Original Research Article

Comparative anti-plasmodial and cytotoxic effect of *Annickia affinis* (Exell) Versteegh & Sosef leaves, stem bark and roots methanolic extracts.

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ABSTRACT

Aims: This study aimed to evaluate and compare the *in vitro* anti-plasmodial and cytotoxic effect of the methanolic extracts from leaves, stem bark and roots of *Annickia affinis*.

Study design: This is an experimental study.

Place and Duration of Study: The work was conducted at the Pharmacochemistry and Natural Substances Laboratories of the Faculty of Medicine and Pharmaceutical Sciences, University of Douala for the extraction and at the Biomedical Chemistry Research Center of Rhodes University in South Africa for the antimalarial and cytotoxic essay. All the experiments were carried out from the 15th October 2019 to the 31th July 2020.

Methodology: The anti-plasmodial test was performed on *Plasmodium falciparum* sensitive strains 3D7 while cytotoxicity was evaluated on HeLa cell line.

Results: The anti-plasmodial tests revealed that the roots and the stem bark exhibited a moderate anti-plasmodial effect with IC_{50} of 19.7 ± 0.8 and 12.1 ± 0.8 µg/ml respectively. The anti-plasmodial effect of the leaves were classify as low (33.7 ± 1.9 µg/ml). At up to 50 µg/ml, all the extract showed high rate of survival among the Hela cells. No effect was observed with the leaf's extracts (100% of survival).

Conclusion: This is first report on cytotoxic study and comparative anti-plasmodial effect of *Annikia affinis*. It highlights the potential of *Annickia affinis* as an important source of anti-plasmodial drug with less cytotoxic *in vitro*. In agreement with the use in traditional medicine, the stem bark was more active than woods, while leaves showed low activity.

Keywords: Annikia affinis; anti-plasmodial activity; cytotoxicity; Plasmodium falciparum 3D7; HeLa; Medicinal plant.

1. INTRODUCTION

Malaria is one of the most death-leading diseases caused by a mosquitoes-borne parasite. Despite efforts to eradicate malaria, the disease is globally rather causing an increasing

number of deaths. In fact, according to the WHO there is an increasing number of malaria cases 227 million in 2019 to 241 million in 2020 in 85 malaria-endemic countries. This disease affects mostly children under 5 years and pregnant women [1]. This is said to be due to the resistance of the parasite to the available drugs, and the weakness of the distribution and surveillance strategy in the endemic countries. This dark figure led to the death of 627.000 people in 2020. This number is going up in comparison with the number already hight reported in 2019 [1]. There was a positive correlation between the increase and the presence of the Covid 19 pandemic [1]. With regards to that and in addition to the fact that since the first drug against malaria natural compounds has globally delayed the appearance of resistance, there is an emergent need for new natural drugs from the endemic countries. In that way, interest has been focused on plants used in traditional medicine to treat the symptoms of malaria [2-17].

Annikia affinis is a plant from the Annonaceae family, that is also called *Enantia affinis* Exell (1926) The vernacular names are African yellow wood, and yellow wood (in Englis). Moambe Jaune (in French). *Annickia affinis* is highly used by the Tropical Africa rural communities for many purposes. Regarding the medicinal uses, the plants are used traditionally for the management of malaria and malaria-like symptoms, the gut and the respiratory tract and skin infections. It is said to be a tonic. Recently, Erhunse et al. in 2023 showed that the stem bark of *A. affinis* possessed anti-plasmodial activity [18]. To the best of our reading, we did not find a scientific report on anti-plasmodial activity of the leaves and root, and cytotoxicity of this plant. In parallel, *Annikia chlorantha*, a closely related plant used traditionally for the same purpose have shown to contain chemicals and possessed antiparasitic, antifungal, antibacterial, cytotoxic activities among other [19,20]. This work therefore aimed at evaluating the comparative *in vitro* anti-plasmodial and cytotoxic effect of the methanolic extracts from leaves, stem bark and roots of *Annickia affinis*.

