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In vitro Pharmacological Properties and Phenolic Contents of Stem Barks Extracts of *Piliostigma reticulatum* (DC) HOCHST and *Piliostigma thonningii* (SCHUM) MILNE-REDH. (Caesalpiniaceae)

Ouédraogo Noufou[1*], A. M. Emmanuel Thiombiano[2], Sawadogo W. Richard[1,3], Tibiri André[1], Martin Kiendrebeogo[2], Lompo Marius[1], Diederich Marc[3,4] and Guissou I. Pierre[1]

[1]Département de Médecine-Pharmacopée Traditionnelle/Pharmacie (IRSS/CNRST) 03 BP 7192 Ouagadougou 03, Burkina Faso.
[2]Laboratory of Biochemistry and Applied Chemistry (LABIOCA), UFR/SVT, 09 BP 848 Ouagadougou 09, University of Ouagadougou, Burkina Faso.
[3]Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, 9, rue Edward Steichen, L-2540 Luxembourg, Luxembourg.
[4]College of Pharmacy, Seoul National University, Building 29 Room 223, 1 Gwanak-ro, Gwanak-gu Seoul, 08826, Korea.

Authors' contributions

This work was carried out in collaboration between all authors. Authors ON and AMET designed and conducted experimental work. Authors TA and SWR managed the analyses of study. Authors MK, LM and DM performed the literature searches and corrected the first draft. The work was done under the supervision of author GIP. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: arnoufou2@yahoo.fr;*

ABSTRACT

Aims: This study aimed to investigate the cytotoxic, antioxidant and lipoxygenase inhibition activities of extracts from stem barks of *Piliostigma reticulatum* and *Piliostigma thonningii*.
Methodology: Methanol extract and decoction from stem barks of *P. reticulatum* and *P. thonningii* were evaluated for their lipoxygenase inhibitor effect and antioxidant properties using 2, 2'-azino-bis (acid 3-ethylbenzothiazoline-6-sulfonique (ABTS) and lipid peroxidation. Cytotoxic effect on leukemia cell lines (K562) was evaluated by trypan blue assay. Total phenolic, tannins and flavonoids contents in the extracts were determined with standard methods.
Results: *P. thonningii* and *P. reticulatum* methanol extracts inhibited lipoxygenase activity with IC_{50} values of 7.80 ± 0.57 and 12.86 ± 1.47 µg/mL respectively. With ABTS scavenging method, *P. thonningii* methanol extract showed the strongest activity with IC_{50} value of 3.96 ± 0.03 µg/mL, followed by *P. reticulatum* methanol extract (IC_{50} = 4.90 ± 0.07 µg/mL). Both decoctions from these plants had scavenged $ABTS^{+}$ radical. Methanol extracts from *P. thonningii* and *P. reticulatum* exhibited significant activity against lipid peroxidation and K562 cells viability. Phytochemical analysis showed the presence of phenolic compounds including tannins and flavonoids in methanolic extracts and decoctions from *P. reticulatum and P. thonningii*.
Conclusion: The extracts from *Piliostigma reticulatum* and *Piliostigma thonningii* stem barks exhibited lipoxygenase inhibitor, antioxidant and cytotoxic effects.

Keywords: Piliostigma reticulatum; Piliostigma thonningii; antioxidant; lipoxygenase; cytotoxicity.

1. INTRODUCTION

Plants have a long history of use in the treatment of several diseases and play an important role in the traditional medicine practice [1]. Most of the people living in developing countries, use medicinal plants to diagnostic, prevent and treat several diseases. These plants produce high diversity of natural products which constitute a natural source to discover, hemi-synthesize or synthesize some therapeutic compounds [2].

Medicinal plants contain bioactive compounds such as polyphenols which have antioxidant properties. Recently scientific investigations on natural products increased up to find natural antioxidant to replace synthetic antioxidant [3]. Antioxidant effect of phenolic compounds plays an important role in reducing risk of metabolic diseases including cancer, diabetes, hypertension. Antioxidant drug acts as a defensive system against harmful production of free radical as reactive oxygen species (ROS). ROS formed during natural metabolism play an important role in several biological processes and cell function. However, excessive production of ROS is harmful products and induce oxidative stress that is known to involve in the pathogenesis of inflammatory pathologies, cancer, diabetes and cardiovascular diseases [4,5,6]. Lipoxygenase(LOX) is key enzymes in leukotrienes biosynthesis which are pro-inflammatory mediators, and lipoxygenase activation generates ROS during arachidonic acid oxidation [7]. Leukotrienes and ROS are involved in cancer initiation and development. Literature reported high expression of lipoxygenase, ROS andleukotriene (LTB4) receptors in cancer such as pancreatic cancer, prostate cancer [8,9].

