CxImage Application)

Mitotic Index

Mitotic index was determined by the examination of 1000 cells as described by Samuel *et al*. (15).

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells counted}} \times 100$$

Mitotic Inhibition

The percentage of mitotic inhibition was calculated using the following formula:

$$\text{Mitotic inhibition} = \frac{\text{mitotic index in control} - \text{mitotic index in test solution}}{\text{mitotic index in control}} \times 100$$

Phase Index

The phase index was calculated using the following formula:

$$\%\ \text{Phase index} = \frac{\text{number of cells on each mitotic phase}}{\text{number of dividing cells (16)}} \times 100$$

Scoring for Chromosomal Aberrations

The calculation of the frequency of aberrant cells (cm) was conducted based on the number of aberrant cells per total cells scored for each *S. latifolius* (17).

Percentage (%) of Chromosomal Aberration

To determine the percentage of chromosomal aberration, the number of total aberration was divided by the total dividing cells and then was multiplied by 100. It was calculated as follows:

$$\text{Percentage aberration} = \frac{\text{number of total chromosomal aberration}}{\text{total number of dividing cells}} \times 100$$

Statistical Analysis

Student's t-test was utilized for statistical analysis and the results were expressed as Mean±SEM. Results were considered significant when $P<0.05$.

Results and Discussion

Antispasmodic Effects

Table 1 and 2 show the impact of the antiplasmodial activity of *Sarcocephalus latifolius* on mice.

Suppressive Test

With regard to the suppressive test, the results of *in vivo* chemosuppressive antimalarial assays of the graded doses of the ethanolic extract of *Sarcocephalus latifolius* using *P. berghei* on infected mice are indicated in Table 1. There was a dose dependent rise in activity (percentage suppression of parasitemia) with 4.9%, 16.1%, and 72.7% accompanying the administration of *S. latifolius* extracts at 25, 50, and 100 mg/kg, respectively. The standard drug chloroquine (5mg/kg) resulted in the production of 76.4% suppression in *S. latifolius* extract. The extracts and chloroquine did not cause any remarkable ($P<0.05$) decrease in parasitemia compared to the untreated control group.

Curative Test

Following the establishment of parasitaemia for 3 days post inoculation, the mice administered with *Sarcocephalus latifolius*, showed dose-dependent activities against *P. berghei*. The mean percentage parasitemia in groups that had been administered with

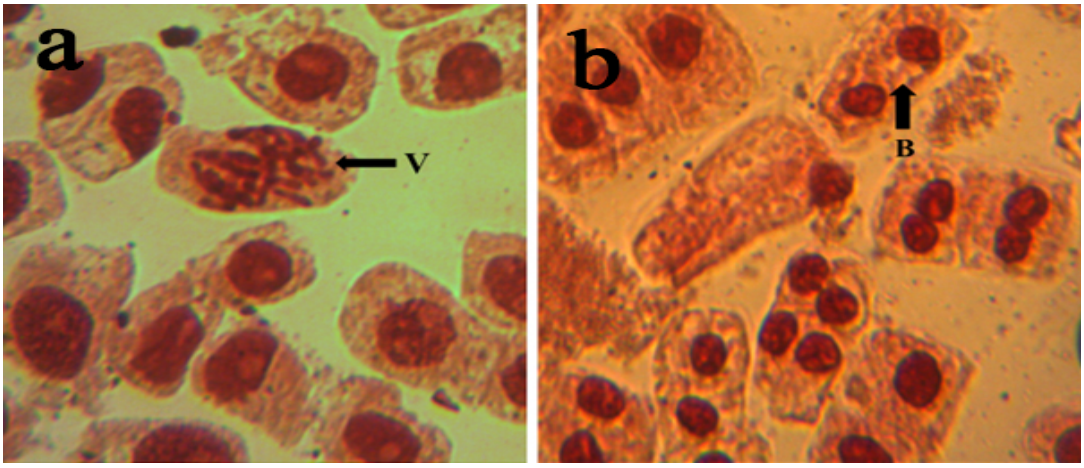

Figure 1. (a) V-vagrant metaphase in 25mg after 24 hours; (b) B-binucleated in 50mg after 48 hours.