2. MATERIALS AND METHODS

2.1 Plant material

The plant materials were collected in January 2020 at 9 Am in Mount Kalla situated in Mbankomo locality in Méfou-et-Akono Division, centre region of Cameroon. The plants were formally identified by Mr Victor Nana, an ethnobotanist at the Cameroon National Herbarium where a voucher specimen was deposited under the identification number 6420/HNC.

2.2 Preparation of extract

After harvesting, the plants material including leaves, stem bark and roots were washed with tap water, chopped in piece and air dried out of sunlight. The dried material was ground into powder and 500 g were soaked in 95% methanol and let for 72 hours maceration. Afterward, the preparations were filtrated and dried under a rotary evaporator. The marc was soaked back in the solvent and the operation was repeated as until the supernatant become colourless showing an exosted extraction. The extracts obtained were mixt together and stove in the oven for 7 days at 45° to remove traces of solvent.

The extraction yields were calculated according to the following formula.

$$yield = \frac{\text{weight of dried extract}}{\text{weight of start material}} \times 100$$

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Extract stock solution (40 mg/ml) were prepared in pure DMSO and store in the fridge. Prior to test a mother solution (2 mg/ml) were prepared in the testing culture media. The mother solution was prepared the same days the tests were performed.

2.3 Anti-plasmodial activity

The anti-plamodial tests were performed on chloroquine-sensitive 3D7 *Plasmodium falciparum* strains following the procedure previously describe by Desjardin et al. [21], with the modification as described by our team [9,12,13]. Briefly, the *P. falciparum* maintained in sealed T75 culture flasks containing RPMI 1640 supplemented with L-glutamine (2 mM), HEPES (25 mM) Albumax II (5%),Glucose (20 mM),hypoxanthine (650 µM), gentamycin (60 µg/ml), and human red blood cells (2-4% haematocrit) in at 37 ºC under an atmosphere of 5% CO_2, 5% O_2, 90% N_2 in sealed T75 culture flasks. 2.5 µl of the mother extract solution were added to a 96-microplate filled with 97.5 µl of the infested culture medium. The final concentrations of the extracts were 100 µg/ml, DMSO were then 0.25%. After 48 h in a 37ºC CO_2 incubation, the cell viability was assessed using the quantification of the parasite Lactate Dehydrogenase (pLDH). To that end, 20 µl of each well were collected and add in corresponding well of a new 96-wells plates filled with 125 µl of Malstat/NBT/PES solution. After homogenisation, the plate was red at 620 nm against blank containing the parasite culture medium and the Malstat/NBT/PES prepared in the same condition. The control positive (chloroquine and emetine at 10 µM final concentration) and negative (antiparasitic drug replace with culture medium) were prepared in the same condition as the tests.

2.4 Cytotoxic activity

The The cytotoxicity of the extracts were assessed on HeLa (human cervix adenocarcinoma) cells using the protocol as previously describe by our team [9,12,13]. Briefly, 100 µl containing $2x10^4$ HeLa cell in Dulbecco's Modified Eagle's Medium (DMEM) with 5 mM L-glutamine (Lonza), supplemented with 10% foetal bovine serum (FBS) and antibiotics (penicillin/streptomycin/fungizone - PSF) were dispense into wells of the flat bottom 96 well plate. The plates were preincubated for 24 hours in a 5% CO_2 humidified incubator. This allowed the cell to adhere in the bottom of the well. The culture medium (supernatant containing death cells)was removed afterward and replace with 200 µl of the same fresh culture medium containing plants extracts at the maximum concentration of 50 µg/ml. the plates were designed in such a way that each concentration of each plant's extracts is prepared in duplicate. The prepared plate was incubated in the same condition for 48 hours. The cell viability was monitored by checking the mitochondria functionality using the resazurin based tests. The test is based on the reduction of the oxidized non-fluorescent blue resazurin to a red fluorescent dye (resorufin) by the mitochondrial respiratory chain in live cells. The resorufin was read in the fluorescent mode with Excitation/Emission (560 nm/590 nm). The intensity of the fluorescence is directly proportional to the number of living cells. The following formula was used to calculate the percentage of living cells.

