

***In vitro* antiplasmodial activity and phytochemical screening of *Alstonia boonei* De wild used in treating malaria-associated symptoms**

T.A Enemakwu^{*}, M.M Adeyemi¹ and L. Salihu²

Department of Chemistry, Nigerian Defence Academy Kaduna, Nigeria.

Corresponding author*

T.A Enemakwu

Department of Chemistry,

Nigerian Defence Academy Kaduna, Nigeria

E-mail: enemkwuthomas@gmail.com

Abstract

Background & objectives: This study was undertaken to investigate the *In vitro* antiplasmodial activity and phytochemical screening of the leaves from *Alstonia boonei* to support its traditional use as a remedy for malaria.

Methods: The crude ethanol extract (21.662g) from the medicinal plant (AB_{.01}) was fractionated into ethyl acetate (AB_{.01-01}), chloroform (AB_{.01-02}) and n-hexane (AB_{.01-03}) fractions. These were subjected to *In vitro* antiplasmodial assays using confirmed *Plasmodium falciparum* infected blood sample from malaria patients. Phytochemical screenings of ethyl acetate, chloroform and n-hexane fractions from the plant were further carried out.

Results: Plant's leaves extracts and fractions were found to be active against *Plasmodium falciparum* with percentage elimination range of 40.90 to 81.81%. Alkaloids, saponins tannins, flavonoids, steroids and glycosides were detected in the extracts of the plant. The analysis of the results of the extract and that of the standard (positive control) using Mann-Whitney U test indicate that there is no significant difference between them. Spearman rank correlation analysis of the plant samples with the positive control samples showed that there is correlation and the degree of relationship between the antiplasmodial activities of the plant samples and positive control samples is positively perfect ($r_s = 1$).

Conclusion: This study has proven that the medicinal plant used for this study is active against *Plasmodium falciparum* parasite. Tannins, Saponins, Flavonoid, Steroids, Alkaloids and Cardiac glycoside were found to be present in the medicinal plant. Further studies need to be done to isolate and characterize the most active constituents of extract from the plant.

Keywords: *In-Vitro* antiplasmodial, Phytochemical, *Alstonia boonei* De Wild

1. Introduction

Plants have been used as a source of medicines that form the backbone of human healthcare from ancient times. Today, medicines from medicinal plants form the basis of primary healthcare for majority of the people living in rural and urban areas [1]. *Alstonia boonei* De Wild (*Apocyanaceae*) has a history of use in traditional medicine in Nigeria for malaria treatment and prevention of disease [9]. Malaria is a worldwide public health concern. The World Health Organization (WHO) [16], reported an estimate of 207 million cases and 627,000 deaths globally in 2012 alone. It has been reported that, over 70 countries are malaria endemic and globally, an estimated 3.4 billion people are at risk of malaria infection. Despite the substantial progress made in the treatment of parasitic diseases, malaria remains a significant therapeutic challenge. Malaria treatment failures are due to *P. falciparum* developing reduced sensitivity to Artemisinin based Combination Therapy (ACTs) [15], and the parasites recrudescence after treatment with ACTs [4]. Therefore, lack of new antimalarial drugs in the pipeline and reduced sensitivity, highlight the urgent need to search for new antimalarial drugs to replace the currently used or to expand the antimalarial drug arsenal.

2. Materials and Methodology

2.1 Sampling

The fresh morphological part of the plant was collected from Eke-Avrugo Community in Igalamela-Odolu LGA of Kogi State identified and authenticated by Prof. M.O Nwosu of Department of Plant Science and Biotechnology University of Nigeria, Nsukka as *Alstonia boonei* De Wild (UNH No_{.12a}).

