British Journal of Pharmaceutical Research
9(3): 1-8, 2016, Article no.BJPR.21453
ISSN: 2231-2919, NLM ID: 101631759

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In vitro Antiplasmodial Activity and Phytochemical Screening of *Newbouldia laevis* Used in Treating Malaria-associated Symptoms

Enemakwu Thomas Andrew[1*], M. M. Adeyemi[1] and L. Salihu[1]

[1]Department of Chemistry, Nigerian Defence Academy Kaduna, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author ETA designed the study, wrote the protocol and the first draft of the manuscript and conducted the research work. Author MMA supervised and guided the work. Author LS contributed in the interpretation and analysis of the results. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2016/21453
Editor(s):
(1) Syed A. A. Rizvi, Department of Pharmaceutical Sciences, College of Pharmacy, Nova Southeastern University, USA.
Reviewers:
(1) Aina Oluwagbemiga Olanrewaju, Nigerian Institute of Medical Research, Nigeria.
(2) Abdullahi M. Nuhu, Kaduna Polytechnic, Nigeria.
Complete Peer review History: http://sciencedomain.org/review-history/12190

Original Research Article

Received 18th August 2015
Accepted 12th September 2015
Published 9th November 2015

ABSTRACT

This study investigated the *In vitro* antiplasmodial activity and phytochemical constituents of the leaves from *Newbouldia laevis* used in the local treatment of malaria. The crude ethanol extract from the medicinal plant (NL_{-01}) was fractionated into ethyl acetate (NL_{-01-01}), chloroform (NL_{-01-02}) and n-hexane (NL_{-01-03}) fractions. NL_{-01-03} has the highest weight of 1.7 g followed by NL_{-01-01} (0.9 g). SL_{-01-02} has the least weight of 0.7 g. *In vitro* antiplasmodial assays and phytochemical screening of ethyl acetate, chloroform and n-hexane fractions from the plant were further carried out. Alkaloids, saponins tannins, flavonoids, steroids and glycosides were detected in the extracts of the plant. Plant's leaves extracts and fractions were found to be active against *Plasmodium falciparum* with percentage elimination range of 43.18 to 79.54%. The results showed that percentage elimination increases as the concentrations of the extract and fractions increase. At 0.5 mg/cm^3, SL_{-01-01} has the highest activity of 54.54% while SL_{-01-02} has the least percentage elimination of 43.18%. At concentration of 5.0mg/cm^3, NL_{-01} has the highest activity of 79.54% while NL_{-01-02} has the least activity of 72.72%. The analysis of the results of the extract and that of the standard (positive

**Corresponding author: Email: enemakwuthomas@gmail.com;*

control) using Mann-Whitny U test indicate that there is no significant difference between them 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 between the antiplasmodial activities of the plant samples and positive control samples is positively perfect ($\gamma_s = 1$).
TLC screening of the ethyl acetate fraction using ethyl acetate/n-hexane mixture in the ratio 1:4 revealed four spots on the plate.

Keywords: Newbouldia laevis; In-vitro antiplasmodia; phytochemical.

1. INTRODUCTION

Malaria is a vector borne disease, caused by protozoan parasites of the genus *Plasmodium* transmitted from the blood of an infected person and passed to a healthy human by a female anopheles mosquito bites. They are single celled organisms that cannot survive outside of their host(s) [1]. There are four main malaria causing parasites namely: *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium falciparum*. Additionally, in some parts of Southern Asia there are reports of *P. knowlesi* infecting human beings, [2] and [3]. The parasites multiply within red blood cells (RBC) causing symptoms that include anaemia as well as other general symptoms such as fever, chills, nausea and flu-like illness and in severe cases coma and death. Malaria is a worldwide public health concern. The World Health Organization (WHO) Malaria World Report, [4] 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), [5,6] and the parasites recrudescence after treatment with ACTs, [7]. It has been reported that ACT's reducing sensitivity has spread to Africa as well, [8].

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. According to the WHO, [9] more than 80% of people living in developing countries still depend on herbal medicines to treat common diseases including malaria [10]. Evidently, more than 62% of new small chemical drugs approved in the United States of America between 1981 and 2007 were isolated from medicinal plants or synthesized from compounds isolated form plants.

2. EXPERIMENTAL SECTION

2.1 Sampling

The fresh leaves 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 *Newbouldia laevis* (UNH No_340b).

2.2 Preparation of the Plants Materials

The leaves of the plant, *Newbouldia laevis* 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.

2.3 Maceration

A weight of 200 g of the powdered plant sample was macerated in 95% ethanol (1 litre) at room temperature for 2 weeks. This was then filtered using a no.1 whatmans filter paper. The solvent was evaporated using a rotary evaporator (R110) at 40℃. The *Newbouldia laevis* ethanol extracts was weighted (22.739g) and labeled as NL_{-01}, [11].