$$Viability(\%) = \frac{(fluorescence\ of\ test - fluorescence\ of\ negative\ control)}{(fluorescence\ of\ negative\ control)} \times 100$$

2.5 Cytotoxic Single concentration screening and dose response assays

The initial evaluation of the anti-plasmodial and cytotoxicity testing, the corresponding cell culture were incubated with tests sample at a fixed concentration of 50 µg/ml in triplicate. The percentage of growth inhibition were calculated relative to untreated control cultures

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using the upstated formulas. The validation of the tests was done using a positive control at 10 µM chloroquine (anti-plasmodial test) and emetine (for cell apoptosis).
The inhibitory parameter was the concentration that inhibits 50% of cell growth (IC_{50}) and was obtained through dose-response assays. The extracts were tested in a range of concentration ranging from 50 to 0.046 µg/ml using a 3-fold serial dilution for anti-plasmodial. The dose-response curves of % cell viability vs. Log[compound] were plotted and the IC_{50} were determined by non-linear regression using GraphPad Prism (Version 5.02). Chloroquine was used as positive control in the anti-plasmodial assays. IC_{50} values for cytotoxicity were not determined due to the low inhibition observed in the single concentration screening.
All measurements were performed using the Biotek Synergy MX microplate reader.

3. RESULTS

3.1 Yield of extraction

The extracts were obtained with different yields (table 1). The highest yield was obtained with roots (7.62%) and the stem bark has mathematically the least yield (4.23%).

Table 1. Extraction yield of the methanolic extracts of stem bark, leaves and roots of *Annikia affinis* (exell).

Vegetal material	Amount of start material (g)	Crude extract (g)	Yield (%)
Stem bark	2500	105.97	4.23
Leaves	2000	115.01	5.75
Roots	500	38.12	7.62

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3.2 Anti-plasmodial activity

The analysis of the results shows that the methanolic extracts of the leaves, the roots and the stem bark of *A. affinis* reduced the viability of *Plasmodium falciparum* 3D7 cells by 92.2, 87.9 and, 83.6% respectively (figure 1).

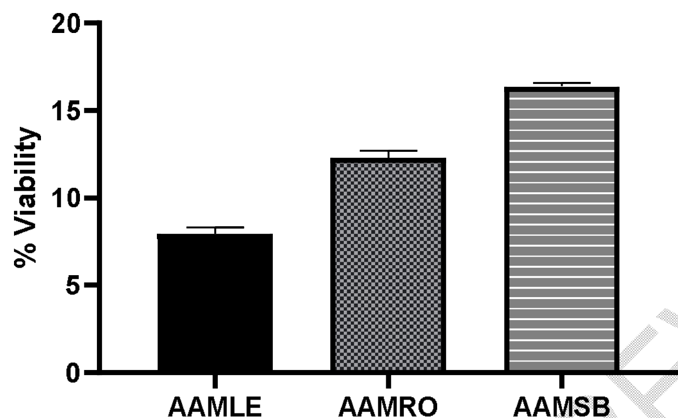

Fig. 1. Percentage of viability of *Plasmodium falciparum* 3d7 cells on *Annikia affinis* methanolic extracts
AAMLE *= Methanolic extract of Annikia affinis leaves,* ***AAMRO*** *= Methanolic extract of Annikia Affinis roots,* ***AAMSB*** *= Methanolic extract of Annikia affinis stem bark;* ***% viability*** *= percentage viability.*

All these extracts have successfully inhibited the chloroquine sensitive *plasmodium falciparum* (figure 2). The most efficient extract was that of stem bark (12.1 µg/ml), followed by root extract (19.7 µg/ml) and extract with the poorest activity was that of leaves with the ic_{50} of 33.7 µg/ml.

