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Anti-inflammatory and antioxidant effect of *Ceratonia siliqua L.* methanol barks extract

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ABSTRACT

Ceratonia siliqua L. has been used in Moroccan traditional medicine as antidiarrheal and diuretic. The infusion of carob leaves is used as an emetic for acute poisoning. The present investigation was designed to determine the anti-inflammatory potential of methanolic barks extract of Ceratonia siliqua in rodents using female Swiss mice (20-30 g) and Wistar male rats (150-250 g). The acute toxicity studies were carried out based on OECD 423 guidelines. The LD_{50} of Ceratonia siliqua was found to be more than 5 g/kg and did not produce mortality or changes in general behavior of the tested animals. Methanolic barks extract of Ceratonia siliqua was investigated for anti-inflammatory properties by using carrageenan and experimental trauma-induced hind paw oedema in rodents at the dose of 50, 100, 200 mg/kg p.o. respectively, Indomethacin at 10 and 20 mg/kg was used as standard. From the results obtained, the methanolic barks extract of Ceratonia siliqua showed significant reduction ($P \leq 0.001$) and inhibition of oedema comparable to the control and reference drug used in both models. This study showed the justification of the use of Ceratonia siliqua in the treatment of inflammatory disease conditions in Morocco.

Key words: *Ceratonia siliqua* L., methanolic extract, polyphenols, antioxidant activity, anti-inflammatory activity.

INTRODUCTION

Inflammation is a complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells or irritants (physical or chemical). It is a defense mechanism aimed to remove the injurious stimuli and initiate the tissue healing process [1, 2]. Inflammation is characterized by five cardinal clinical signs, namely redness, swelling, pain, heat, and loss of function [3]. Inflammation can be acute and chronic. Many degenerative diseases such as rheumatoid arthritis, shoulder tendonitis, gouty arthritis, polymyalgia rheumatica, heart disease, asthma, cancer, and inflammatory bowel disease are often associated with inflammatory processes [4-6]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the commonly prescribed medications, with an estimated 70 million number of prescriptions annually written and 6.8 billion $ spent worldwide in the attainment of this drug class [7, 8]. Thus employment of anti-inflammatory agents may be helpful in the therapeutic treatment of those pathologies associated with inflammatory reaction. However, the side effects of currently available anti-inflammatory drugs pose a major problem in their clinical use. For instance, some non steroidal anti-inflammatory drugs (NSAIDs) my cause gastric ulceration and renal damage [9, 10], and with adverse effects at the level of digestive tract, ranging from dyspeptic symptoms, gastrointestinal erosions and peptic ulcers to more serious complications, such as over bleeding or perforation [11].

Therefore to overcome the toxicity of NSAID, the development of new anti-inflammatory drugs is still necessary and the natural product such as medicinal plants could potentially serve as leads in the production of new drugs for treating inflammation with reduced or no side-effects.

The carob tree (*Ceratonia siliqua* L.), also called algarroba, is an evergreen tree which grows throughout the Mediterranean area, mainly in Morocco, Spain, Italy and Portugal [12]. The species belongs to the Cesalpinaceae sub-family of the family Leguminoseae (syn Fabaceae) [13]. It's been reported that the genus of Ceratonia, consists of three species, Carob trees may be thus male, female, and hermaphrodite [14-16]. Carob has been cultivated for thousands of years as a forage crop or food for human consumption [17]. Recently, this species has attracted much attention and became economically important. Pods and seeds are used as row material in food, pharmaceutical and cosmetic industries [18]. Everywhere in Morocco, pods are used to fight diarrhea in infants, children and adults [19]. Infusion of carob leaves is used as an emetic for acute poisoning [20]. In Turkish folk medicine, leaves and barks of carob tree are used as an antidiarrheal and diuretic [21, 22]. The fruits of this plant are traditionally used as an antitussive and against warts [23, 24].