2.4 Merceration of the Crude Extracts (NL_{-01})

The ethanol extract (NL_{-01}) was extracted successively with chloroform, n-hexane and ethyl acetate according to the protocols described by Fatope et al. [12].

2.5 Phytochemical Screening

A weight of 10 g of the pulverized plant sample (*Newbouldia laevis*) was macerated in 50 cm^3 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 [13-15].

2.5.1 Test for tannins

1 cm^3 each of the fractions was separately added to 1 cm^3 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

1 cm^3 each of the fractions were separately mixed with 4 cm^3 of distilled water and shaken vigorously for a stable persistent froth.

2.5.3 Test for flavonoid

1 cm^3 of 10% NaOH was mixed with 1 cm^3 each of the fractions, shaken vigorously and observed for the development of a yellow colouration.

2.5.4 Test for cardiac glycosides

1 cm^3 of concentrated sulphuric acid was gently poured on the walls of an inclined test tube containing 1 cm^3 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, 1 cm^3 each of the fractions were separately dissolved in 10 cm^3 chloroform and equal volume of concentrated sulphuric acid (H_2SO_4) 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

2.5.6.1 Preparation of wagner's reagent

This was prepared by dissolving 1.0 g of I_2 crystals and 3.0 g of KI in distilled water and making up to the mark in 50cm^3 volumetric flask.

2.5.6.2 Test

4 cm^3 of 1% hydrochloric acid was added to 1 cm^3 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 1 ml 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 10 mg/cm^3 of the extract and fractions were prepared by dissolving 0.02 g of the dried extract and fractions separately in 2 cm^3 of 1% Dimethyl sulphoxide (DMSO). The following concentrations 0.5 mg/cm^3, 1.0 mg/cm^3, 2.0 mg/cm^3 and 5.0 mg/cm^3 were prepared from the stock solutions of each of the extract and fractions by serial dilution using:

$$C_1V_1 = C_2V_2$$

C_1 = concentration of the stock solution, C_2 = final or expected concentration
V_1 = volume of stock solution, V_2 = final volume (that is V_1 + volume of solvent).

0.5 mg/cm^3 was prepared by diluting 0.05 cm^3 of the stock solution with 0.95 cm^3 of DMSO. 1.0 mg/cm^3 was prepared by diluting 0.1 cm^3 of the stock solution with 0.9 cm^3 of DMSO. 2.0 mg/cm^3 was prepared by diluting 0.2 cm^3 of the stock solution with 0.8 cm^3 of DMSO. 5.0 mg/cm^3 was prepared by diluting 0.5cm^3 of the stock solution with 0.5 cm^3 of DMSO.

Artemetther lumefantrine anti-malaria drug was used as Positive control prepared in the same way as test solutios. RPMI 1640 and 1% DMSO don't affect the development of P. *falciparum* in the culture medium, [16].

2.6.2 Sourcing of malaria parasites for the assay

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, mixed thoroughly and then transported to the Microbiology laboratory at Bayero University in a thermo flask containing water maintained at 4℃ as demonstrated by [17].

2.6.3 Determination of *Plasmodium falciparum* (positive blood samples) using the thin smear method

The method described by Dacei and Lewis was adopted. Using a clean capillary tube, a small drop of the blood sample was placed at the centre of a clean glass slide at least 2 mm from one end. A cover slip was placed at angle 45° in front of each drop and drawn backward to make a contact with each drop. The drop was run along the full length of the edge of the cover slip. Smears were formed by moving the cover slip forward on each glass slide. The thin smears were dried and immersed in methanol for few seconds. Geimsa's stain was dropped on each smear, and allowed to stay for about 10 minutes. Excess stain was washed with clean tap water. The smears were dried in air by hanging the glass slides inverted in a rack. Each dried smear was microscopically observed under a high power objectives (x100) using oil immersion. An average parasitaemia was determined using the reading of 3 microscopic fields.

2.6.4 Separation of the erythrocytes (0.5% *Parasitaemia*) from the serum of the blood samples

50% dextrose solution (0.5 cm^3) was added to each of the blood samples (5 cm^3) defibrinated, and then centrifuged at 2500 rpm for 15 minutes in a spectra merlin centrifugation machine. Supernatant layers were separated from the sediments. The latter was diluted with normal saline [17] and centrifuged at 2500 rpm for 10 minutes. The resulting supernatants were discarded. Samples with higher parasitaemia (above 0.5%) were diluted with fresh malaria parasite negative erythrocytes [18].

2.6.5 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.