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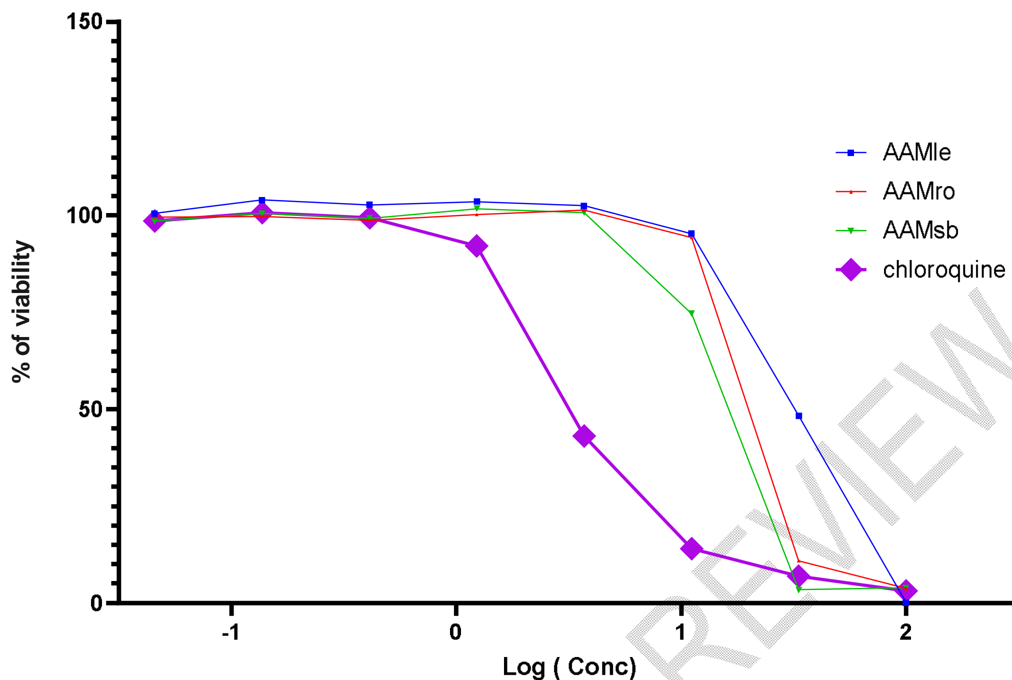

Fig. 2. Dose-reponse curves of the anti-plasmodial assay of *Annikia affinis* methanolic extracts

AAMle* =** *Methanolic extract of Annikia affinis leaves,* ***AAMro = *Methanolic extract of Annikia affinis roots,* ***AAMsb =*** *Methanolic extract of Annikia affinis stem bark;* ***%*** *= Pencentage;* ***Log (conc) =*** *log (concentration in ug), Chloroquine = reference drug.*

3.3 Cytotoxic activity

The methanolic extracts of the stem bark, roots and leaves of *A. affinis* were subjected to the cytotoxicity test in the presence of HeLa cells at 50 µg/ml but only the percentages of viability were determined. Figure 3 gives the values of the percentages of viability of the various extracts tested.

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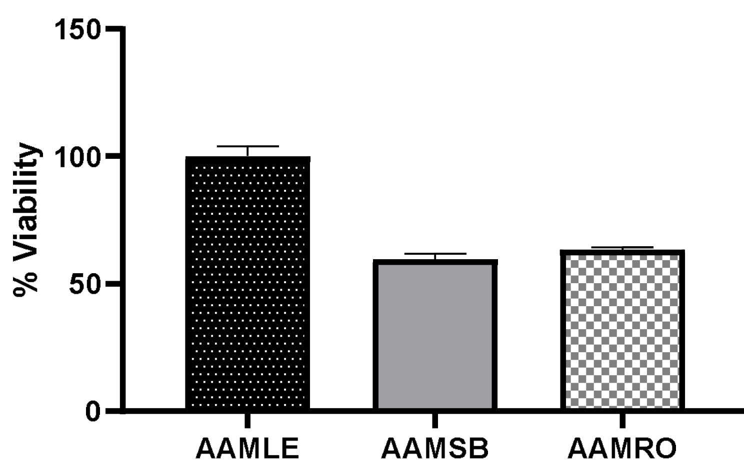

Fig. 3. Percentage of viability of HeLa cells on *Annikia affinis* methanolic extracts
AAMle = *Methanolic extract of Annikia affinis leaves,* ***AAMro =*** *Methanolic extract of Annikia affinis roots,* ***AAMsb =*** *Methanolic extract of Annikia affinis stem bark;* ***% viability*** *= Pencentage viability.*

The methanolic leaves extract showed zero cytotoxic activity with a percentage viability of 100.0%. The methanolic extracts of stem barks and roots showed weak cytotoxic activity with respective viability percentages of 63.3 and 59.5%.

