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Research Article

Chelation Activity, Hydroxyl Radicals and Hydrogen Peroxide Scavenging and Anti-Inflammmatory Properties of Algerian *Xanthium Strumarium* Leaf Extracts

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ABSTRACT

Xanthium strumarium L., commonly known as cocklebur, is a species of annual plant belonging to the Compositae family. This plant is prescribed to treat bacterial infections, urticaria, arthritis, chronic bronchitis, rhinitis, allergic rhinitis, edema, lumbago, and other ailments. The aim of this study is to evaluate the antioxidant properties of leaf extracts from *Xanthium strumarium* and the anti-inflammatory effect of its crude extract. In the present study, four extracts were prepared : crude extract (CrE), chloroformic extract (ChE), ethyl acetate extract (EAE) and aqueous extract (AqE). Their antioxidant activity was evaluated *in vitro* by the use of chelation of ions and trapping hydroxyl radicals and hydrogen peroxide tests. Anti-inflammatory activity was tested by the induction of ear edema using two different molecules; xylene and croton oil. Carrageenan was used to induce paw edema in rats. Doses administrated in this test were 200, 400 and 600 mg / Kg. Results showed that, in the case of antioxidant activity, CrE showed high efficiency in the iron chelation test (IC_{50} = 0.47 ± 0.008 mg / ml). In the test of scavenging hydroxyl radicals, EAE displayed the most potent antioxidant capacity (IC_{50} = 0.240 ± 0.0158 mg / ml). Whereas in the test of trapping hydrogen peroxide CrE extract was the more efficient (IC_{50} = 0.114 ± 0.001 mg / ml). Treatment with the crud extract of *Xanthium strumarium* shows significant inhibition of the ear edema induced by xylene, this effect is comparable to that of aspirin. In addition, significant inhibition of ear edema induced by croton oil was observed in mice with values significantly greater than that of indometacin. In the case of carrageenan-induced paw-edema in the rat, administered doses of *Xanthium strumarium* generally reduced edema. These results show that *Xanthium strumarium* possess antioxidant properties, and high anti-inflammatory activity, which explains its use in traditional medicine.

INTRODUCTION

Traditional medicine based on medicinal plants has long been used as an alternative therapy [1, 2]. The genus *Xanthium* (Family Asteraceae) is represented by 25 species in the world. *Xanthium strumarium* L. has traditionally been used as an herbal medicine in China, Indo-China, Malaysia, America, and Europe [1]. The whole plant is used to cure nasal sinusitis, headache, urticaria, and arthritis [3], and has been shown to possess curative effects against chronic bronchitis, rhinitis, allergic rhinitis, lumbago, constipation, diarrhea and vomiting [1, 4].

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The most important chemical constituents of *X. strumarium* include phenolic compounds as thiazolidinediones, chlorogenic acids, ferulic acids [5], 1,3,5-tri-*O*-caffeoyl quinic acid, 1,5-di-*O*caffeoyl quinic acid, caffeic acid [6], as well as isoprenoids such as strumasterol, β-sitosterol [7], monoterpene and sesquiterpene hydrocarbons [8], triterpenoid saponins and xanthanolide sesquiterpene lactones [9].

Phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins, are present in different parts of the plant (roots, leaves, branches / stems, barks, flowers and fruits). Phenolic compounds are powerful antioxidants due to their hydroxyl groups that donate hydrogen to free radicals [10-

[12], their ability to chelate metals, quenching of singlet oxygen [13, 14] and the stabilization of lipid peroxidation [15]. In addition, polyphenols were previously reported to have anti-inflammatory effects by modulating the enzymatic inflammatory cascade [16, 17].

According to the bibliography, fruits of *Xanthium strumarium* L. known as *Xanthii Fructus* were the part of plant the most used in the traditional medicine and studied [18]. In the present study, the *in vitro* antioxidant activities of different fractions, prepared from *Xanthium strumarium* leaves methanolic extract and *in vivo* anti-inflammatory activities were examined.

MATERIALS AND METHODS

Plant Material

Leaves of *Xanthium strumarium* (XS) were collected from Beni Aziz (Setif) Algeria. The plant was autentified by Pr. Oudjhih Bachir, Elhadj Lakhdar University, Batna, Algeria, with the reference number 164/ISVSA/DA/UHLB1/13.

