IN VITRO BIOCHEMICAL EVALUATION OF THE ANTIPLASMODIAL ACTIVITIES OF VARIOUS FRACTIONS OBTAINED FROM PHYLLANTHUS NIVOSUS LEAF EXTRACT

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Abstract - Resistance to anti malarial medicines has been a threat to global efforts toward malariaeradication and elimination. Highly effective anti malarial agents are necessary requirementsfor overcoming this challenge and this requires continuous efforts towards development of new drugs to replace the old ones as they lose effectiveness. Phyllanthus nivosus leaf extract has been shown in our previous study to possess antimalarial potential through its inhibition of Plasmodium Lactate Dehydrogenase (pLDH) activityin vitro. In this study, various fractions of the leaf extract were screened for antiplasmodial activity. Plasmodium falciparum infected erythrocytes were incubated at 37^0C in RPMI 1640 culture media (modified with L-glutamine, sodium bicarbonate and HEPES) in the presence of varying concentrations (12µg/ml, 6µg/ml and 3µg/ml) of ethanolic extract, hexane, ethyl acetate, butanol and alkaloid rich fractions of Phyllantus nivosus leaf. Same concentrations of Chloroquine and Artemether Lumefantrine (ACT) were used as standards. Plasmodium Lactate dehydrogenase activity was determined after 72 hours as a measure of parasite growth. pLDH activity was reduced in all cases with IC50 values of 1.74µg/ml, 1.76µg/ml, 1.53µg/ml,5.14µg/ml and 2.10µg/ml respectively, as compared with 2.09µg/ml and 2.18µg/ml for chloroquine and ACT respectively. All the fractions were found to contain either alkaloids or terpenes or both, except the butanol fraction which also displayed the least antiplasmodial activity. It is therefore suggested that the antimalarial activity of this plant may be attributed to the presence of alkaloids and terpenes.

Index Terms – Anti malarial, Plasmodium falciplarum, Plasmodium lactate dehydrogenase, Phyllanthus nivosus.

I. INTRODUCTION

Elimination and Eradication is an important component of the Global Strategy for combating malaria and anti malarial drugs are essential tools at all stages of this process [1]. They are required for treatment of malaria illness, prevention of both infection and disease caused by Plasmodia, elimination of sexual stages from blood and dormant parasites from the liver, and hence, prevention of malaria transmission. The malaria research agenda for eradication therefore include research aimed at developing such drugs [2]. The overall goal of this study is therefore to isolate compounds with antimalarial activity from Phyllanthus nivosus leaf, a plant known for its natural antioxidant, analgesic, anti-inflammatory and antimicrobialactivity [3]. Itis abundant in Nigeria where it is being used traditionally to treat malaria. The antiplasmodial activity of this plant was demonstrated in our preliminary study [unpublished] and it was selected as a possible source of antimalarial agent. In this study, the ethanolic extract, hexane, ethyl acetate, butanol and alkaloid rich fractions of the leaf were screened for antiplasmodial activity

II. MATERIALS AND METHODS

A. The Plant Sample

Fresh leaves of Phyllanthus nivosus were collected at the Senior Staff Quarters of the University of Jos, Jos, Nigeria. It was authenticated at the Department of Plant Science and Technology of the same University. The leaves were spread under shade at 25^0C until they were completely dried.

B. Preparation of extract and fractions

The Powdered leaf was dissolved in 70% ethanol at room temperature, stirred till well mixed and allowed to stand for 24hours. The mixture was filtered using a fine sieve and the filtrate wasallowed to dry in an oven at 60^oC to obtain the ethanol extract. A portion of the ethanol extract was used for solvent partitioning to obtain hexane, ethyl acetate and butanol fractions [4]. For the alkaloid rich fraction, 2% H_2SO_4 was added tothe mixture of powdered leaf and 70% ethanol at room temperature. It was allowed to stand for a while and 10% NH_4OH was added, followed by chloroform to form two layers. Anhydrous $NaSO_4$ wasadded to the upper layer to obtain the alkaloids-rich fraction which was dried in an oven at 60^oC. The fractionswere finely capped in airtight containers and stored in the refrigerator prior to use.Phytochemical screening of the extract and fractionsobtained was carried out using standard qualitative procedure [5, 6].

C. Plasmodium falciparum culture and maintenance

O+ Plasmodium falciparum infected erythrocyteobtained from Faith Alive Foundation Hospital, Jos, Nigeria was cultured in vitro according

to the method described by Trager and Jensen with some modifications [7], in RPMI 1640 media (Sigma-aldrich), containing 2g/L of glucose, 1mg/L of L-glutamine, 2g/L of NaHCO3, Amino Acids, Vitamins, Inorganic Salts, Phenol Red and supplemented with 0.01 mg/mL of gentamicin (Sigma-aldrich), 25 of mMN-2-hydroxyethylpiperazine- N-2-ethanesulfonic acid (HEPES) buffer (Sigma-aldrich), and 5% of lipid-enriched bovine albumin preparation (Albumax®).