To provide scientific evidence for its use in the folk medicine for some anti-inflammatory diseases, the main purpose of the present study was to investigate the effect of methanolic barks extract of spontaneous male of *Ceratonia siliqua* on experimental models of acute inflammation (Carrageenan-induced oedema of the hind paw, and physical trauma induced inflammation), in an attempt to validate its ethno-medical use. First, *Ceratonia siliqua* methanol barks extract was subjected to preliminary phytochemical screening test for various constituents. Total phenolic contents and the antioxidant activity of the same extract were also carried out using spectrophotometric methods. To the best of our knowledge, there are no such pharmacological studies concerning anti-inflammation activity for this plant.

EXPERIMENTAL SECTION

This work was conducted on unproductive Dkar (spontaneous male) of carob barks. Plant materials were collected in 2011 from the province of Chefchaouen (NW of Morocco) and identified by Professor A. Ennabili. Authenticated voucher specimens N° INP212 was deposited in the Herbarium of National Institute of Medicinal and Aromatic Plants, Sidi Mohamed Ben Abdellah University, Fez, Morocco.

Reagents and chemicals

The Carrageenan and the Triton X were purchased from Fluka Biochemika, sodium chloride was purchased from Solvachim Laboratory (ref 1480), and Indomethacin was purchased from (Pharma 5 Pharmaceutical Laboratories (Lot 5496)).

Preparation of the extracts

Plant was air dried at room temperature, with no direct sunlight, and then pulverized using an electrical grinder. 260 g dried coarse powder of the bark was placed into the extractor of a Soxhlet. The extraction was carried out by using solvents of increasing polarity starting from hexane, dichloromethane, ethyl acetate and methanol. At the end of the extraction, the respective solvents were concentrated by evaporation to dryness under reduced pressure. Final methanolic extract of *Ceratonia siliqua* was a dark brown semi-solid in percentage dray weight 15%. For assuring stability, the methanolic extracts were stored at 4°C until use.

Phytochemical screening

The methanol extract was screened for phytochemical constituents (Alkaloids, Flavonoids, Saponins and Tannins) using simple qualitative methods of Paris and Nothis [25]. Sterols and Terpenes, Coumarins, Quinones, Cardiac glycosides, and Mucilages were screened using the protocol as described in the study of Diallo [26].

Total phenolic contents

The total phenolic contents were determined spectrophotometrically using the Folin-Ciocalteu method. This reagent, based on the Slinkard and Singleton method [27], and the early work of Singleton & Rossi [28], is a colorimetric oxidation/reduction method for phenolic compounds.

Determination of free radical scavenging activity by DPPH method

Free radical scavenging activity of the sample extracts was determined spectrophotometrically using the method of Blois [29]. This method is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. Briefly, 100 µL of various concentrations of the extract in methanol were added to 10 mL of a methanol solution of DPPH (1.01×10^{-4} M). The mixture was vigorously shaken and then allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 517 nm by using a double-beam UV-visible

Camspec M550 spectrophotometer. A mixture of 100 µL of methanol and 10 mL of DPPH solution was used as the control. The scavenging activity on the DPPH radical was expressed as inhibition percentage using the following equation:

$$\% \text{ Inhibition} = [(A_B - A_S)/A_B] \times 100 \qquad [29]$$

where A_B is the absorbance of the control reaction (containing all reagents except the test compound), and A_S is the absorbance of the test compound. Butylatedhydroxytoluene (BHT) was used as positive control. The tests were carried out in triplicate. The extract concentration providing 50% inhibition IC_{50} was calculated from the graph of inhibition percentage plotted against extract concentration (4.0, 2.0, 1.0, 0.5 and 0.25 mg/L).