In genus of *Piliostigma* two species namely *Piliostigma reticulatum* and *Piliostigma thonningii* are employed in Burkina Faso folk medicine to treat several diseases such as inflammatory and infectious illness. Babajide et al. [10] showed the antimicrobial and cytotoxic activities of flavonols and an oxychromonol which isolated from leave extracts of *P. reticulatum*. Literature reported anti-ulcerogenic properties of methanolic extract from *Piliostigma reticulatum* roots and *in vivo* antioxidant of *P. thonningii* leaves [11,12,13].

Based on our research to appreciate Burkina Faso folk medicine through medicinal plants with antioxidant activity, the present study aimed to investigate antioxidant, anti-inflammatory and cytotoxic activities of aqueous and methanolic extracts of stem barks of *Piliostigma reticulatum* and *Piliostigma thonningii* ; and to determine total phenolic, tannins, total flavonoids and flavonols contents found in these extracts.

2. MATERIALS AND METHODS

2.1 Plant Materials

The stem bark of *Piliostigma reticulatum* and *Piliostigma thonningii* were collected in March

2014 at Gampela, 15 km from Ouagadougou (Burkina Faso). The plants were identified by Dr Ouédraogo Amadé at forest department of institut de l'environnement et de recherche agricole (INERA-CNRST). Voucher specimen were deposited at National herbarium of centre national de recherche scientifique et technologie (CNRST) under number PT ID 16880 (*P. thonningii*) and PR ID16880 (*P. reticulatum*).

2.2 Chemicals

Lipoxygenase (type I-B) enzyme, ABTS (2, 2'-azino-bis (acid 3-ethylbenzothiazoline-6-sulfonique), Folin-Ciocalteu reagent (FCR 2N), aluminum chloride, Sodium tetraborate, boric acid, linoleic acid and potassium persulfate were purchased from Sigma-Aldrich. Trichloroacetic acid and 2-thiobarbituric acid were from Flukachemica.

2.3 Extraction

Fifty grams (50 g) of dried powder of *Piliostigma reticulatum* and *Piliostigma thonningii* bark stems were extracted by decoction using 500 mL of water for 30 min. The aqueous solution was concentrated and then lyophilized to yield aqueous extracts (7.2 g for *P. reticulatum* and 6.4 g for *P. thonningii*).

50 g of dried powder of *P. reticulatum* and *P. thonningii* bark stems were extracted by maceration using 500 mL methanol during 24 h at room temperature. After filtration, methanol solutions were concentrated in vacuum at 40℃ using a rotary evaporator Büchi. Dried methanol extracts were powdered and exposed to laboratory during 24 hours at room temperature to completely eliminate methanol. Methanol extracts of *P. reticulatum* (5.1 g) and *P. thonningii* (4.7 g) were obtained.

2.4 Lipoxygenase Inhibition Assay

Lipoxygenase inhibition activity of methanol extracts of *P. reticulatum* and *P. thonningii* stem barks was assessed spectrophotometrically as described by Lycklander and Malterud [14] with slight modifications. Briefly 142.5 µL of the enzyme solution (at the final concentration of 200 U/mL) was prepared in boric acid buffer (0.2 M; pH 9.0), mixed with 3.75 µL of extract solution (from 100 to 6.125 µg/mL in boric acid buffer) and then incubated at room temperature for 3 min. Controls were performed under the same conditions in the extract absence. Reaction was initiated by the addition of 150 µL of substrate solution (linoleic acid, 250 mM). Absorbance was recorded at 234 nm. The lipoxygenase inhibitor effect was expressed as the reduction percentage of lipoxygenase activity compared to those in the control test.

$$\%\ \text{inhibition} = \frac{(\mathrm{E} - \mathrm{S}) * 100}{\mathrm{E}}$$

where E = absorbance of solution at 234 nm without a test sample, and S = absorbance of solution at 234 nm with a test sample.

2.5 2, 2'-azino-bis (acide 3-ethylbenzothiazoline-6-sulfonique) (ABTS) Assay

Scavenging $ABTS^{\bullet+}$activity of extracts from *P. reticulatum* and *P. thonningii* stem barks was used to evaluate the antioxidant capacity of extracts according to Re et al. [15]. ABTS radical cation was produced by mixing ABTS solution with potassium persulfate and keeping it in the dark at room temperature for (12 – 16 h). Before the test the reagent, solution was diluted in ethanol to an absorbance of 0.7 at 734 nm. Twenty microliter (20 µL) of extract concentration or reference substance (Trolox) were mixed to 200 µL of ABTS radical cation solution in 96 wells-microplate. After 30 min of incubation in the dark at 25℃, absorbance was measured at 734 nm using spectrophotometer BioRad (Model 680).

