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Research Article

Radical Scavenging and Antiplasmodial Activity of *Polygonum senegalense* of Benin

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Abstract

Drug resistance developed by *Plasmodium falciparum* doubled their rates of infection in the last two decades and encourages efforts to further explore natural materials that can be used as a new antimalarial drug. Thus, present study aims to evaluate "*in vitro*" antioxidant (DPPH test) and antiplasmodial activities of ethanolic and aqueous ethanolic extracts (30/70) of Benin's *Polygonum senegalense* leaves. Compared with the ethanolic extract, only the hydroethanolic extract has a good anti-radical activity with an IC_{50} (0.18 mg/mL) similar to that of quercetin (0.1 mg/mL) which is one of references antioxidants used in this study. This activity would be due to the presence of phenolic compounds identified during the phytochemical screening of Polygonum leaves and their high presence in the aqueous ethanolic extract (81.76 mg/g against 17.83 mg/g for the ethanol extract) revealed by quantitative analysis. Both extracts investigated showed good antiplasmodial activity (IC_{50} < 50 µg/mL) and were more active on the Dd2 chloroquine resistant strain of *Plasmodium falciparum* than the reference medicinal product which is Artemisinin-based combination (IC_{50} > 50 µg/mL). The plant material studied is therefore a potential source of phenolic compounds, antimalarial and anti-radical molecules.

1. INTRODUCTION

Major parasitic disease, malaria is found in tropical and subtropical regions encompassing mainly countries with a low Gross Domestic Product (GDP)[1] such as Benin. It is a pandemic for which mankind pays a heavy price. It is transmitted by the bites of Anopheles mosquitoes infected with Plasmodium, of which five species are indexed in the human malaria: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium knowlesi*. Among the causes of this infection, free radicals are implicated in the anemic state of paludal[2]. They are also slow and initial causes of many chronic diseases by oxidative stress they generate and their paralyzing action on the immune system. According to the World Health Organization (WHO), malaria affected 207 million people around the world in 2012, causing over 627000 deaths[3]. In Africa sub-Saharan, the most affected region by this pandemic, one child dies of this disease every minute. The resistance of plasmodium to antimalarial drugs (chloroquine-resistant strains in most endemic areas and strains resistant to artemisinin in Cambodia, Thailand and Vietnam)[4] as well as the vector to insecticides increase the difficulties in controlling and eradicating malaria and make it a public health problem. Thus, prospecting for new sources of natural substances gifted with antimalarial activity is a priority. In fact, these substances are potential sources of such molecules as was the case for quinine. In this thinking, the present study aims to evaluate "*in vitro*", the antiplasmodiale and anti-radical potentials of concentrated extracts of *Polygonum senegalense* (Polygonaceae) of Benin after exploration of its chemical composition.

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P. senegalense is a perennial herb erected height 1 m, scabrous peduncle with pink and white flowers. It is found in swamps and rivers throughout tropical Africa and Egypt[5].

2. MATERIALS AND METHODS

2.1 Plant Material

Leaves of wild plants of *P. senegalense* (Polygonaceae) were collected at Godomey (South Benin). After collection, they were dried at room temperature (25°C) and away from direct sunlight until stabilization of their mass, then cut and powdered.

2.2 Reagents

Gallic acid, Butyl Hydroxy Anisole (BHA), quercetin, RPMI 1640, and catechin were purchased from Sigma Chemical Co. (St. Louis, MO), while the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent obtained from Acros Organics (Morris Plains, NJ). L-glutamine, albumax and gentamicin are obtained Gibco BRL and Artemisinin-based Combination Therapy (reference tablet) was purchased at the pharmacy.

2.3 Parasite

Dd2 strain of *Plasmodium falciparum* chloroquine-resistant (IC_{50} = 114 nM).

2.4 Phytochemical Study

2.4.1 Test tubes characterization

The powder obtained was analyzed according to various staining and precipitation reactions to determine the qualitative chemical composition of plant material studied. The different classes of active compounds have been identified by standard procedures. Table 1 shows the desired metabolites as well as specific reagents for each metabolite[6-11].

