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Phytochemical Investigation of Algerian *Ceratonia siliqua* L. Leaves Extract, Evaluation of the Antioxidants, and Analgesic Effects

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Abstract

The present study reports on the investigation of phenolic compounds and the determination of the biological activities, toxicity and analgesic effect *Ceratonia siliqua*, phenolic content was spectrophotometrically quantified with the microplate method, while the bioactivities of methanolic and aqueous extracts were assessed by determining of antioxidant activity. The methanolic extract showed high contents of total flavonoids and exhibited the highest *in vitro* antioxidant potential (DPPH, ABTS, Superoxide, FRAP, CUPRAC, and phenanthrolin). In the acute toxicity, no death was reported in a dose of 1000, and 1500mg /Kg BW, however the dose of 2000mg/kg BW showed many signs of toxicity, so the DL_{50} is higher than 2000mg/Kg BW.

In analgesic *in vivo* assay, the methanolic extract showed a very important capacity to reduce pain, whether central or peripheral, with a certain dose-dependent relationship. For the three tests (hot plate, tail flick and acetic acid assay), the effective dose was 500mg/kg and the effective duration was 30min to 1 hour.

This study demonstrate interesting *in vitro and in vivo* effects of carob leaves extracts and suggests that could be used as an alternative to chemical compounds. According to the extracts concentration and nature, it is very interesting to use like a source of natural antioxidants and analgesic compounds that might be more widely used in pharmaceutical industries.

Keywords: Antiradical activities, antalgic, Acute Toxicity, Carob extract.

Introduction

Reactive oxygen species (ROS), formed normally during the cellular processes like cellular respiration, include superoxide radical ($O^{\bullet}_2^-$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($O^{\bullet}$) and H_2O_2 [1]. Overproduction of ROS induces the oxidation of cellular components, which in turn leads to damage of the affected tissues. The human body can attenuate ROS damage by its own antioxidant defense systems which include superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and others [2]. This is why a great interest has been focused on using natural antioxidants. The use of medicinal plants is a common practice in traditional medicine in Algeria. Its wide use is justified by the richness of the Algerian flora, particularly endemic species and by the diversity of the know-how. This richness is due to country's geographical situation, climate, and soil composition [3].These plants are considered a natural reservoir of biologically active substances, which can be integrated into several kinds of drugs, also are endowed with a very important antioxidant power that is necessary to maintain the balance of ROS in the organism. They provide protection against the deleterious effects of these free radicals, the origin of various diseases such as neurodegenerative problems, inflammatory diseases, cancer and diabetes …[4,5,6].

Pain is a very common symptom of diseases; it is a natural defense phenomenon of living organism [7]. Anti-inflammatory and analgesic drugs are used to block pain, by reducing inflammation and repairing tissue damage if possible [8]. Most of these drugs has side effects leaving various unwanted effects in the body such as ulcers... [9,8]. For these reasons, it's necessary to look for bioactive

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molecules with analgesic properties which are devoid of negative effects on health.

Ceratonia siliqua L. is a large tree belonging to the leguminous family (Fabaceae) which can reach up to 20m in height. It is a common tree in countries Mediterranean, generally known as the carob tree. It occupies the upper arid to upper semi-arid thermo-Mediterranean, and demonstrates qualities of adaptation to terrain [10,11]. In folk medicine, *C.siliqua* was used both as food and for the treatment of several disorders [12,13]. Also, this tree has great economic, ecological and industrial importance, it is considered a major source of carbohydrate exploitation production of bioethanol [14]. On the other hand, its wood is used for making charcoal, the bark and roots are used in tanning [15,16].

In traditional medicine, different parts leaves, bark and fruits of *C. siliqua* tree are used for the treatment of gastrointestinal infections, and against warts, also they are considered as diuretic and anti-diarrheic agents [17]. Leaves of *C.siliqua* have been traditionally investigated for treatment of various health disorders due to their important biological properties such as antioxidant, antimicrobial, antitumor and anti-proliferative, as demonstrated in several studies to reduce, manage, prevent and treat many human health problems and diseases [18,19,20,17,14].