2.2 Materials

The materials and reagents that were used for this study are outlined below. *Alstonia boonei*, Infected *Plasmodium falciparum* blood, Ethanol, methanol, n-hexane, chloroform, ethyl acetate, dimethyl sulfoxide (DMSO), Giemsa's

stain, Roswell Park Memorial Institute (RPMI) 1640 medium, gentamicin, ferric chloride, hydrochloric acid, sodium hydroxide, silica gel, Round bottom flasks, conical flasks, beakers, glass rod, measuring cylinder, weighing balance, test tubes, Rotary evaporator, glass slides, cover Slip, microscope, EDTA bottles and incubator.

2.3 Preparation of the plants materials

The leaves of the plant, *Alstonia boonie* were collected, washed and air-dried for two weeks after which they were grinded, subsequently sieved into powder form and stored in air tight containers for further use.

2.4 Maceration

A weight of 200g of the powdered plant sample was percolated in 95% ethanol (1litre) at room temperature for 2 weeks. This was then filtered using a no.1 Whatman filter paper. The solvent was evaporated using a rotary evaporator (R110) at 40°C. The *Alstonia boonie* ethanol extracts was weighted and labeled as AB_{.01}. The ethanol extract (AB_{.01}) was extracted successively with chloroform, n-hexane and ethyl acetate according to the protocols described by Fatope *et al.* [5].

2.5 Phytochemical Screening

A weight of 10g of the pulverized plant sample was macerated in 50cm³ each of the following solvents: Ethyl acetate, Chloroform, n-hexane and Ethanol for one (1) week. The filtrates obtained from the solvent fractions were subjected to phytochemical screening. The phytochemicals screened include alkaloids, saponins tannins, flavonoids, steroids and glycosides [7,12,14]

2.5.1 Test for Tannins:

1cm³ each of the fractions were separately added to 1cm³ of distilled water in test tubes. A few drops of 10% ferric chloride was added and observed for brownish green or a blue-black colouration.

2.5.2 Test for Saponin:

1cm³ each of the fractions were separately mixed with 4cm³ of distilled water and shaken vigorously for a stable persistent froth.

2.5.3 Test for Flavonoid: (Shinoda's test)

Three pieces of magnesium chips was added to about 1cm³ each of the fractions, followed by few drops of conc. HCl, shaken vigorously and observed for the development of a pink, orange, or red to purple colouration.

2.5.4 Test for Cardiac Glycosides:

1cm³ of concentrated sulphuric acid was gently poured on the walls of an inclined test tube containing 1cm³ each of the fractions separately. 10% Ferric chloride solution was added in drop wise and a brown ring was observed.

2.5.5 Test for steroids:

To test the presence of steroid phytochemicals, 1cm³ each of the fractions were separately dissolved in 10cm³ chloroform and equal volume of concentrated sulphuric acid (H₂SO₄) added by sides of test tube. The upper layer turned red and sulphuric acid layer turned yellow with green fluorescence. This indicated the presence of steroids.

2.5.6 Test for Alkaloids:

Preparation of Wagner's reagent: This was prepared by dissolving 1.0g of I₂ crystals and 3.0g of KI in distilled water and making up to the mark in 50cm³ volumetric flask.

Test: 4cm³ of 1% hydrochloric acid was added to 1cm³ each of the fractions in separate test tubes, mixed and the solutions warmed in a warm bath. The presence of turbidity or precipitate after the addition of 1ml of Wagner's reagent confirmed the presence of alkaloid.

2.6 Malaria parasite assay

2.6.1 Preparation of Test Solution

A stock solution of 10mg/cm³ of the extract and fractions were prepared by dissolving 0.02g of the dried extract and fractions separately in 2cm³ of 1% Dimethyl sulphoxide (DMSO). The following concentrations 0.5mg/cm³, 1.0mg/cm³, 2.0mg/cm³ and 5.0mg/cm³ were prepared from the stock solutions of each of the extract and fractions by serial dilution. *Artemether lumefantrine* anti-malaria drug was used as Positive control prepared in the same way as test solutions. RPMI 1640 and 1%DMSO don't affect the development of *P. falciparum* in the culture medium [8].

