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Anti-Inflammatulatory Activity of Hydro-Ethanolic Bark Extracts of *Piliostigma reticulatum* Hochst (*Caesalpiniaceae*) and Analysis by HPLC-DAD and HPLC-MS of its Methanol Fraction

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ABSTRACT

Use of plants as remedies dates back to the time of the oldest civilizations. Among these active ingredients, polyphenols play an important role. *Piliostigma reticulatum*, a plant whose barks are rich in condensed tannin is often used as an anti-inflammatory. The purpose of this work was to correlate the anti-inflammatory activity of extracts with their chemical composition through chromatography fractionation analyses (HPLC-DAD and HPLC-MS)**.** The barks were extracted by moderate hydroethanol decoction followed by silica gel splitting with successively ethyl acetate, methanol and water. Anti-inflammatory activity was evaluated by the method of carrageenan induced hind paw edema in Wistar rats. The chemical study was carried out by HPLC-DAD and HPLC-MS after isolation of the compounds by fractionation on Sephadex LH-20 gel. The bark hydroethanolic extract had shown ability to inhibit significantly the inflammation induced by carraghenan. Among the hydroethanolic bark extract fractions that methanol fraction had the best activity at 10 mg/kg with inhibition percentages similar to those of acetylsalicylic acid with 48.51% ± 2.26; 54.71% ± 5.13; 64.19% ± 6.70 vs. 54.69% ± 7.23; 57.83% ± 9; 65.13% ± 8.44 respectively. The HPLC-DAD analysis showed that the SF5 sub-fraction presented the best chromatogram with several peaks, three of which were high intensity. The latter would correspond to monomer, dimer and trimer of catechin according to the weights [M-H] + m / z: 291.07; 579.18; 867.27 obtained by HPLC-MS. The compounds responsible for anti-inflammatory activity would be condensed tannins. The latter would consist mainly of catechin oligomers.

Keywords: *Piliostigma reticulatum, bark, a*nti-inflammatory activity, HPLC-DAD, HPLC-MS

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INTRODUCTION

The use of plants as remedies originates in the oldest civilizations. To this day, despite considerable advances in modern medicine and the pharmaceutical industry, medicinal plants still remain important in the medical care of populations. Pharmacies and pharmaceutical industries continue to use them in dried form or as raw materials for the manufacture of medicines. Au Sénégal, les plantes médicinales sont très présentes dans les marchés urbains et constituent semble-t-il la principale source d'automédication des populations. This is certainly due to the many pharmacological properties granted to plants but also to the cost well accessible to the population. In recent years, there have been numerous studies to justify the use of plant-based products to prevent or treat certain diseases by using pharmacological tests and identification of the active ingredients responsible for their effect.

Among these active ingredients, the polyphenol family occupies an important place. They are believed to be responsible of many related biological activities of plants such as anti-inflammatory, antimicrobial, antiradical activities [1]. Polyphenols are known to be excellent antioxidants especially through their ability to trap free radicals responsible for oxidative stress [2], [3]. They prevent diseases that are often associated with them such as cancer, coronary heart disease, thrombosis, osteoarthritis, atherosclerosis and aging [4,5]. This is the case for phenolic compounds such as anthocyanins, flavonols, catechin etc.[6].

In addition, the barks of *Piliostigma reticulatum* (DC) Hochst (*Caesalpiniaceae* ; Synonym: *Bauhinia reticulata*) are very rich in polyphenols made up mainly of condensed tannins [7]. This is how we became interested in the evaluation of anti-inflammatory activity as well as the study of the nature of the tannins of its barks. *Piliostigma reticulatum* is a well-known plant of Senegalese traditional practitioners who use the bark of the plant to treat toothache, gingivitis, diarrhea and over-infected wounds [8, 9, 10].

The objective of this work was to correlate the anti-inflammatory activity of extracts with their chemical composition through fractional analyses by chromatography (HPLC-DAD and HPLC-MS).

MATERIAL AND METHODS

Plant material

The barks of *Piliostigma reticulatum* were harvested in Diourbel, central Senegal. The plant has been identified at the Pharmacognosy and Botany Laboratory of the Medicine, Pharmacy and Odontology Faculty of the Cheikh Anta Diop University in Dakar. The identification herbarium number of this plant is 1641. The barks were washed for about 15 minutes with tap water and then dried at room temperature for two weeks in the dark and in an airy room of the laboratory before being reduced to powder by a grinder (Brabender®, granulometry : 1.5 mm). The powder was used for extraction.

Animals

Adult male and female Wistar rats (160 to 220 g) were used to assess anti-inflammatory activity. Male and females rats were raised separately in the pet shop of the Laboratory of Pharmacology of the Faculty of Medicine, Pharmacy and Odontology. They had free access to food and water until 16h before the experiments.

Reagents and solvents

The solvents and reagents that have been used are: Ethanol (VWR Chemicals BDH, France), Methanol (Carlo ERBA, France), Ethyl acetate (VWR Chemicals BDH, France), distilled water, silica gel (Scharlau GE 0048,60A- 0.04-0.06 mm), Carrageenan (Sigma Aldrich, Allemagne), acetyl salicylic acid (Sigma Aldrich, Chine),physiological water 0,9%, Sephadex LH-20 (Sigma-Aldrich, USA), silica CCM plates (TLC Silica gel - F_{254}, Merck), formic acid (VWR Chemicals BDH, France), aluminum silica plates (TLC Silica gel - F_{254}, Merck).

