In vitro activity of defatted crude extracts of *Solanum pseudocapsicum* leaves against *Plasmodium falciparum*

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ABSTRACT

Background: *Plasmodium falciparum* is the most lethal and major malaria parasite that is widely spread in Africa. It has led to high mortality rate especially in children below the age of 5years and also caused a serious economic and social burden to this region. The lack of an effective vaccine and increase in drug resistance and cost to the currently used antimalarial drugs has complicated malaria therapy and necessitate the search for new effective and affordable alternative antimalarials. *Solanum pseudocapsicum* commonly called Jerusalem cherry is a plant with a lot of traditional medicinal claims.

Objective: The aim of the study was to screen the methanol and dichloromethane extracts of *Solanum pseudocapsicum* for their antimalarial activities.

Methods: Extraction was carried out using cold maceration with methanol (polar) and dichloromethane (non polar) solvents after defatting with petroleum ether. Infected blood collected from symptomatic patients was cultivated in vitro in a candle jar set up using RPMI 1640 medium. Monoculture of *P. falciparum* was established by PCR protocol using *P. falciparum* specific primers. *In vitro* antiplasmodial studies involved sub cultivation of already synchronised culture (using 10% sobitol) and further cultivation of the parasite with extracts at concentrations in triplicates on a 96 well micro titre plate using RPMI 1640 supplemented with 15% O^+ human serum. The antimalarial effects of the extracts were expressed as mean of percentage inhibition of parasite growth relative to control wells (0µg/ml) and mean values were statistically compared using MS-Excel add in (version 1.11) with those of chloroquine treated control group. Phytochemicals were screened using conventional screening methods.

Results: Phytochemical screening of the 3 extracts revealed the presence of cardiac glycosides, steroid and triterpenes. Tannins were detected in the methanol and dichloromethane extracts while
saponins, flavonoids and alkaloids detected in the methanol extract and anthraquinone was absent in all extracts. *In vitro* antiplasmodial screening shows that the methanol extracts of *S. pseudocapsicum* leaves had the highest activity with IC_{50} of 6.31 *µg/ml. The dichloromethane fraction of S. pseudocapsicum* leaves was moderately active with IC_{50} of 22.39. Statistical analysis reveals increasing activity with increasing concentration which shows significant antiplasmodial activity at P value ≤ 0.01. The petroleum ether extracts of *S. pseudocapsicum* leaves were in active with IC_{50} values greater than 50 *µg/ml.*

Conclusion: To our knowledge, this is the first report of the antimalarial study of this plant and it is thought that further identification of active constituents through further bioguided assay could lead to possible drug development.

Key words: Antimalarial drugs, In vitro antiplasmodial assay, Malaria, PCR, *Plasmodium falciparum.*

Activité *in vitro* des extraits bruts dégraissés des feuilles de *Solanum pseudocapsicum* contre *Plasmodium falciparum*

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RESUME

Contexte: Le *Plasmodium falciparum* est le parasite le plus mortel et majeur du paludisme largement répandu en Afrique. Il a entrainé un taux de mortalité élevé, en particulier chez les enfants de moins de 5 ans, et a également causé un fardeau économique et social grave pour cette région. L'absence de vaccin efficace et la croissance de la résistance aux médicaments et des coûts pour les médicaments antipaludiques actuellement utilisés ont compliqué le traitement contre le paludisme et nécessitent la recherche de nouveaux antipaludiques alternatifs efficaces et abordables. Le *Solanum pseudocapsicum* communément appelé cerise de Jérusalem est une plante avec beaucoup de revendications médicales traditionnelles.

Objectif: Le but de l'étude était de filtrer les extraits de méthanol et de dichlorométhane de *Solanum pseudocapsicum* pour leurs activités antipaludiques.