2.6.2 Sourcing of Malaria Parasites for the Assay

Confirmed malaria parasites infected blood sample of patients were collected from Hematology Department Bayero University Hospital, Kano. The samples were immediately transferred into K3-EDTA disposable plastic sample bottles corked and transported to the Microbiology laboratory at Bayero University in a thermo flask containing water maintained at 4°C as demonstrated by Dacie and Lewis [2].

2.6.3 Separation of the Erythrocytes from the Serum of the Blood Samples

50% dextrose solution (0.5cm³) was added to each of the blood samples (5cm³) defibrinated, and then centrifuged at 2500rpm for 15minutes in a spectra merlin centrifugation machine. Supernatant layers were separated from the

sediments. The latter was diluted with normal saline [1] and centrifuged at 2500rpm for 10minutes. The resulting supernatants were discarded. Samples with higher parasitaemia (above 0.5%) were diluted with fresh malaria parasite negative erythrocytes [6].

2.6.4. Determination of *Plasmodium falciparum* initial count.

The method described by Dacei and Lewis was adopted. Using a clean dropper pipette, a small drop of the defibrinated erythrocyte blood sample above was smeared on microscopic slide and stained by Giemsa's staining techniques. The mean number of erythrocytes appearing as blue discoid cells containing life rings of the parasite (that appeared red pink) was estimated as the initial count of the parasite before incubation.

2.6.5 Preparation of *Plasmodium falciparum* Culture Medium

Venous blood (2cm^3) from the main vein of white healthy rabbits pinnae was withdrawn using a disposable 5cm^3 syringe (BD 205 WG). This was defibrinated by allowing it to settle for at least one hour [2]. The defibrinated blood was centrifuged at 1500rpm using spectre merlin centrifuge for 10minutes and the supernatant layer was collected in a sterilized tube. The sediment was further centrifuged at 1500rpm for five minutes, and the supernatant layer was added to the first test tube. The sediments were discarded and the serum collected was supplemented with the salt of RPMI 1600 medium (KCl 5.37mM, NaCl 10.27mM, MgSO_4 0.4mM, NaHPO_4 17.73mM, $\text{Ca}(\text{NO}_3)_2$ 0.42mM, NaHCO_3 2.5mM, and glucose 11.0 mM. (BDH Ltd, UK) as demonstrated by Devo *et al*[3]. The medium was sterilized with $40\mu\text{g}/\text{cm}^3$ gentamicin sulphate [13].

2.6.6 In-Vitro Assay of the Activity of the Extracts and Fractions on *Plasmodium falciparum* Culture

A 0.1cm^3 of test solution and 0.2cm^3 of the culture medium were added into a tube containing 0.1ml of 0.5% parasitaemia erythrocytes and mixed thoroughly. The sensitivity of the parasites to the samples was determined microscopically after incubation for 48 hours at 37°C . The incubation was undertaken in glass bell jar containing a lighted candle to ensure the supply of required quantity of CO_2 about 5% O_2 gas, 2% and nitrogen gas 93% as demonstrated by [10].

2.6.7 Determination of the Activity

At the end of the incubation periods 48 hours, the sample was centrifuged to remove the culture media and a drop of the sample (red blood cell only) was smeared on microscopic slides and stained by Giemsa's staining techniques. The mean number of erythrocytes appearing as blue discoid cells containing life rings of the parasite (that appeared red pink) was estimated and the average percentage elimination by the samples was determined. The activity of the test samples was calculated as the percentage elimination of the parasites after incubation period of 48 hours, using the formula below;

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

Where, % = Percentage activity of the test sample; N = Total number of parasites eliminated by the sample; N_x = Total number of parasitized RBC.