Extraction

The hydroethanol extract (HEE) of the barks was obtained by moderate decoction under reflux of the powder at about 70° C for 30 minutes. Thus 600 g (4x150 g) of powder was extracted with 6 liters (4x1.5 L) of ethanol-water mixture v/v (80:20). After cooling and filtration on Whatman No 1 filter paper, the solvent was removed using a rotary evaporator at 60° C. The resulting dry extract was powdered and sealed in a jar.

Silica column fractionation

The fractionation was done by adapting the method of Labourel and Péaud-Lenoel [11]. A cylindrical glass column 3 cm in diameter was filled with 100 g of silica mixed with 300 ml of ethyl acetate while avoiding trapping air. The silica was then washed three times in a row with 200 ml of ethyl acetate generating a separation height of 31 cm with a dead volume of 195 ml. Then 2 g of powdered hydroethanol bark extract and 2 g of silica homogeneously mixed by triturating with methanol before evaporating it in the boil at 40° C,

were deposited at the head of the column so that the surface was horizontal, then protected with cotton to avoid depressions caused by elution. Elution was made with successively 0.5 L of ethyl acetate, 0.5 L of methanol and 0.5 L of Milli-Q water with an average flow of 12.3 ml/min. The dead volume was eliminated after each change of solvent. The three fractions thus collected (Methanol Fraction, FM; Ethyl acetate fraction, FAE; Water Fraction, FA) have been evaporated and dried by the same process as the EHH previously described.

Sephadex LH-20 column fractionation

A Sephadex LH-20 fractionation of the methanol fraction was carried out using the slightly modified method described by Yang et al. [12]. A 3 cm diameter glass cylindrical column was carefully filled with 20 g of Sephadex LH-20 gel mixed with 200 ml of methanol while avoiding any trapping air. The column was then washed twice in a row with 200 ml of methanol. Elution was made with a solvent system water/methanol of different proportions (100/0; 70/30; 50/50; 20/80; 0/100, v/v). The eluent was collected in test tubes at a volume of about 5 ml per tube at a rate of one drop per second.

At the end of the elution, all test tubes were analyzed by Thin Layer Chromatography (TLC) with the Methanol/Ethyl Acetate/Hexane mixture as a migration solvent (2/1/1.5, v/v). The revelation was made with sulphuric vanillin (0.1 g of vanillin - 28 ml of methanol - 1 ml of H_2SO_4). The tubes having the same profile were combined leading thus to six sub-fractions named SF1 to SF6.

Anti-inflammatory activity

The anti-inflammatory activity of the bark extract and its fractions was evaluated by the method of rat hind paw edema induced by carrageenan [13, 14].

Rats were divided into 5 lots of 5 each: one group per dose administered and fasted 16 hours before the experiment. The extracts were prepared with physiological water and administered by gavage at different doses:

– Lot 1 (control): physiological water (10 ml/kg, *per os*);

– Lot 2 (reference): acetylsalicylic acid (10 mg/kg, *per os*) ;

– Lot 3 and 4: hydroethanol extract (100 and 300 mg/kg, *per os*) ;

– Lot 5: fractions from hydroethanol extract (1 mg/kg, *per os*);

– Lot 6: fractions from hydroethanol extract (3 mg/kg, *per os*);

– Lot 7: fractions from hydroethanol extract (10 mg/kg, *per os*).

An edema under the plantar pad of the rats' right paw was induced by injection of 100 μl of a 1% carrageenan solution (m/v) 1 hour after feeding the rats. The thickness of the leg was measured before the induction of the edema and then at 1, 3 and 5 hours after induction using an electronic calliper.

The importance of edema was appreciated by determining the average percentage increase (% API) in the thickness of the rat leg following the formula:

$$\%API = \frac{TRh - TR0}{TR0} \times 100$$

TRh : Thickness of rat's paw at T-time

TR0 : Thickness of the rat's paw at 0-time

The anti-inflammatory activity of the samples was expressed as a percentage of edema inhibition (% INH) calculated according to the following formula:

$$\%INH = \frac{\% \, APIcontrol - \% \, APIsample}{\% \, APIcontrol} \times 100$$

HPLC-DAD analysis of Subfraction (SF1 to SF6)

The tests were carried out on an Ultimate 3000 (Thermofisher) chain equipped with an automatic injector, a thermostatized compartment at 35°C and a UV-DAD detector. The samples were prepared by dissolution or dilution in the HPLC grade methanol. All samples were filtered on 0.22 μm PTFE membrane. The extracts and the standard (catechin) were injected due to 10 μl per sample. Chromatographic separation was obtained at 40°C on an C18 reverse phase column (Luna 3u PFP (2) 100A ; 150x3.0 mm (Phenomenex)) preceded by a pre-column. Elution was made under pressure between 112 -145 bars with a flow of 0.3 mL/min of the moving phase according to a gradient mentioned on Table 1. The moving phase was composed of a mixture of solvent A (0.1% solution of formic acid in MilliQ water) and solvent B (a solution of 0.1% formic acid in HPLC grade methanol). The detection of separate compounds was made at 254 nm and 280 nm.

Table 1 : Gradient of elution of the mobile phase of the samples

Time (min)	Water 0,1% formic acid	Methanol 0,1% formic acid	Flow (ml/min)
0	85	15	0.3
15	80	20	0.3
35	60	40	0.3
40	35	65	0.3
45	30	70	0.3
50	30	70	0.3
51	85	15	0.3
55	85	15	0.3

The treatment of chromatograms was carried out by Chromeleon 6.8 software.