4. DISCUSSION

This work aimed at evaluation the anti-plasmodial effect and cytotoxicity of different parts of the methanolic extracts of *Annikia affinis*, a traditional medicine native in Central Africa region where it is used to manage traditionnaly many diseases including, parasitic infection, inflammation and fever, the main symptom of malaria. To the best of our reading, Erhunse et al. in 2023 showed that the stem bark of *A. affinis* possessed anti-plasmodial activity [18]. In the present study, the plant was located, collected and identified with the help an ethnobotanist that is use with the collection of both plants in other research project [22].

4.1 Extraction

Methanol were used as solvent as it said to be the best solvent to extract most of the secondary metabolites present in plants [4,9-13,15]. Using that solvent, the extracts were obtained with different yield from the organs involved in this study. The difference in yields can be explained the fact that, secondary metabolites are store in different amount in different plants organs. In this plant the secondary metabolites are mostly stored in the roots of the plants, probably to help fight against invader from the soil. These yields are higher compared to that obtained from bark *Annikia chlorantha* Oliv, a plant for the same genus [22,23].

4.2 Evaluation of the anti-plasmodial activity

The anti-plasmodial effect of the roots (IC_{50} = 7.62 µg/ml) and stem bark (IC_{50} = 12.1 µg/ml) extracts can be classify as moderate with the regard to the classification of the anti-plasmodial effect of the plant extract ($5 < IC_{50} \leq 30$ µg/ml) [19,24]. These results are not

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similar to those of Erhunse et al. in 2023, who reports an ic_{50} value of 1.49 ug/ml for the stem bark, higher activity (IC_{50} ≤ 5 µg/ml). This can be justified by the place and the period of harvest as their study having been carried out in Nigeria and on an extract of a different nature as in the study of Erhunse et al., it was an aqueous extract while in our study, we have a methanolic extract [18]. These results describe in the literature regarding *Annickia chlorantha* goes in the same line [1,8,22-24]. From the same available classification, the extract from the leaves (IC_{50} = 33.7 µg/ml) can be classified as low activity.

4.3 Evaluation of the cytotoxic activity

The cytotoxicity of *Annickia affinis* is studied here for the first time and revealed that the extracts from roots followed by stem bark and leaves (percentage viability of 59.5, 63.3, 100.0% respectively) are not cytotoxic for the HeLa cells. As the percentages of cell viability remained high (> 50%) for all tested extracts (figure 3), their IC_{50} values were not assessed. Hence, extracts of roots, leaves and stem bark of *A. affinis* and were considered non-cytotoxic. We did not find in the literature authors that tested the effect of *Annickia affinis* for their cytotoxicity effect. But these results revealed that the extracts of *Annickia affinis* are safer in comparison to that of Mussuyu et al [24]. In fact, these authors reported that the aqueous extract of the *Enanthia chloranta* stem bark have an IC_{50} of 5.66 µg/ml on MRC-5 cell line. These results goes in the same line with that describe Imieje et al. in 2017 which total methanolic extract hexane fraction, ethyl acetate fraction, butanol fraction and the aqueous fraction of *Enanthia chloranta* stem bark have an IC_{50} > 4.760 µg/ml on VERO cells [25]. This observation might be an indicator of their safety as drugs used in pharmacopeia.