Note: RBC = Red Blood Cells [10]

3. Results

The results of all the experiment conducted are presented as given below:

Table 1: Results of ethanol extract and its macerated fractions

Fractions	Colour of the solution	Weight of Extracts (g)	Percentage yield (%)
AB-01	Deep green	21.662	10.83
AB _{.01-01}	Deep green	1.1	5.08
AB _{.01-02}	Dark green	1.6	7.39
AB _{.01-03}	Light yellow	1.5	6.92

AB_{.01} = ethanol extract, AB_{.01-01} = Ethyl acetate fraction from ethanol extract. AB_{.01-02} = Chloroform fraction from ethanol extract. AB_{.01-03} = hexane fraction from ethanol extract.

Table 2: Results of phytochemical screening of the various fractions of plants

Phytochemicals	Ethyl acetate fraction AB _A	Chloroform fraction AB _C	Ethanol fraction AB _E	Hexane fraction AB _H
Tannins	-ve	-ve	+ve	-ve
Saponins	-ve	+ve	-ve	+ve
Flavonoid	-ve	+ve	+ve	NI
Steroids	+ve	+ve	+ve	+ve
Alkaloids	+ve	NI	+ve	-ve
Cardiac glycoside	+ve	+ve	+ve	+ve

+ve = present; -ve = absent; NI = not intense

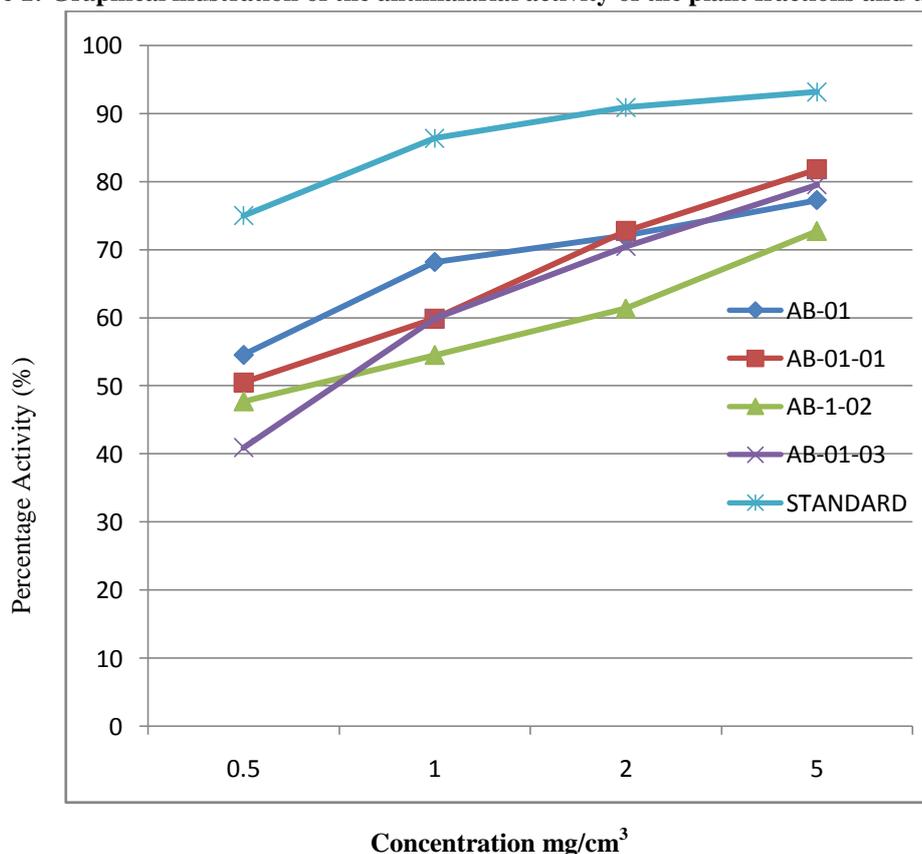
Table 3: Results of Antimalarial activity of fractions from the leaves of *Alstonia boonie*

