Original Research Article

PHOSPHOLIPASE A2 INHIBITION AND ANTIINFLAMMATORY ACTIVITY OF F4 FRACTION OF TOTAL ETHEREAL LEAF EXTRACT OF *ANNONA SENEGALENSIS* PERS. (ANNONACEAE)

Abstract

Annona senegalensis Pers. (ANNONACEAE) is a plant which is used in african traditional medicine for the treatment of various diseases. This study aimed to investigate the analgesic and anti-inflammatory activity of total ethereal leaf extract fractions of *A. senegalensis*. Compounds of methanolic fractions of ethereal leaf extract of *A. senegalensis* were separated by gel sephadex chromatography, in five fractions (F1, F2, F3, F4, F5). Experiments were performed in acetic acid-induced contortions in mice, carrageenan rat paw edema and phospholipase A2 inhibitory test. The methanolic fraction of total ethereal leaf extract (10 mg/kg, *per os*) significantly prevented the carrageenan inflammatory edema. The variation of edema is 22.31±3.35 %, 49.66±13.50 %, 52.10±10.02 % respectively at T1h, T3h and T5h. The increased edema after oral administration of F4 fraction administered at 300 µg/kg and 1 mg/kg *per os* is respectively 52.77±7.36 % and 33.81±6.94 %. The variation of edema in betamethasone group (1 mg/kg, *per os*) is 23.46±3.99 %. F4 fraction at 300 µg/kg, significantly inhibited 16.39 % of phospholipase A2 enzyme activity. F4 fraction (300 µg/kg, *per os*) also significantly prevented acetic acid-induced pain in mice. The number of abdominal contortions is 21 versus 72 in control group. F4 fraction compounds have a powerful analgesic and anti-inflammatory activity that involves phospholipase A2 inhibition, comparable to betamethasone profile on pain and inflammation.

Keys-words: *Annona senegalensis*, Leaf extract, Pain, Inflammation, Phospholipase A2.

Introduction

Inflammatory syndrome is frequently encountered in clinical practice. The inflammatory response is linked to various diseases such as infection, cancer, thromboembolic and degenerative diseases [1-5].

The deleterious effects of inflammatory process justify the treatment with analgesic and anti-inflammatory drugs. However, its use is limited by the incidence of occurrence of digestive, renal and cutaneous adverse effects [6]. The valorisation of medicinal plant extracts with analgesic and anti-inflammatory activity could be an alternative to develop drugs which have a better selectivity towards the targets of inflammatory reaction and therefore likely to cause less adverse effects.

Annona senegalensis Pers. (ANNONACEAE), is a very widespread plant in the Sudano-Guinean savannas, extended from Senegal to Sudan and all along the East African coast and Madagascar [7]. The fruit of *A. Senegalensis* was consumed for many years without any obvious complaints or toxicity. Previous studies had shown that the root bark extracts of *A. senegalensis* are safe at the lower dose tested, and calls for caution in use at higher doses in treatment [8].

A. Senegalensis extracts are used in traditional medicine for treatment of nociceptive and inflammatory processes [9]. Previous studies had shown anti-inflammatory activity of total ethereal leaf extract of *A. senegalensis* leaves [10].

The aim of that study was to investigate phytochemical characteristics, analgesic and anti-inflammatory activity of total ethereal leaf extract fractions of *A. senegalensis* on carrageenan-induced paw edema in rats and acetic acid contortions in mice.

Materials and methods

Drugs, chemicals and solvents

Carrageenan, acetyl salicylic acid, betamethasone, acetic acid and extraction solvents were obtained from Sigma/BES-Senegal. sPLA2 (type V) inhibitor screening assay kit came from Cayman chemicals (Bertin Pharma, France).

Plant material

A. senegalensis leaves were collected from Pout, in Senegal. Botanical samples were identified at Botany and Pharmacognosy Department of the Faculty of Medicine and Pharmacy of Cheikh Anta DIOP University of Dakar, where the voucher specimen (DPB-15-03) was deposited.

The leaves had been dried in the shade at room temperature (25 °C) for 4 weeks before being pulverized.

Animals

Adult Wistar KYOTO strain rats of 140 g and mices of 22 g body weight were used. The animals had free access to food and water. The experimental protocols were conducted in accordance with the guidelines on the care and use of laboratory animals (Senegal National Ethical Committee for Health Research). All animals had received human care and its use was approved (11/12/2015) by the Research Ethical Committee of Cheikh Anta DIOP University of Dakar (approval n° 0136/2015/CER/UCAD).

