American Journal of BioScience
2020; 8(1): 1-5
http://www.sciencepublishinggroup.com/j/ajbio
doi: 10.11648/j.ajbio.20200801.11
ISSN: 2330-0159 (Print); ISSN: 2330-0167 (Online)

Antiplasmodial Activities of Flavonoids from Leaves of *Securidaca longepedunculata* Fresen (Polygalaceae)

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To cite this article:

Issa Karama, Moussa Compaoré, Abdoulaye Djandé, Orokia Traoré, Latifou Lagnika, Martin Kiendrebeogo. Antiplasmodial Activities of Flavonoids from Leaves of *Securidaca longepedunculata* Fresen (Polygalaceae). *American Journal of BioScience*. Vol. 8, No. 1, 2020, pp. 1-5. doi: 10.11648/j.ajbio.20200801.11

Received: January 29, 2020; **Accepted**: February 19, 2020; **Published**: February 28, 2020

Abstract: The discovery of new molecules for fighting against malaria is still relevant to overcome *Plasmodium* sp resistance. Phenolic compounds from medicinal plants have shown antiplasmodial properties. In addition, the targets of flavonoids on *P. falciparum* are multiple. This work aimed to identify the antiplasmodial compounds from methanol extract of *Securidaca longepedunculata* leaves. The inhibition of β-hematin formation was used to detect antiplasmodial compounds through a bio-guided chromatographic fractionation procedures. W2 strain was inhibited by flavonoids fractions Fc1 and Fb4 with 6.98 and 10.39 µg/mL as IC_{50} respectively. Also, fractions of phenol acids have shown good activities on the inhibition of β-hematin formation. The HPLC analyze showed that *S. longepedunculata* leaves extract contained quercetin, 3-β-quercetin, luteolin, chrysin, isorhamnetin, hyperoside, rutin, gallic acid, ellagic acid, chlorogenic acid, tannic acid and ferulic acid. Among these compounds identified, some had shown antiplasmodial and inhibitory activities on the formation of β-hematin. The antimalarial activity of the leaves of S. longepedunculata would be due in part to phenolic acids and flavonoids. The antiplasmodial activity observed in this work would be due in part to the ability of flavonoids from S. longepedunculata leaves to inhibit the formation of β-hematin. This finding could justify partially the *S. longepedunculata* uses in malaria treatment in Burkina Faso.

Keywords: *Securidaca longepedunculata*, Flavonoids, Phenolic Acids, β-hematin, Antiplasmodial

1. Introduction

The number of malaria cases recorded in 2016 was 216 million, an increase of 5 million compared to the previous year. Effective treatment of Malaria remains a challenge for modern medicine as part of its eradication. The complexity of development cycle and the ability of *P. falciparum* to resist against new antiplasmodial molecules posed a threat to the global health system, especially in sub-Saharan Africa [1]. Despite the progress made in the search for a vaccine against malaria, the observation is that none has passed the last experimental phase [2]. One of alternative to conventional medicine, is to promote heavily the use of medicinal plants for the malaria treatment [3, 4]. These plants have always constituted an investigation source to research new interest compound [5]. Indeed, molecules belonging to different chemical groups with very interesting antiplasmodial activities have been isolated from antimalarial plants [6, 7].

Securidaca longepedunculata Fresen (Polygalaceae) is a plant used to treat malaria in African traditional medicine [8]. The dichloromethane leaves extracts were inhibited the sensitive *P. falciparum* 3D7 strain grow with 6.69µg/mL as IC_{50} [9, 10]. The roots of this plant are very used [8]. In previous studies it was shown that after leaves extract defatted, the methanol extract had a very high antiplasmodial activity on the chloroquine-resistant strain K1 (IC_{50}=2.33 µg/mL) [11].

This work consisted in a bio-guided fractionation of leaves methanolic extracts by using β-hematin formation inhibition assay. The results could help on the knowledge of the active compounds and one of the possible mechanisms of action.