According to the literature, several biological activities remain unexplored in the leaves of *C.siliqua* L. The present study aims the investigation of the antioxidant capacity of the methanolic and aqueous extract, the acute toxicity and the analgesic effect of *C.siliqua leave*s.

II. Material and methods

1. Plant collection

The leafy branches of *C.siliqua* L. were collected from Constantine, Algeria eastern, on the period of March 2019. A voucher specimen N°CS2019146 was deposited in the herbarium of the Pharmacological and Toxicological laboratory in Veterinary Science Institute. Constantine 1 university-Algeria.

2. Extraction process

Fresh leaves of *C.siliqua* were shade-dried for several days at room temperature, under laboratory conditions. The dried leaves were ground to a coarse powder of small particles of size 1- 2 mm.

Extraction was carried out according to the method described by Zeghad [21] and assisted with the ultrasonic method. In brief, 500 g of *C.siliqua* L. leaves powder was extracted by maceration in methanol or water to obtain methanolic (MeOH) and aqueous (AQ) extracts, respectively. The extraction was assisted by ultrasound (Fisher scientific fb 15046, Leicestershire, England) (>20kHz in frequency) for 2 hours at room temperature and repeated three times under identical conditions. The filtered extracts were combined and evaporated in a rotary evaporator (Buchi, Switzerland) under vacuum at low temperature (<40°C) to afford crude extracts which were subjected immediately to lyophilization (Christ Alpha 2e4 LD plus, Osterode am Harz, Germany). Finally, freeze-dried extracts were kept under low temperature (-25°C) until required for further experiments.

3. *Invitro* assay

3.1 Total phenolic content

The total polyphenol content was estimated by the Folin-Ciocalteu reagent according to the protocol of Singleton and Rossi [22], this quantification was achieved by the microplate assay method described by Muller [23]. The FCR reagent consisting of a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMO_{12}O_{40}$), which is reduced during the oxidation of phenols. The blue coloration produced is proportional to the content of total phenols and has a maximum absorption around 750 -765 nm. The protocol consist to mix 20 µl of extract dissolved in distilled water with 100 µL of FCR (10%) and 75 µL of sodium carbonate solution (0.7M) in the wells of 96-well microplate. After 2 hours of incubation at room temperature in the darkness, the absorbance was evaluated using microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Inc., Massachusetts, USA) at 765 nm against a blank. The tests were performed in triplicate. The concentration of total phenolic compounds is estimated from a calibration curve established with gallic acid as standard, it expressed as µg gallic acid equivalents/g dried extract weight (µg GAE/ g of extract). The tests were performed in triplicate ($n=3$), which was based on the calibration curve of gallic acid with the linearity range from 10 to 100 µg/ mL (R > 0.99). Y= 0,0034 Gallic acid (mg) + 0,1044 (R^2 = 0,997).

3.2 Total Flavonoid content

The determination of the flavonoids amount in extracts based on the formation of a complex between aluminum trichloride ($AlCl_3$) and flavonoids. The method of Topçu [24] was used with light modifications on a 96-well microplate. The protocol consisted to mix a volume of 50 µl of the diluted extract with 130 µl of methanol followed by 10 µl of potassium acetate (CH_3COOK) (1M) and 10 µl of aluminum nitrate (Al $(NO_3)_2$,$9H_2O$) (10%) After 40 min, the absorbance is read at 415 nm using a microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Inc., Massachusetts, USA). A sample blank is prepared by replacing the reagents with methanol (50 µl extract + 150 µl methanol). All extracts were performed in triplicate ($n=3$). TFC was expressed as µg of quercetin equivalents per mg of dried extract weight (mg QE/g of extract) on the

calibration curve of quercetin with the linearity range from 25 to 200 µg/mL ($R > 0.99$).

3.3 Antioxidant assessment

3.3.1 DPPH radical scavenging activity

The scavenging capacity of the extracts/fractions against the DPPH radicals was determined using the method of Blois [25] with some modifications using of a microplate system.

The DPPH solution was prepared by dissolving 6 mg of DPPH in 100 ml of methanol and kept at -20°C in darkness.

The protocol stated to put in a 96-well microplate 160 µL of the methanolic DPPH solution with 40µL of the samples in methanol at different concentrations (12.5–800 µg/mL).