Animals

Female Swiss mice (20-30 g) (Offa-credo, France) were used in the acute toxicity, and Wistar male rats (150-250 g) in the pharmacological tests (anti-inflammatory activities). The animals were obtained from the Animal Experimental Centre of Mohammed V University, Faculty of Medicine and Pharmacy, Rabat. All animals were kept in a room maintained under environmentally controlled conditions of (temperature 23 ± 1°C) and relative humidity (50 ± 5%), with dark and light cycles 12 hours. All animals had free access to water and standard diet *ad libitum*. They were acclimatized at least one week before the experiments were started. The animals submitted to oral administration of the extracts or drugs were fasted for 18h before the experiment (water was available). All procedures used in the present study followed the "Principles of Laboratory Animal Care".

Acute toxicity

According to organization for Economic Co-operation and Development (OECD) guidelines No 423 [30], acute toxicity study was carried out to determine the LD_{50} value of methanolic extract of *Ceratonia siliqua*. Four different groups of female mice (n = 12, 3 in each group). The animals were kept fasting for overnight providing only water, after which the methanolic extract of *Ceratonia siliqua* was administered orally at the single oral dose at different concentrations (2000, 5000 mg/kg, p.o.). The control group received only the distilled water. The animals were placed in individual clear plastic cages and all were observed for possible mortality cases (24h) and any behavioral changes such as increased-decreased motor activity, ataxia, tremors, convulsions, sedation and lacrimation, or any instant death, followed by daily weight monitoring for 14 days. The aim of performing the acute toxicity study is to establish the therapeutic index of a particular drug and to ensure its safety *in vivo*.

Anti-inflammatory activity

The evaluation of the anti-inflammatory activity of methanolic bark extract of *Ceratonia siliqua*, was carried out by using two different methods that used chemical stimuli (Winter Test) and mechanical stimuli (Riesterer and Jacques Test) induced paw oedema in rats.

Carrageenan induced rat paw oedema

The anti-inflammatory activity was assessed on the basis of inhibition oedema induced by the injection of carrageenan an oedematogenic agent into the sub plantar region of the left hind paw of the rat [31]. For the experiment, the male Wistar rat (150-250 g) were divided into four different groups (n = 6). The animals were fasted overnight (18h) prior to the start of the experiment, providing only water *ad libitum*. The control group received (5 mL/kg of distilled water p.o.). The standard group received the reference drug (indomethacin 10 mg/kg, p.o.), and the testing groups received different concentration of methanolic extract of *Ceratonia siliqua* (50, 100, 200 mg/kg p.o., respectively). One hour after oral administration of different substances, fresh carrageenan suspension (0.05 mL of 1%) was injected to all animals in the left hind paw. The right hind paw is not treated; it is taken as a witness. The difference in the foot pad volume between the left and right foot was taken as the inflammation oedema induced by carrageenan. The value of this oedema was measured by using Plethysmometer Digitals 7500, at 1h30, 3h and 6h after induction of inflammation [32, 33]. Mean differences of treated groups were compared with the mean differences of the control group. The percentages of inhibition of inflammation were calculated according to the following formula:

$$\% \text{ of inhibition} = [v_{Left} - v_{Right}]_{Control} - [v_{Left} - v_{Right}]_{Treated} \ / \ [v_{Left} - v_{Right}]_{Control} \times 100$$

(v_{Left} means volume of oedema on the left hind paw and v_{Right} means volume of oedema on the right hind paw).

Experimental trauma in rat induced paw oedema

The anti-inflammatory activity was evaluated according to the method described by the Riesterer and Jacques test 1970 [34]. For the experiment the male Wistar rats (150-250 g) were divided into four different groups, 6 in each group (n = 6). The animals were fasted overnight (18h) prior to the start of the experiment, providing only water *ad*

libitum. The control group received (5 mL/kg of distilled water p.o.). The standard group received the reference drug (indomethacin 20 mg/kg, p.o.). The testing groups received different concentration of methanolic extract of *Ceratonia siliqua*, (50, 100, 200 mg/kg, p.o.). In this test, the substances to be tested were administered by stomach tubing 1 hour before eliciting the traumatic oedema. A weight of 50 g was made to fall onto the dorsum of the left hind-paw of all animals. The right hind paw is not treated; it is taken as a witness. The difference in the foot pad volume between the left and right foot was measured and taken as the oedema value by using a Plethysmometer Digitals 7500 at 1h30, 3h and 6h after induction of inflammation [32, 33]. Mean differences of treated groups were compared with the mean differences of the control group. The percentages of inhibition of inflammation were calculated according to the following formula:

$$\% \text{ of inhibition} = [v_{Left} - v_{Right}]_{Control} - [v_{Left} - v_{Right}]_{Treated} \ / \ [v_{Left} - v_{Right}]_{Control} \times 100$$