2.6 Lipid Peroxidation Inhibition Assay

Thiobarbituric acid method was used to determine inhibitor effect of methanol extracts of *P. reticulatum* and *P. thonningii* stem barks on lipid peroxidation (LPO) [16]. $FeCl_2$-H_2O_2 induced the liver homogenate peroxidation. 40 µL of extract at the concentration of 10 mg/mL was mixed with 200 µL of liver homogenate (1 %, w/v), then 10 µL of $FeCl_2$ (0.5 mM) and 10 µL of H_2O_2 (0.5 mM) were added. After 60 min of incubation at room temperature, 200 µL of trichloroacetic acid (15%) and 200 µL of 2-thiobarbituric acid (0.67 %) were added and the mixture was heated up in boiled water for 15 min. the absorbance were red at 532 nm using spectrophotometer Agilent 8453. Quercetin was used as positive control.

2.7 Cytotoxic Assay

Leukemia cells (K562) were cultured in RPMI medium supplemented with serum FCS (10%) and antibiotic (1%) at 37℃ and 5% CO_2 atmosphere, in humidified incubator. Briefly cytotoxic activity of extracts from *P. reticulatum* and *P. thonningii* stem barks were assessed on K562 cells. Trypan blue cytotoxic assay against cancer cell lines was used to determine the cytotoxic effect of extracts. After pre-incubation of cells in 24-well plates (2 x 10^5 cells/mL) for 24 h, cells in 24-well plates were treated or not with each extract (10 and 50 µg/mL) and incubated for 72 h. After incubation, 20 µL of sample per well and 20 µL of trypan blue solution were put on Malassez blade for microscope reading. Dead cells appearing blue from treated wells were recorded and compared to control wells.

2.8 Determination of Total Phenolic Content

According to Singleton et al. [17], Folin-Ciocalteu method was used to determine total phenolic contents of extracts from *P. reticulatum* and *P. thonningii* stem barks. Extract (10 µL) was added to Folin-Ciocalteu reagent (2.5 mL) and sodium carbonate solution (cott). The mixture was incubated for 2 h. After incubation, absorbance of the reaction mixture was recorded at 760 nm using spectrometer Agilent equipped (Agilent 8453) equipped with UV-visible ChemStation software. Tannic acid was used as a reference standard, and total phenolic contents were expressed as milligram tannic acid equivalent (mg TAE)/g of extract.

2.9 Determination of Tannins Content

Tannins content of extracts from *P. reticulatum* and *P. thonningii* stem barks was determined using the Folin-Ciacalteu method as above, after reaction of the phenolic with PVPP (polyvinylpolypyrrolidone). 100 mg of PVPP was added to 1 mL of each extract in test tube. After 15 min at 4℃, tubes were vortexed and centrifuged for 10 min at 3000 g. Two (2) mL of supernatant each tube were sampled and been used to determine phenolic content as described above (method of phenolic content determination). Tannins contents were calculated subtracting from total phenolic contents and these are expressed as tannic acid equivalent [18]. The amount of tannins was determined as the difference between total phenolic (containing tannins) and the total phenolics (in absence of tannins).

2.10 Determination of Total Flavonoids Content

Aluminum chloride colorimetric method was used to measure the total flavonoid content of extracts from *P. reticulatum* and *P. thonningii* stem barks, according to Abdel-Hameed et al. [19]. 0.1 ml of extract was mixed with 0.3 mL of sodium nitrite. 0.5 mL of aluminium chloride and sodium hydroxide were added to mixture. After 30 min of incubation, the absorbance was measured at 515 nm against a blank using spectrophotometer (Agilent 8453) equipped with UV-visible ChemStation software. Total flavonoid content was determined using standard curve of quercetin. The calibration curve was constructed using rutin. Total flavonoid content was expressed as milligrams of rutin equivalent (mg RE) per g of extract.

2.11 Statistical Analysis

Data were expressed as means ± SEM, and analysed using Graph Prism version 5.0 software. The statistical analysis was performed by One way ANOVA followed by Dunnett's test. The differences were considered significant compared to the control test at $P< 0.05$.

3. RESULTS AND DISCUSSION

The results of this work indicate that the methanol extracts and decoction from stem barks of *Piliostigma reticulatum* and *Piliostigma thonningii* possessed antioxidant, lipoxygenase inhibitor and cytotoxic effects. In addition, the extracts contain phenolic compounds (tannins and flavonoids).