After 30 minutes of incubation at room temperature in darkness, the absorbance was recorded at 517 nm against a blank using a microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Inc., Massachusetts, USA).

BHA, BHT and α-tocopherol were used as comparison while methanol was used as a negative control. The percentage of inhibition of radical scavenging activity was calculated using the following equation :

% Inhibition = [(AC – AS) /AC)]*100

Where *AC* is the absorbance of the control reaction and *AS* is the absorbance of samples. The IC_{50} was determined as the concentration of the test sample required to neutralize 50% of free radicals. It is calculated graphically by the linear regression (Y= a X + b), of the plotted graphs which are formed by the percentages of inhibition versus different concentrations of the fractions. The IC50 of each extract is calculated: $IC_{50} = (Y- b) /a$

3.3.2 CUPRAC assay (cupric reducing antioxidant capacity)

The CUPRAC method following the protocol established by Apak [26] is possible to evaluate the capacity of the extract to reduce the copper ions. This protocol consisted of mixing 50 ml of $CuCl_2$ (10 mM), 50 ml of Neocuproine (7.5 mM in ethanol) and 60 ml of CH_3COONH_4 (1M) with 40 ml of the sample at different concentrations in a microplate of 96 wells. The absorbance was measured at 450 nm after 40 min. A0.5 value was evaluated from the absorbance curves. BHT and BHA were used as standards.

3.3.3 Reducing power assay

The reducing power capacity of different extracts was performed as described by Oyaizu [27] with some modifications. The reducing power capacity of the sample was determined by increase in absorbance of the sample.

The protocol consists of mixing 10 µL of different concentrations of the sample with 40 µL of phosphate buffer (200 mM, pH 6.6), and 50 µL of potassium ferricyanide (1%). After incubation at 50°C for 20 min, 50 µL of trichloroacetic acid (10%), 40 µL of distilled water, and 10 µL of ferric chloride solution (0.1%) were added to the mixture. Absorbance was measured at 700 nm using a-tocopherol as standard.

3.3.4 ABTS assay

This method was evaluated using the protocol of Re [28]. The ABTS solution was prepared by reacting ABTS cation (19.2 mg) and potassium persulfate $K_2S_2O_8$ (3.3 mg) in aqueous solution. The mixture was protected from light 12- 16 Hours. The absorbance of the solution thus obtained is adjusted by (Ethanol or H_2O) to 0.700 ± 0.020 at 734 nm before use. Mix 160 µl of ABTS $^+$ solution to 40 µl of diluted extract, and after 10 min the absorbance was taken at 734 nm. ABTS + activity was expressed as a percentage and calculated by the following equation: % Inhibition = [(AC – AS) /AC)*100.

Results were compared to the standards BHA and BHT.

3.3.5 Superoxide scavenging activity

In this assay, the superoxide radical was generated by the addition of sodium hydroxide to air-saturated DMSO (Alkaline Dimethyl Sulfoxide). The generated superoxide remains stable in solution and reduces blue tetrazolium (NBT) by coloring Formazan at room temperature which can be measured at 560 nm [29].

Put 40µl extract and 130 µl alkaline DMSO (20mg of NaOH is dissolved in 100 ml DMSO) were added to 30 µl NBT (10 mg in 10 ml of distilled water), the absorbance was read at 560 nm. The percentage inhibition of superoxide anion generation was calculated by using the following formula : % Inhibition = [(AC – AS) /AC)*100.

3.3.6 Phenanthroline

The phenanthroline method was based on the reduction of Fe^{3+} to Fe^{2+} ion by an antioxidant, the Fe^{2+} ion formed which then reacted with ortho-phenanthroline to form a red-orange complex. According to the protocol of Szydlowska-zerniaka [30], a volume of 10µl extracted was added to 50 µl Ferric chloride $FeCl_3$ (0.2%) and 30 µl Phenanthroline (0.5%) then 110µl MeOH. After incubation for 20 min at 30°C in the dark, the absorbance was measured at 510 nm, and BHT was used as a standard. The percentage of inhibition

was calculated according to the following formula % Inhibition = [(AC – AS) /AC)*100. The results were presented as IC_{50} value in µg/ml.