Méthodes: L'extraction a été effectuée à l'aide d'une macération à froid avec des solvants au méthanol (polaire) et au dichlorométhane (non-polaire) après dégraissage avec de l'éther de pétrole. Le sang infecté recueilli auprès de patients symptomatiques a été cultivé *in vitro* dans une culture sous jarre à l'aide du RPMI 1640. La monoculture de *P. falciparum* a été établie par protocole PCR (amplification en chaîne par polymérase) en utilisant des apprêts spécifiques de *P. falciparum*. Des études anti-plasmodium *in vitro* impliquaient une sous-culture de la culture déjà synchronisée (utilisant 10% de sorbitol) et une autre culture du parasite avec des extraits à des concentrations en triplicata sur une plaque de micro-titre 96 puits en utilisant du RPMI 1640 additionné de 15% de sérum humain O^+. Les effets antipaludiques des extraits ont été exprimés en pourcentage d'inhibition de la croissance du parasite par rapport aux puits de contrôle (0µg/ml) et les valeurs moyennes ont été comparées statistiquement à l'aide de MS-Excel add (version 1.11) avec celles du groupe témoin traité à la chloroquine. Les produits phytochimiques ont été testés à l'aide des méthodes de dépistage classiques.

Résultats: Le dépistage phytochimique des 3 extraits a révélé la présence de glycosides cardiaques, de stéroïdes et de triterpènes. Des tanins ont été détectés dans les extraits de méthanol et de dichlorométhane tandis que les saponines, les flavonoïdes et les alcaloïdes détectés dans l'extrait de méthanol et l'anthraquinone étaient absente dans tous les extraits. Le dépistage anti-plasmodium *in vitro* montre que les extraits de méthanol des feuilles de *S. pseudocapsicum* ont eu la plus grande activité avec IC_{50} de 6,31 µg/ml. La fraction de dichlorométhane des feuilles de *S. pseudocapsicum* était modérément active avec de 22,39. L'analyse statistique révèle une activité croissante avec une concentration croissante qui montre une activité anti-plasmodium significative à la valeur $P \leq 0,01$. Les extraits d'éther de pétrole des feuilles de *S. pseudocapsicum* étaient actifs avec des valeurs de IC_{50} supérieures à 50 µg/ml.

Conclusion: À notre connaissance, il s'agit du premier rapport de l'étude antipaludique de cette plante et on pense qu'une identification plus poussée des constituants actifs grâce à un autre essai bio-guidé pourrait conduire à un éventuel développement de médicaments.

Mots clés: médicaments anti-malaria, essai anti-plasmodium in vitro, malaria, PCR, *plasmodium falciparum*.

INTRODUCTION

Malaria being an ancient disease is documented to occur in a Chinese document from about 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC and Hindu texts as far back as the sixth century BC.[1] This disease is caused by protozoan parasites of the genus *Plasmodium* transmitted to humans from infected female *Anopheles* mosquitoes of which *Plasmodium falciparum* being the most lethal.[2] Malaria is one of the most important parasitic diseases in the world and it remains a major public health problem in Africa. An estimated 212 million cases of malaria and 429000 deaths were recorded in 2015 with sub Saharan Africa countries having 90% of the malaria cases and 92% of malaria deaths.[3] Africa accounts for about 80% of malaria cases in the world with high mortality rates especially in infants below the age of 5years and it is estimated that about 12 billion Euros are paid for the annual cost due to malaria disease in developing countries.[4] Because of this devastating nature of the disease there is an urgent need to develop new drugs or vaccines for the treatment, prevention and management of the disease.

In most developing countries traditional medicine serves as the first line of therapy in ailment, this is due to its easy accessibility and affordability in such developing countries.[5] Traditional medicines comprises the sum total of skills, practices and knowledge based on the theories and experience indigenised to different cultures in order to maintain good health and about 25% of the currently available orthodox medicines are obtained from herbal medicines [6,7] Hence there is a board consensus for the need for development of new agents and owing to the fact that the two current most important anti malarial drugs for treatment of severe *Plasmodium falciparum* malaria i.e. quinine and artemisinin were derived from natural products.[8]

Solanum pseudocapsicum is a shrub belonging to the family Solanaceae and genus Solanum, it is commonly called Jerusalem plant or Jerusalem cherry or winter cherry, is widespread in the north western part of Nigeria. It is used by the indigenous people for the treatment of acute abdominal pain. In southern part of Africa it is used in treatment of boils, gonorrhoea and as an aphrodisiac and Phytomedical investigations revealed that *S. pseudocapsicum* possesses hypertensive and anti-spasmodic, antiviral, hepatoprotective, antifungal properties and antibacterial activity and antioxidant properties.[9,10]

In this study, the antiplasmodial activity of defatted extracts of *Solanum pseudocapsicum* leaves was evaluated *in vitro* using RPMI 1640 medium in a candle jar set up.