2.6.6 Preparation of *Plasmodium falciparum* culture medium

Venous blood (2 cm^3) from the main vein of white healthy rabbits pinnae was withdrawn using a disposable 5 cm^3 syringe (BD 205 WG) into EDTA bottles. This was defibrinated by allowing it to settle for at least one hour [17]. The defibrinated blood was centrifuged at 1500 rpm using spectre merlin centrifuge for 10 minutes and the supernatant layer was collected in a sterilized tube. The sediment was further centrifuged at 1500 rpm 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 5.37 mM, NaCl 10.27 mM, $MgSO_4$ 0.4 mM, $NaHPO_4$ 17.73 mM, $Ca(NO_3)_2$ 0.42 mM, $NaHCO_3$ 2.5 mM, and glucose 11.0 mM. (BDH Ltd, UK). The medium was sterilized with 40 µg/cm^3 gentamicin sulphate [19].

2.6.7 *In-Vitro* assay of the activity of the extracts and fractions on *plasmodium falciparum* culture

A 0.1 cm^3 of test solution and 0.2 cm^3 of the culture medium were added into EDTA bottle containing 0.1 ml 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 Muktar [20].

2.6.8 Determination of the activity

At the end of the incubation period of 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 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 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;

$$\% = \frac{\mathrm{N}}{\mathrm{N}x} \times 100$$

Where,

% = Percentage activity of the test sample
N = Total number of parasites eliminated by the sample,
Nx = Total number of parasitized RBC.
Note: RBC = Red Blood Cells [20]

2.7 Thin Layer Chromatography (TLC)

TLC was conducted on the ethyl acetate fractions of the medicinal plant. This was carried out using aluminium plate silica gel (20x20). Ethyl acetate and chloroform solvent mixture was used in the following ratio 1:1, 1:4, 1:9, 0.5:9.5, 2:3; and ethyl acetate and n-hexane solvent mixture in the following ratio, 1:1, 1:4, 1:9, 4:1, 2:3.

3. RESULTS

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

Table 1. Results of ethanol extracts of leaves

Plants	Colour of solution	Weight of extracts (g)	Percentage yield (%)
NL-01	Deep green	22.739	11.37

NL-01 = Ethanol extract of Newbouldia leavis

Table 2. Results of macerated fractions from crude ethanol extract of the plant

Fractions	Colour of the solution	Weight of extracts (g)	Percentage yield (%)
NL_{-01-01}	Deep green	0.9	7.92
NL_{-01-02}	Dark green	0.7	6.17
NL_{-01-03}	Greenish yellow	1.7	14.95

NL_{-01-01} = Ethyl acetate fraction of Newbouldia laevis from crude ethanol extract; NL_{-01-02} = Chloroform fraction of Newbouldia laevis from crude ethanol extract; NL_{-01-03} = n-hexane fraction of Newbouldia laevis from crude ethanol extract

Table 3. Results of phytochemical screening of the various fractions of plants

Phytochemicals	NL_A	NL_C	NL_E	NL_H
Tannins	-ve	-ve	+ve	-ve
Saponins	-ve	NI	-ve	+ve
Flavonoid	+ve	+ve	+ve	+ve
Steriods	+ve	+ve	+ve	+ve
Alkaloids	+ve	NI	+ve	-ve
Cardiac glycoside	+ve	+ve	+ve	+ve

+ve = present, –ve = absent; NI = not intense; NL_A = Newbouldia laevis ethyl acetate fraction; NL_C = Newbouldia laevis chloroform fraction; NL_E = Newbouldia laevis ethanol fraction; NL_H = Newbouldia laevis n-hexane fraction

Table 4. Results of Antimalarial activity of fractions from the leaves of *Newbouldia laevis*

Fraction	Concentration (mg/cm^3)	Average no. of parasite before incubation	Average N number of parasite after 48 hrs	Percentage elimination at the end of incubation. (%)
NL_{-01}	0.5	44	21	52.27
	1.0	44	15	65.91
	2.0	44	12	72.72
	5.0	44	9	79.54
NL_{-01-01}	0.5	44	20	54.54
	1.0	44	18	59.9
	2.0	44	15	65.91
	5.0	44	11	75.00
NL_{-01-02}	0.5	44	25	43.18
	1.0	44	19	56.81
	2.0	44	16	63.63
	5.0	44	12	72.72
NL_{-01-03}	0.5	44	22	52.00
	1.0	44	18	59.90
	2.0	44	13	70.45
	5.0	44	10	77.27

Table 5. Results of antimalarial activity of positive control

Control	Concentration (mg/cm^3)	Average No. of parasite before incubation	Average Nnumber 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

Table 6. Results of TLC of ethyl acetate fractions of the leaves of the plants Ethyl acetate/hexane solvent mixture

Plants	Ethyl acetate/hexane ratio	Number of spots	Retention factor (R_f)
NL	1:4	4	0.09, 0.36, 0.53, 0.98

NL= Newbouldia laevis

4. ANALYSIS OF RESULTS

Mann-Whitney U Test and Spearman' Rank Correlation where adopted for the analysis of the results.