Experimental procedures

Extraction

Powder leaves (300 g) of *A. senegalensis* was mixed with petroleum ether (2 L). The mixture had been boiled (70 °C) for 2 hours after cooling filtered. The fractionation of the total ethereal leaf extract (TEE) with methanol gave methanolic fraction (MF) and residual ethereal fraction [10].

The dry residue of the methanolic phase was fractionated on a Sephadex LH20 gel column [11]. A sample of 0.5 g of methanolic dry extract was dissolved in 5 mL of methanol and deposited on the upper part of the gel using a syringe. The column was eluted with the moving phase consisting of methanol. Eluates are collected in numbered vials, taking into account their colour. This operation was repeated until the sample was exhausted. A total of 9.5 g of dry extract was thus fractionated. Between two chromatographies, the column was rinsed with methanol to wash and recover the gel. Thin-layer chromatography was performed for each eluate. Eluates with a similar chromatogram after observation with the naked eye and UV were grouped to obtain a total of 5 fractions F1, F2, F3, F4, and F5

Phytochemical study

The phytochemical characterization was performed by thin layer chromatography (TLC). Ferric chloride ($FeCl_3$) was used for the detection of tannins. Flavonoids were characterized by 5 % of aluminum chloride in Water/Methanol (1:1). Dragendorff reagent was used for the detection of alkaloids. Sterols and triterpenes were revealed with the Libermann-Buchard reagent.

Carrageenan induced rat paw edema

The anti-inflammatory study was carried out following the method described by Winter [12]. The rats were divided into 11 groups of 5. Then, They had been fasted for 12 hours before the tests.

Before treatment, the initial volume (V_0) of the left hind paw was measured using a water plethysmometer (APELEX 05-7150), Allinde, Bagneux, France.

The fractions F1, F2, F3, F4, F5, were tested at a dose 10 times lower than that of the methanolic parent fraction that is active at 10 mg/kg per os.

- Group 1: Normal saline (NS) (10 mL/kg, *per os*)
- Group 2: Acetyl salicylic acid (ASA) (1 mg/kg, *per os*)
- Groups 3 and 4: Betamethasone (300 µg/kg and 1 mg/kg, *per os*)
- Group 5: Methanolic fraction (MF) (10 mg/kg, *per os*)
- Groups 6, 7 and 8: F1, F2 and F3 fractions (1 mg/kg, *per os*)
- Groups 9 and 10: F4 fraction (300 µg/kg and 1 mg/kg, *per os*)

The rat paw edema was induced by injection of carrageenan solution 1 % (100 µL) underneath the planter region of left hind paw of the rats 1 h after oral administration with the different solutions. The increased edema was measured using water plethysmometer 60, 180 and 300 minutes (T_{1h}, T_{3h} and T_{5h}) after carrageenan injection.

The importance of edema was assessed by determining the mean percentage increase (% INC) of volume of rat paw according to the following formula:

$\% \mathbf{INC} = (\mathbf{Vt} - \mathbf{Vo}) \times \frac{\mathbf{100}}{\mathbf{Vo}}$ **;** $\mathbf{Vt}$: Paw volume at t time, $\mathbf{V_o}$: Initial paw volume

Phospholipase A2 inhibition assay

The phospholipase A2 enzyme inhibitory effect was measured using the Cayman sPLA2 (Type V) inhibitor screening assay kit (Cayman Chemical - Bertin France). The solution of F4 fraction was prepared by dissolving in methanol and diluting in 25 mM Tris-HCl assay Buffer - pH 7.5 (Item No. 765010). sPLA2 (10 µL), in 25 mM Tris-HCl / Item No. 10004913, was additionned with 10 µL of F4 fraction (10 - 300 µg/mL). The reaction was initiated by the addition of 200 µL of substrate solution (Diheptanoyl Thio-PC, 1.44 / Item No. 75014). The plate had shaked for 30 seconds, covered and incubated for 15 min at 25°C. Finally, 10 µL DTNB (Item No. 765012) was added to each well to stop enzyme catalysis. The plate had shaked for 10 seconds and the absorbance was measured at 405 nm after one minute using a microplate reader. The percentage of sPLA2 inhibition was then determined.