D. In vitro antiplasmodial assay

Plant extracts were assessed for anti-plasmodial activity in vitro using modified parasite lactate dehdrogenase (pLDH) assay [8, 9].Plasmodium falciparumculture was incubated at 37^0C in the presence of varying concentrations (12µg/ml, 6µg/ml and 3µg/ml) of ethanolic extract, hexane, ethyl acetate and alkaloid rich fractions of Phyllantus nivosus leaf. Same concentrations of Chloroquine and Artemether Lumefantrine (ACT) were used as standards. After 72 hours of incubation, 20 µL were removed and added to 100µL of Malstat reagent (Prepared by dissolving 0.11% of Triton X -100, 115.7mM of L-lactate, 30.27mM of Tris buffer, and 0.62mM of 3-acetyl pyridine adenine dinucleotide in deionized water, the pH was adjusted to 9.2 with hydrochloric acid) present in a flat-bottomed 96-well micro titer plate in triplicates. 25 µL of NTB/ PES (prepared by dissolving 1.96 mM of Nitro Blue Tetrazolium and 0.24mM of Phenazine Ethosulfate in deionized water) were then added to each well, thereby initiating the lactate dehydrogenase reaction. Absorbance was read at 630nm using aspectrophotometric microplate reader. The optical density values from control (untreated) wells were referred to as having 100% pLDH activity. The IC50values of each extract or standard drug were obtained by regression analysis, using a non-linear dose-response curve [10].

III. RESULTS AND DISCUSSION

The ethanolic extract and the four fractions used in this study were found to bring about inhibition in the activity of pLDH (figure 1) which is an indication of Plasmodium falciparum suppressive effect [11]. pLDH is a terminal enzyme in the glycolytic pathway of the malaria parasite. It is essential for ATP generation and one of the most abundant enzymes expressed by P. falciparum [12]. pLDH levels have been seen to reduce in the blood soon after treatment.The pLDH antigen is considered a specific marker for the presence of viable Plasmodium in blood, and is used for screening in malaria-endemic countries. pLDH is therefore suggested to be an important tool in the design of novel antimalarial drugs [11]. IC50, the half maximal inhibitory concentration is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. Adrug is said to be potentially effective as an antimalarial agent if its IC50 is below 10µM and 5µM is used as a cut-off level to reduce the number of false positive results [10].

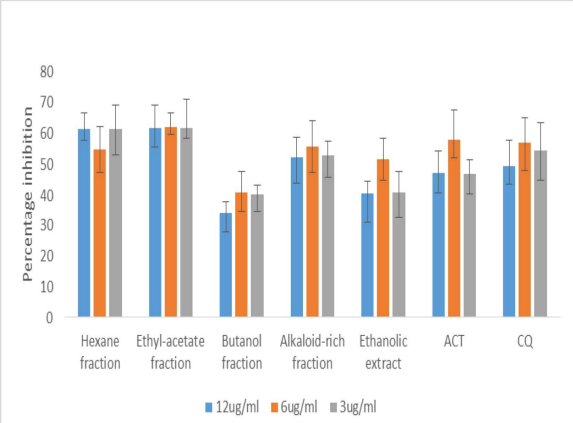

Figure 1 - Percentage inhibition of pLDH activity by extract and fractions of phyllanthus nivosus leaf and the standard drugs ACT and Chloroquine (CQ). Values are expressed as mean ± SD of triplica As shown in table 1, IC50 values obtained were 1.74µg/ml, 1.76µg/ml, 1.54µg/ml, 5.13µg/ml and 2.10µg/ml for ethanolic extract, hexane, ethyl acetate, butanol and alkaloid rich fractions respectively, as compared with 2.09µg/ml and 2.18µg/ml for chloroquine and ACT respectively.

Table 2 shows the result of the phytochemical screening of the extracts and fractions of Phyllanthus nivosus leaf. It was observed that, highly effective samples with IC50 values ranging from 1.53µg/ml to 2.10µg/ml possess either alkaloids or terpenes or both. The butanol extract which gave the least antiplasmodial activity with an IC50 value of 5.14µg/ml contains neither alkaloids nor terpenes. This important observation suggests that these compounds might be involved in the antimalarial activity of Phyllanthus nivosus leaf.

Alkaloids are one of the major classes of natural products that exhibit antimalarial activity. The first antimalarial drug quinine is an alkaloid [13]. It was isolatedin 1820, by the French scientists Pelletier and Caventou from the bark of the Cinchona spp. (Rubiaceae) tree that was used by Peruvian Indians. The structure was established by Rabe in 1908, and its synthesis was carried out by Woodward and Doering in 1944. Quinine was used as a template for the synthesis of Chloroquine in 1940 [13]. Chloroquine which was, until recently, the only drug available for malaria chemotherapyhas been reported to induce gametocytogenesis, which is not only linked with the prevalence of drug resistance, but also encourages transmission of malaria parasite from humans to mosquito vectors [1]. The antimalarial activity of terpenes (sesquiterpenes, triterpenes, diterpenes, and miscellaneous terpenes) has also been reported[14]. Artemisinin (Qinghaosu), one of the most potent and effective antimalarials to date, discovered by Chinese chemists in the 1970's 'project 523'is a sesquiterpene trioxane lactone. Artemisinin

is effective against multi-resistant strains of P. falciparumas well as,asexuals and stage I-III gametocytes. However, clinical resistance to artemisinin based combinations (ACTs) has been recently reported in Cambodia [1], hence the need for development of new drugs. Isolation and characterization of Phyllanthus nivosus alkaloids and terpenes might lead to the development of new highly effective antimalarial drugs.