Animals

Male Nmris mice weighing 25-30 g each and female wistar rats weighing 170 to 210 g each were purchased from the Pasteur Institute of Algeria. They were kept with free access to food and water and on a 12 h light/dark cycle. This study complied with current ethical regulations on animal research of our university and All procedures were performed in compliance with laws and institutional guidelines. The animals were housed for a period of at least seven days for acclimatization before the experiments.

Methodology

Extraction and Fractionation Procedure

Leaves of *Xanthium strumarium* were air-dried in the dark, then powdered. Extraction was carried out three times with Methanol 98 % at room temperature with a ratio of 1:10 W/V. The macerates were evaporated until dryness under reduced pressure on a rotavapor at 40ºC. Fractionation of the extract was effectuated using liquid-liquid extraction [19]. Crude extracts were successively extracted with different solvents of increasing polarity: hexane, chloroform and ethyl acetate. The obtained organic layer of each partition was evaporated under reduced pressure on a rotavapor at 40ºC to dryness. Determination of total phenols and flavonoids contents in extracts was applied according to Aouachria *et al* [20]. The results of the extraction yields and the content of polyphenols and flavonoids are cited in Guemmaz *et al* [19].

Antioxidant Activities

Ferrous-Ion Chelating Activity

The ability of the extracts to chelate irons is measured by following the inhibition of the formation of Fe (II)-Ferrozine, after incubation of the extracts with divalent iron according to the method of Belkhiri *et al* [21]. Briefly, extract samples at different concentrations were added to a solution of 0.6 mM $FeCl_2$ (100 µl). The reaction was initiated by the addition of 100 µl of ferrozine (5 mM) and the mixture was shaken vigorously and left for 10 minutes at room temperature. This allows for the formation of a red compound, Fe (II)-Ferrozine, that absorbs at 562 nm. Ethylene diamine tetraacetic (EDTA) was used as a positive contrôle. The Fe^{2+} chelating activity of the extracts was calculated as :

$$\% \text{ Chelation} = [(A_C - A_E) / A_C] \times 100$$

A_C : The absorption of the test that contains all the solutions except the extract

A_E : Absorption in the presence of the extract

Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity of the extracts was estimated by the method of Sudha *et al* [22]. The reaction mixture contained 1 ml of $FeSO_4$ (1.5 mM), 0.7 ml of H_2O_2 (6 mM), 0.3 ml of sodium salicylate (20 mM) and varied concentration of the different extracts. These mixtures were incubated for 1 hour at 37 °C. The absorbance of the hydroxylated salicylate complex was mesured at 562 nm. 2, 6 di-tert-butyl-4-methyl phenol (BHT) was used as a positif control. The percentage of hydroxyl radicals scavenging activity was calculated by the following equation:

$$I\ \% = [1-(A_1-A_2) / A_0] \times 100$$

A_0 is absorbance of the control without extract

A_1 is absorbance in the presence of the extract

A_2 is absorbance without sodium salicylate

Hydrogen Peroxide Scavenging Activity

The ability of extract to quench hydrogen peroxide (H_2O_2) was determined as Bhatia and Paliwal [23]. To different concentrations of each extract (1 ml) was mixed 2.4 ml of phosphate buffer solution (0.1 M, pH 7.4) and then mixed with 0.6 ml of 120 mM solution of H_2O_2. 2, 6 di-tert-butyl-4-methyl phenol (BHT) was used as a positif control. Absorbance of H_2O_2 was

determined 10 minutes later in a spectrophotometer at 230 nm. The ability of extracts to scavenge the hydrogen peroxide was calculated according to the following equation:

$$I\ \% = [(A_C - A_E) / A_C] \times 100$$

A_C is absorbance of H_2O_2 solution (120 mM).
A_E is absorbance in the presence of the extract

Anti-Inflammatory Activities
Xylene-Induced Ear Edema in Mice
Xylene-induced ear edema test was performed as described by Kou *et al* [24]. Animals were treated orally with the extract (200, 400 and 600 mg/kg), aspirine (100 mg/kg) and distilled water. One hour later, each animal received 30 µl of xylene on the anterior surface of the right ear lobe. The left ear was considered as control. Two hours later, animals were sacrificed by cervical dislocation and both ears were removed. The thikeness of circular sections with a diameter of 6 mm were measured. The edema was expressed as an increase in the ear thickness due to xylene application.