F4 fraction, which is more potent to prevent increased edema than other fractions (F1, F2, F3, F5), was selected for phospholipase A2 inhibition assay and acetic acid induced writhing in mice.

Acetic acid induced writhing in mice

The writhing test in mice was used [13]. Contortions were induced by intraperitoneal injection of acetic acid 3 %. Animals were divided into groups of 5 mice. They had been then fasted for 12 hours before the tests.

Mice were stuffed with the following solutions:

- Group 1 : Normal saline (NS) (10 mL/kg, *per os*)
- Groups 2 and 3 : Acetyl salicylic acid (ASA) (1 and 100 mg/kg, *per os*)
- Group 4 : Betamethasone (300 µg/kg, *per os*)
- Group 5 : F4 Fraction (300 µg/kg, *per os*)

Intraperitoneal injection of 3 % acetic acid solution was performed 1 h after gavage. The sensitivity to pain was evaluated by the contortions number counted during 30 min after latency time.

Statistical analysis

The experimental results are expressed as mean ± standard error of mean (SEM). Significance was evaluated using the student's t-test. Values of $p<0.05$ were significantly different. n is the number of experiences.

Results

Phytochemical analysis

Phytochemical study shows the presence of tannins, flavonoids, sterols, and triterpenes in the methanolic fraction. A similar phytochemical composition was observed with the F4 fraction. Flavonoids are seen in all fractions. The alkaloids were only revealed in the F5 fraction. **(Table I)**

Induction of rat paw inflammatory edema in control group

The administration of carrageenan 1 % in rat paw after pretreatment with normal saline induced edema. The significant increase of rat paw is 45.23±10.73; 81.13±12.83 and 103.46±8.95 % respectively at T_{1h}, T_{3h}, and T_{5h} after carrageenan administration ($p<0.05$ vs baseline, n=5). **(Figure 1)**

Effect of fractions, acetylsalicylique acid (ASA) and betamethasone on carrageenan induced inflammatory edema in rat

The administration of MF (10 mg/kg, *per os*) significantly prevented carrageenan induced inflammatory edema. The variation of edema is 22.31±3.35; 49.66±13.50 and 52.10±10.02 % (n=5), respectively at T_{1h}, T_{3h}, and T_{5h} after carrageenan administration. These results are significantly different from the control group ($p<0.05$). **(Figure 1)**

Oral administration of ASA (1 mg/kg) significantly prevented the development of inflammatory edema following injection of carrageenan. The variation of paw volume is 29.08±10.74; 37.52±9.91 and 54.72±11.82 % respectively at T_{1h}, T_{3h}, and T_{5h} ($p<0.05$ vs control, n=5). **(Figure 1)**

Betamethasone (300 µg/kg, *per os*) significantly prevented carrageenan induced rat paw edema. The increased edema is 17.57±2.14; 9.26±2.79 and 22.62±3.36 % respectively at T_{1h}, T_{3h}, and T_{5h} ($p<0.05$ vs control, n=5). The same variations are obtained at 1 mg/kg of betamethasone. **(Figure 2)**

The F4 fraction induced anti-inflammatory effect in dose-dependent manner. Administration of F4 fraction (300 µg/kg, *per os*) significantly prevented inflammatory edema. The variation of paw volume is 24.39±4.07; 37.84±6.61; and 52.18±7.36 % respectively at T_{1h}, T_{3h}, and T_{5h} after carrageenan administration ($p<0.05$ vs control, n=5). **(Figure 2)**

The F4 fraction induced prevention of inflammatory edema is more effective at 1 mg / kg *per os*. The variation of rat paw volume is 18.22±5.32; 22.64±1.67 and 33.82±6.95 % (n=5) to T_{1h}, T_{3h}, and T_{5h}. This variation is not significantly different to betamethasone group. **(Figure 3)**

The oral administration of F5 methanolic fraction (1 mg/kg) showed a tendency towards prevention of carrageenan induced inflammatory edema. The variation of paw volume is 29.59±1.58 %; 35.52±5.11 % and 56.29±8.52 % (n=5) respectively at T_{1h}, T_{3h}, and T_{5h} after carrageenan administration. **(Figure 3)**