2. Material and Methods

2.1. Chemical and Origin of Plasmodium Falciparum W2 Strain

All solvents used within this study were analytical or HPLC grades. RPMI-1640 (liquid without L-Glutamine), NaOH, Hypoxanthine, Albumax II, Hepes buffer, L-glutamine, Gentamicin, 3-acetylpiridine adenine di-nucleotide (APAD), Nitro Blue Tetrazolium (NBT), Phenazine ethosulfate (PES), Lithium L-Lactate, Trizma base, Hemin chloride, Triton X100, Chloroquin, were provided from Sigma-Aldrich (Belgium 2015). TLC glass plates (Silicagel $60F_{254}$) and Silica gel 60 (230-400 mesh) were from Merk. Gallic acid, Syringic acid, Chlorogenic acid, Caffeic acid, Tannic acid, Ferulic acid, Hyperoside, Rutin, Ellagic acid, 3β-quercetin, Quercetin, Luteolin, Isorhamnetin, and Chrysin used in HPLC were analytical grade from Sigma-Aldrich. *Plasmodium falciparum* W2 strain was provided from BEI Resources (USA, 2015).

2.2. Samples Collection

Leaves from *Securidaca longepedunculata* Fresen (Polygalaceae) were harvested in Badara locality (Region of "Haut Bassin") in April 2014. Voucher specimen (CI: 16713) was identified and deposited in the herbarium of "Université Joseph KI-ZERBO ", Burkina Faso.

2.3. Extraction and Fractionation Procedure

Dried leaves powder was defatted with petroleum ether then extracted by methanol. Methanol extract (10g) was subjected to an open column chromatography (Silica gel 60, 230-400 mesh, Merck). The column was eluted with hexane (100%), hexane-ethyl acetate (50:50), ethyl acetate, ethyl acetate-methanol (50:50) and methanol (100%). Twelve fractions were collected, concentrated until dryness and examined for their chromatographic profile capacity to inhibit β-hematin formation according to Akkawi et al. methods [12]. Thin layer chromatography analysis was performed on precoated silica gel plate (GF_{254} 10 x 10 cm MERK) under UV (254 and 365 nm) detection and NEU reagent.

2.4. Chromatographic Analysis

The Fractions with interesting inhibition activity were selected for the flash chromatography (Puriflash 215; column 50 STD - 25.0 g, 15 bars). The sub-fractions resulting from flash chromatography were grouped according to their thin layer chromatography profile followed by the assessment of their inhibitory activity. The active sub-fractions were re-fractionated by using preparative thin layer chromatography for isolating the compounds fractions with β-hematin inhibitory activity.

Main bioactive fraction was analyzed using an HPLC-UV system (Ultimate 3000, C18 column (150mm×4.6mm), λ=254 or 365 nm). Data from HPLC was integrated by Ultimate 3000 software and results were obtained by comparison with external standards retention time (t_R) and UV spectral. Mobile phase consisting of solvent A (Water +Formic acid 0.2%), solvent B (methanol+ Formic acid 0.2%) and at a flow rate of 15mL/min. The isocratic elution program was used for chromatograms development.

2.5. Inhibition of β-hematin Formation

The inhibition of β-hematin formation was assessed by according to methods from the literature [12, 13]. Freshly prepared hemin chloride solution (100 µL, 0.5mg/ mL in DMSO) was mixed with sodium acetate buffer (200 µL, 0.5M in water, pH 4.4) and test sample at 100µg/mL in mixture. Test mixture was incubated (37°C for 18 to 24h) and centrifuged (4000 rpm for 10 min) for discarding supernatant. Total pellet was suspended in DMSO (400 µL) and centrifuged (4000 rpm for 10 min) for dissolving the remaining hemin chloride. β-hematin pellet within tubes is solubilized with NaOH (400 µL, 0.1M). The negative control contained water. The absorbance (Abs) of β-hematin solution was read at 405nm. Data were expressed as the percentage of inhibition of β-hematin formation and calculated by using the equation (1).

$$\text{Inhibition (\%)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}) / (\text{Abs}_{\text{control}})] \times 100 \quad (1)$$