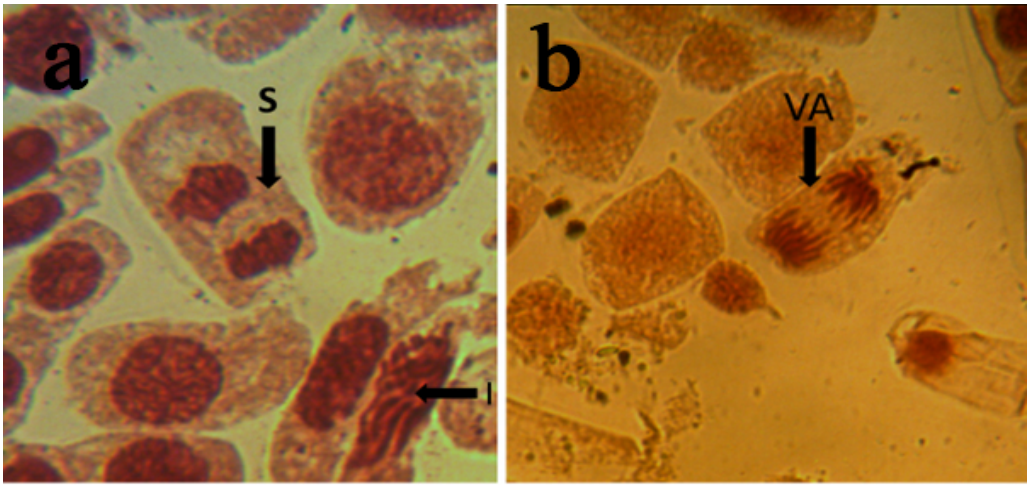

Figure 2. (a) S-sticky telophase, I-irregular prophase showing decompressed chromosome. In 50mg at 48hours; V-vagrant anaphase. In 2mmg at 72 hours.

the doses of the leaf extract and chloroquine reduced all along the period of observation, while an increase was observed in the control group administered with distilled water. There was no mean percentage parasitaemia for the groups treated with 25, 50 and100mg/kg on the eighth and the ninth days, while all the mice in the control died on these days. The groups treated with 25 mg/kg, 50mg/kg and 100mg/kg leaf extract, and chloroquine (5mg/kg) had percentage parasitemia of 1.5±0.5, 1.1±0.2, 0.6±0.1 and 0.00±0.00 respectively on the fourth day. As the days of infection increased, however, the groups administered with chloroquine (5mg/kg) were observed to have 100% clearance. The percentage parasitemia in groups administered with the doses of the leaf extract and chloroquine decreased throughout the period of observation, while an increase in mean percentage parasitemia was observed in the negative control group administered with distilled water. All the extracts and chloroquine brought about remarkable ($P<0.05$) decrease in parasitemia compared to the untreated control group.

Cytological Study

Tables 3-7 indicate the results obtained from *Allium cepa* assay. The microscopic and macroscopic analyses of the impact of *Sarcocephalus latifolius* on cell division indicated that it could interfere with mitosis. There was a dose response correlation in the

Table 1: The Impact of the Suppressive Activity of *Sarcocephalus latifolius* on Parasitemia Count of *P. berghei* Infected Mice (5-day test).

Treatment (mg kg-1)	Parasitaemia	Chemosuppression (%)
Control (Distilled water)	4.1±1.7	0.0
25	3.9±2.7	4.9
50	3.4±1.1	16.1
100	1.1±0.2	72.7
Chloroquine	0.9±0.5	76.4

Values are expressed as Mean ± SEM (n=5)

Table 2: The Impact of the Curative Activity of *Sarcocephalus latifolius* on *P. berghei* Infection in Mice (10-day test).

	Days									
Treatment (mg kg^{-1})	0	1	2	3	4	5	6	7	8	9
Control	3.4±1.0	4.8±0.8	5.4±0.8	5.7±0.8	6.3±0.8	7.7±0.8	7.9±0.7	8.3±1.1	All died	All died
25	3.6±0.8	3.3±1.0	2.7±0.8^{b}	2.2±0.7^{b}	1.5±0.5^{b}	1.1±0.3^{b}	1.1±0.3^{c}	0.8±0.2	0	0
50	2.6±0.4	1.7±0.4^{a}	1.5±0.3^{b}	1.3±0.2^{b}	1.1±0.2^{b}	0.7±0.2^{b}	0.7±0.2^{b}	0	0	0
100	2.1±0.6	1.7±0.6^{a}	1.0±0.3^{b}	0.7±0.2^{b}	0.6±0.1^{b}	0.6±0.2^{b}	0.6±0.2^{c}	0	0	0

Values are expressed as Mean ± SEM. Significance relative to control $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$; $^{d}p<0.0001$, n=5

Table 3: The Effect of *S. latifolius* on Root Length.