(v_{Left} means volume of oedema on the left hind paw and v_{Right} means volume of oedema on the right hind paw).

Statistical analysis

Data were entered into Excel (Microsoft, USA) and analyzed on PASW Statistics 18 (IBM, Chicago, IL, USA). Quantitative variables were described as mean ± standard deviation when the distribution followed the normal law of Laplace-Gauss, or as median (Interquartile range Q1-Q3) if appropriate. Qualitative variables are described as numbers and percentages. We used the Student's t-test for comparison between groups. Analysis of Variance (ANOVA) was used to compare different variables between the groups followed by Post hoc Bonferroni Test. A P value < 0.05 was considered to be statistically significant.

RESULTS

Phytochemical screening

As shown in Table 1, phytochemical screening of the crude methanolic carob tree barks revealed the presence of flavonoids, tannins, sterols, quinones, cardiac glycosides, and mucilages. But alkaloids, coumarins, terpenes and saponins were not detected. Results of the polyphenols content and DPPH scavenging assay of the methanolic extract of *Ceratonia siliqua* bark are summarized in Table 2.

Table1. Phytochemicals detected in methanolic extract of spontaneous male carob barks

Varieties	Phytochemicals	Methanolic extract
Spontaneous male	Alkaloids	-
	Flavonoids	+
	Tannins	+
	Saponins	-
	Sterols	+
	Terpenes	-
	Quinones	+
	Coumarins	-
	Mucilages	+

Key: (+) = *Present;* (-) = *Absent*

Table 2: Total polyphenolic contents and *DPPH radical scavenging ability* (IC_{50} (g/L)) value of methanol extract of spontaneous male carob barks, comparatively with BHT used as standard

Categories	Sample	Total phenolic content (g/L GAE)	DPPH IC_{50} (g/L)	BHT
Spontaneous male	MeOH	0.76	0.7	0.2

Acute Toxicity

In folk medicine of *Ceratonia siliqua* we found no scientific references about its toxicity. Therefore, in order to evaluate the safety of this plant, the methanolic extract was orally administered at different doses (2000 and 5000 mg/kg p.o.). No significant difference in body weight was noted between the control and any of the treated groups at any period time. Besides, the extract did not produce death nor symptoms associated with toxicity such as convulsion, ataxy, diarrhea or increased diuresis occurred during and no abnormal behavior in the tested animals at the tested doses during the 72h observation period. Therefore, we can conclude that the oral LD_{50} is greater than 5 g/kg in mice. These results indicate the effectiveness and relative safety of *Ceratonia siliqua*.

Inflammatory activity

Carrageenan-induced rat paw oedema

The results of the acute anti-inflammatory effect of the investigated methanol bark extract of *Ceratonia siliqua* on carrageenan-induced oedema in hind paws of rats are shown in Figures 1 and 2. Carrageenan induced paw oedema remained even 3h after its injection into the sub-plantar region of rat paw. All tested extracts of *Ceratonia siliqua* showed a significant reduction in the oedema paw volume in a dose-dependent manner ($P < 0.05$). Interestingly, the methanol extract of *Ceratonia siliqua* at the dose of (50 and 100 mg/kg p.o.) exhibited the greatest anti-inflammatory effect where it inhibited the oedema formation to the extent of 47.69 % and 51.45% respectively. All these results were compared to the control and the positive control (Indomethacin). In control group, the carrageenan increases the development of oedema of the rat-paw. The indomethacin, at a dose of (10 mg/kg, p.o.) produced a significant inhibitory effect to an extent of 71% at 3h ($P < 0.005$). In addition, the concentration 200 mg/kg, of the methanol extract of *Ceratonia siliqua* showed a maximum inhibition of the oedema formation to the extent of 68.70% at 3h and the oedema were found to be significantly reduced to 0.20 ± 0,01 ($P = 0.001$) comparatively to the indomethacin 0,19 ± 0,01 ($P \leq 0.001$) as reference drug, at the same time.