MATERIALS AND METHODS

Collection and Extraction of Plant Materials: Fresh leaves *Solanum pseudocapsicum* were collected in February, 2015 from Zaria, Nigeria and was identified and botanically authenticated by the taxonomist of the Department of Biological sciences herbarium, Ahmadu Bello University Zaria and a sample voucher specimen was deposited for future reference with voucher number 1054.

The fresh leaves of *S. pseudocapsicum* was separated and dried under shade until fully dried and subsequently size-reduced using a milling machine to produce a coarse powder. Defatting was firstly carried out by adding 500ml of petroleum ether in 280g of powdered material contained in closed amber coloured bottles and was allowed to stay for 24-48 hours with constant shaking of the bottles. The defatting solvent was strained off using a muslin cloth, clarified by filtration using 0.22µm membrane filter and the subsequent filtrate was evaporated in open air. A sequential extraction was then carried out using equal volumes of both polar (methanol) and non polar (dichloromethane) solvents. All the extract obtained was concentrated to dryness on a water bath at 40°C, weighed and stored in a refrigerator at 4°c in amber coloured well-tight containers.[11]

Phytochemical Screening of Extracts: the preliminary phytochemical analysis of the plant extracts was carried out to detect the presence of secondary metabolites using standard screening test in accordance to standard procedures.[12]

Collection of Blood Sample and Molecular Characterization: Approval to carry out the study was obtained from the ethical committee of Kaduna State Ministry of Health. Blood samples were collected from symptomatic patients referred for malaria parasite test at the haematology department of Barau Dikko Specialist hospital Kaduna in the month of April 2015. Prepared slides (thin smear) are stained for 10 minutes with 10% Giemsa solution prepared in phosphate buffer of pH 7.3 after fixation with methanol for 10 seconds and examined microscopically for parasites.[13]

200 µl of whole blood was added into a 1.5ml Ependoff tubes. 400µl of lysis buffer and 10µl of protinase k was added and the tube was placed on a heating block which was set at 60°C and allowed to heat for 1hr. 400µl of phenol: chloroform (1:1) was added to the lysate and vortex briefly for 2 minutes. Contents were centrifuged

at 10000rpm for 10mins to separate the phases. The upper layer was carefully removed with a micro-pippete and transferred into a 1.5ml tube. 400µl of chloroform was carefully added and votex briefly before spinning at 10000rpm for 10 minutes. The upper clear layer was carefully transferred to another 1.5ml tube without extracting the interface. Equal volume of 100% ethanol and 20µl of 3M sodium acetate was added and mixed carefully by gently shaking the tubes before incubating at 20°C for 24 hours. After incubation, the tube was centrifuged at maximum speed for about 20min, the ethanol portion was removed and 400µl of 70% ethanol was added and centrifuged at maximum speed for 5min at 4°C. The resultant DNA was dried out by leaving the tube opened for 3-10 minutes.

PCR primers were purchased from Integrated DNA Technologies, Inc. (IDT), Coralville, Iowa, USA. Primers [*Pf1* (5' GGA ATC TTA TTG CTA ACA 3') *Pf2* (5'AAT GAA GAG CTG TGT ATC3')] that are specie specific for *Plasmodium falciparum* (Pf1 and Pf2) were used for the amplification to confirm the *P. falciparum* status. Amplification was carried out in a DNA thermal cycler GeneAmp® PCR System 9700 Applied Biosystems®. 2µl of the extracted DNA was resuspended in a 20µl (final volume) reaction Accupower® Hotstart PCR premix containing 1unit of Hot-start DNA polymerase, 1× PCR buffer, 250 µM of each dNTPs and 1µl Pf1/Pf2 (Pf PCR). The amplification step included a 95°C "pre-denaturation" for 5 minutes, 1 minute "denaturation" at 94°C, 1 minute "annealing" step at 56°C *P. falciparum* PCR for 30 cycles and lastly, a 1 minute "elongation" step at 72°C. The thermal cycler temperature was maintained at 4°C after the final cycle, until the samples were removed.[14]