Table 1: Metabolites desired and specific reagents

Metabolites		Reagent and Reaction
Polyphenols		$FeCl_3$ (2%) → Greenish coloration
Tanin	Total	$FeCl_3$ → Blackish blue color
	Gallic	Stiasny reaction, $FeCl_3$ (2%) → Blackish blue color
	Catechic	Stiasny reaction
Flavonoids	Total	NaOH (10%) → Yellow-orange coloration
	Freed	Cyanidin Reaction
	Anthocyanin	Color change depending on acidic or basic nature of the medium
	Leucoanthocyanin	Cyanidin reaction without magnesium shavings
Saponins		Determination of moss index I (positive if I >100)
Mucilage		Absolut ether → Flocculent precipitate
Alkaloids		Bouchardat → Black-brown precipitate Mayer → White precipitate
Coumarin		NaOH (10%) → Fluorescence under UV light
Proteins		$CuSO_4$ → Violet coloration
Reducing sugars		Fehling reagent → Brick-red precipitate

2.4.2 Extraction and determination of phenolic compounds

a)Extraction

2 g of dried powdered leaves of *Polygonum senegalens* were extracted with 50 mL of ethanol or ethanol-water (70/30) for 24h at room temperature (25°C). The extracts obtained were filtered on Buchner and concentrated under reduced pressure. The extraction yield was calculated using the following formula:

$$Y = \frac{Mext}{Mhd} X100$$

Y: yield (%); M_{ext}: extract mass; M_{hd}: herbal drug mass

b) Determination of Phenolic Compounds

i. Total Polyphenols

The total phenolic content was determined by a Folin-ciocalteu assay[12,13] using Gallic acid (GA) as the standard. The mixture of the sample solution (50 µL), distilled water (3 ml), 250 µL of Folin-ciocalteu's re-agents solution and 70% Na2CO3 (750 µL) was vortexed and incubated for 8min at room temperature. Then, a dose of 950 µL of distilled water was added. The mixture was allowed to stand for 2 hours at room temperature. The absorbance was measured at 765 nm against distilled water as a blank. The total phenol content was expressed as Gallic acid equivalents (mg of GAE/g sample) through the calibration curve of Gallic acid.

ii. Flavonoids

Total flavonoid content was determined using a colori- metric method described previously [14]. Briefly, a dose of 0.25 ml of extract or quercetin standard solution was mixed with 1.25 ml of distilled water in a test tube, followed by adding 75 µL of a 50% NaNO2 solution. After 6 min, 150 µL of a 10% $AlCl_3{\cdot}6H_2O$ solution was added and was allowed to stand for another 5 min before adding 0.5 ml of 1 M NaOH. The mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the sample) at 510 nm. The results were calculated and expressed as micrograms of quercetin equivalents (mg of QE/g sample) using the calibration curve of quercetin.

iii. Anthocyanins

The principle of this assay is based on the decolorization of the reaction medium by sodium bisulfite ($Na_2S_2O_5$ to 150g/L)[15]. Reading was made in a spectrophotometer at 520 nm. The anthocyanin content is expressed in mg equivalent of cyanidin per gram of dry matter (mg CE / g DM).

iv. Condensed Tannins

Analysis of condensed tannin content was carried out according to the method of Broadhurst and Jones[16] and as modified by Xu and Chang[17]. To 50 µL of the suitably diluted sample, 3 ml of a 4% methanol vanillin solution and 1.5 ml of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min and the absorption was measured at 500 nm against methanol as a blank. The amount of condensed tannin was calculated and expressed as mg catechin equivalents (mg of CE/g sample) using the calibration curve of (+) – catechin.

v. Determination of antioxidant activity

The DPPH (2,2-diphenyl-1-pycrilhydrazil hydrate) radical scavenging activity of extracts was estimated according to the method reported by Agbangnan et *al.*[18]. Briefly, 0.1 mM of DPPH solution in methyl alcohol was prepared and 1 ml of this solution was added to 3 ml of sample or standard. Discolorations were measured at 517 nm after incubation for 30 min at 25°C in the dark. Low absorbance of the reaction mixture indicates higher free radical scavenging activity. The percentage of $DPPH^{\bullet}$ scavenged ($\%DPPH^{\bullet}_{sc}$) was calculated using:

$$\%DPPH^{\bullet}_{sc} = (A_{cont} - A_{samp}) \times 100/A_{cont}$$

Where, A_{cont} is the absorbance of the control and A_{samp} is the absorbance of the sample.