HPLC-MS analysis of the Subfraction SF5

The analyses were carried out on a chain of HPLC Ultimate 3000 (Thermofisher Scientiific) coupled with mass spectrometry (Bruker HPL071) in positive ionization mode. This series-connected chain is equipped with a UV detector and an Amazon SL ion trap mass detector with an electropray ionization interface (ESI). The extract was prepared by dissolution in the HPLC grade methanol of the dry extract of subfraction SF5. The resulting solution was filtered on 0.22 μm PTFE membrane before injection. The chromatographic separation was obtained on Kinetec EVo C18 column (150x2,1 mm, 2,6 μm, Phenomenex) at a flow of 0.3 ml/min at 35°C, with UV detection at 250, 280 and 330 nm for mass with spectrum recording from 50 to 2000 m/z. The voltage of the capillary of the ESI source is adjusted to 4.5 KV. The pressure of the nebulizer was set at 40 psi nitrogen. For desolvation, the temperature was 200°C and the gas had a flow of 9 l/min. The elution gradient was identical to that used for HPLC-DAD and data processing was carried out with Hystar and Data analysis software.

Statistical analyses

The data were expressed as average ± SEM. The significative analyses was carried out by Fisher's test using StatView version 4.55 software. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Extraction and fractionation

From 600 g of bark powder, 70 g of dry hydroethanolic extract were obtained representing a yield of 11.66%. Silica gel fractionation of 2 g of hydroethanolic extract gave the fractionation yields mentioned in Table 2.

Table 2 : Yields of ethyl acetate, methanol and aqueous fractions

	EAF	MF	AF
Dry extract (g)	0.044	1.616	0.209
Yield (n: 3 ; %)	2.19 ± 0.1	80.80 ± 3.3	10.47 ± 0.13

EAF: Ethyl acetate fraction, MF: Methanol Fraction ; AF: Aqueous fraction

Anti-inflammatory activity

The evaluation of the inhibition of increased edema induced by bark hydroethanol extract (HEE) yielded the results illustrated by Fig. 1. Compared to the control lot, HEE caused a dose-dependent inhibition of the induced inflammation. At the dose of 100 mg/kg, inhibition of increased edema was not significant except for the 5th hour (41.36% ± 6.69; 67.16% ± 3.76; 64.09% ± 2.71 respectively at 1h, 3h and 5h) with inhibition percentages (%INH) corresponding to 13.73% ± 10.2; 17.19% ± 5.84; 29.42% ± 4.61. However, at 300 mg/kg, HEE inhibits significantly manner the increase inflammatory edema at every hour with 11.05% ± 1.84; 44.66% ± 5.62 and 62.23% ± 2.9 respectively at 1h, 3h and 5h. The inhibition percentages of inflammation obtained at this dose were 77.01% ± 4.71; 44.74% ± 8.01; 31.10% ± 6.25 respectively at 1h, 3h and 5h.
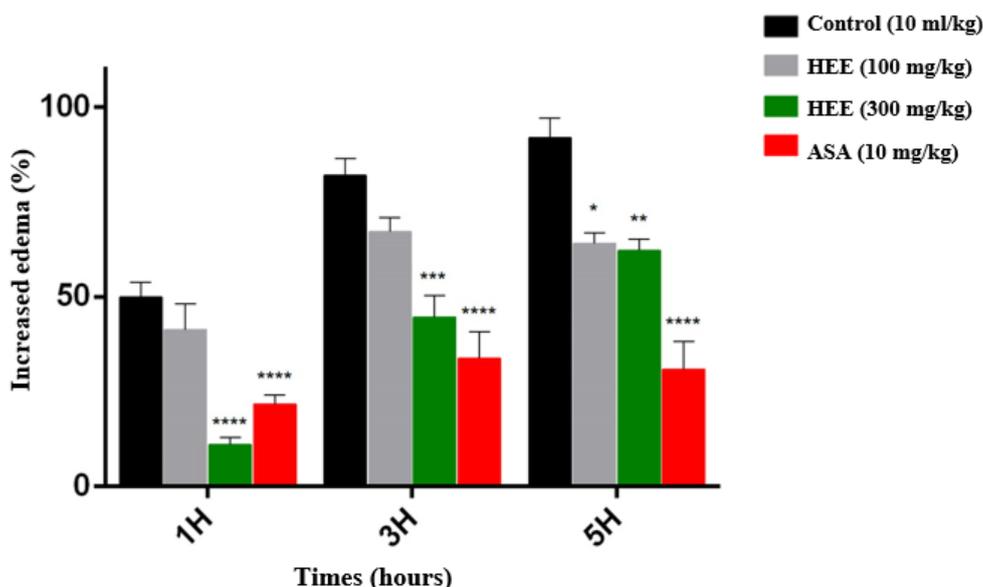
Fig. 1 : Inhibition of increased inflammatory edema by bark hydroethanol extract at different doses and acetylsalicylic acid.