Prior oral administration of F1 fraction (1 mg/kg) did not prevent carrageenan induced inflammatory edema. The variation of rat paw volume is 15.09±2.33; 48.41±4.72 and 63.64±10.26 % respectively at T_{1h}, T_{3h}, and T_{5h} after carrageenan administration. The pretreatment with F2 and F3 fractions did not also prevent rat paw edema. **(Figure 3)**

Inhibitory effect of F4 fraction (10, 30, 100, 300 µg/mL) on phospholipase A2 (sPLA2)

The F4 fraction (10, 30, 100, 300 µg/mL) showed a significant and concentration-dependent phospholipase A2 inhibitory activity ($p<0.01$). The percentages of inhibition were respectively 4.79 %, 5.50 %, 10.89 %, and 16.39 %. **(Figure 4)**

Analgesic activity of acetylsalicylic acid (ASA), betamethasone and F4 fraction on acetic acid induced contortions in mice

In control group, the number of contortions after intra-peritoneal administration of 3 % acetic acid in mice is 72±6.

The administration of ASA (100 mg/kg, *per os*) significantly prevented the occurrence of contortions in mice. The number of contortions is 26±4 ($p<0.05$ vs control, n=5). Betamethasone (300 µg/kg, *per os*) also significantly prevented acetic acid induced contortions in mice.

The F4 fraction significantly prevented contortions induced by intraperitoneal administration of 3 % acetic acid in mice.

The analgesic effect of F4 fraction (300 µg/kg, *per os*) is similar to that observed with betamethasone administered with the same dose. The number of contortions after F4 fraction administration is 21 ± 2 versus 24 ± 4 in betamethasone group. **(Figure 5)**

Discussion

Previous studies had shown the existence of an anti-inflammatory activity of total ethereal leaf extract of *A. Senegalensis* and its methanolic fraction. This fraction is more potent than total ethereal extract to prevent increased edema [10].

In this study, the methanolic fraction of total ethereal leaf extract of *A. Senegalensis* is also more effective in preventing carrageenan-induced rat paw edema than total ethereal extract and ASA. The dose of 1 mg/kg of F1, F2 and F3 fractions, derived from the methanolic extract did not prevent rat paw inflammatory edema induced by carrageenan. Conversely, low doses of F4 fraction (300 µg/kg and 1 mg/kg) prevented inflammatory edema in a dose-dependent manner. Anti-inflammatory activity of F4 fraction is more effective than ASA group and similar to betamethasone prevented rat paw edema.

A similar profile of activity was observed in mice contortions induced with acetic acid. In fact, F4 fraction is more effective than ASA to prevent contortions in mice pretreated with acetic acid.

Phytochemical study revealed the presence of tannins, sterols and triterpenes in methanolic extract and its F4 fraction, while flavonoids were present in all fractions. The alkaloids were exclusively found in F5 fraction.

The absence of a real anti-inflammatory activity with F1, F2 and F3 fractions, suggests the non-involving of flavonoids to prevent carrageenan induced edema.

Previous studies had attributed analgesic and anti-inflammatory effects of some species of ANNONACEAE family to the presence of sterols and triterpenes. The 18-acetoxy-ent-kaur-16-ene, a terpenic compound, extracted from the bark of *Annona squamosa*, is analgesic in acetic acid pain model in albino mice, and anti-inflammatory on carrageenan-induced paw edema in rat [14]. Berenjenol is a triterpenic molecule isolated from *Oxandra xylopioides*, a species of the ANNONACEAE family is anti-inflammatory on models of acute and subchronic inflammation [15].

The presence of sterols and triterpenes in methanolic and its F4 fractions could explain the analgesic and anti-inflammatory properties of those fractions to prevent pain and inflammation. The present study shows that methanolic and F4 fractions, which contain sterols and triterpenes, are more effective than F1, F2 and F3 fractions lacking these compounds, to prevent pain and inflammatory edema.

The analgesic and anti-inflammatory activity of F4 fraction is more potent than type 2 cyclooxygenase (COX2) inhibitor, blocking only the production of prostanoids (prostaglandins, prostacyclines). Previous studies of Geetha and Varalakshmi [16] had also suggested a mechanism of different actions between triterpenes and non-steroïdial anti-inflammatory drugs (NSAIDs).

The analgesic and anti-inflammatory activity of F4 fraction containing sterols and triterpenes is similar to glucocorticoid compounds such as betamethasone. The latter blocks more upstream the production of mediators of inflammation and pain such as prostanoids and leukotrienes [17]. Those arguments were supported by the structural analogy between some triterpenes and steroids used in anti-inflammatory therapy [18].