4. CONCLUSION

This work highlights the potential of *Annikia affinis* as an important source of anti-plasmodial drug with less cytotoxic *in vitro*. In agreement with the use in traditional medicine, the stem bark was more active than woods, while leaves showed low activity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors declare that 'ethical clearance was obtained from the Institutional Ethics Committee of the University of Douala for the conduct of this study and for the publication of this article'. All experiments were reviewed and approved. A copy of the ethical clearance is available for review by the editorial office/editor/editorial board members of this journal.

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REFERENCES

1. WHO. Word Malaria Report 2021; 2021.
2. Mustofa, Valentin A, Benoit-Vical F, Pélissier,à Y, Koné-Bamba D, Mallié M. Antiplasmodial activity of plant extracts used in west African traditional medicine. J Ethnopharmacol. 2000;73(1-2):145-151.

DOI:10.1016/S0378-8741(00)00296-8
3. Ene AC, Atawodi SE, Ameh DA, Kwanashie HO, Agomo PU. *In vivo* antiplasmodial effect of chloroform extracts of *Artemisia maciverae* Linn and *Artemisia maritima* Linn. Afr J Biotechnol. 2009;8(23):6612-6616. DOI:10.4314/ajb.v8i23.66362
4. Bagavan A, Rahuman AA, Kamaraj C, Kaushik NK, Mohanakrishnan D, Sahal D. Antiplasmodial activity of botanical extracts against *Plasmodium falciparum*. Parasitol Res. 2011;108(5):1099-1109.
DOI:10.1007/s00436-010-2151-0
5. Inbaneson SJ, Ravikumar S, Suganthi P. *In vitro* antiplasmodial effect of ethanolic extracts of coastal medicinal plants along Palk Strait against *Plasmodium falciparum*. Asian Pac J Trop Biomed. 2012;2(5):364-367.
DOI:10.1016/S2221-1691(12)60057-4
6. Donkor AM, Oduro-Mensah D, Ani E, et al. *In vitro* anti-plasmodial activity of aqueous and ethanolic extracts of *Moringa oleifera* and *Phyllanthus amarus*. Int J Biol Chem. 2015;9(4):198-206.
DOI:10.3923/ijbc.2015.198.206
7. Nworu CS, Ejikeme, TI Ezike AC, et al. Anti-plasmodial and anti-inflammatory activities of cyclotide-rich extract and fraction of *Oldenlandia affinis* (R. & s.) D.C. (Rubiaceae). Afr Health Sci. 2017;17(3):827-843.
DOI:10.4314/ahs.v17i3.26
8. Berthi W, González A, Rios A, Blair S, Cogollo Á, Pabón A. Anti-plasmodial effect of plant extracts from *Picrolemma huberi* and *Picramnia latifolia*. Malar J. 2018;17(1):1-12.
DOI:10.1186/s12936-018-2301-x
9. Mbosso TJE, Siwe NX, Nguemfo EL et al. Biological activities of plant extracts from *Ficus elastica* and *Selaginella vogelli*: An antimalarial, antitrypanosomal and cytotoxity evaluation. Saudi J Biol Sci. 2018;25(1):117-122. DOI:10.1016/j.sjbs.2017.07.002
10. Asmare M, Mohammedbrhan A, Dessalegn A, Gelayee A. *In-vivo* anti-malarial activity of crude extract and solvent fractions of the roots of *Clematis simensis* Fresen .(Ranunculaceae) in *Plasmodium berghei* infected mice. J Pharm Biol Sci. 2019;14(5):52-62.
DOI:10.9790/3008-1405015262
11. Fenta M, Kahaliw W. Evaluation of antimalarial activity of hydromethanolic crude extract and solvent fractions of the leaves of *Nuxia congesta* R. Br. Ex fresen (Buddlejaceae) in *Plasmodium berghei* infected mice. J Exp Pharmacol. 2019;11:121-134.
DOI:10.2147/JEP.S230636