*HEE : Hydroethanol extract; ASA : Acetylsalicylic acid; * : p < 0.05*

The fractions also showed dose-dependent activity. Of all the fractions from EHE, the methanol fraction at the 10 mg/kg dose is the most active. The variation in the inflammatory edema which it induced was 25.62% ± 2.11; 36.36% ± 2.28 and 31.62% ± 3.88 respectively at 1h, 3h and 5h (see Fig. 2). At this dose, its activity was statistically similar to that of the reference, acetylsalicylic acid (21.79 ± 2.27 at 1h; 33.77 ± 7.08 at 3h; 30.96 ± 7.25 at 5h) as shown in Fig. 3.
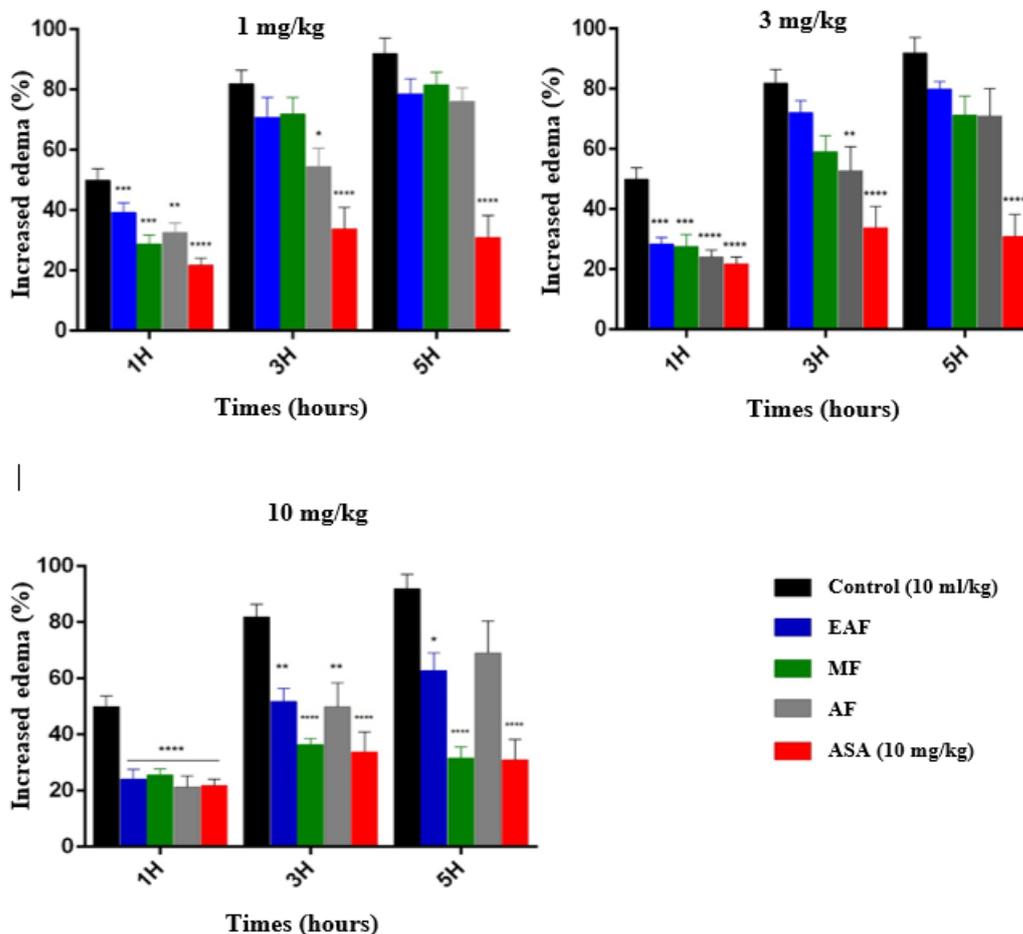

Fig. 2 : Inhibition of increased inflammatory edema by fractions of bark hydroethanol extract at different doses and by acetylsalicylic acid.

*EAF: Ethyl acetate fraction ; MF: Methanol Fraction; AF: Aqueous fraction; ASA : Acetylsalicylic acid ; * : p < 0.05*
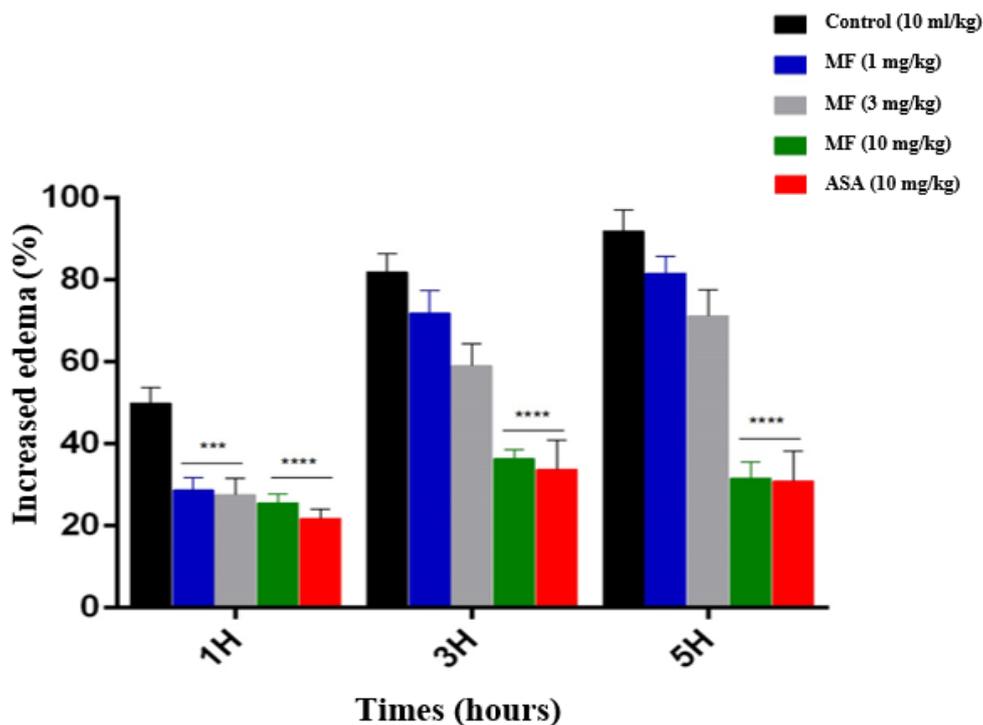
Fig. 3 : Inhibition of increased edema by methanol fraction at different doses and acetylsalicylic acid at 10 mg/kg