2.6. Inhibition of Plasmodium Falciparum Parasite Growth

The antiplasmodial activity was determined according to Makler et al. method [14]. Test sample) was mixed within culture medium (O^+ blood: 2% hematocrit, parasitaemia: 1.5%). After incubation (72 hours, 37°; 5% CO_2, 2% O_2, 93% N_2 and 95% of humidity) parasite growth was measured with Malstat reagent and NBT/PES solution. The absorbance (Abs) was recorded at 650nm against a blank (culture medium without test sample). Data were expressed as inhibition percentage of parasite growth and calculated by the equation (2). The concentration inhibiting 50% of the parasite growth (IC_{50}) was determined.

$$\text{Inhibition (\%)} = [1-(\text{Abs}_{\text{test}} - \text{Abs}_{\text{blank}}) / (\text{Abs}_{\text{control}})] \times 100 \quad (2)$$

2.7. Statistical Analyses

The results were expressed as mean value of three (n=3) independent experiments ± standard deviation. Table curve 2D v.2 was used to determine IC_{50}. Statistical analysis of data was performed with the XLSTAT pro 7.5. ANOVA for $p<0.05$.

3. Results and Discussions

3.1. Inhibition Activity of β-hematin Formation

The ability of crude extract and 12 fractions to inhibit the

formation of β-hematin was summarized in the Figure 1. The percentages of inhibition were ranged from about 5.97 to 82.96%. The extract inhibited 53.24% of β-hematin formation *in vitro*. The Fractions with ameliorated inhibiting effect were F4 (73.79%), F5 (81.96%), F6 (71.66%), F7 (82.96%), F8 (72.71%) and F11 (53.57%). All activities from fraction were less than chloroquine inhibitory activity (91.51%). This increased inhibition activity in some fractions suggested that the increasing of bioactive compounds concentration in these fractions. The fractionation by flash chromatography of one gram of the mixture of F7 and F8 gave twenty-seven (27) sub-fractions (F1'-F27'). According to their TLC chromatographic profile, these sub-fractions were grouped into 4 groups which were Fa (F1'-F'7), Fb (F'8), Fc (F9'-F'25) and Fd (F26'- F27') which were submitted to the β-hematin inhibition assay and the result was indicated by the Figure 2. The high activity was recorded with the sub-fractions Fb and Fc that showed 76.52% and 75.06% as inhibition percent respectively. Again, the best sub-fraction inhibition activity was less than Chloroquine inhibition activity. The observed activities of fractions could be due the presence of flavonoids and phenolic acids detected by TLC analysis. Particularly, flavonoid can form complex with iron (iii) ions and induced the non-formation of β-hematin [15, 16].

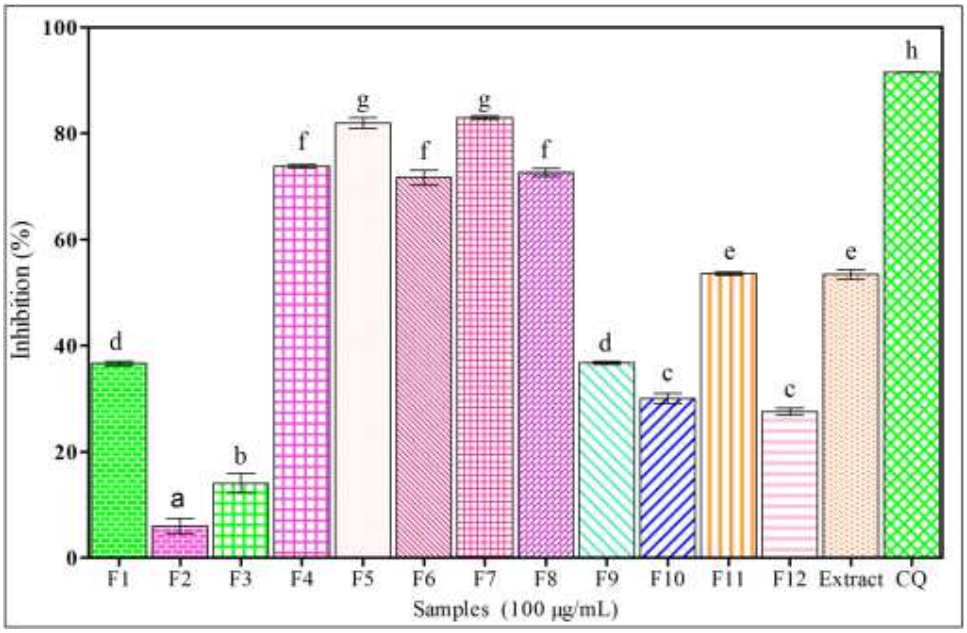

Figure 1. *Inhibition percentage of β-hematin formation of fractions (F1-F12).*

The data were expressed as the percentage of inhibition. Data followed by same letter are not significantly different at p>0.05.