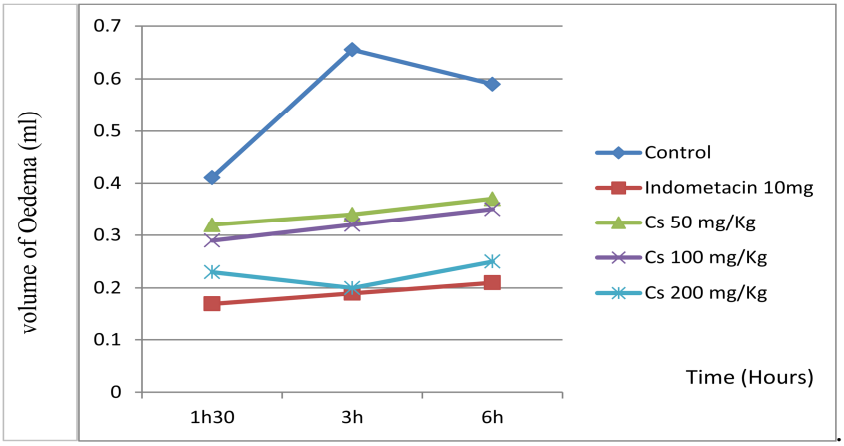

Figure 1: Anti-inflammatory effect of *Ceratonia siliqua* on carrageenan induced hind paw oedema in rats

The data represent the mean volume of oedema (paw left-paw right) ±standard error mean (Mean ±S.E.M) , Control (vehicle)-distilled water, Ceratonia siliqua methanolic extract (50, 100 and 200 mg/kg, p.o.), $P \leq 0.001$ compared with control and reference drug (indomethacin 10 mg/kg, p.o.).

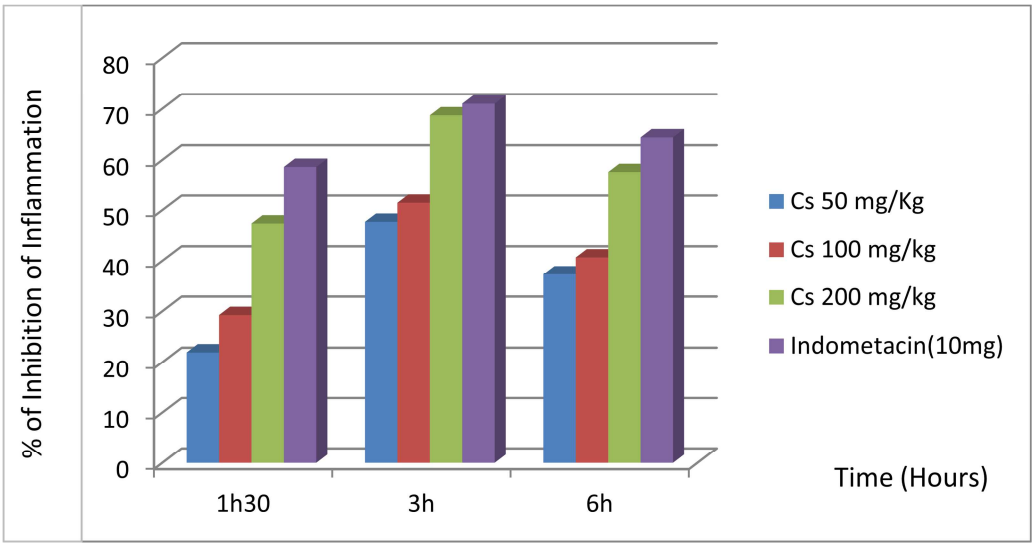

Figure 2: Percent of inhibition of inflammation of *Ceratonia siliqua* using carrageenan induced paw oedema in rats

N = 6; these results compared with standard drug (indomethacin 10 mg/kg, p.o.) were administered by the oral route.