2.5 Evaluation of antiplasmodial activity

Chloroquine-resistant Dd2 strain of *Plasmodium falciparum* was grown in 96-well plates as described by Trager and Jensen[19]. Blood cells were washed three times with RPMI 1640 before use in culture. Erythrocytes were then suspended in RPMI supplemented with l-glutamine (4.2 mM), HEPES (25 mM), bovine foetal serum (10%: v/v), streptomycin (100 g/ml) and penicillin (100 IU/ml). The haematocrit was 5%. The 'Lumate forte' (Artemisinin-based Combination Therapy) was used as positive control.

The in vitro antimalarial tests were performed by light microscopy using Giemsa-stained smears as described by Rieckmann *et al.,*[20]. Plant extracts as well as 'Lumate forte' were diluted with sterile physiological saline. The aliquots of drug solutions were added in duplicate. A control experiment was performed separately to check the effect of solvents on parasite maturation. Drug concentrations in the wells ranged from 6.25 µg/mL to 1 mg/mL for the ethanolic extracts, hydroethanolic extracts and reference compound. The plates were incubated at 37◦C in a candle jar for a total period of 96 h. Each 24 hours blood smears were made and stained with May-Grünwald Giemsa to assess parasitemia and determine the 50% inhibitory concentration (IC_{50}) of the growth of *P. falciparum*. Finally parasitaemia was calculated using the following formula:

$$\text{Parasitaemia} = \frac{\text{(Parasitized red blood cells x 100)}}{\text{Parasitized red blood cells + Healthy red blood cells}}$$

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

Phytochemical screening helps to identify different chemical groups in study samples. Table 2 shows the different metabolites identified in the powder plant material studied.

Table 2: Metabolites identified in *P. senegalense* leaves powder

		P. Senegalense
Polyphenols		+
Tanins	Total	+
	Catechic	+
	Gallic	-
Flavonoids	Total	+
	Anthocyanin	+(x)
	Free	+(a)
	Leucoanthocyanin	+
Saponosids	Foam height	+
	Foam index	150
Mucilages		+/-
Alkaloids (Mayer, Bouchardat)		+
Coumarins		+
Reducing sugar		+
Proteins		+

(a): Flavones; (x): 3-deoxianthocyanin; +: Presence; +/-: Doubt; - : negative

This test showed the presence of phenolic compounds (catechol tannins, flavonoids), alkaloids, saponins, coumarins, proteins, and reducing sugars in the leaves of *P. senegalense* marked by the absence of Gallic tannins. Our results are consistent with those obtained by Abdel-Gawad and El-Zait[21] in Egypt, Maradufu Ouma[22] and Midiwo *et al*.,[23] in Kenya, which have reported the presence of flavonoid compounds in the extracts of the leaves of *P. senegalense*. Contrary to the results obtained by Bothon (2012)[24] on the leaves of *P. senegalense* of Adjohoun (Benin), our samples harvested at Godomey (Bénin) contain anthocyanins, leucoanthocyanins, flavones and reducing sugars.

3.2 Extraction yield of phenolic compounds

The extraction yields of phenolic compounds are shown in Table 3.

Table 3: Yield of phenolic compounds extraction

Ethanolic extract (%)	Hydroalcoholic extract (%)
18	24

The ethanolic extract has a weak extraction yield unlike the hydroethanolic extract (30/70) (Table 3). This observation was made by Agbangnan et *al.*[25] Which showed that the mixture ethanol-water extract phenolic compounds better than any of the solvents considered separately. The plausible explanation is that the phenolic compounds are polar which justifies their affinity for water/ethanol mixture which is more polar than ethanol.

3.3 Phenolic content

Figure 1 shows the levels of phenolic compounds in ethanolic and hydroethanolic extracts of *P. senegalense* leaves.