The *in vitro* anti-inflammatory activity of methanol extracts of the two *Piliostigma* species were evaluated using lipoxygenase inhibition test. Both methanol extracts significantly ($p< 0.05$) exhibited lipoxygenase activity (Table 1). *P. thonningii* methanol extract expressed the more activity with IC_{50} value of 7.80 ± 0.57 µg/mL, against lipoxygenase than methanol extract (IC_{50} = 12.86 ± 1.47 µg/mL) from *P. reticulatum*. Lipoxygenase involves in inflammation process by biosynthesis of leukotrienes which are pro-inflammatory mediators. Leukotriene B4 is chemo-attractant and is involved in the production of reactive oxygen species (ROS) and NF-κB activation [8]. It suggests that inhibitor

effect of extracts may have anti-inflammatory properties by inhibiting leukotrienes and ROS biosynthesis.

ROS inhibition effect of extracts was evaluated by determining antioxidant activity of extracts from *P. reticulatum* and *P. thonningii* using ABTS and lipid peroxidation inhibition methods. The results obtained are summarized on Table 1. Antioxidant capacity of methanol extract and decoction extract from the two plants was evaluated with ABTS assay. In this method, the decoction extract of *P. thonningii* was more active than *P. reticulatum* decoction, however *P. thonningii* methanol extract showed the strongest activity with IC_{50} = 3.96 ± 0.03 µg/mL, followed by *P. reticulatum* methanol extract (IC_{50} =4.90 ± 0.07 µg/mL). Free radical scavenging effect of extracts contribute to the lipoxygenase inhibitor mechanism [20].

ROS can damage cells directly or indirectly via lipid peroxidation leading to DNA damage and cancer. The ability to inhibit lipid peroxidation (LPO) of methanol extracts from two medicinal plants was evaluated. The inhibitor activity of *P. thonningii* methanol extract and *P.reticulatum* methanol extract against lipid peroxidation respectively was 50.00±1.02 and 53.57±0.51 at 100 µg/mL. Both extracts have shown an antioxidant property; however, there is not a significant difference between these extracts in LPO assay. The obtained results indicated that extract effect against lipid peroxidation inhibition was less important than lipoxygenase inhibitor effect and ABTS radical scavenger; this is in agreement with the investigations reported by Czapski et al. [21]. Kim et al. [22] reported that substances possessing LOX inhibitor effect and ROS scavenging activity, inhibit the release of pro-inflammatory cytokines (TNF α, IL 1β) and NF-κB activation.

In the present study, cytotoxic activity of *P. reticulatum* and *P. thonningii* extract (decoction and methanol) were determined by trypan blue assay on Leukemia cells (K562). The results indicated that these two extracts exhibited a moderate cytotoxic effect against K562 cells (Fig. 1). The presence of phenolic compounds in the extracts may explain the antioxidant and cytotoxic activities [23]. Campos et al. [24] showed that phenolic compounds in propolis from Stingless Beeof *Tetragoniscafiebrigi* were responsible of cytotoxic activity against K562 (leukemia) cells and others cancer cell lines.

Total phenolic and tannin contents of extracts were determined with Folin-Ciocalteu reagent test; and flavonoid contents was performed using aluminium chloride assay (Table 2). The total phenolic content of extracts was in the following order: *P. thonnigii* methanol extract >*P. reticulatum* methanol extract >*P. thonnigii* decoction >*P. reticulatum* decoction. *P. thonnigii* methanol extract exhibited the highest amount of total phenolic (608.3 ± 8 mg TAE/100 g dw) and tannins (433.02 ± 4 mg TAE/100 g dw).