Experimental trauma-induced rat paw oedema

In experimental trauma-induced paw oedema in rats, the methanolic extract of *Ceratonia siliqua* was evaluated in this test at concentrations of 50, 100, and 200 mg/kg p.o. *Ceratonia siliqua* extracts with the doses of 50, 100 mg/kg significantly reduced the experimental trauma induced paw oedema formation in rats in third hour after the induction by 57.15%, 60.17% in a dose-dependent manner respectively as showed in Figures 3 and 4. These results

are comparable to those of the control and standard drug indomethacin (20 mg/kg p.o.). The experimental trauma induced paw oedema remained between 3-6 h after induced paw oedema into the sub plantar region. The *Ceratonia siliqua* extract at the dose (200 mg/kg, p.o.) inhibited oedema formation to the extent of 70% at (3h) and the oedema were found to be reduced to 0.20 ± 0.01 ($P \leq 0.001$) comparable with indomethacin 0.18 ± 0.01 ($P \leq 0.001$) as a reference standard drug that inhibited the oedema formation due to experimental trauma to an extent of 74.3% at 3h ($P < 0.005$).

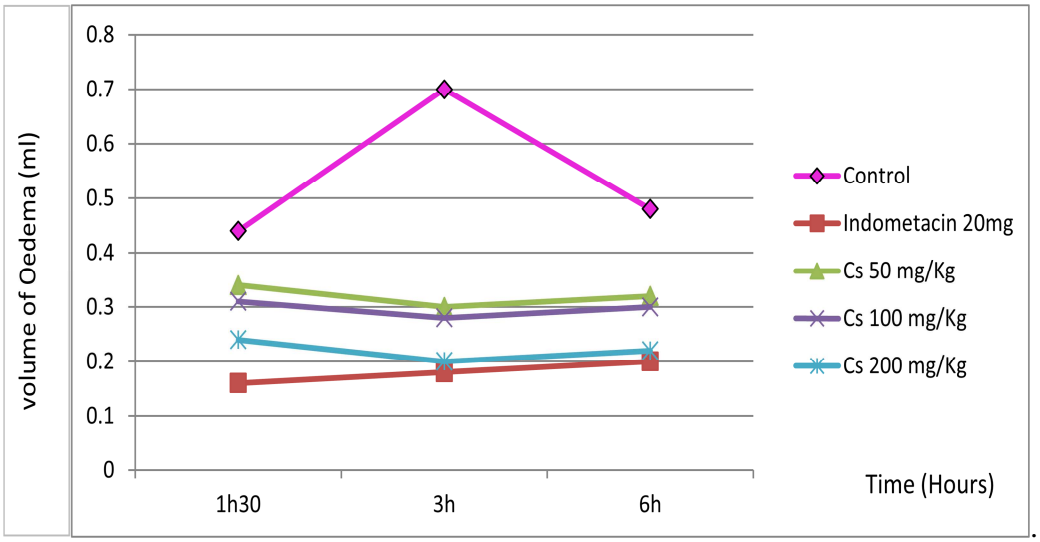

Figure 3: Anti-inflammatory effect of *Ceratonia siliqua* methanolic extract on experimental trauma induced hind paw oedema in rats
The data represent the mean volume of oedema (paw left-paw right) ±standard error mean (Mean ±S.E.M) , Control (vehicle)-distilled water, Cs: Ceratonia siliqua L, (50, 100 and 200 mg/kg, p.o.), $P \leq 0.001$ compared with control and reference drug (indomethacin 20 mg/kg, p.o.).