CONCLUSION

Alkaloids and/or terpenes containing fractions obtained from Phyllanthus nivosus leaf displayed considerable antimalarial activity comparable to chloroquine and ACT. The antimalarial potential of this plant could therefore be attributed to the presence of these compounds.

Sample	Ethanolic Extract	Ethyl acetate Fraction	Hexane Fraction	Butanol Fraction	Alkaloid-rich fraction	Chloroquine	ACT
IC50 value (µg/ml)	1.74	1.53	1.76	5.14	2.10	2.09	2.18

Table 1 - IC50 (µg/ml) values of extract and fractions of phyllanthus nivosus and the standards drugs

Sample	Ethanolic Extract	Ethyl acetate Fraction	Hexane Fraction	Butanol Fraction	Alkaloid-rich fraction
Phytochemicals	Alkaloids Tannins Flavonoids Cardiac glycosides Steroids Terpenes	Tannins Flavonoids Steroids Terpenes	Steroids Cardiac glycosides Terpenes	Steroids Tannins Flavonoid	Alkaloids

Table 2 - Phytochemical contents of the extracts and fractions obtained from Phyllanthus nivosus leaf

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REFERENCES

[1] T.O. Johnson. “Transmission Blocking Strategy for Malaria Eradication: the role of Antimalarial Agents”. International Journal of Pharmacognosy. Vol. 2, no. 10, pp. 484-93. Oct. 2015.

[2] The malERA Consultative Group on Drugs. “A Research Agenda for Malaria Eradication: Drugs.” PLoS Medicine 8.1 (2011): e1000402. PMC. Web. 26 Aug. 2017.

[3] F.A. Onyegbule, I.O Ilouno,C. Ikeh, B.O. Umeokoli and P.M. Eze.“Evaluation of phytochemical constituents, analgesic, anti- inflammatory, antimicrobial and antioxidant activities of extracts of Breynia nivosa leaves” Planta Med. 80 Oct.2014.

[4] Z. Liu“Preparation of Botanical Samples for Biomedical Research” Endocr Metab Immune Disord Drug Targets. Vol. 8, no. 2, pp.112–121. 2008.

[5] Sofowora. “Medicinal Plants and Traditional Medicine in Africa” (2nd Edition). Spectrum Books Ltd, Ibadan, Nigeria 1993; 289.

[6] G.E Trease and W.C Evan“Pharmacognosy” 11th edn. Bailliere Tindall, London 1986; 45-50.

[7] W. Trager, J.B. Jensen “Human malaria parasites in continuous culture”. Science Vol. 193, no. 4254, pp. 673-675.Aug. 1976.

[8] M.T. Makler andD.J. Hinrichs: Measurement of the lactate dehydrogenase activity of Plasmodium falciparum as an assessment of parasitemia. Am J Trop Med Hyg. Vol. 48, pp. 205-210.Feb. 1993.

[9] M.T. Makler, J.M. Ries, J.A. Williams, J.E. Bancroft JE, Piper, B.L. Gibbins, D.J. Hinrichs. Parasite Lactate Dehydrogenase as an Assay for Plasmodium falciparum Drug Sensitivity. Am J Trop Med Hyg.Vol. 48, pp. 739-741.June 1993.

[10] K. Junior, A. Ezekiel, W. Noel, A. Ukpe , A. Francis, T.O Johnson, et al. Synthesis and Antimalarial Properties of Three Substituted Trinitro- Tribenzylamine. Organic & Medicinal Chem IJ. Vol. 1, no. 3, pp. 555-561.Oct.2016.

[11] D.k. Shoemark, M.J Cliff, R.B. Sessions and A. R. Clarke. Enzymatic properties of the lactate dehydrogenase enzyme from Plasmodium falciparum. FEBS Journal Vol. 274, pp. 2738-2748. June 2007.

[12] D.L Vander Jagt, L.A. Hunsaker andJ.E. Heidrich. "Partial purification and characterization of lactate dehydrogenase from Plasmodium falciparum". Mol Biochem Parasitol. Vol. 4,no.5–6,pp.255–264. Dec. 1981.

[13] A.B Oliveira, M.F. DolabelaI, F.C. BragaI, R.L.R.P. JácomeI, F.P. VarottiI and M.M. Póvoa. "Plant-derived antimalarial agents: new leads and efficient phythomedicines. Part I. Alkaloids" An. Acad. Bras. Ciênc., Rio de Janeiro. Vol. 81, no 4, pp. 715-740. Dec. 2009.

[14] R. Batista, A. Jesus Silva Júnior and A.B. Oliveira. "Plant-Derived Antimalarial Agents: New Leads and Efficient Phytomedicines. Part II. Non-Alkaloidal Natural Products" Molecules. Vol. 14, pp. 3037-3072. Aug. 2009.

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