4. *Invivo* assay

Animals used in this experiment were male albino *Wistar* rats with a weight between 180g to 220g and aged (5-6) weeks. They were kept in standard polypropylene cages at room temperature (24 ± 2°C)

for 12 hours of light/dark cycle. They have free access to a standard pellet diet and water *ad libitum.* These rats were placed in laboratory conditions for 10 days before beginning the experiment. The living conditions were placed according to the guidelines of the principals of Laboratory Animal Care "Guide for the Care and Use of Laboratory Animals" (Protocol no: 2016-AA10).

Regarding obtained results in *invitro* tests, the methanolic extract was chosen for *in vivo* assessment.

4.1 Acute toxicity

After an overnight fast, healthy animals were weighed and randomly distributed into five groups of six animals each (one control group and four treated groups). Rats from the four treated groups received serially diluted doses (0.5-1.0-1.5 and 2.0 g/kg). After oral administration (gavages) of water and reconstituted extract in distilled water for the negative and treated groups with methanolic extract of *C.siliqua* , respectively, the rats were observed every 30 minutes for 8 hours on the first day and every day for 14 days to monitor any signs of delayed toxicity [31]. They were also weighed to follow the evolution of their weight. Death or changes in general behavior and other physiological activities were noted. At the end of the experiment, animals were sacrificed and their internal organs including the heart, liver, kidneys, lungs, and spleen were examined and visually checked for any abnormality [32].

4.2 Analgesic assessment

4.2.1 Peripheral Analgesic assay

Peripheral analgesic effects of *C.siliqua* methanolic leaves extract were evaluated using the acetic acid-induced writhing following the protocol of Zeghad [21] in albino male *Wistar* rats. Experimental rats were randomly assigned to five groups with each consisting of 6 animals. Groups I, II, and III received an oral treatment of 0.1 g/kg, 0.3 g/kg and 0.5 g/kg, respectively, of the studied plant extract. On the other hand, groups IV and V received 0.1 g/ kg of acetylsalicylic acid (Aspirin) and 1 ml of distilled water orally as positive and negative controls, respectively. After 30 minutes, writhing was induced in each experimental rat with an intraperitoneal injection (*i.p*) of 1% v/v acetic acid. All the drugs were administered at a volume of 1 ml. Experimental rats were monitored individually, and the number of writhes was counted after 5 minutes of writhing induction, for 20 minutes, and recorded.

The percentage inhibition of writhing were calculated as an indicator of analgesic activity following the equation :

The Index of Pain Inhibition (IPI) was expressed by the present formula :

$$\mathbf{IPI} = \frac{\boldsymbol{Nc} - \boldsymbol{Nt}}{\boldsymbol{Nc}}\mathbf{x100}$$

*N*c: the number of writhes observed for the control group, and *N*t : number of writhes in tested groups (*C.siliqua* L. extract, acetylsalicylic acid).

4.2.2 Central Analgesic assay

A. Hot plate test

The hot plate test was used to measure response latency time according to the method described by Zeghad [33, 21]. Five groups of rats (6 rats per group) were used. Treated groups received, one hour before test methanolic extract of *C.siliqua* at different concentrations (0.1, 0.3 and 0.5 g/kg, single dose, *per os*), acetylsalicylic acid (0.1g/kg, *per os*) and distilled water (control). Rats were placed on a hot metal plate at 55.0±0.5°C. The reaction time to the thermal stimulus was recorded as the time interval from the introduction of the animal to the plate until the first lick of the limbs or the first jump of the animals. The responses were measured at 0, 0.5, 1.0, 2.0 and 3.0 hours. Only rats that showed an initial nociceptive response within 30 seconds were selected and used for the study. The cut-off time for the hot plate latencies was set at 30 secs [34].