In fact, F4 fraction leaves of *A. Senegalensis*, showed a significant and concentration-dependent PLA2 inhibitory activity.

Several studies had already described inhibitory activity of triterpenes on inflammatory mediators production. In fact, cyclomargenyl-3-O-β-caffeoyl ester, a triterpenic molecule isolated (cycloartanes group) from *Krameria pauciflora* inhibits, concentration-dependent manner, the PLA2 activity [19]. Similar results were reported by Bernard and al. [20] with betulinic acid, and by Vishwanath and al. [21], with aristolochic acid, isolated from plants of ARISTOLOCHIACEAE family.

The sPLA2 inhibition explained more important analgesic and anti-inflammatory actions of F4 fraction similar to betamethasone.

Alkaloids compounds were exclusively found in F5 fraction. In addition, the sterols and triterpenes found in F4, were absent in F5 fraction. The F5 fraction induced anti-inflammatory action. However this effect is less observed with F4 fraction group. It could probably be attributed to the presence of alkaloids in this extract.

Previous studies had described a probable existence of a relationship between the presence of alkaloids in some extracts and anti-inflammatory activities. In fact, evodiamine and rutaecarpine, molecules belonging to the group of alkaloids, inhibit the pro-inflammatory prostaglandins E2 production. In this same study, goshuyuamide II (alkaloid) was shown to be an inhibitor of 5-lipoxygenase (5-LOX), enzyme that transforms arachidonic acid into pro-inflammatory leukotrienes [22].

The alkaloids of F5 fraction could probably, like that evodiamine and goshuyamide II, have as proteic target, enzymes involved in the production of inflammatory mediators such as COX2 or 5-LOX.

The synergistic action of anti-inflammatory molecules on different targets could explain a better efficacy of some plants extracts such methanolic fraction of total ethereal leaf extract in inflammatory edema prevention.

Conclusion

A. senegalensis leaf extracts possess analgesic and anti-inflammatory activity on acetic acid pain model and carrageenan inflammatory edema. This activity is correlated with the presence of sterols and triterpenes in the extracts. The analgesic and anti-inflammatory effect of F4 fraction involves PLA2 inhibition.

References

1. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860-67.

2. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends in immunol*. 2004;25:4-7.

3. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 2006;124:823-35.

4. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. *Cell* 2010;140:918-34.

5. Makki KF. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. *ISRN inflamm*. 2013;2013:p.139239.

6. Muster D. Médicaments de l'inflammation. *EMC-Stomatologie* 2005;1:21-29.

7. Matig OE, Ndoye O, Kengue J, Awono A. Les fruitiers forestiers comestibles du Cameroun. Cotonou: *Bioversity International* 2006, p204.

8. Okoye TC, Akah PA, Ezike AC, Okoye MO, Onyeto CA, Ndukwu F et al. Evaluation of the acute and the sub acute toxicity of *Annona senegalensis* root bark extracts. Asian Pac J Trop Med. 2012:277-282.

9. Adzu B, Amos S, Adamu M, Gamaniel K. Anti-nociceptive and anti-inflammatory effects of the methanol extract of *Annona senegalensis* root bark. *JNR* 2003;3:63-67.

10. Sene M, Barboza FS, Sarr A, Outouen DT, Wele A, Bassene E et al. Analgesic and anti-inflammatory activity of methanolic fraction of total ethereal leaf extract of *Annona senegalensis* Pers. (Annonaceae). *Afr. J. Pharm. Pharmacol*. 2017;11:120-24.

11. Wang YQ, Tang X, Li JF, Wu YL, Sun YY, Fang MJ et al. Development of an on-line mixed-mode gel liquid chromatography×reversed phase liquid chromatography method for separation of water extract from flos carthami. *J Chromatogram A.* 2017;1519:145-51.

12. Winter CA, Risley FA, Nuss G. Carrageenan induced oedema in hand paw of the rat as assays anti-inflammatory drugs. *Proc. Soc. Exp. Biol. Med*. 1962;111:544-47.

13. Koster R. Anderson M, Beer E. Acetic acid for analgesic screening. *Proceedings* 1959;18-412.

14. Chavan M, Wakte P, Shinde D. Analgesic and anti-inflammatory activities of 18-acetoxy-ent-kaur-16-ene from Annona squamosa L. bark. *Inflammopharmacol*. 2011;19:111-15.