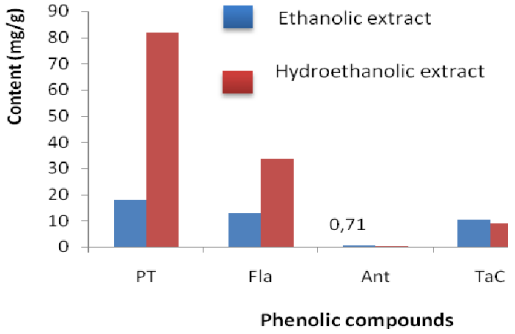

Figure 1: Phenolic compounds content of ethanolic and hydroethanolic extracts.

PT: Total Polyphenols; Fla: Flavonoids; Ant : Anthocyanins ; TaC : Condensed Tannins

The mixture of solvents, ethanol-water, extracts respectively four and twice over of total polyphenols and flavonoids from P. senegalense leaves than ethanol, whereas the two solvents extracted anthocyanin and condensed tannins in the same proportion with a slight increase in ethanol. Comparing our results with those of Bothon[24] shows that the hydroalcoholic extract of leaves of P. senegalense harvested at Adjohoun is richer in total polyphenols (126 ± 8 mg EAG/g DM) and condensed tannins (488 ± 52 mg EC/g DM) than our hydroethanolic extracts but less rich in flavonoids (8.4 ± 1.0 mg EQ/g DM against 33.83 mg EQ/g DM). The differences between our results and those of Bothon may be related to methods of extraction and quantification, which are two factors that may affect the phenolic content of plant species[26].

It appears from these results that the hydroethanolic extract is richer in phenolic compounds than ethanolic extract, which confirms the extraction yields obtained. The same observation was made by Naczk et al.,[27] and Agbangnan et al.,[25] who reported that the pure solvents have low extractives capacities. This observation would be justified by the fact that the phenolic compounds are polar with a low affinity for water due to their many functions and their phenolic aromatic rings.

3.4 Anti-radical activity

The DPPH test provides information on the reactivity of the test compounds with a stable free radical. DPPH gives a strong absorption band at 517nm in visible region. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution is decolourised as the colour changes from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract. Figure 2 presents the IC_{50} (Inhibitory Concentrations of 50% of DPPH) of ethanolic and hydroethanolic extracts of leaves of P. senegalense compared with those of standards.

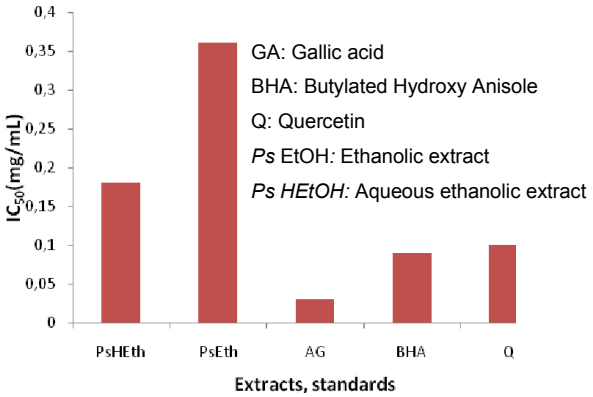

Figure 2: Inhibitory Concentration of 50% of DPPH

Evaluation of anti-radical property of our extracts shows that they contain compounds able to trap the DPPH. The antioxidant activity of each extract is expressed as concentration of substrate which inhibits 50% of DPPH activity (IC_{50}). The antioxidant capacity of an extract is considerable when its IC_{50} is low.

In view of the results obtained, only hydroalcoholic extract (0.18 mg/mL) has an IC_{50} close to that of quercetin (0.1 mg/mL) which is the reference antioxidant used. We note a correlation between anti-radical activity of our extracts and their phenolic content. This observation corroborates those already made earlier by Chevalley[28] in 2000; Djeridane *et al*,[29] (2006) and Wojdylo *et al*,[30] (2007).

3.5 Antiplasmodial activity

For this activity, it has been tested both ethanolic and hydroethanolic extracts from leaves of Polygonum senegalense and positive control, 'Lumate forte' (artemether & lumefantrine 40/240 mg), which is a reference drug, Artemisinin-based Combination Therapies (ACTs) for treatment of malaria in endemic countries. Table 4 shows the concentrations that reduced at least 50% parasitemia of Dd2 strain of P. falciparum in culture.