MF: Methanol Fraction ; ASA : *Acetylsalicylic acid; *: p< 0.05*

The inhibition percentages of inflammation of methanol fraction and acetylsalicylic acid at 10 mg/kg were 48.51% - 2.26, respectively; 54.71% - 5.13; 64.19% - 6.70 *versus* 54.69% - 7.23; 57.83% - 9; 65.13% - 8.44 (*p* < 0.05 every hour).

HPLC-DAD analysis of subfractions (SF1 to SF6)

The resulting chromatograms showed the most important UV absorption at 280 nm. Of all the samples injected, the SF5 had the most complex chromatographic profile with the presence of several peaks, among which three had a high absorption: peak 3, 5 and 6 (see figure 3). The chromatogram data are summarized in table 3.
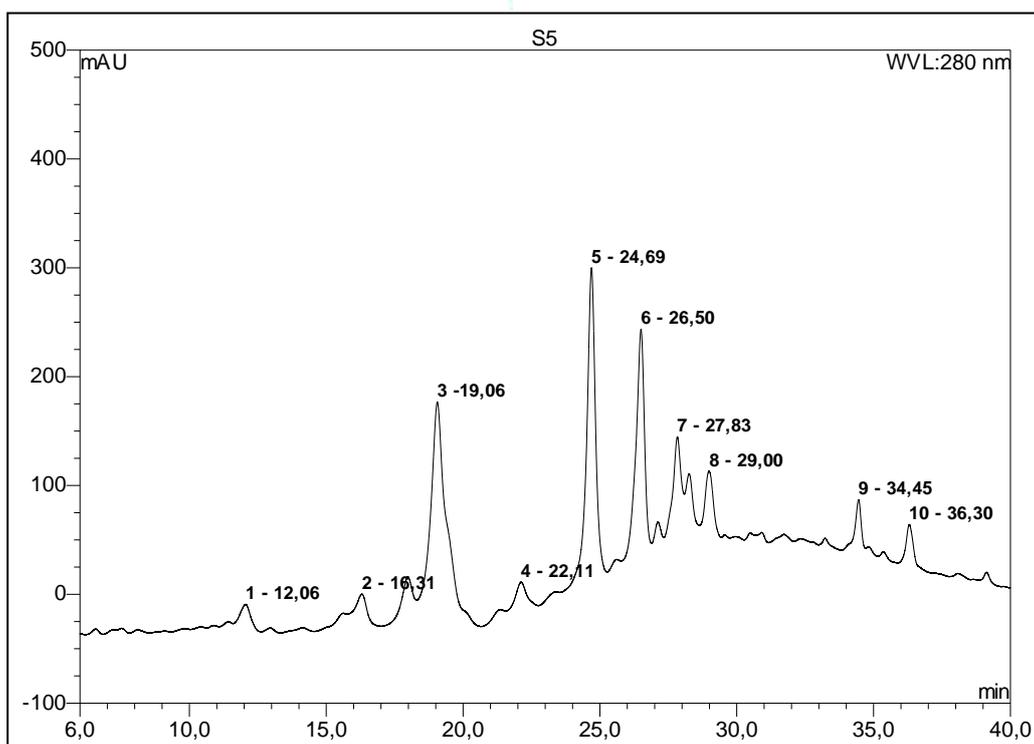

Fig. 4 : SF5 Chromatogram obtained by HPLC-DAD analysis

Table 3 : SF5 HPLC-DAD chromatogram data

No. peak	Ret.Time	Height	Area	Rel.Area
	min	mAU	mAU*min	%
1	12.06	24.712	14.822	3.69
2	16.31	29.363	19.873	4.94
3	19.06	189.198	113.805	28.31
4	22.11	24.112	10.683	2.66
5	24.69	292.212	112.825	28.06
6	26.50	210.588	77.611	19.30
7	27.83	58.743	14.872	3.70
8	29.00	55.037	17.862	4.44
9	34.45	42.886	9.449	2.35
10	36.30	36.396	10.245	2.55
Total:		963.247	402.049	100.00