Mann-Whitney U Test Analysis of antiplasmodial results of *Newbouldia laevis* 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.

5. DISCUSSION

Ethanol extract of the *Newbouldia laevis* medicinal plant yielded 22.739 g as shown in above Table 1. Table 2 shows the weights of ethyl acetate, chloroform and n-hexane fractions from crude ethanol extract of the plant. It also shows the colour of the solutions of the fractions. NL_{-01-03} has the highest weight of 1.7 g followed by NL_{-01-01} (0.9 g). NL_{-01-02} has the least weight of 0.7 g.

Table 3 shows the result of phytochemical screening of the various fractions of the medicinal plant. Six phytochemicals (alkaloids, saponins tannins, flavonoids, steroids and glycosides) with promising antiplasmodial activity were screened for. From the results, it showed that the leaves of the plant sample contained the phytochemicals screened for in one or more of the solvent fractions. Tannins were absent in less polar solvent such as n-hexane, chloroform and ethyl acetate fractions except in ethanol fraction. Saponins were found to be present mainly in the less polar solvent fractions such as NL_C and NL_H although not intense in NL_C. Alkaloids were found to be present in more polar fractions such as ethanol and ethyl acetate; and absent in n-hexane fraction. Flavonoids, steroids and cardiac glycoside were found to be present in all the fractions.

Table 4 above show the antiplasmodial (antimalarial) activities of fractions from the leaves of *Newbouldia laevis*. Their percentage activities were investigated at various concentrations of 0.5 mg/cm^3, 1.0 mg/cm^3, 2.0 mg/cm^3 and 5.0 mg/cm^3. 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. At 0.5 mg/cm^3, NL_{-01-01} has the highest activity of 54.54% followed by NL_{-01} (52.27%) while NL_{-01-02} has the least percentage elimination of 43.18%. At concentration of 5.0 mg/cm^3, NL_{-01} has the highest activity of 79.54% followed by NL_{-01-03} (77.27%) while NL_{-01-02} has the least activity of 72.72%.

Table 5 above shows the results of antimalarial activity of the positive control. At 5.0 mg/cm^3, the percentage elimination activity is 93.18% and at 0.5 mg/cm^3, the percentage activity is 75%. Table 6 above shows the result of thin layer chromatography of large scale ethyl acetate extracts of the leaves of the medicinal plant. Also, TLC screening of the ethyl acetate fraction using ethyl acetate/n-hexane in the ratio 1:4 revealed four spots on the plate. The results of antiplasmodial activities of the positive control were compared with that of the results of the antiplasmodial activities 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 7.

Table 7. Results of antiplasmodial activity of positive control and resized results of antiplasmodial activity of the plant samples

Concentration mg/cm^3	0.5	1.0	2.0	5.0
SL Test sample	52.27	61.36	75.00	84.10
Positive Control	75	86.36	90.90	93.18

The analysis of the results of the extract and that of the standard (positive control) using Mann-Whitny U test indicated that there is no significant difference between them 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 between the antiplasmodial activities of the plant samples and positive control samples is positively perfect ($\gamma_s = 1$).

Table 6 shows the result of thin layer chromatography of ethyl acetate extract of the leaves of the medicinal plant. The result reveals ethyl acetate/n-hexane solvent mixture 1:4 has the highest number of spots and better retention factor compared to the rest hence will be used for further chromatographic work in order to isolate the most active antimalarial compound(s) and subject them to structural elucidation.

6. CONCLUSION

This study as a contribution to the evaluation of ethnomedicinal plants for search of new chemical constituents with known antiplasmodial properties has proven that the medicinal plant used for this study is active against *Plasmodium falciparum* parasite. Tannins, Saponins, Flavonoid, Steriods, Alkaloids and Cardiac glycoside were found to be present in the medicinal plant screened.

The phytochemical screening and *in vitro* antiplasmodial activity of the ethanol extracts as well the fractions from the plant supported the use of this plant to treat malaria associated symptoms in traditional settings. Data analysis of the results of the plant's samples and that of the standard (positive control) using Mann-Whitny 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 ($\gamma_s = 1$).

The identification and isolation of the bioactive components of this plant will serve as a basis for in-depth pharmacological evaluation of these bioactive phytochemicals for antimalarial drugs development. Additionally, this can also assist in herbal drug formulations of the selected plants.

Further works will include actual identification of single chemical entity responsible for antiplasmodial activity from the leaves of the medicinal plant, *Newbouldia laevis,* using ethyl acetate fraction.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/12190