B. Tail flick assay

The antinociceptive (analgesic) activity of *C.siliqua* L. leaves methanolic extract was evaluated by the tail-flick method described by Zeghad [21] with some modifications. Five groups of rats (6 rats per group) were used. They received, one hour before testing *C.siliqua* L. methanolic extract at different concentrations (0.1, 0.3 and 0.5 g/kg, single dose, *per os*), acetylsalicylic acid (0.1g/kg, *per os*) and distilled water (control). About 5 cm from the distal end of the tail of each rat was immersed in warm water maintained at 50°C. The reaction time (in seconds) was the time taken by the rat to flick its tail due to pain recorded (using a chronometer). The reaction time was recorded before (0 min) and at 0.5, 1.0, 2.0, and 3.0 hours after the administration of the treatments. The maximum reaction time was fixed at 15 sec to prevent any tail tissue injury. If the reading exceeds 15 sec, it would be considered as maximum analgesia [35].

5- Statistical analysis

Data for each test are the average of triplicate experiments ± standard deviation (SD) and all the results were analyzed by the test of *Tukey* (*HSD)*. A value of $P<0.05$ was considered to be significant. The results obtained were compared to the control group. *XL Stat* version was used to realize statistical analyses.

III. RESULTS AND DISCUSSION

***In vitro* assay**

1. Total phenolic and flavonoid contents

The amounts of phenolic and flavonoid coumpounds of the methanolic (MeOH) and aqueous (AQ) *C.siliqua* leaves extracts are shown in Table 1. The aqueous extract recorded the highest total phenolic content with a value of (379,76 ± 0,04 mg GAE g^{-1})followed by methanolic extract (162,1 ± 0,02 mg GAE g^{-1}). Regarding flavonoids, the content of these later was evaluated by $AlCl_3$ method, quercetin was used as the standard reference. As depicted in Table 1, the highest quantity was recorded by methanolic extract with 58,32 ± 0,01 mg QE g^{-1} followed by aqueous extract with 41,66 ± 0,04 mg QE g^{-1}. This finding could eventually be explained by the fact that MeOH extract contains other classes of flavonoids not extracted by water [36]. These results are in agreement with the findings of previous studies.

Many studies also reported that flavonoids were higher in hydroalcolic extract of *C.siliqua* L leaves extract [37, 38,14]. It is necessary to mention that the technique and the extraction solvent play a very important role in the quantification of polyphenols and flavonoids as previously reported by several studies [39,40,41,42].

2. Antioxidant assessment

The aim of the present work was to evaluate the antioxidant activity of methanolic and aqueous *C.siliqua* leaves extracts, performing two types of typical assays ;

The first category of antioxidant activity measures the ability of plant extract to scavenge free radicals (stable free radicals like DPPH, ABTS and Superoxide) and the second category measures the potential of the plant extract to reduce ions or oxidants (by acting as reducing agents) like ferric ion (FRAP, Phenanthrolin), cupric ion (CUPRAC). The increased antioxidant activity is mainly associated with the presence of phenolic compounds, and flavonoids, whereas this activity was attributed as reported in previous studies to their ability to absorb and neutralize free radicals [43]. The results of antioxidant activities were represented in Table 1.

The data showed that the antioxidant levels were the highest in methanolic extract, which IC_{50} ranged from 07.10 ±0.93 to 15.85 ± 0.05 µg/ml for the scavenge free radicals tests (DPPH, ABTS, Superoxide) and EC_{50} from 08.47±0.08 to 23.12±0.21 µg/ml for the reducing ions tests (FRAP, CUPRAC and Phenanthrolin). Whereas, the aqueous extract exhibited the lowest levels.

Othman and collaborators [14] reported in their studies that hydroalcolic extract of leaves has a great antioxidant activity compared with aqueous extract with 178.33± 2.12 g TE 100 g−1 DW, which is in agreement with our results. Many studies confirm the antioxidant effect of different parts especially pods of *C.siliqua* L.[37,44,17,13].

Extracts with higher flavonoid content generally show higher antioxidant activity, and good correlations have been found among these parameters [45,46] . Suggesting that flavonoids may be the major contributor of methanolic *C.siliqua* L. leaves extracts, they show strong antioxidant and radical scavenging activities, and the use of flavonoid-containing drugs is associated with a reduced risk for certain chronic diseases, some cardiovascular disorders, and certain types of cancerous processes [47]. Their antiradical properties stem from targeting ROS.