Fraction	Concentration (mg/cm ³)	Average No. of parasite before incubation	Average No. of parasite after 48hrs	Percentage elimination at the end of incubation (%)
AB ₋₀₁	0.5	44	20	54.54
	1.0	44	14	68.18
	2.0	44	12	72.72
	5.0	44	10	77.27
AB ₋₀₁₋₀₁	0.5	44	22	50.00
	1.0	44	18	59.90
	2.0	44	12	72.72
	5.0	44	8	81.81
AB ₋₀₁₋₀₂	0.5	44	23	47.73
	1.0	44	20	54.54
	2.0	44	17	61.36
	5.0	44	12	72.72
AB ₋₀₁₋₀₃	0.5	44	26	40.90
	1.0	44	18	59.90
	2.0	44	13	70.45
	5.0	44	9	79.55

Table 4: Results of Antimalarial activity of Positive control

Control	Concentration (mg/cm ³)	Average No. of parasite before incubation	Average Number of parasite after 48hrs	Percentage elimination at the end of incubation (%)
Positive	0.5	44	11	75
	1.0	44	6	86.36
	2.0	44	4	90.90
	5.0	44	3	93.18

Figure 1: Graphical illustration of the antimalarial activity of the plant fractions and the Standard



3.1 Analysis of Results

Table 5: Results of antiplasmodial activity of positive control and resized results of antiplasmodial activity of the three plant samples.

Concentration mg/cm ³	0.5	1.0	2.0	5.0
AB Test sample	40.90	54.54	72.72	81.81
Positive Control	75	86.36	90.90	93.18

Mann-Whitney U Test and Spearman's Rank Correlation were adopted for the analysis of the results [10].

(ai) Mann-Whitney U Test Analysis of antiplasmodial results of *Astonia boonie*

Mann-Whitney U Test is used to determine whether there is a difference at 0.05 significance level between the results of the positive control and the test samples.

Mann-Whitney U Test is given by the equation 1 below:

$$Z = \frac{U - \mu_u}{\sigma_u} \quad \text{--- eqn1, } U = N_1N_2 + \frac{N_1(N_1+1)}{2} - R_1 \dots \text{eqn2}$$

$$\mu_u = \frac{N_1N_2}{2} \text{-----eqn3,} \quad \sigma_u = \sqrt{\frac{N_1N_2(N_1+N_2+1)}{12}} \text{-----eqn4}$$

X ₁	75.00	86.36	90.90	93.18
X ₂	40.90	54.54	72.72	81.81

X₁ = results of the positive control samples, X₂ = results of the test samples

Step 1: Rank X₁ and X₂ from the lowest to the highest.

40.9	54.54	72.72	75.00	81.81	86.36	90.90	93.18
1	2	3	4	5	6	7	8

Step 2: Separate the samples with their ranks

X ₁	Rank	X ₂	Rank
75.00	4	40.9	1
86.36	6	54.54	2
90.90	7	72.72	3
93.18	8	81.81	5
Sum = 25		Sum = 11	

Step 3: N₁ = N₂ = 4 and R₁ = 25 while R₂ = 11

Substituting the values N and R in eqn. 2, we have

$$U = N_1N_2 + \frac{N_1(N_1+1)}{2} - R_1 = (4)(4) + \frac{4(4+1)}{2} - 25 = 1$$

$$\mu_u = \frac{N_1N_2}{2} = \frac{(4)(4)}{2} = 8$$

$$\sigma_u = \sqrt{\frac{N_1N_2(N_1+N_2+1)}{12}} = \sqrt{\frac{(4)(4)(4+4+1)}{12}} = 3.46$$

$$Z = \frac{U - \mu_u}{\sigma_u} = \frac{1 - 8}{3.46} = -2.02$$

Test Statistics

H₀: There is no significant difference between X₁ and X₂

H₁: There is significant difference between X₁ and X₂

Decision Rule

Reject H₀ if Z_{cal.} > Z_{tab} otherwise do not reject H₀

Using a two tail test, t = $\frac{1 - \alpha}{2}$ where α = 0.05,

$$t = \frac{1 - 0.05}{2} = 0.475$$

Therefore, the Z_{tab} value -1.96.