Peak 3 at a retention time of 19.06 min could correspond to the catechin as shown by the overlay of the two chromatograms (see fig. 5).
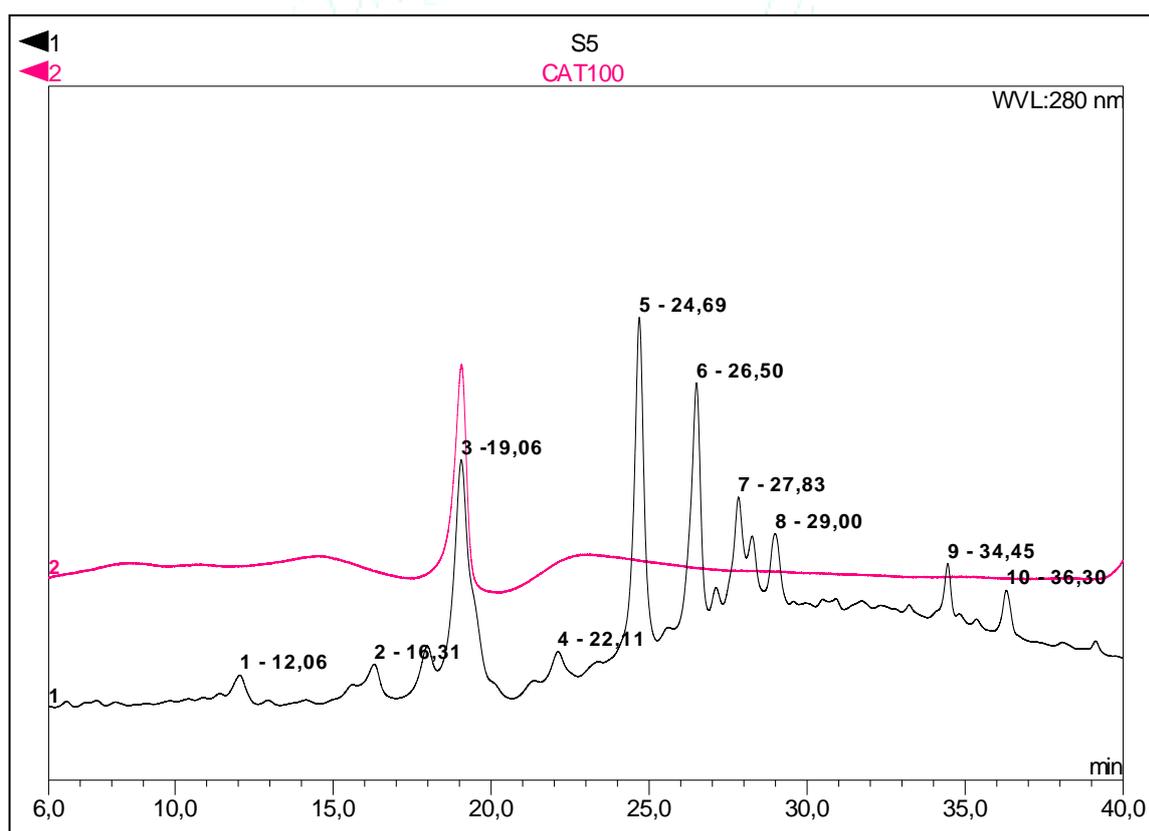
Fig. 5 : Chromatogrammes HPLC-DAD de la SF5 (1) et de la catéchine (2)

HPLC-MS Analysis of SF5

Positive ionization made it possible to obtain molecular ions [M + H] +. The resulting chromatograms yielded several molecular ion weights (m/z) of which 3 were frequently present with a high signal/noise ratio (S/N) as shown in fig. 6. Mass spectrum data was summarized in table 5.
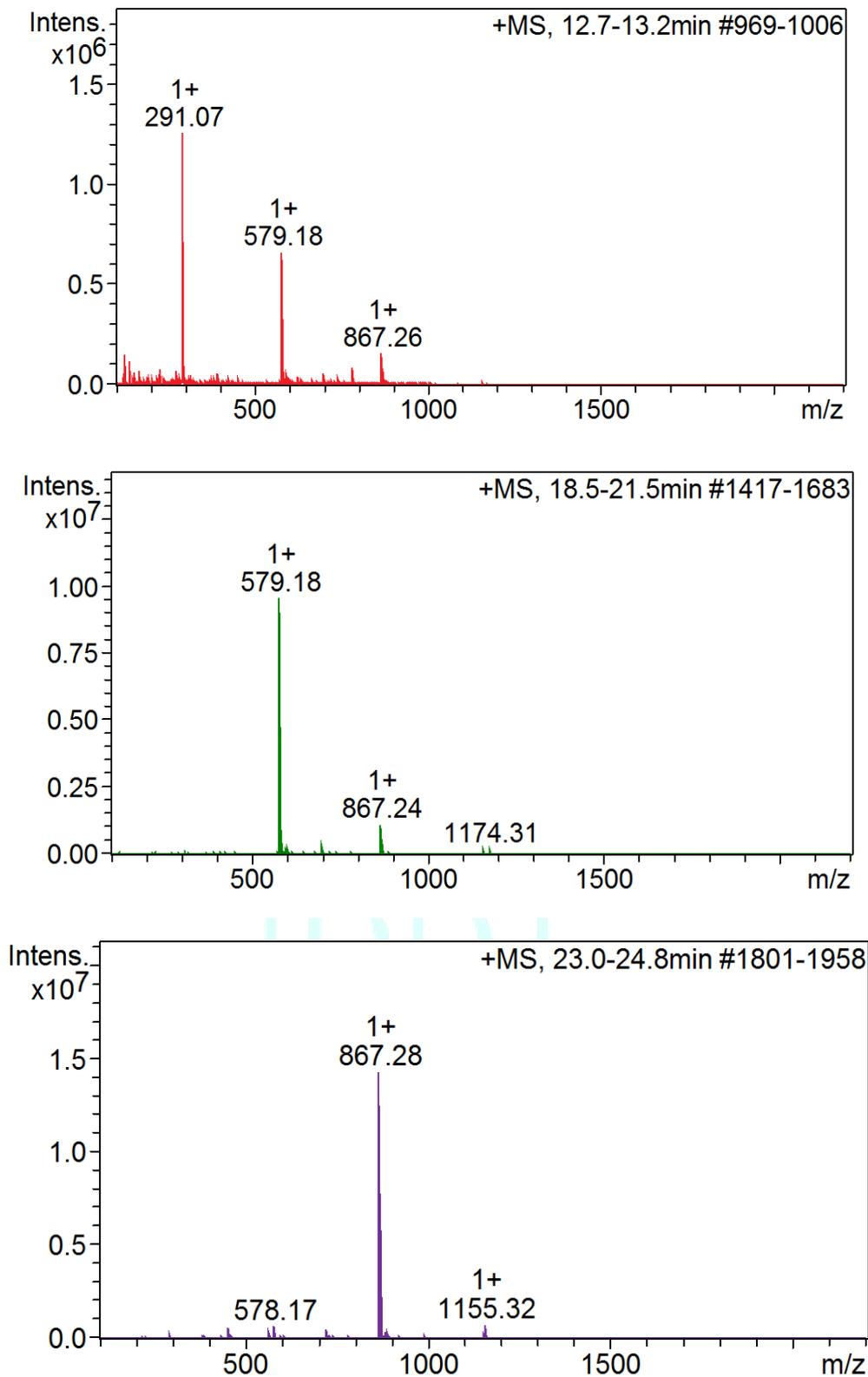