Moreover, flavonoids are soluble chain-breaking inhibitors of the peroxidation process. They scavenge intermediate peroxyl and alkoxyl radicals and chelate metal ions, the latter of which are among the major components in the initiation of radical reactions [47].

This is the first time that Algerian carob leaves have been investigated for different antioxidant activity. Taken together with results for the phenolic profile and antioxidant activities, our results suggest that the methanolic leaves extract possess exploitable antioxidants, and that they could be a source of flavonoids with potential antioxidant activity. Our findings, therefore, serve as a prelude to *in vitro* and *in vivo* studies that may lead to verification of the efficacy of the biological activities of carob leaves extract against various human diseases.

Table 1. Total phenolic (TPC), flavonoid (TFC) contents of methanolic and aqueous *C.siliqua* L extracts as well as their antioxidant activities measured by the DPPH, ABTS, FRAP, CUPRAC, Superoxide and Phenanthrolin assays.

Samples standards	TPC (mg/g) GAEg^{1-}	TFC (mg/g) QE g^{1-}	DPPH IC_{50}(µg/mL)	ABTS IC_{50}(µg/mL)	Superoxide IC_{50} (µg /mL)	FRAP EC_{50}(µg/mL)	CUPRAC EC_{50}(µg/mL)	Phenanthrolin EC_{50}(µg /mL)
MeOH	162.10±0.02	58.32± 0.01	10,30 ± 0.03	07.10 ±0.93	15.85 ±0.05	23.12±0.21	14.00 ±0.07	08.47±0.08
AQ	379.76±0.04	41.66± 0.04	73,08± 0.02	45.08± 0.93	NA	46.37± 0.08	22.00±0.11	297.00±0.02
BHT	/	/	06,82 ± 0.49	01.59 ±0.03	NT	NT	08.33 ±0.87	00.93±0.07
BHA	/	/	07,29 ± 0.49	01.03 ±0.01	NT	NT	02.24 ±0.19	02.24±0.17
Tannic acid	/	/	NT	NT	<3.125	05.39±0.91	NT	NT
α-tocopherol	/	/	NT	NT	<3.125	NT	NT	NT

GAE g^{-1}: gallic acid equivalent, QE g^{-1}: quercetin equivalent. Values are expressed as Mean ± SD (*n=3*). NT: not tested, NA: no activity

***In vivo* assay**

1. Acute toxicity

The oral administration of doses of 1000mg/kg and 1500mg/kg methanolic extract (treated groups I and II, respectively), caused no death in the rats. No lethal effects were noted throughout the short and long-term observation period. No toxicity signs were observed in the animals throughout the 14 days study period.

Rtibi and Ben Ayache [44,13] assessed the toxicity profile of *C.siliqua* L. extract at doses of 20g/Kg and 2g /Kg respectively. They also found that pod's aqueous extracts of *C. siliqua* didn't show any toxicological signs on mice. Qasem [48] reported that also methanolic extract of carob pods did not have a toxicological effect on rats at a dose of 5g/Kg.

However, many signs of toxicity and clinical symptoms have been reported such as: difficult breathing, rapid and severe cessation of activity, change in eye shape and color, diarrhea, and decreased appetite, after dosing animals with methanolic *C.siliqua* L. extract at dose of 2000mg/kg. These signs persisted for 72 hours. From the 4th day, rats show a slight improvement in health. It is from the 5th day when rats have recovered their normal states and disappearance of signs of toxicity: activity, form, appetite. 2000mg / kg remains a dose causing significant signs of toxicity but no death of rats was noted. Therefore, the extract may be safe at these doses and the oral LD_{50} is considered greater than 2000 mg/kg in rats. Substances presents LD_{50} higher than 500 mg/kg administered by oral route can be considered practically non-toxic[21].

In the absence of reagents and adequate type of equipments for the microscopic analysis of the organs, the study was concentrated only on the macroscopic aspect. The macroscopic examination performed later on the main organs (heart, liver, kidneys, lungs, spleen) also revealed that there was no abnormality. The animal treated with the extract don't showed significant changes in body weight when compared with the control group.