Table 4: Inhibitory Concentration (µg/mL) of 50% of *P. falciparum* Dd2 strain based on the number of days.

	Positive control	*Polygonum senegalense*	
	"Lumate forte"	Ethanolic	Hydroethanolic
Day 1	54,00	**10,00**	**13,33**
Day 2		**< 6,25**	
Day 3	240,00	99,00	96,00
Day 4		< 6,25	

The classification of the antiplasmodial activity according to the IC_{50} made by Mbatchi *et al*,[31] (IC_{50} <10µg/mL good antiplasmodial activity; IC_{50} <50µg/mL moderate antiplasmodial activity; IC_{50} ≥ 50µg/mL low antiplasmodial activity and IC_{50} ≥ 100 µg/mL no antiplasmodial activity) allowed us to determine the antiplasmodial activity of our extracts.

After 24 hours of incubation, the parasite of the negative control (without extract) increased from 3.38% to 4.16%. This justifies that the conditions are met for normal proliferation of the parasite. Thus, the inhibition of Plasmodium observed in the tests and the positive control would be due only to antiplasmodial activity of extracts investigated and Artemisinin-based Combination Therapy (ACT). *P. senegalense* leaves extracts showed moderate antiplasmodial activity (IC_{50} <50µg/mL), while the positive control has a weak antiplasmodial activity (IC_{50}>50µg/mL). These results indicated that the extracts were more active than the Artemisinin-based Combination Therapy (Lumate forte) used as a reference in this study.

After 48 hours of incubation, all extracts, as well as the positive control, showed good antiplasmodial activity with less than 6.25 µg/mL as IC_{50} (6.25 µg/mL is the lowest concentration applied in this study).

After 72 hours of contact between extracts or ACT and the Plasmodium, there has been a proliferation of the parasite. Thus, 48 hour is the maximum duration of action of investigated extracts and positive control.

The fourth day, the IC_{50} obtained is less than 6.25 µg/mL and a significant drop of the parasitemia of the negative control showing parasitemia smaller than the day zero is observed. This finding could be attributed either to the depletion of the nutrient medium that would lead to the fall of parasitaemia either a mass effect which is due to the large population of parasite in the medium after 72 hours. It is therefore necessary to renew the culture medium as well as the extract or reference product after 48 incubation hours at best or every 24 hours.

In Kenya in 2007, Midiwo et al.,[23] have observed that flavonic and chalconique fractions of aerial part of P. senegalense showed a good antiplasmodial activity against strains D6 chloroquine-sensitive (3.1 ± 0.8) and W2 chloroquie-resistant (2.4 ± 0.3) of P. falciparum. Many factors could be the basis of the differences observed between the IC_{50} of extracts studied and observed values in the literature. According to some authors, the polarity of the solvent used in conjunction with its solubilizing capacity of certain plant substances, the period of collection of plant material, the vegetative stage of the plant and soil[32] are all factors capable of influencing the antiplasmodial power of extracts. We do not note a relationship between anti-radical and antiplasmodial activities of the extracts investigated. Thus, *P. senegalense* leaves don't necessarily act by neutralizing the free radicals generated by Plasmodium[2].

4. CONCLUSION

The importance of the present study lies not only in the promotion of Benin flora and search for new antimalarial drugs but also in the need for a scientific monitoring of traditional health care in Africa. In fact, this study dedicated to the determination of the chemical composition and evaluation of anti-malaria and anti-radical activities of P. senegalense extracts revealed that aqueous ethanol is most suitable solvent for the extraction of phenolic compounds from this plant materiel. Extracts studied showed a better antiplasmodial activity than the Artemisinin-based Combination Therapies (ACTs), the reference drug most used nowadays for the treatment of malaria. Therefore Polygonum senegalense is a credible alternative to effective fight against Plasmodium, the malaria pathogen, and free radicals incriminated in several diseases. We recommend tradipractitioners which are seeking talented antioxidant activities of extracts to treat diseases caused by free radicals, the binary ethanol-water as the most suitable solvent for extraction.

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