Croton oil -Induced Ear Edema in Mice
Croton-induced ear edema test was performed as described by Manga *et al* [25]. Animals were treated orally with the extract (200, 400 and 600 mg/kg), indomethacin (50 mg/kg) and distilled water. One hour later, cutaneous inflammation was induced on the inner surface of the right ear by application of 15 µl of acetone–water (1:1) solutions containing 80 µg of croton oil as irritant. On the left ear, 15 µl of acetone–water (1:1) solutions were applied. After 6 h, the mice were sacrificed and a disc (5 mm in diameter) was cut from both ears. Inflammation was measured as edema formation and quantified by the thickeness difference between the right and the left ear. The anti-inflammatory activity was expressed as a percentage of the edema reduction in treated mice compared to the control mice.

Carrageenan- Induced Paw Edema in Rats
This test is based on the method described by Bouriche *et al* [26], female wistar albino rats were used they were divided into six groups of five animals each. The test groups received orally 200, 400 and 600 mg/kg of the extract and 50 mg/kg of indomethacin, while the control group received distilled water. After 1 h, 0.1 ml (1% w/v) carrageenan suspension in physiological solution was injected into the subplantar tissue of the right rat paw. The paw thickness was measured at hourly interval for 6 h using a digital caliper

Statistical Analysis
All values obtained are expressed as mean ± SD. The results were statistically analyzed by ANOVA one-way test followed by Tukey test. Graphpad Prism v.5.0 was used in curves and statistical analyzes. The values of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ are statistically significant compared to the control groups.

RESULTS AND DISCUSSION
Antioxidant Activities
Ferrous-Ion Chelating Activity
The obtained data revealed that the ferrous ion chelating effect of fractions and EDTA was concentration dependent. From the IC_{50} (Fig.1), the potency of ferrous iron chelating ability of all fractions was very lower than that of EDTA (IC_{50} = 0.0061 ± 0.00 µg/ml). The results show that the crud extract possess the highest chelation potential with an IC_{50} = 0.47 ± 0.008 mg / ml, Whereas, ChE had the weakest ability to chelate with an IC_{50} = 2.9 ± 0.07 mg / ml.

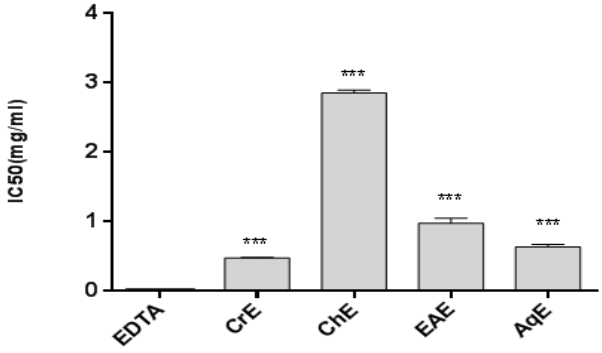

Figure 1: The IC_{50} values of ferrous iron chelating activity of CrE, fractions and EDTA. Values were expressed as mean ± SD (n = 3). CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract and EDTA : ethylene diamine tetra acetic acid ($p < 0.001$).

An important mechanism of antioxidant activity is the ability to chelate / deactivate the transition metals, which catalyze the Fenton reaction [21]. Therefore, it was considered of importance to screen the iron (II) chelating ability of extracts. In the presence of chelating agents, ferrozine-Fe2+ ion complex is disturbed, resulting in a decrease in color of the complex. Measurement of the color reduction allowed for estimate the

metal chelating activity for the coexisting chelator [27].

CrE, EAE and AqE exhibited a chelating activity (Fig. 1). This result indicates that the constituents of these extracts are capable of inhibiting the formation of ferrous complexes with the reagent ferrozine, suggesting the chelating activity of these extract and capture of the ferrous ions before ferrozine.