15. Aquila S, Rojano B, Recio MC, Giner RM, Schinella GR, Debenedetti SL et al. Anti-inflammatory activity of berenjenol and related compounds. *Planta med*. 2009;75:18-23.

16. Geetha T, Varalakshmi P. Anti-inflammatory activity of lupeol and lupeol linoleate in rats. *J. Ethnopharmacol*. 2001;76(1):77-80.

17. Holte K, Kehlet H. Perioperative single-dose glucocorticoid administration: pathophysiologic effects and clinical implications. *J Am Coll Surg.* 2002;195(5):694-712.

18. Krief S. Métabolites secondaires des plantes et comportement animal: surveillance sanitaire et observations de l'alimentation des chimpanzés (Pan troglodytes schweinfurthii) en Ouganda. Activités biologiques et étude chimique de plantes consommées. Thèse de doctorat. *MNHN* 2003; Paris.

19. Ramírez-Cisneros MÁ, Rios MY, Ríos-Gómez R, Aguilar-Guadarrama AB. Cycloartanes from Krameria pauciflora and their in vitro PLA2, COX-1, and COX-2 enzyme inhibitory activities. *Planta med.* 2012;78(18), 1942-48.

20. Bernard P, Scior T, Didier B, Hibert M, Berthon JY. Ethnopharmacology and bioinformatic combination for leads discovery: application to phospholipase A2 inhibitors. *Phytochem*. 2001;58(6), 865-74.

21. Vishwanath BS, Fawzy AA, Franson R C. Edema-inducing activity of phospholipase A2 purified from human synovial fluid and inhibition by aristolochic acid. *Inflamm*. 1988;12(6),549-61.

22. Choi YH, Shin EM, Kim YS, Cai XF, Lee JJ, Kim HP. Anti-inflammatory principles from the fruits of Evodia rutaecarpa and their cellular action mechanisms. *Arch Pharma Res*. 2006;59:293-97.

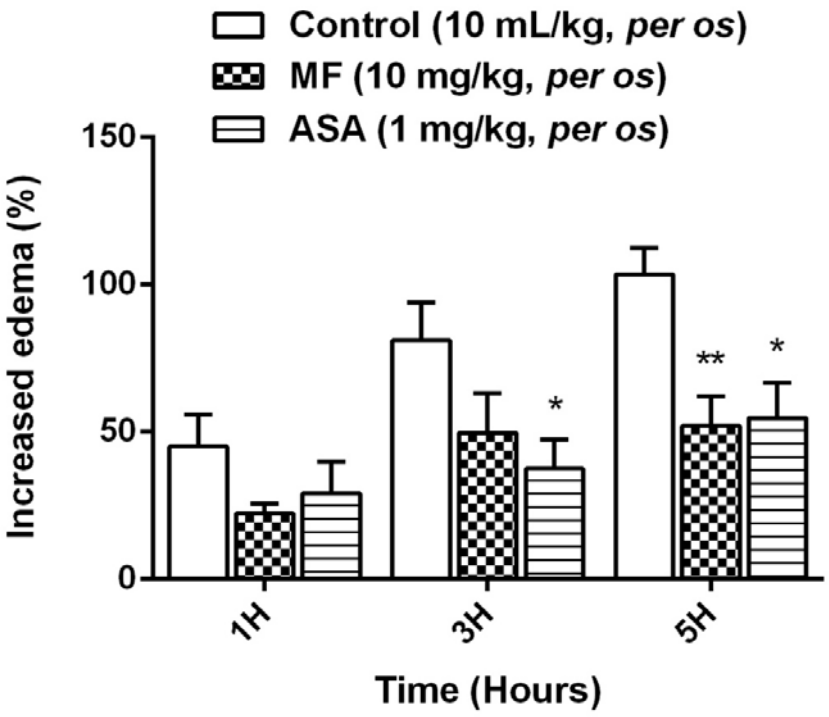

Figure 1: Effect of methanolic fraction of total ethereal extract (MF) on carrageenan-induced inflammatory edema in rats. *p<0.05 versus control group, **p<0.01 versus control group. n=5