Treatment (mg/ml)	24hours	48hours	72hours
Control	1.47±012	1.51±0.09	1.75±0.17
25mg	1.28±0.11	1.33±0.16	1.24±0.13^{a}
50mg	1.20±0.09^{a}	1.02±0.07^{b}	1.05±0.15^{b}
100mg	1.19±0.07^{a}	0.96±0.07^{b}	0.88±0.07^{c}

Values are expressed as Mean ± SEM. Significance relative to control $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$; $^{d}p<0.0001$, n=5

Table 4: Chromosomal Aberration of *S. latifolius* on Cells of *Allium cepa*.

Treatment (mg/ml)	Mean±SEM			Percentage Root Length			Percentage Root Inhibition		
	24hours	48hours	72hours	24hours	48hours	72hours	24hours	48hours	72hours
Control	1.47±0.12	1.51±0.01	1.75±0.17	100	100	100	0	0	0
25	1.28±0.11	1.33±0.16	1.24±0.13	86.90	88.28	70.86	13.10	11.72	29.14
50	1.2±0.1	1.02±0.07	1.05±0.15	79.47	67.55	61.14	18.53	32.45	38.86
100	1.19±0.07	0.96±0.07	0.88±0.07	80.58	63.58	50.29	19.42	36.42	49.71

growth of *Allium cepa* root in the leaf extract of *S. latifolius*. The fact that all the concentrations (25, 50 and 100mg/ml) caused chromosome aberrations was statistically significant ($p<0.05$) from the control.

Macroscopic Effect

The effect of *S. latifolius* on the root growth of *Allium cepa* suggests their level of toxicity using growth inhibition as a determining factor. The mean root length of *Allium cepa* grown in *S. latifolius* water after the time interval of 24 hours, 48 hours and 72 hours are shown in table 3 and 4. The mean root length of *Allium cepa* bulb at 25, 50, and 100mg/ml of *S. latifolius* water increased as time increased compared to the onion bulbs grown in the control.

Percentage Root Length and Root Inhibition

The mean root length of *Allium cepa* grown in *Sarcocephalus latifolius* water was compared to the control length, and expressed as a percentage of the control values. There is an inverse relationship between the percentages of both root length and root inhibition. As the value of percentage root length for treatment after 24, 48 and 72 hours' decreases, the value for percentage root inhibition increases variably. As shown in table 4, after 72 hours, for concentration at 25, 50, and 100mg/ml, a reduction occurs in

Table 5: Chromosomal Aberration of *S. latifolius* on Cells of *Allium cepa*.

Concentration (mg/ml)	Number of dividing cell	Binucleated	Sticky	Vagrant	Percentage Chromosomal aberration
24 hrs					
25	35±4.0	21±2.0	10±0.5	5±0.3	102.85
50	32±1.0	15±1.2	11±1.1	3±0.1	90.63
100	28±0.0	6±0.3	17±1.3	4±0.0	89.29
48hrs					
25	27±2.5	12±1.0	17±1.4	10±1.0	144.44
50	23±2.0	18±1.5	11±1.5	13±0.5	182.61
100	16±1.5	11±1.0	22±3.0	9±1.6	262.50
72hrs					
25	17±1.6	5±0.5	12±1.0	4±0.2	123.53
50	6±0.2	5±0.2	3±0.0	2±0.0	166.67
100	5±0.2	4±0.0	4±0.0	1±0.0	180.00

N=5. Values are expressed as Mean ± SEM except % chromosomal aberration

percentage root length of 70.86, 61.14 and 50.29% respectively, while for percentage root inhibition, there is an increase of 29.14, 38.86, and 49.71% respectively.

Chromosomal Aberration

Most aberrations were recorded after 72 hours for 100mg/ml concentration that showed 180% aberration and a total of 9 aberrant cells (Table 5). Binucleated, sticky and vagrant cases were the chromosomal aberrations observed with *S. latifolius*. The different chromosomal aberrations are shown in figures 2 and 3.

Mitotic Index and Mitotic Inhibition

The mitotic index decreased as concentration of the treatment increased, while for 72 hours the mitotic inhibition increased with concentration of 100mg/ml showing 48.6% mitotic inhibition. Meanwhile, there was a drop in the value at the 25mg/ml concentration that only inhibited mitosis to 45.20%. There was an inverse relationship between the mitotic index and the mitotic inhibition so that as the concentration increased, the mitotic index decreased whereas the mitotic inhibition increased (Table 6).