Polyphenols are characterized by their chelation of minerals [28]. According to this study, there appears to be no correlation between the extracts content of polyphenols and flavonoids, results are shown in Guemmaz *et al* [19], and the ability to chelate, these results correspond to those mentioned by Bouaziz *et al* [29]. On the other hand, the polarity of the extracts appears to be related to their iron chelating activity; The more polar extracts were characterized by higher efficacy than weak polarity. These results are consistent with those reported by Bouaziz *et al* [30], as the extracts stimulate chelating effect is directly proportional to the polarity of their solvents.

Hydroxyl Radical Scavenging Assay

All the tested extracts exhibited Hydroxyl radical scavenging activity, but with varying efficiencies. EAE extract possessed the highest scavenging effect with IC_{50} = 0.240 ± 0.0158 mg / ml. Whereas, the extracts CrE and AqE were characterized by the weakest scavenging ability with close values of IC_{50} = 0.42 ± 0.08 mg / ml and IC_{50} = 0.447 ± 0.032 mg / ml, respectively (Fig. 2). The EAE extract the richest in polyphenols and flavonoids showed the highest scavenging ability.

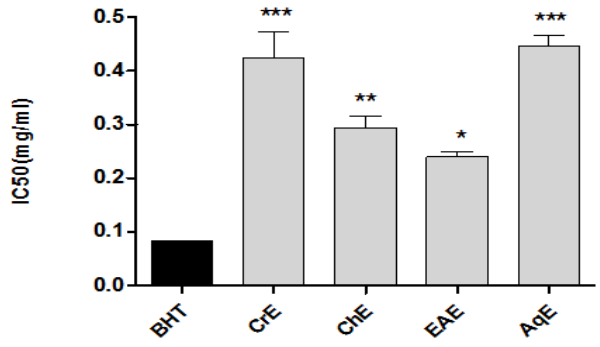

Figure 2: The IC_{50} values of hydroxyl radicals scavenging of CrE, fractions and BHT. Values were expressed as mean ± SD (n = 3). CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract. (p < 0.001).

The superoxide anion and hydrogen peroxide can be removed by superoxide dimutases and catalases / peroxidases, respectively. However, no enzyme allows the elimination of hydroxyl radicals [31]. On the other hand, the hydroxyl radicals are the most active and reactive free radicals, and they cause severe damage to neighboring molecules such as DNA, lipids and proteins [29]. As a result, trapping of hydroxyl radicals is necessary to protect cells from damage.

The powerful trapping effect of EAE extract is promising for the use of *Xanthium strumarium* as a source of natural antioxidant in oxidative stress caused by the hydroxyl radical.

Hydrogen Peroxide Scavenging Assay

A significant decrease in the amount of Hydrogen peroxide was observed due to the scavenging ability of the fractions and BHT. CrE, ChE, and EAE extracts exhibited a similar scavenging values of IC_{50} = 0.114 ± 0.001 mg / ml, IC_{50} = 0.113 ± 0.002 mg / ml, and IC_{50} = 0.105 ± 0.002 mg / ml, respectively. They were more efficience than BHT (p < 0.001).

Whereas, the AqE extract was characterized by the weakest scavenging capacity of IC_{50} = 0.307 ± 0.01 mg / ml) (Fig. 3). Otherwise, all fractions and BHT scavenge Hydrogen peroxide in dose dependent manner.

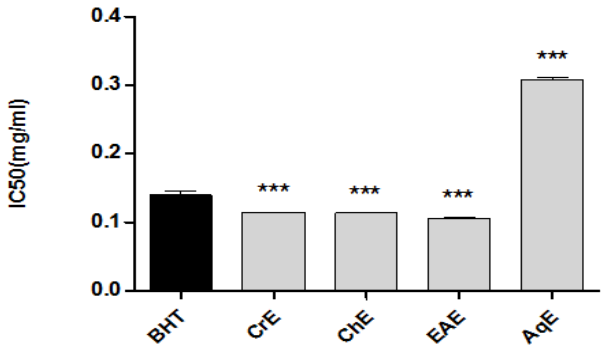

Figure 3: The IC_{50} values of hydroxyl radicals scavenging of CrE, fractions and BHT. Values were expressed as mean ± SD (n = 3). CrE : crude extract, ChE : chloroform extract, EAE : ethyle acetate extract, AqE : aqueous extract. (p < 0.001).