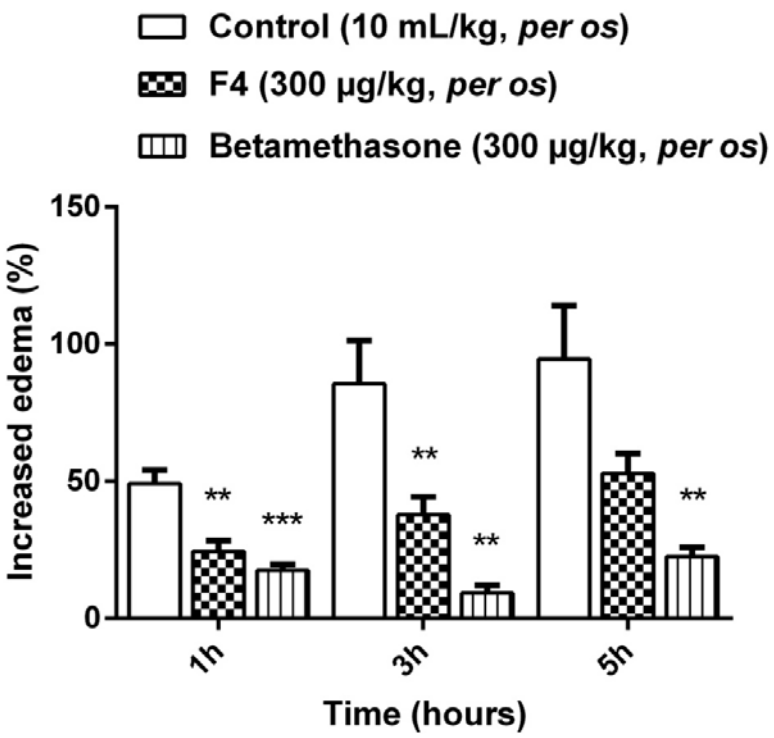

Figure 2: Effect of F4 fraction on carrageenan-induced inflammatory edema in rats.
**p<0.01 versus control group, ***p<0.001 versus control group. n=5

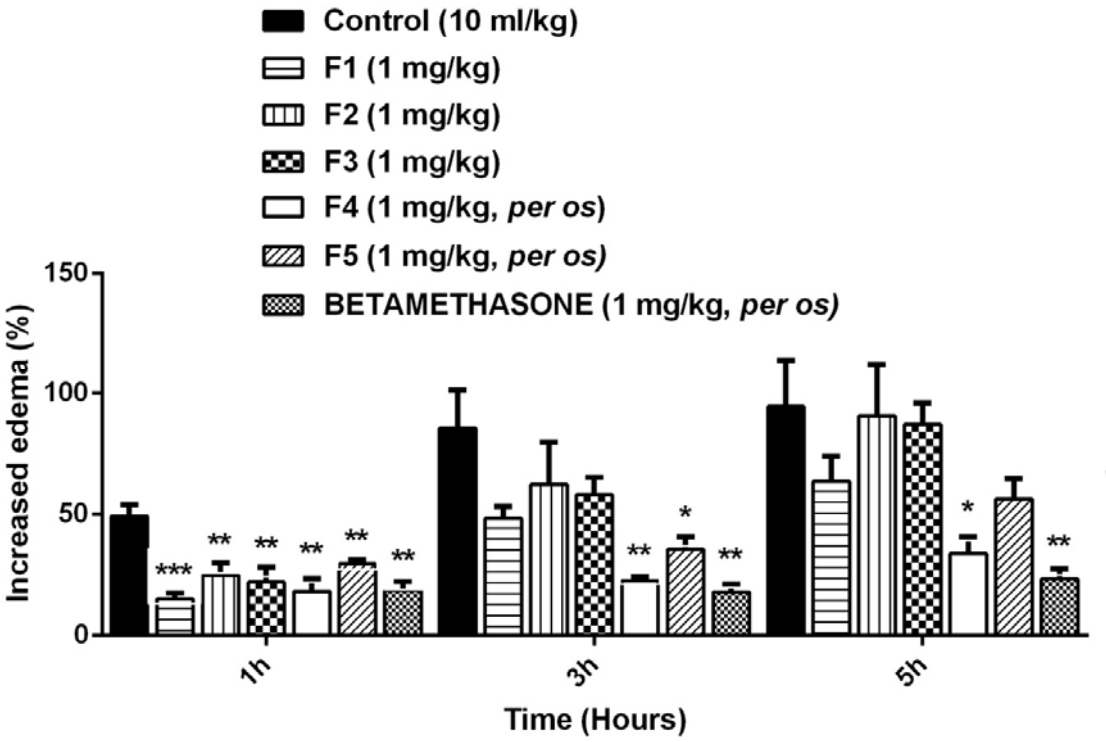

Figure 3: Effects of F4 and F5 fractions on carrageenan-induced inflammatory edema in rats. *p<0.05 versus control group, **p<0.01 versus control group. n=5