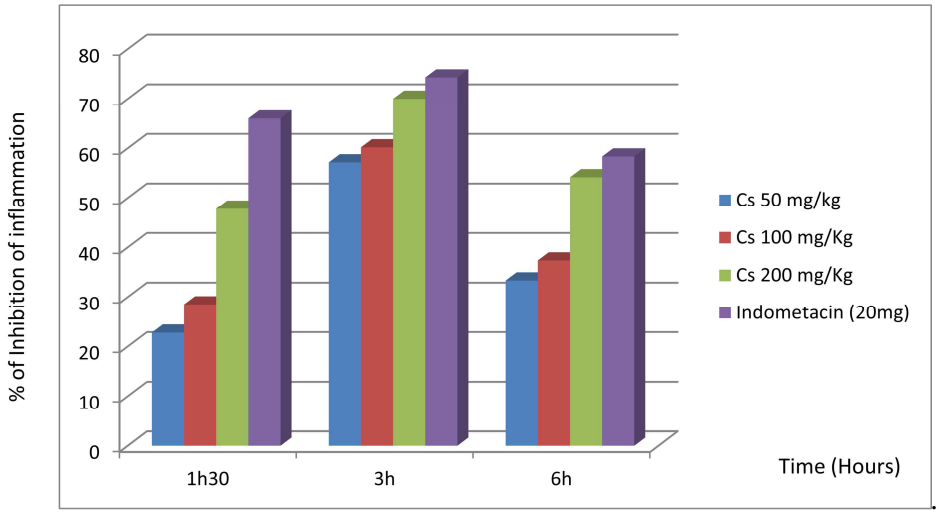

Figure 4: Percent of inhibition of inflammation of *Ceratonia siliqua* using experimental trauma induced paw oedema in rats
N = 6; these results compared standard drug (indomethacin 20 mg/kg, p.o.) were administered by the oral route.

DISCUSSION

Inflammation is a normal protective mechanism adopted by the body to get rid of offending stimuli, but if not properly treated may result to a more damage with exuberance to create chronic inflammation [35, 36]. The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair [37]. During inflammatory responses, the activation of phospholipase A2 induces the mobilization of fatty acids, in particular arachidonic acid from the membrane lipid pool [38]. Arachidonic acid is then oxidized by constitutive cyclooxygenase-1 (COX-1) or inducible cyclooxygenase-2 (COX-2) enzymes, leading to the production of prostaglandins [38, 39], precisely PGE2 and PGF2a, found in high concentration at the inflammatory site [40]. The released PGs either stimulate pain receptor or sensitized pain receptors to the action of other pain producing substances such as histamine, 5-hydroxytryptamine (5HT), and

bradykinin which initiate and cause the nerve cells to send electrical pain impulse to the brain [41]. These different reactions in the inflammatory response cascade are therapeutic targets which anti-inflammatory agents interfere with to suppress exacerbated inflammatory responses usually invoked in such disorders such as rheumatoid arthritis, infection or injury. NSAIDs are the widely anti-inflammatory drugs used with over 30 million daily users who depend on this class of medications that permits these patients to have relief from the respective ailments that are plaguing them [42]. Their ease of accessibility has created one of the most widely used drug classes for reducing mild to moderate pain [43], but with undesirable side effects like: nausea, indigestion, bleeding from the stomach, peptic ulcer, and bronchopasm [44]. The search for new drugs that effectively interfere with inflammation and painful processes is currently of great relevance. Efforts have been made to discover and develop new and promising anti-inflammatory and analgesics from natural sources. The carrageenan and experimental trauma induced inflammation model is a significant predictive test to evaluate the anti-edematous effect of natural products in acute phase of inflammation [32]. The oedema induced by carrageenan injection involves three phases of chemical mediator release in an orderly sequence [45]. The first phase (1h) involves the release of histamine and serotonin and is characterized by increase in vascular permeability. The second phase (2h) is mediated by release of bradykinin, an important chemical mediator of both pain and inflammation. Release of prostaglandins and cyclooxygenases products takes place in the third and final phase (3h) [46].