Hydrogen peroxide is not itself highly reactive, but it may be toxic to the cell. Its high ability to cross cellular membranes allows the formation of hydroxyl radicals when Hydrogen peroxide interacts with Fe^{2+} or superoxide anions in cells

[32]. Thus, Hydrogen peroxide scavenging is very important as an anti-oxidant mechanism in cells [33]. The ability of an extract to trap hydrogen peroxide can be attributed to the structure of its active compound which determine its ability to give it an electron and therefore to neutralize its effect by converting it into water molecules [34].

There seems to be no correlation between the ability to trap hydrogen peroxide and the content of polyphenols and flavonoids. In terms of polarity, the most polar AqE extract has the lowest scavenging capacity.

Anti-Inflammatory Tests

Induction of Ear Edema with Xylene

Results obtained from xylène-induced ear edema are shown in Fig. 4. The crud extract of *Xanthium strumarium* reduced the edema with a percentage of 74.54 ± 0.002 % in the case of the concentration 600 mg / kg. On the hand, aspirine used as standard anti-inflammatory drug, inhibits the edema with a percentage of 89.10 ± 3.15 % ; statistically, difference between the dose 600 mg / kg and aspirine is not significant ($p < 0.05$). The effect of *Xanthium strumarium* was dose dependent.

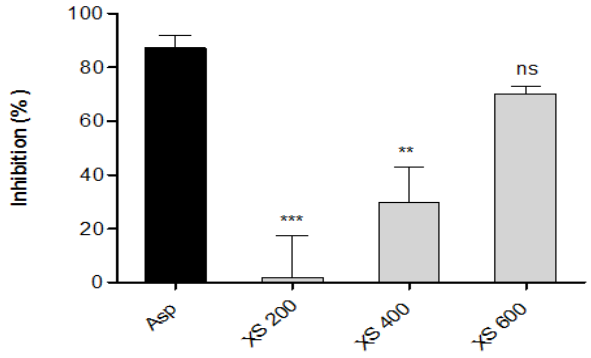

Figure 4 : Anti-inflammatory activity of *Xanthium strumarium* in ear oedema induced by xylène. Asp : aspirine, which was used as reference drug. Values were expressed as mean ± SEM (n = 6) $p < 0.05$.

Xylene-induced edema in mice is a model of acute inflammation, as xylene causes vasodilation and skin level changes [35]. The xylène- induced mouse ear edema method has certain advantages for natural product testing and good predective values for screening of anti-inflammatory agents [36, 37]. Xylene-induced ear edema causes severe vasodilation and increases vascular permeability associated with substance P and the CGR (Calcitonin Gene Related Peptide); This allows fluid accumulation and edema [38]. Substance P, as a neurotransmitter, spreads all over the CNS. Furthermore, substance P generates neurogenic inflammation when peripherally stimulated. Xylene-induced swelling is followed by innate immunity response of the skin, a cytotoxicity reaction of activated T cells and then migration of polymorphonuclear leukocytes which augment swelling and thickness of the ear [39]. The effect of the extract may be due to the inhibition of the secretion of substance P or to its inhibition.

Induction of Ear Edema with Croton Oil

In this study, the thickness of the right ear and the thickness of the left ear were measured 6 hours after topical application of croton oil, this allowed an evaluation of the anti-edema extract effect (Fig. 5). This extract reduced the rate of edema with a rate of 50.66 ± 11.32% in the case of a concentration of 400 mg / kg. This ratio is close to that observed with indomethacin (48.00 ± 4.29 %) used as reference drug.