Fig. 6 : HPLC-MS chromatogram at different retention times of SF5

Table 4 : HPLC-MS spectrum data of SF5

Tr (min)	[M-H] m/z	z	I	FWHM	S/N
2.6	330.20	1+	1022309	0.39	401.9
5.2	577.18	1+	281895	0.43	143.1
9.4	453.18	1+	356798	0.43	137.8
9.9	741.24	1+	443343	0.42	195.7
12.8	291.08	1+	1257750	0.38	500.5
14.4	579.19	1+	1364623	0.42	576.6
17.8	867.24	1+	414503	0.42	188.6
21.0	579.19	1+	12309845	0.42	2601.3
22.0	291.08	1+	3038763	0.39	654.7
22.5	865.27	1+	1178800	0.42	268.0
23.7	867.28	1+	14312367	0.44	2411
24.2	1155.32	1+	1776091	0.46	307.1
25.0	772.21	1+	1824975	0.29	266.0
26.3	520.39	1+	7209310	0.41	721.5
28.4	652.47	1+	6857386	0.43	607.4
30.0	428.32	2+	968002	0.31	56.8
36.0	313.33	1+	7199566	0.42	156.5

Tr : Retention time ; Z : Proton number   ; I : Intensité ; FWHM : Full width at half maximum ; S/N : Signal/Noise ratio

DISCUSSION

Hydroethanol bark extract showed, at high dose (300 mg/kg) and at a short period of time, a significant anti-inflammatory activity greater even than that of acetylsalicylic acid, but this activity decreased in intensity over time. On the other hand, it inhibited in a dose-dependent manner induced inflammation since a decrease of the administered dose resulted in a sharp decrease of anti-inflammatory activity with inhibition percentages (IP) less than 30% every hour.

At all doses tested, the fractions inhibited induced inflammation in a dose-dependent manner from 1 to 10 mg/kg. Unlike hydroethanol extract, fractions showed low-dose activity with 10 mg/kg as the maximum active dose. The fractionation therefore appeared to increase the anti-inflammatory effect of the extract.

Of all the fractions tested, the methanol was the most active and inhibited in long time manner the induced inflammation at 10 mg/kg with IP greater than 50% at all times. Its activity has been statistically similar to that of acetylsalicylic acid, Which suggesting on the one hand that they have the same mechanism of action. On the other hand, the active compounds responsible for the anti-inflammatory activity of the barks extract have a greater affinity for methanol than for other solvents. This has been the case with the work of Nariman et al.[15] where silica column fractionation showed that the methanol fraction contained the active biological compounds responsible for the antibacterial activity of the aerial parts of *Xanthium brasilicum* and *Trachyspermum copticum.* This seems to be confirmed by the fractionation yields that were obtained with 80.80% for the methanol fraction.

As a result, the methanol fraction was split again on Sephadex LH-20 gel in order to isolate and identify the active compounds responsible for its anti-inflammatory activity. In addition, our previous results showed that the barks of the plant were composed mainly of condensed tannins (72.18%)

and that more than 56% of these tannins were isolated by fractionation with methanol [7]. These condensed tannins appeared to be the support of the biological activity of the barks. These were flavan-3-ols polymers (proanthocyanidols) subdivided into several subgroups. The most common was the subgroup of procyanidines where the constituent unit is most often catechin or epicatechin. They have been isolated and identified in all plant families [16,17,18]. This justifies the use of catechin as standard for this study. The Sephadex LH-20 is a hydroxypropyled and reticulated dextran gel with an average diameter of dry beads 18-111 μm. The latter swell in methanol to give diameters between 27-163 μm. It is used to isolate polyphenolic molecules such as flavanolic monomers as well as oligomers such as dimers, trimers or even tetramers [19,20].