Conclusion since $Z_{\text{cal}} = -2.02 < Z_{\text{tab}} = -1.96$ at $\alpha = 0.05$, we do not reject H_0 , therefore there is no significant difference between samples X_1 and X_2 at 95% confidence interval.

(iii) Spearman Rank Correlation analysis of antiplasmodial results

Spearman rank correlation denoted by r_s is used to investigate if there is any relationship between samples X_1 and X_2 and the degree of the relationship.

X_1	X_2	R_1	R_2	$R_1 - R_2$ (d)	d^2
75.00	40.9	1	1	0	0
86.36	54.54	2	2	0	0
90.90	72.72	3	3	0	0
93.18	81.81	4	4	0	0

$\sum d^2 = 0$

$$r_s = 1 - \frac{6\sum d^2}{n(n^2 - 1)} = 1 - \frac{6(0)}{4(4^2 - 1)} = 1$$

Since $r_s = 1$, the degree of correlation is positively perfect.

Test Statistics: $H_0: \rho = 0$ (there is no correlation), $H_1: \rho \neq 0$ (there is correlation)

Decision rule: Reject H_0 if $Z_{\text{cal}} > Z_{\text{tab}}$ at 10% level of significance

$$Z = r_s \sqrt{n+1} = 1 \cdot 4 \sqrt{1} = 2.24$$

Since $Z_{\text{cal}} = 2.24 > Z_{\text{tab}} = 1.65$, we therefore rejected H_0 and concluded that there is correlation between X_1 and X_2 at 10% level of significance.

4. Discussion

The yield of ethanol extract and its macerated ethyl acetate, chloroform and n-hexane fractions of the medicinal plant is shown in Table 1. Comparing the fractions, AB₀₁₋₀₂ has the highest weight of 1.6g while AB₀₁₋₀₁ has the least weight of 1.1g.

The result of phytochemical screening of the various fractions of the plant is shown in Table 2. The phytochemicals include alkaloids, saponins, tannins, flavonoids, steroids and glycosides with promising antiplasmodial activity. From the results, it showed that the leaves of the plant sample contain the phytochemicals screened for in one or more of the solvent fractions. Tannins were rarely present in the less polar solvent. Saponins were found to be present mainly in the less polar solvent fractions. Flavonoids and alkaloids were found in almost all the fractions while steroids and cardiac glycoside were found to be present in all the fractions.

Tables 3 show the antiplasmodial activities of the fractions. Their percentage activities were investigated at various concentrations of 0.5mg/cm³, 1.0mg/cm³, 2.0mg/cm³ and 5.0mg/cm³. It was found that all fractions were active against the *plasmodium* parasite. The results showed that percentage elimination increases as the concentrations of the extract and fractions increase. These were also illustrated in graphical form. At 0.5mg/cm³, AB₀₁ has the highest activity of 54.54%. At concentration of 5.0mg/cm³, AB₀₁₋₀₁ has the highest activity of 81.81%.

Table 4 shows the results of antimalarial activity of the positive control. At 5.0mg/cm³, the percentage elimination activity is 93.18% and at 0.5mg/cm³, the percentage activity is 75%. The results of antiplasmodial activities of the positive control were compared with that of the plant test samples. Random sampling technique was used to resize the results of the antiplasmodial activities of the plants to the corresponding number of results in the positive control as shown in Table 5. The data analysis of the antiplasmodial results of the plant test samples with the standard samples (positive control) was conducted using Mann-Whitney U test and Spearman Rank Correlation. The results of the data analysis showed that there is no significance difference between the antiplasmodial activities of the plant samples and the positive control samples at 95% confidence interval. Spearman rank correlation analysis of the plant samples with the positive control samples showed that there is correlation and the degree of relationship is positively perfect ($r_s = 1$).