12. Fouokeng Y, Feumo HM, Mbosso TJE, et al. *In vitro* antimalarial, antitrypanosomal and HIV-1 integrase inhibitory activities of two Cameroonian medicinal plants: *Antrocaryon klaineanum* (Anacardiaceae) and *Diospyros conocarpa* (Ebenaceae). South Afr J Bot. 2019;122:510-517.
DOI:10.1016/j.sajb.2018.10.008
13. Mbosso TJE, Siwe NX, Zeh MJE, et al. Compound isolation and biological activities of *Piptadeniastrum africanum* (hook.f.) Brennan roots. J Ethnopharmacol. 2020;255:112716.
DOI:10.1016/j.jep.2020.112716
14. Okaiyeto K, Okoh AI. *In vitro* assessment of antiplasmodial and antitrypanosomal activities of chloroform, ethyl acetate and ethanol leaf extracts of *Oedera genistifolia*. Appl Sci. 2020;10(19):1-13.
DOI:10.3390/app10196987
15. Atsbha GH, Balasubramanian R, Gebre AK. Antimalarial effect of the root of *Silene macrosolen* A. Rich (Caryophyllaceae) on *Plasmodium Berghei* -Infected Mice. Evidence-based Complement Altern Med. 2021;2021. DOI:10.1155/2021/8833865
16. Tajbakhsh E, Kwenti TE, Kheyri P, Nezaratizade S, Lindsay DS, Khamesipour F. Antiplasmodial, antimalarial activities and toxicity of African medicinal plants: a systematic review of literature. Malar J. 2021;20(1).
DOI:10.1186/s12936-021-03866-0
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17. Chaniad P, Phuwajaroanpong A, Techarang T, Viriyavejakul P, Chukaew A, Punsawad C. Antiplasmodial activity and cytotoxicity of plant extracts from the Asteraceae and Rubiaceae families. Heliyon. 2022;8(1):e08848. DOI:10.1016/j.heliyon.2022.e08848
18. Erhunse N, Omoregie SE, Sahal D. Antiplasmodial and antimalarial evaluation of a Nigerian hepta-herbal Agbo-iba decoction: Identification of magic bullets and possible facilitators of drug action. J Ethnopharmacol. 2023;301:115807.
DOI: 10.1016/j.jep.2022.115807
19. Olivier DK, Van Vuuren SF, Moteetee AN. *Annickia affinis* and *A. chlorantha* (*Enantia chlorantha*) - A review of two closely related medicinal plants from tropical Africa. J Ethnopharmacol. 2015;176:438-462.
DOI:10.1016/j.jep.2015.10.021
20. Kémeuzé VA, Nkongmeneck BA. *Annickia affinis* (Exell) Versteegh & Sosef. [Internet] Record from PROTA4U. 2011. Published 2007. Accessed August 3, 2022.
http://www.prota4u.org/search.asp
21. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. Quantitative Activity Semiautomated Technique. Antimicrob Agents Chemother. 1979;16(6):710-718.
22. Fekam BF, Madiesse E, Tepongning R, et al. Antiplasmodial activity of extracts from seven medicinal plants used in malaria treatment in Cameroon. J Ethnopharmacol. 2009;123:483-488.
DOI:10.1016/j.jep.2009.03.008
23. Kemgne EAM, Mbacham WF, Boyom FF, Zollo PHA, Tsamo E, Rosenthal PJ. *In vitro* sensitivity of *Plasmodium falciparum* field isolates to extracts from Cameroonian Annonaceae plants. Parasitol Res. 2012;110(1):109-117. DOI:10.1007/s00436-011-2456-7
24. Musuyu MD, Fruth BI, Nzunzu LJ, et al. *In vitro* antiprotozoal and cytotoxic activity of 33 ethonopharmacologically selected medicinal plants from Democratic Republic of Congo. J Ethnopharmacol. 2012;141(1):301-308. DOI:10.1016/j.jep.2012.02.035
25. Imieje V, Zaki AA, Fasinu P, Ali Z, Khan AI, Tekwani B, Khan IS, Nosa OE, Falodun A. Antiprotozoal and Cytotoxicity Studies of Fractions and Compounds from *Enantia chlorantha*. Trop J Nat Prod Res. 2017;1(2):89-94.
DOI: 10.26538/tjnpr/v1i2.8
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