Phase and Percentage Phase Index

The phase index of the mitotic cells per concentration after 24, 48 and 72 hours is indicated in Table 5. There was a higher level of mitotic arrest at prophase than at any other phase of mitosis. Telophase recorded a

Table 6: Mitotic Index and Mitotic Inhibition of *S. latifolius* on the Cells of *Allium cepa*.

Concentration	Cell Counted	Number of Dividing	Mitotic Index(%)	Mitotic inhibition
24hours				
control	1000	500	50	0
25mg	875	35	4	42.00
50mg	853	32	3.8	42.40
100mg	818	28	3.4	43.20
48hours				
25mg	782	27	3.5	43.00
50mg	773	23	3.0	44.0
100mg	731	16	2.2	45.6
				45.6
72hours				
25mg	712	17	2.4	45.2
50mg	680	6	0.9	48.2
100mg	670	5	0.7	48.6

Table 7: Phase and Percentage Phase Index.

Concentration	Number of dividing cell	PHASE				Percentage Phase (%) Index			
		P	M	A	T	P	M	A	T
24hours									
control	500	200	150	100	50	40	30	20	10
25mg	35	16	8	7	4	45.71	22.86	20.00	11.43
50mg	32	12	9	6	5	37.50	28.13	18.75	15.63

generally low mitotic arrest. Moreover, after 72 hours there was no mitotic arrest recorded for telophase at 25 and 50mg/ml.

In this study, the impacts of the antiplasmodial and cytological activities of *S. latifolius* on mice and *Allium cepa* were evaluated. *Plasmodium berghei*, a rodent parasite has been used in investigating the activity of potential antimalarials in mice and rats (12, 18). The results, which showed that the leave extract of *S. latifolius* could have noticeable antiplasmodial activity as evident from the chemosuppression obtained during the suppressive and curative phase, may suggest the plant to be a potential drug-lead entity for malarial infection. Reports have indicated that some metabolites in plants have been implicated with antiplasmodic activities (19, 20). Pharmacognostic studies have shown the presence of metabolites such as tannins, flavonoids, carbohydrates, cardiac glycosides (21) phenols, anthraquinones, glycosides, alkaloids, saponins (7) and terpenes (22) in *S. latifolius*. The death of the plasmodium parasites has been associated with the presence of monoterpene indole alkaloids from medicinal plants which elicit endoperoxidation by generating reactive oxygen species which culminates in the death of the parasite (23, 24). The detection of flavonoids in this plant may also contribute to the antimalarial activities as these metabolites have been proven to possess potential immune modulatory effect in other plants and antioxidant effect, which might play a role in diseasing the resistance (25). Hence, the presence of alkaloids and flavonoids in *S. latifolius* may have elicited endoperoxidation thereby causing death of the parasite.

The regular and significant decrease in root tip growth at increasing concentration might imply the existence of toxicity which could cause the significant chromosomal aberrations, consequent gradual and significant reduction in mitotic index value. This drop in the value of mitotic index indicates interference in cell cycle (26). Fiskesjo (12) described vagrant chromosomes and C-mitotis as indicative of the risk of aneuploidy while sticky chromosomes exhibit a highly toxic, irreversible effect that might lead to cell death. There is a hypothesis that stickiness of chromosomes may result in the incomplete separation of daughter chromosomes due to cross-linkage chromoproteins (27). Similarly, stickiness is an indicator of cell death and may be caused by physical adhesion of proteins to the chromosomes (28). The presence of these abnormalities in the chromosomes of the test plant used for the study indicates that organisms exposed to this leaf extract at the tested concentration may suffer from cell death or have the risk of non-disjunction of chromosomes.

Conclusion

This study, therefore, has exhibited in an animal model of malaria the efficiency of *S. latifolius* traditionally used in Abeokuta, southwestern Nigeria, for chemotherapy of *Plasmodium falciparum* infection in humans. Hence, the traditional use of these plants in the treatment of malaria is based on a real anti-parasitic activity. However, the doses can still be worked upon as some chromosomal aberrations were observed that might be indicative of the signs of toxicosis. To a reasonable extent, this plant is safe with regard to the tested parameters. Nevertheless, caution must continue to be applied in the use of crude plant extracts. Tests must continue to be conducted on other indices relevant to the assessment of toxicity of herbal materials.

Acknowledgment

Non.

Conflict of Interest

The authors declare that they have no conflict of interest.

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