5. Conclusion

This study has proven that the medicinal plant used for this study is active against *Plasmodium falciparum* parasite. Tannins, Saponins, Flavonoid, Steroids, Alkaloids and Cardiac glycoside were found to be present in the medicinal plant.

The phytochemical screening and *in vitro* antiplasmodial activity of the ethanol extracts as well the fractions from the plant supported the use of these plants to treat malaria associated symptoms in traditional settings. Data analysis of the results of the extract and that of the standard (positive control) using Mann-Whitney U test indicate that there is no significant difference between them. Spearman rank correlation analysis of the plant samples with the positive control samples showed that there is correlation and the degree of relationship is positively perfect ($r_s = 1$).

Reference

- [1] Balick, M.J. and Cox, P.A. Plants, People, and Culture: The Science of Ethnobotany. Scientific American Library. New York 1996.
- [2] Dacie JV and Lewis SM. *Practical Haematology* fourth edition Black well Publishers, USA, PP 1968: 65-66.
- [3] Devo AA, Groary TG, Davis NL, Jonson J.B. Nutritional Requirements of *Plasmodium falciparum* in Culture necessary for continues growth. *I. Protozoal* 1985; 32:59-64.
- [4] Dondorp, A. M., Yeung, S., White, L., Nguon, C., Day, N. P., Socheat, D., & von Seidlein, L. Artemisinin resistance: current status and scenarios for containment. *Nature Reviews Microbiology*, 2010; 8 (4): 272-280.
- [5] Fatope, M. O; Ibrahim H. and Takeda Y. Screening Higher Plants Reputed as Pesticides Using the Brine Shrimp Lethality Assay: *Inter. J. of Pharmacognosy*, 1993; 31 (4): 250-256.
- [6] Hanne I.Z; Dan S; Jette C, Lars H; Henri Jerzy WJ. *In-vitro P. falciparum* sensitivity assay. *Antimicrobial agents and Chemotherapy*, 2002; 42(6): 1441-1446.
- [7] Harbone JB (ed.). *Methods of plants analysis. Phytochemical Methods* Chapman and Hall London 1973.
- [8] Josué W.P, Nadia N.A.C; Claire K.M; Jeannette Y, Mbida M. *In Vitro* sensitivity of Plasmodium *falciparum* field isolates to methanolic and aqueous extracts of *Cassia alata* (Fabaceae). *Altern Integ Med* 2014; 3: 159. doi:10.4172/2327-5162.1000159
- [9] Majekodunmi SO, Adegoke OA, Odeku OA. Formulation of the extract of the stem bark of *Alstonia boonei* as tablet dosage form. *Trop J Pharm Res.* 2008; 7:987-94.
- [10] Mukhtar MD, M Bashir and Arzai A.H. Comparative invitro studies on antiplasmodial acticity of some Nigerian and foreign brands of chloriquine oral formulation marketed in Kano. *African Journal of Biotechnology*, 2006; 5(24): 2464-2468.
- [11] Murray R. Spiegel and Larry J. Stephens. *Theory and problems of statistics.* Fourth edition 2008.
- [12] Sofowora A.E. *Medicinal and traditional medicine in Africa.* Vol. 2 Spectrum Books Ltd. 1993; 142-146.
- [13] Trager W. Cultivation of Malaria Parasites. *British Mee Bull* 1982; 38(2):129-31.
- [14] Trease, G.E. and Evans, W.C. *Pharmacognosy.* 15th Ed. Saunders Publishers, London. pp. 2002; 42-44, 221-229, 246-249, 304-306, 331-332, 391-393.
- [15] Whegang, S. Y., Tahar, R., Foumane, V. N., Soula, G., Gwét, H., Thalabard, J. C., & Basco, L.K. Efficacy of non-artemisinin and artemisinin-based combination therapies for uncomplicated *falciparum* malaria in Cameroon. *Malaria Journal*, 2010; 9: 56.
- [16] WHO. (2013). *World malaria report.* Geneva Switzerland.