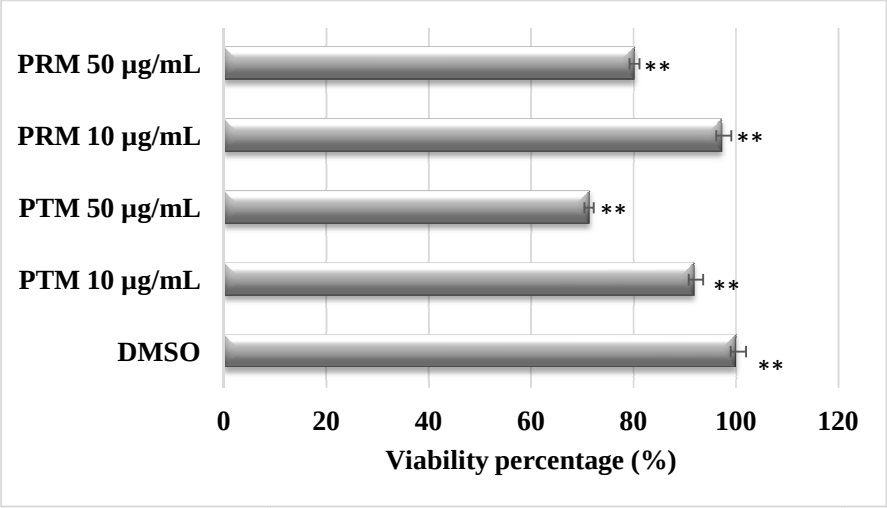

Fig. 1. Cytotoxic effect of extracts from stem barks of *Piliostigma reticulatum* and *Piliostigma thonningii*. PRM: Methanol extract of *Piliostigma reticulatum* and PTM: Methanol extract of *Piliostigma thonningii*

*Values are mean ± S.E.M. n = 8 ; ** : P = 0.01significantfrom control (one way ANOVA analysis followed by Dunnett's test)*

Table 1. Antioxidant activity and inhibitor effect on lipoxygenase of extracts from stem barks of *Piliostigma reticulatum* and *Piliostigma thonningii*

Samples	ABTS (IC_{50} µg/mL)	LPO (% inhibition) at 100 µg/mL	Lipoxygenase inhibition (IC_{50} µg/mL)
P. reticulatum MeOH	4.90 ± 0.07**	53.57±0.51*	12.86 ± 1.47*
P. reticulatum DEC	55.53 ± 7.4**	---	---
P. thonningii MeOH	3.96 ± 0.03**	50.00±1.02*	7.80 ± 0.57**
P. thonningii DEC	49.62 ± 2.4*	---	---
Trolox	0.004 ± 0.001**	---	---
Quercetin	---	46.98 ± 0.2*	
Zeluiton	---	---	1.1 ± 0.3**

*MeOH : methanolextract and DEC : decoction. Values are mean ± S.E.M. for triplicate ; * : P = 0.05 and ** : P= 0.01significantfrom control (one way ANOVA analysis followed by Dunnett's test)*

Table 2. Total phenolics, tannins, flavonoids and flavonols contents in extracts from stem barks of *Piliostigma reticulatum* and *Piliostigma thonningii*

Samples	Total Phenolic content (g TAE/100 g dw)	Tannins (g TAE/100 g dw)	Total flavonoids (g QE/100 g dw)
P. reticulatum DEC	265.1 ± 17.31*	215.43 ± 25.96*	12.9 ± 2*
P. reticulatum MeOH	470.3 ± 2.3*	316.1 ± 12*	7 ± 0.5*
P. thonningii DEC	307.36 ± 12*	262.01 ± 8.8*	6.6 ± 1.3*
P. thonningii MeOH	608.3 ± 8	433.02 ± 4**	4.6 ± 0.6**

*MeOH : methanol extract and DEC : decoction. Values are mean ± S.E.M. for triplicate ; * : P = 0.05 and ** : P =0.01 significant from control (one way ANOVA analysis followed by Dunnett's test)*

The obtained data of this study indicated that methanol extracts of both species were the most active comparing to decoction. Interestingly, *P. thonningii* methanol extract was the most active against lipoxygenase as well as antioxidant and cytotoxicity activities. This means that phenolic and tannins contents could be responsible of the investigated pharmacological activities. Inhibitor effect of extracts against lipoxygenase activity could be explained by the presence of phenolic compounds in the extracts [25]. Despite the antioxidant capacity of *P. thonningii* stem barks has been shown in this study, Awhin et al. [26] reported that the chronic consumption of *Piliostigma thonningii* stem barks increased the level of aspartate aminotransferases (ALT) and alamine aminotransferases (ASL), which has a potential toxicity on the liver. Literature reported that ethanol extract of *P. reticulatum* stem bark had provoked an increase of cholesterol and creatinine levels but in reversible manner after two weeks of treatment at 1000 mg/kg at rats [27].

4. CONCLUSION

The obtained results of the present study indicate that extracts from *Piliostigma reticulatum* and *Piliostigma thonningii* stem barks possessed lipoxygenase inhibitor effect, antioxidant activity and moderate cytotoxic effect. This study demonstrated that stem barks of *P. reticulatum* and *P. thonningii* contained phenolics, tannins and flavonoids. These scientific data help to enhance the use of *P. reticulatum and P. thonningii* stem barks in traditional medicine.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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