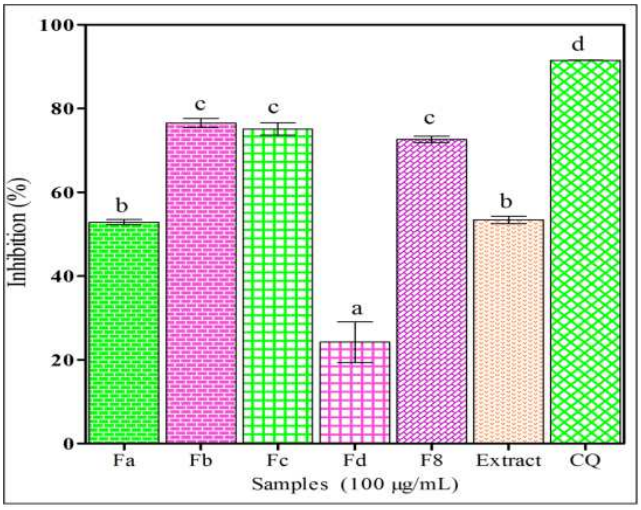

Figure 2. *Inhibition of β-hematin formation by sub-fractions (Fa-Fd).*

The data were expressed as the percentage of inhibition. Data followed by same letter are not significantly different at p>0.05.

3.2. Sub-fractions Antiplasmodial Activities

The figures 3 and 4 showed the inhibition of β-hematin formation and W2 growth of fractions from sub-fractions Fb and Fc. The best sub-fractions were Fb4 (78.14%), Fc1 (72.09%), Fb3 (66.68%), Fc4 (64.82%) and Fb2 (61.94%) concerning the inhibition of β-hematin formation. But their activity was less than chloroquine inhibition effect. Fc1 and Fb4 were able to inhibit significantly the W2 development according to the figure 4 with IC_{50} as 6.98 and 10.39 µg/mL respectively. Fc1 was twice active than fraction F8 and 8 times active than the methanol extract according to this data. These indications confirmed the presence of antiplasmodial compound in the extract and its fractions. In TLC, Fb4 and Fc1 are flavonoid fractions having the same frontal references as quercetin (Rf = 0.91) and 3-βquercetin (Rf = 0.56) respectively.

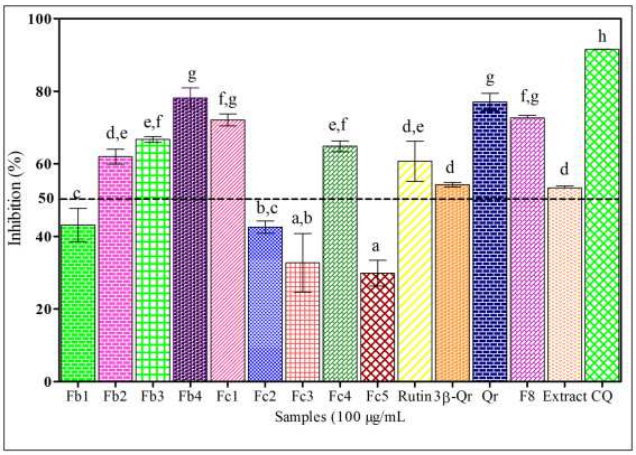

Figure 3. *Sub-fractions and standards compound inhibition activity on β-hematin formation. Data followed by the same superscript letter are not significantly different at p>0.05. Fb1-Fb4: fraction from sub-fraction Fb; Fc1-Fc5: fraction from sub-fraction Fc*