2. Analgesic assessment

2.1 Peripheral Analgesic assay

In this study, the results revealed a dose-dependent reduction in writhing corresponding to an increase in parentage inhibition of acetic acid-induced writhing in rats (Table 2).

Upon administration of the methanolic *C.siliqua* L. leaves extract (0.1, 0.3 and 0.5 g/kg, *peros*), the percentage inhibition of writhing significantly ($p < 0.0001$) increased in a dose-dependent manner compared to the control group (as shown in (Table 2). The percentage inhibition were 71.21, 82.74, 91.50 and 92.37 for the extract (0.1, 0.3 and 0.5 g/kg, *per os*) and acetylsalicylic acid (0.1 g/kg, *per os*), respectively. Comparatively, the extract at equal doses (3.0 and 5.0 mg/kg) showed no significant differences compared to the group treated by the drug reference.

2.2 Central Analgesic assay

The results of the central analgesic effect of *C.siliqua* L. leaves methanolic extract using a hot plate and tail immersion methods are presented in Table 3. The results showed that there was no significant difference in the thermal stimulus in rats treated compared to control group in the tail immersion test, except for doses of 0.3 and 0.5 g/kg of *C.siliqua* L. It was noted that leaves methanolic extract has a significant reaction time at 1.00 H.

However, the hot plate reaction time was increased by *C.siliqua* L. After the administration of the extract, the increases were statistically significant ($P<0.0001$), especially at doses of 0.3 and 0.5 g/kg (Table 3).

From the obtained results by the three analgesic tests, it was clear that the extract of this plant was endowed with an increased power to reduce pain, whether central or peripheral, with a certain dose-dependent relationship. For the three tests, the effective dose was 500mg/kg and the effective duration was 30min to 1 hour.

Table 2. Antinociceptive effect of *C.siliqua* L.extract and acetylsalicylic acid on acetic acid-induced pain in rats.

Groups	Dose (g/kg)	Number of writhings* (IPI%)
Control (distilled water)	-	112.33 ± 20.42■■ (-)
C.siliqua L.	0.1	31.00 ± 6.23#■ (71.21%)
	0.3	19.00 ± 2.19# (82.74%)
	0.5	9.33 ± 91.50# (91.50%)
Acetylsalicylic acid	0.1	8.33± 1.63# (92.37%)

* Values are expressed by mean ± SD (*Tukey HSD-test, n =6*); #*P<0.0001*:vs. control group; ■*P<0.01*, ■■*P<0.0001* vs. acetylsalicylic acid (standard drug) treated group. IPI: Index of Pain Inhibition (%).

Table 3. Central analgesic activity of *C.siliqua* L. extract, measured by hot plate and tail immersion tests.

Compound and Plant extract	Dose (g/kg)	Reaction time (seconds) * Hot plate test				Tail immersion test			
		0.50H	1.00H	2.00H	3.00H	0.50H	1.00H	2.00H	3.00H
Control (distilled water)	-	0.94± 0.10	0.98± 0.04	0.92±0.06	1.01±0.03	5.26±0.34	5.08±0.47	5.39±0.51	5.18±0.48
C.siliqua L.	0.1	1.51±0.34	2.51±0.38$^{\#\#\#}$	1.12±0.14 ■■	0.86±0.09 ■■	4.88±0.23$^{\#}$	5.30±0.38	4.30±0.59	3.43±0.96 ■
	0.3	1.59±0.29	2.36±0.46$^{\#\#\#}$	2.20±0.59$^{\#\#}$	1.50±0.08$^{\#}$	4.37±0.32	5.54±0.15$^{\#}$	5.21±0.44	4.63±0.41
	0.5	2.52±0.63$^{\#\#\#}$	4.02±0.74$^{\#\#\#}$■	2.15±0.15$^{\#\#}$	1.64±0.41$^{\#}$	5.41±0.52	5.97±0.39$^{\#\#\#}$■■	5.02±0.59 ■■	4.31±0.42 ■■
Acetylsalicylic acid	0.1	2.12±0.45$^{\#\#\#}$	3.09±0.40$^{\#\#\#}$	2.61±0.22$^{\#\#}$	1.80±0.31$^{\#\#}$	5.60±0.88	6.67±0.73	5.55±0.56$^{\#}$	4.77±0.59$^{\#\#}$

* RT in second expressed as mean ± SD, *Tukey (HSD)-test* n=6; $^{\#}$ *P < 0.05,* $^{\#\#}$ *P < 0.01,* $^{\#\#\#}$*P < 0.0001*: compared to control group; ■ *P < 0.05,*■■ *P < 0.01,* ■■■ *P < 0.0001*: compared to acetylsalicylic acid treated group.