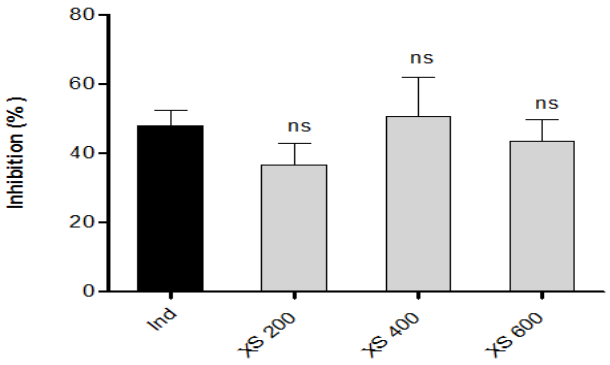

Figure 5 : Anti-inflammatory activity of *Xanthium strumarium extract* in ear oedema induced by croton oil. Ind : indomethacin, which was used as a standard anti-inflammatory agent. Values were expressed as mean ± SEM (n = 6) $p < 0.05$.

Croton oil is an inflammatory agent extracted from the fruits of *Croton tiglium* L. This skin irritating oil contains phorbol esters, the most important of which is 12-O-tetradecanoylphorbol-13-acetate (TPA). The latter induces an acute inflammatory reaction characterized by dilatation of blood vessels, infiltration or penetration of polymorphonuclear leukocytes and an increase in interleukin 1ß, and thus edema forms. On the other hand, This oil increases the activity of the enzyme xanthine oxidase and the oxidation of lipids [40, 41].

These changes are caused by protein kinase C (PKC) which activates phospholipase A2 (PLA2).

[42]. PLA2 hydrolyzes membrane phospholipids to arachidonic acid, the latter interfering with the synthesis of eicosanoids; prostaglandins and leukotrienes, this reaction represents the first step in the inflammatory reaction [43]. On the other hand, PKC stimulates the secretion and activation of several immune media such as cytokines and chemokines that raise the inflammatory response at the skin [44].

Indomethacin, used as standard anti-inflammatory drug, inhibits the two forms of cyclooxygenases 1 and 2, the formation of exudate and the production of the pro-inflammatory mediators such as TNF α and IL-6. [45]. The activity observed with the studied extracts is probably due to the presence of active substances such as flavonoids and polyphenols which are likely candidates for this effect [46, 47].

The effect of the extract may be due to : 1) their strong antioxidant activity, since the treatment of the skin with PKC-inducing substances stimulates the formation of free radicals. 2) It can directly suppress or decrease PKC activity and thus stop the chain of inflammatory reactions and the formation of free radicals [48].

Induction of Paw Edema with Carrageenan

In this study, the thickness of the rat's paw was measured within 6 hours after the subplantar injection of carrageenan, this allowed an evaluation of the anti-edema extract effect (Fig. 6) and (Fig. 7).

The CrE extract reduced the thickness of the edema compared to the control group during the test. However, the reduction in the thickness of the edema continued until five hours, then the thickness increased to the same level as the control group.

On the other hand, the CrE reduced the edema (14.95- 31.47 %) during the first stage (1- 2 h), this may suggest that this extract may inhibit the effect of histamine and serotonin. As for the second stage (2-4 h), the inhibition rate reached its peak 26.14 ± 9.44% at 3 hours. This may indicate the ability of the extract to inhibit prostaglandins, bradykinin and leukotrienes.

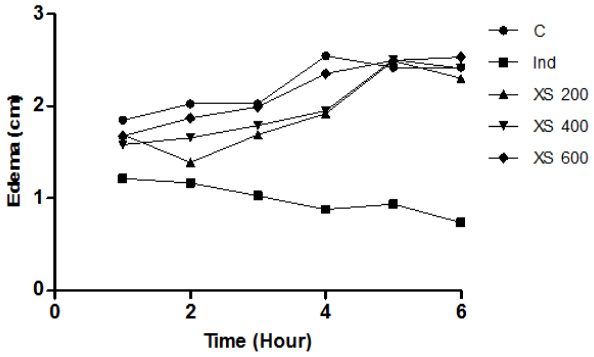

Figure 6 : Effect of *Xanthium strumarium extract* on carrageenan-induced paw edema in rat. C : Control group received only sterile saline solution. Ind : indomethacin, which was used as referebce drug. Each value represents the increase in thickness of the injected paw at different times after injection of carrageenan. Values are means ± SEM (*n* = 5).