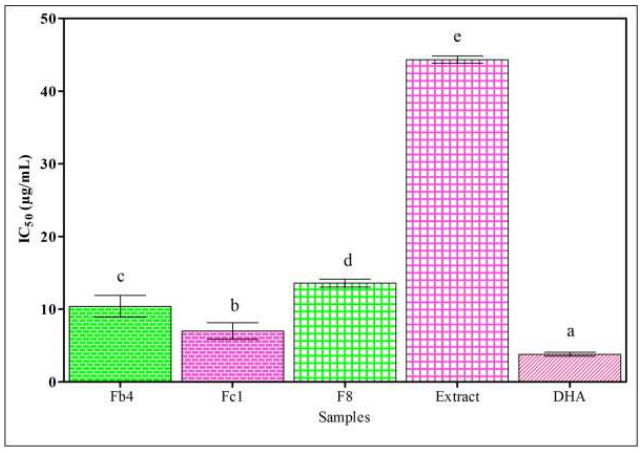

Figure 4. *Antiplasmodial activity on chloroquine resistant strains W2.*

IC_{50}: The concentration inhibiting 50% of the parasite growth. Data followed by same superscript letter are not significantly different at p>0.05. DHA: Dihydroartemisinin (ng/mL). Fb4 and Fc1 are flavonoid fraction from sub-fractions Fb and Fc respectively. F8: fraction 8 from crud extract.

3.3. Compounds Identification by HPLC

In-depth phytochemistry study of the F8 fraction by HPLC analysis allowed to have the data in the table 1. The leaves extract from *S. longepedunculata* contained phenolic acids such as gallic, ellagic, chlorogenic, tannic, ferulic acids and flavonoids such as luteolin, rutin, chrysin, isorhamnetin, hyperoside, quercetin and 3-β-quercetin. The identification of these compounds in the leaves extract of *S. longepedunculata* was reported in the first time. The flavonoids fractions Fb4 and Fc1 would be or would content quercetin and 3-β-quercetin respectively according TLC data and their identification by HPLC analysis in the bioactive fraction. The richest in flavonoids and phenolic acids confirmed the TLC profile and could supported the biological data. So, the ability of quercetin to inhibit β-hematin formation was reported by Manu et al. [17, 18] who found 0.15 µg/mL as IC_{50} and 3-β-quercetin can prevent red blood cell membrane damage [17, 18]. This preventive property would be involved in cell membrane protection against lipid peroxidation induced by free radical during *P. falciparum* infection [19]. The inhibitory activity of 3-β-quercetin on the formation of β-hematin and *P. falciparum* resistant grown is the first to be reported according to our best bibliographic knowledge. Quercetin and luteolin were able to inhibit *falciparum* sp. like 3D7, 7G8 and NF54 [17, 20, 21]. Again, rutin inhibited RIO-2, the *P. falciparum* enzyme involved in cell cycle progression, chromosome stability [22]. The gallic acid were 3D7 antiproliferative compound and tannic acid was inhibited β-hematin formation [23, 24]. The mechanism action of flavonoids and phenolic acids were attributed to their hydroxyl groups [25].

Table 1. *Phenolic acids and flavonoids compound identified in fraction 8 by HPLC.*

Compounds	Retention times (min)	UV λmax (nm)
Gallic acid	2.84	216.4; 270.9
Chlorogenic acid	7.48	216.3; 236.3; 325.3
Tannic acid	9.68	214.3; 278.0
Ferulic acid	12.12	216.7; 234.4; 321.6
Hyperoside	16.52	200.3; 255.6
Rutin	17.98	207.4; 256.0; 354.6
Ellagic acid	19.03	193.9; 252.7; 366.1
3-β-quercetin	21.94	206.6; 255.8; 353.8
Quercetin	23.86	209.8; 254.5; 369.8
Luteolin	24.56	222.3; 253.6; 349.1
Isorhamnetin	27.09	194.4; 252.5
Chrysin	28.32	219.3; 267.0; 312.7

4. Conclusion

The methanol extract and its fractions from leaves of *S. longepedunculata* showed some antiplasmodial potential and this activity was explained partially by the 5 phenolic acids and 7 flavonoid compounds. These compounds could contribute to the leaves antimalarial uses by various mechanisms in synergic action that allow resistance disturbing. The present work results could be a preliminary basis for further studies such as evaluating the effect of *S. longepedunculta* leaves extracts on *P. falciparum* gametogenesis or combinatory activity with some standard antiplasmodial compounds.

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