Amplified PCR products were detected by gel electrophoresis. A standard 1.5% agarose gel was prepared by dissolving 1.5g of agarose powder in 100ml of tris acetate EDTA [stained using a solution of ethidium bromide (0.5 µg/ml)]. 8 µl of each of the samples was loaded and subjected to electrophoresis for 35 minutes at a voltage of 100V and current of 40mA. The gel was visualized with ultraviolet light and photographed using Gel-doc system. The band sizes for the PCR products were observed. Blood sample with mono-infection of *Plasmodium falciparum* after both microscopic and molecular screening were used for the *in vitro* cultivation.[15]

Preparation of Culture Medium and Extract Solution: Culture medium was prepared by addition of 3g of 25 m*M* *N*-2-hydroxthylpiperazine-*N*'-2-ethane-sulfonic acid (HEPES) buffer (sigma) and 0.3ml of gentamicin (gentalek) (from 40mg/ml stock) to 500ml of incomplete RPMI 1640 media supplemented with L-glutamine and sodium bicarbonate was obtained from (sigma aldrich) and stored at 4°c in aliquots of 45ml. Further supplementation with human O^+ serum to 10% of the final volume makes it a complete culture medium.[16,17]

Each methanol (polar) extract obtained from leaves of *S. pseudocapsicum* was dissolved in incomplete culture medium while the dichloromethane and petroleum ether extract was firstly dissolved in 0.5% dimethyl sulphoxide and then diluted with culture medium to obtain a stock solution of 2mg/ml. The stock solution is filtered under sterile conditions and subsequently diluted with culture media to produce different concentrations (1000, 500, 100, 50, 10, 1µg/ml).[18,19]

***In vitro* Cultivation of *P. falciparum* Isolates and Susceptibility Testing**: The assay was performed in triplicate on a 96 wells microtitre plates. The sterile 96 wells tissue culture plate was predosed with 100µl of culture medium containing extracts at various concentrations followed by the addition of 100µl of sub cultured parasite diluted with the O^+ erythrocytes to about 0.5-1% parasitemia. A negative control was maintained with 100µl of *Plasmodium falciparum* culture, 100µl of culture medium and positive control was maintained by the addition of a standard drug chloroquine (40mg/ml) at similar concentrations to that of the plant extracts. The 96 well plates are then incubated in a CO_2 at room temperature in a candle jar for 24-36 hrs.[16] After incubation, contents of each well was harvested after carefully removing the culture media which is at the upper layer then Thin blood smears from each well was prepared on a clean frosted edge microscopic slide. The films were fixed in absolute methanol, stained with 10% Giemsa solution of pH 7.3 and number of infected red blood cells was counted with the aid of a light microscope and an equivalent of 1000 erythrocytes was counted. The counting was done twice for three different parts of the slide and the mean number of infected erythrocytes is obtained.

The control parasite culture freed from extracts was considered as 100% growth and the percentage inhibition per concentration was calculated using the formula:

$$\text{Average \% suppression of parasitemia} = \frac{\text{Average \% parasitemia in control} - \text{Average \% parasitemia in test}}{\text{Average \% parasitemia in control}} \times 100$$

The IC_{50} values, the concentration required to inhibit parasite growth by 50% was determined by linear interpolation from the parasite growth inhibition curves (Log of concentration versus percentage inhibition) generated from each parasite-extract interaction.[17,20]

Statistical Analysis: Microsoft Excel add in (version 1.11) was used to calculate mean parasite growth and percentage parasite inhibition followed by specific post hoc test to analyse the difference and statistical significances were achieved when p≤0.01.

RESULTS

The methanol fraction of *S. pseudocapsicum* had the highest yield of 8.78% while petroleum ether fraction had the least with y 0.67% and the dichloromethane fraction 2.07% (Table 1).

The phytochemical screening of the plant extracts (Table 2) showed the presence of carbohydrates, glycosides, cardiac glycosides, steroids and triterpenes in all extracts, tannins in methanol and dichloromethane fraction, saponoins, alkaloids and flavonoids in methanol fraction. Antraquinones was absent in all extracts.