Different mechanisms actions were provided by the three applied tests; hot plate, tail flick, and acetic acid test.

Hot plate and tail flick tests are realized to confirm analgesic central activity [49]. Methanolic extract proved a very strong analgesic effect during the 1st hour by increasing the reaction time, and the latency period of the sensation of pain on the hot plate and tail flick at 55°C.

The acetic acid method is an effective technique to evaluate peripherally analgesics effect, by evaluating the abdominal contortion response caused by intraperitoneal injection of acetic acid [50]. Methanolic extract presented a very important rate of pain inhibition (88,43%) by reducing the number of contortions caused by acetic acid.

The positive control was used for better comparison and confirmation of extract effect, that many drugs like aspirin and ibuprofen are known by their peripherally analgesic acting, they have been reported to inhibit writhing, which is induced by acetic acid in mice [50,51]. Molecules will express analgesic activity mostly via the interference of prostaglandin synthesis, a peripheral mechanism of pain inhibition [52]. This inhibition may be explained by the suggestion that extract is endowed with an antinceptive action which is expressed by its effects stopping production or releasing pro-inflammatory substances.

Many studies reported that extract contain on total phenolic compounds and flavonoids had a strong relation with analgesic effect and other bioactive activities especially anti-inflammatory effect [53]. That phytochemical compounds alkaloids and flavonoids may exert the analgesic and anti-inflammatory activity via arachidonic acid metabolism pathway [52,54,55].

C.siliqua plant was characterized by the richness in secondary metabolites especially Flavonoids and phenolic acids. Many studies have identified the mainly compounds metabolites in leaves as following : gallic acid, p-hydroxybenzoic acid, chlorogenic acid, coumaric acid, ferulic acid, syringic acid, gentisic acid, (+)-catechin, (−)-epicatechin, (−)-epigallocatechingallate, myricetin, rutin, quercetin, kaempherol, apigenin, isoquercetin, myricetinglucoside, myricetinrhamnoside, 1,6-di-O-galloyl-gucose, 1,2,6- tri-O-galloyl-gucose and 1,2,3,6-tetra-O-galloyl-gucose [19,56,57,58,18,38] reported in Stavrou & Kapnissi-Christodoulou [59]. Other study of Rtibi [60], confirmed presence of flavonoids on percentage like kaempferol (77± 2.43%), tannic acid (13±0.45%), catechin hydrate (4.30±0.34%) and polydatin (0.85 ± 0.22%).

The richness in all these bioactive substances which are known by theirs anti-inflammatory properties perhaps interact with the COX receptors and other inflammatory and neurogenic pain modulators to reduce pain.

From these, it can be suggested that this plant contains very potential compounds, which showed an important analgesic effect [52].

3. Conclusion

C.siliqua has received immense attention because of its versatile medicinal activities. The current work added supplement information about the polyphenolic content and biological activities of *C.siliqua* leaves.

The tested activities included antioxidant capacity via DPPH, CUPRAC and ABTS assays. In addition to FRAP and Phenanthrolin activities. The obtained results revealed that leaves have also been an interesting source of bioactive molecules as well as carob pods and other organs, they have beneficial biological effects, that why have long been used in traditional medicine.

The methanolic extract of *C.siliqua* leaves exhibited significant antioxidant activity compared to the aqueous extract. This is may be explained by the higher flavonoids contains in methanolic extract. The i*nvivo* assay showed that methanolic extract was endowed with an important analgesic effect in both central and peripheral mechanism. Therefore, advanced studies are important to evaluate the *invivo* antioxidant effect and to explore more this pathway to treat different diseases. In addition, purification, isolation, and identification from the crude extract of pure molecules responsible for both activities are very essential and indispensable.

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