We analyzed here the effect of different doses of methanolic extract of *Ceratonia siliqua* for its anti-inflammatory activity. We observed that animals treated with *Ceratonia siliqua* methanolic extract (50, 100 mg/kg p.o.) low reduced and inhibited carrageenan oedema induced in rats. By increasing the dose of *Ceratonia siliqua* to 200 mg/kg, it's showed that *Ceratonia siliqua* reduced and inhibit significantly ($P \leq 0.001$) the oedema in the early and late phases of an acute inflammation with maximum significant effect during (3h) after induction of oedema by the carrageenan. These results were compared with control and indomethacin (10 mg/kg, p.o.) ($P \leq 0.001$). In the experimental trauma induced oedema in rats, the extract of this plant (200 mg/kg p.o.) showed a significant reduction and inhibition ($P \leq 0.001$) of oedema in the different phases of inflammatory response with maximum significant effect during 3h. This result was compared with control and the positive control indomethacin (20 mg/kg, p.o.) ($P \leq 0.001$) a NSAID which is known as a none-selective inhibitor of the enzymes cyclooxygenase [47]. Most of the NSAIDs, for example, Ibuprofen, Indomethacin, and Diclofenac, act through inhibition of enzyme COX, most precisely COX-2 responsible for the biosynthesis of PGs which triggered inflammation thereby preventing the amplification of the pain stimuli [48, 49].

The results obtained suggest that, the methanolic extract of *Ceratonia siliqua* has a significant anti-inflammatory activity and may be related with inhibition of inflammatory mediators like serotonin, histamine, kinin, cyclooxygenase, prostaglandin and cytokine. During inflammation, the leukocytes and macrophages migrating to the site of injury are known to produce the superoxide radicals (O^{2-}), which in turn mediates the generation of hydrogen peroxide [50]. Furthermore, in the presence of suitable transitional elements, hydrogen peroxide may be transformed to the highly reactive hydroxyl radicals. These radicals can also act as secondary messengers, thereby activating the production of other inflammatory mediators [51]. Many plant extracts having antioxidant properties have been shown to scavenge free radicals and thereby act as anti-inflammatory agents. Polyphenols (including phenolic compounds and flavonoids) are plant compounds that can exert significant antioxidant activity, mainly due to their redox properties [52], which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides.

The results of phytochemical screening of methanolic extract of *Ceratonia siliqua* barks showed the presence of polyphenols, flavonoids, tannins, and sterols (Table 1), and have antioxydant effect comparable to the BHT. These compounds have been shown to possess anti-inflammatory, and antioxidant effects [53], and are known to inhibit some molecular targets of pro-inflammatory mediators in inflammatory responses. They also act as antioxidants by scavenging radicals and thereby attenuate the inflammatory process [54, 55].

CONCLUSION

The therapeutic proprieties of *Ceratonia siliqua* are due to the presence of polyphenols, and sterols thus contributing to its potent anti-inflammatory activity and its antioxidant effect that inhibit the free radical release during the inflammatory response process, that would produce an additional therapeutic benefit enhancing its anti-inflammatory effect. The study corroborated the anti-inflammatory effects of this specie, and supported scientifically its ethno-pharmacological use as an anti-inflammatory agent. This result prompted us to study if the extracts of other parts of the plant were able to induce the same effect. In particular we project to use the carob leaves, thought to be a better source of these compounds and in particular of polyphenols. Further studies are necessary to evaluate the molecular basis which underwent the effect of these compounds. Our findings could represent an improvement in the biological and pharmacological characterization of the compounds present in *Ceratonia siliqua*. Moreover, given

the extraordinary ability of barks extracts to inhibit oedema produced by carrageenan and experimental-trauma, our finding could be useful for the future strategies against the anti-inflammatory activity and, therefore, inflammation progression.

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