PCR was carried out with the *P. falciparum* specific primers and product was subjected to gel electrophoresis with lane A containing the ladder of 100bp, lane B containing the blood sample and lane C been the negative control. A band of 300bp was obtained as shown in (Plate 1)

Table 1: Percentage yield of various extracts

Plant parts	Solvent used	Weight of plants (g)	Yield of extract (g)	Percentage yield of extract (%)
Leaves	Methanol	140.00	12.29	8.78
	Dichloromethane	140.00	2.90	2.07
	Petroleum ether	280.00	1.88	0.67

Table 2: Phytochemical components detected in *Solanum pseudocapsicum* leaves extracts

EXTRACTS	CHO	ARQ	GLY	C-GLY	SAP	S. TR	TAN	FLA	ALK
Methanol	+	-	+	+	+	+	+	+	+
Dichloromethane	+	-	+	+	-	+	+	-	-
Pet. Ether	+	-	+	+	-	+	-	-	-

Key: (+) Present(-) Absent

CHO- Carbonhydrates, ARQ- Anthraquinones, GLY- Glycosides, C-GLY- Cardiac glycosides, SAP- Saponins, S.TR- Steriods and Tritrepenes, TAN- Tannins, FLA- Flavonoids, ALK- Alkaloids.

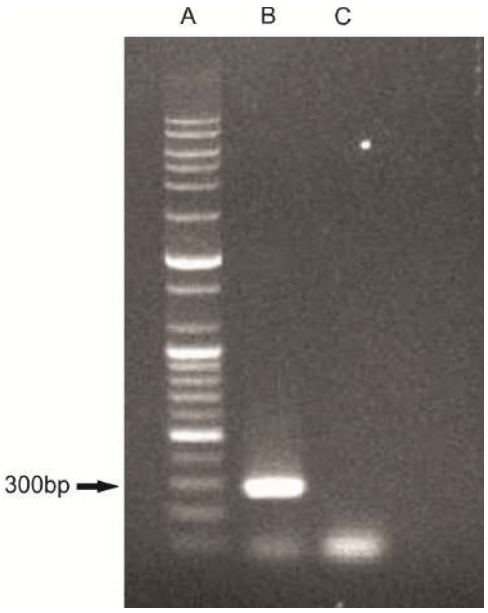

Plate 1: Electrophorograph of PCR Identification of Plasmodium falciparum [A- Molecular ladder, B- Blood sample C- Negetive control

Table 3: Mean parasite growth at various concentrations.

	CONCENTRATION					
	1µg/ml	**10µg/ml**	**50µg/ml**	**100µg/ml**	**500µg/ml**	**1000µg/ml**
Extracts	% mean Parasite growth					
Methanol	32.14±3.73	22.21±3.21	19.68±1.71	17.97±1.19	15.43±3.22	11.19±1.97
Dichloromethane	38.29±4.05	34.01±3.50	13.17±3.88	7.91±1.04	6.39±0.72	3.73±0.19
Pet ether	41.79±1.30	34.75±1.80	26.02±3.80	20.63±1.89	12.71±0.67	7.29±0.73