The chromatogram obtained by HPLC-DAD analysis of the SF5 showed the presence of several compounds, three of which had a maximum absorption corresponding to peak 3: 189,198 mAU respectively; peak 5: 292,212 mAU and peak 6: 210, 598 mAU. The overlay of this chromatogram with that of the catechin showed that peak 3 had the same retention time as the standard. This suggests that the SF5 contained catechin that would correspond to peak 3 (see fig. 7). This was confirmed by the mass spectrum of SF5 where high-intensity peaks were found with molecular ion weights [M-H]+ m/z: 291.07; 579.18 ; 867.27 representing the molecular mass of monomer, dimer and trimer of catechin respectively (m/z: 290 g/mol; 578 g/mol and 866 g/mol). However, it was found that these same weights [M-H] + m/z were found several times at different retention times. It would be due to the presence of an isomer of catechin namely epicatechin. This could lead to several types of oligomers such as for exemple dimers of epicatechin, catechin-epicatechin; trimers of epicatechins, catechin-epicatechin-catechin, catechin-catechechin, epicatechin-epicatechin-catechin, epicatechin-catechin- epicatechin. Low intensity peaks of m/z: 1155.32 were also noted meaning the presence of tetramers trace.
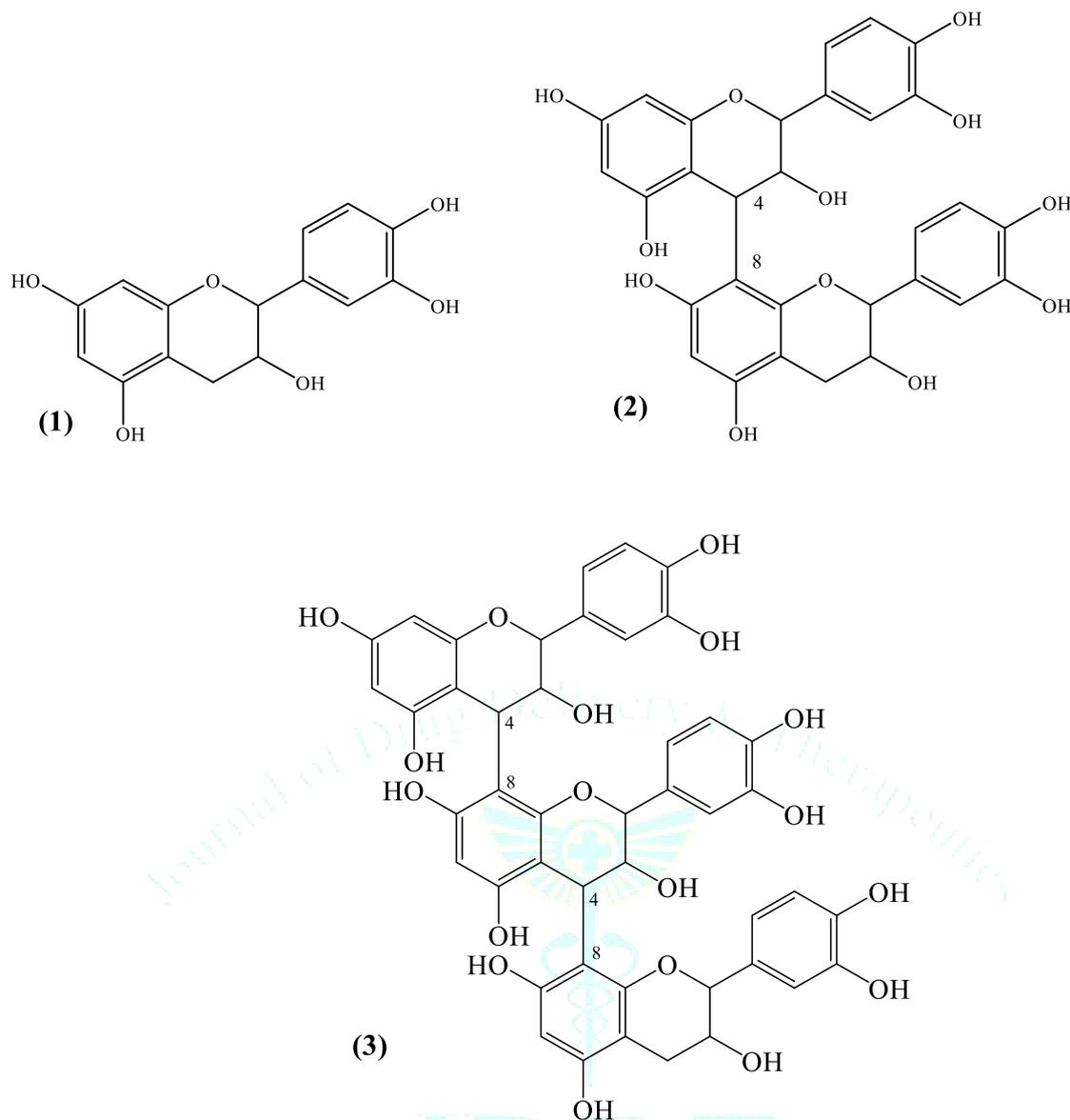
Fig. 7 : Structures of monomers, dimers and trimers of procyanidinol: (1): catechin (epicatechin); (2): example of catechin dimer (epicatechin); (3): example of catechin trimer (epicatechin).

These results suggest thus that condensed tannins widely present in the methanol fraction are partly responsible for the anti-inflammatory activity of the barks. Among which the most frequent oligomers are dimers and trimers. So catechin and/or epicatechin would be the main structural components of the tannins of *Piliostigama reticulatum* barks as such was the case with the proanthocyanidines of the pine barks : *Pinus densiflora* and *Pinus thunbergii* [21].

CONCLUSION

The barks of Piliostigma reticulatum are rich in tannins especially in proanthocyanidines. The latter would be responsible for their anti-inflammatory activity. It would therefore be interesting to assess their toxity for better therapeutic use. It would also be important to fractionate this tannic fraction into monomers, dimers, trimers tetramers etc... and to assess the impact of the polymerization of the latter on the activity. This would require a preparatory analytic fractionation to obtain these different fractions in sufficient quantity for to re-evaluate the anti-inflammatory tests and thus to identify the activities synergies of the extract.

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