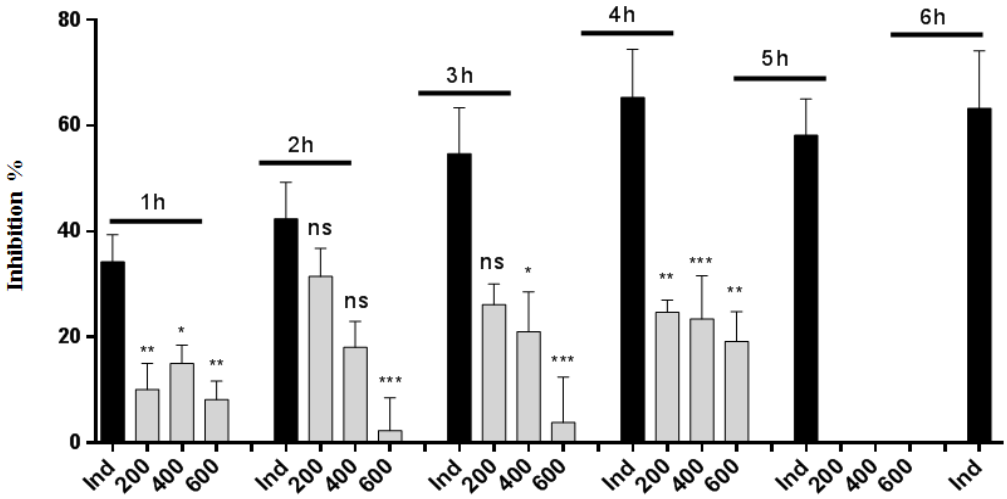

Figure 7 : Anti-inflammatory activity of *Xanthium strumarium extract* in paw oedema induced by carrageenan. Ind : indomethacin, which was used as a standard anti-inflammatory agent. Values were expressed as mean ± SEM (n = 5) $p < 0.05$.

Indomethacin, used as reference anti-inflammatory, inhibits the edema (34.20- 42.35 %) during the two first hour of the test, At the 4th hour, the inhibition reaches the value of 65.36%, which remains almost stable until the 6th hour.

Carrageenan-induced edema is an experimental model of acute inflammation in rats. Edema is measured within 6 hours, it is believed that the inflammatory reaction caused by carrageenan goes through two stages; In the first stage (1-2 h), histamine and serotonin are excreted, while during the second stage (2-6 h) prostaglandins and bradykinin and leukotrienes are secreted, the latter two are produced by macrophages [49].

The anti-inflammatory activity of polyphenols and flavonoids has been reported in several studies. It has been reported that flavon glycosides and flavonols, as well as non-glycated flavonoids, have anti-inflammatory potential in acute or chronic inflammation when given orally or topically applied. Among these flavonoids, apigenin, luteolin, quercetin, kaempferol, myricetin and fisetin. They influence inflammation by inhibiting arachidonic acid metabolism or by inhibiting the activity of cyclo-oxygenase and lypoxygenase enzymes [50, 26].

However, the variation in anti-inflammatory activities of the crud extract, in regards to doses, can be explained by the gastrointestinal metabolism of the bioactive elements and the low bioavailability of the latter. Polyphenols are present in plants as aglycones, glycosides, esters or polymers. Aglycones can be absorbed from the small intestine, while glycosides, esters and polymers must be hydrolyzed by intestinal enzymes, or by the colonic microflora, before they can be absorbed [51]. This may explain the lack or weakness of the effect caused by oral administration of extracts.

CONCLUSION

The majority of research on *Xanthium strumarium* is based mainly on the fruits of this plant, the present study focused on the antioxidant activities of extracts from the leaves of *Xanthium strumarium* and its anti-inflammatory effects. The iron chelation test has shown that the crude extract is the most effective, in the case of scavenging tests of hydroxyl radicals and hydrogen peroxide, the results have shown that the acetate extract ethyl and crude extract were the most effective respectively ; generally they have an efficiency close to that of BHT.

The present study reveals that the crude extract of Xanthium strumarium has potent antiinflammatory activity on acute inflammation induced by xylene, croton oil and Carrageenan.
To better understand the mechanism of action of these extracts as antioxidants and anti-inflammatory agents, it is necessary to determine the composition of the different extracts to understand the structure-activity relationship.

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