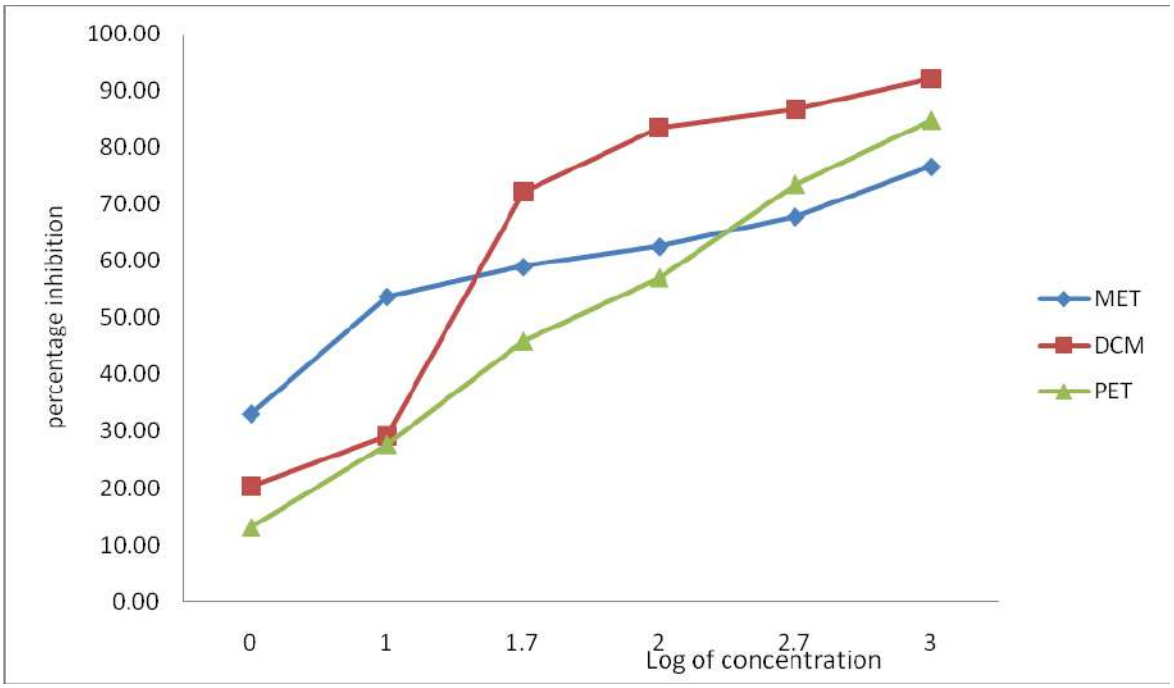

Fig 1. The percentage inhibition of the extracts of *S. pseudocapsicum* leaves showing dose-dependent antiplasmodial activity.

DISCUSSION

The result obtained from extraction showed that methanol (polar) extract of *S. peudocapsicum* leaves has the highest yield; the dichloromethane fraction produced a lower yield. This suggests that the plant contains more polar soluble biochemical components than non polar component. The petroleum ether (Defatting agent) fraction yielded the least; this is in line with what was reported.[10] The defatted plant extract are more active this is because the fat which is a critical part of their cellular membranes and essential oils are extracted hence it indicates that there are less fatty soluble components in the plants.[21] The concentration and proportion of the active compounds obtained in plant extract depend on the plant variety, plant origin, time of collection, solvent used, condition of extraction and storage.[22]

The preliminary phytochemical screening of the various plants extracts revealed that all extracts contained more than one group of constituents in each morphological part tested and pharmacological activity may be due to one or more group of constituents.[23] The active extracts from this study contained tannins, glycosides, cardiac glycosides, carbohydrates, steroids, triterpenes and alkaloids. The antiplasmodial properties of plant extracts may be attributed to these phytochemicals which may be: by inhibition of *P falciparum* merozoite invasion into the erythrocytes and disruption of *P falciparum* rosettes by carbohydrates; inhibition of protein synthesis by triterpenoids; inhibiting the detoxification of heame in RBCs and fatty acid biosynthesis by alkaloids; inhibition of β-heamatin formation, decrease mitochondrial membrane potential, DNA fragmentation and cytoplasmic acidification by steroids.[24]

PCR based assay has been developed for the detection and identification of malaria parasite to overcome some of the limitations of microscopy and has proven to be more specific and sensitive.[25] In this study, PCR was carried out on the blood containing the cultured *P. falciparum* using specific primers and a band size of 300bp was seen thus confirming the presence of the parasite.

In the study, methanol, dichloromethane and petroleum ether fractions of *S. pseudocapsicum* was evaluated *in vitro* for antiplasmodial activity (Table 3 and Fig 1) and among the test extracts the methanol fraction showed the lowest IC_{50} value of 6.31µg/ml, the dichloromethane fraction 22.39 µg/ml and petroleum ether fraction 74.13 µg/ml. Extracts with IC_{50} less than 10µg/ml as highly active. Those with IC_{50} between 10µg/ml and 50µg/ml are moderately active and extracts with IC_{50} greater than 50µg/ml as inactive.[24, 26] Based on this classification, results from this study showed that the methanol extract have a high antimalarial activity, dichloromethane extract is moderately active and the petroleum ether extract is inactive. Although this research was carefully prepared and has reached its aims, there were some unavoidable limitations. First, the RPMI 1640 media was very susceptible to contaminations and even while they were stored in aliquots some had to be discarded due to contaminations and secondly, artefacts were seen on the microscopic slides when determining the parasitemia. Further studies on the in vivo activities of the extracts against *plasmodium berghei* is necessary in determination of it antimalarial activity.

CONCLUSION

It appears for the first time from scientific investigation that the methanol and dichloromethane extracts of *S. pseudocapsicum* may be potential source for the discovery of an antimalarial agent due to their good antiplasmodial effect. These promising results obtained can be a starting point to seek bioactive compounds by bioguided fractionation and biological studies for the development of an antimalarial drug.

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