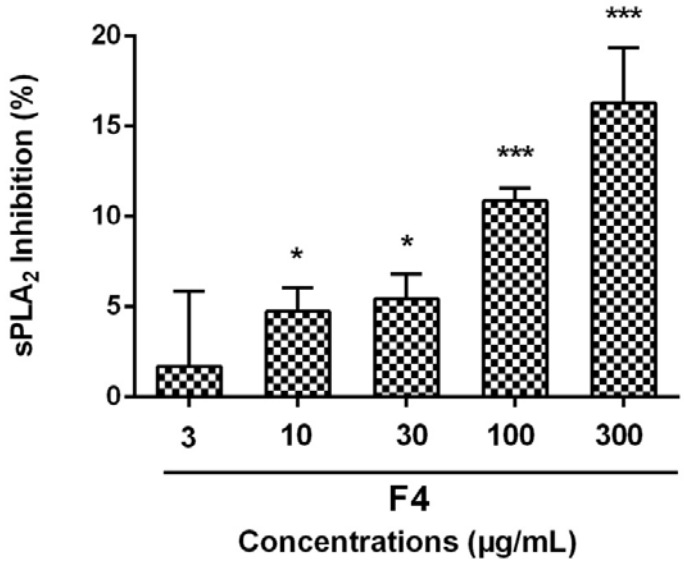

Figure 4: F4 fraction induced sPLA2 inhibition. *p<0.05 versus control, **p<0.01 versus control. n = 5.

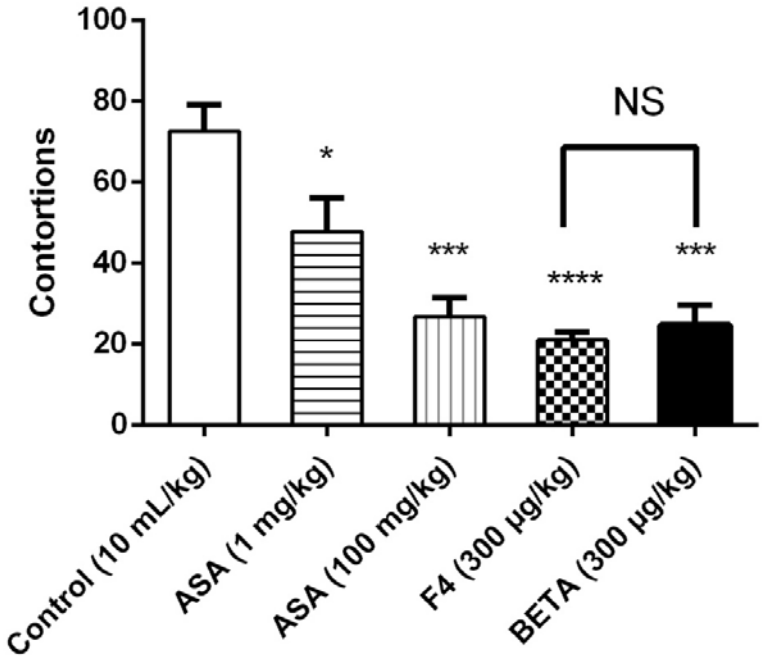

Figure 5: Effect of F4 fraction on contortions induced with acetic acid 3 % in mice.

*p<0.05 versus control group, ***p<0.001 versus control group, ****p<0.0001 versus control group. NS: non significatif. n=5. BETA = betamethasone

Table I: Recapitulation of the chemical constituents in different fractions

	TANNINS	ALKALOIDS	FLAVONOIDS	STEROLS and TRITERPENES
MF	+	-	+	+
F1	-	-	+	-
F2	-	-	+	-
F3	-	-	+	-
F4	+	-	+	+
F5	-	+	+	-

+ = presence **-** = absence, MF: Methanolic fraction, F1, F2, F3, F4, F4, F5: Fractions