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Evaluation of Antioxidant, Antihemolytic Activity, Analgesic and Anti-Inflammatory Potential of *Aristolochia clematitis Extracts*

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ABSTRACT

There is an increasing interest in medicinal plants to find natural remedies relatively safer than synthetic alternatives. The present study reports the evaluation of the *in vitro* and *in vivo* biological properties of crude extract (CrE), hexane (HeE), ethyl acetate (EAE) and aqueous (AqE) fractions of *Aristolochia clematitis*. The polyphenol and flavonoid content were determined and varied from 15.77 ± 0.28 to 93.62 ± 1.63 mg EGA/g, and 5.71 ± 0.1 to 13.53 ± 0.15 µg EQ/mg, respectively. Crude extract (CrE) and ethyl acetate (EaE) showed the highest scavenging activity of DPPH radicals (IC_{50} = 50.66 ± 1.41 and 69.57 ± 0.38 µg/mL) and hydroxyl radicals (IC_{50} = 0.894 ± 0.042 and 0.905 ± 0.890 mg/mL), reducing power (113.85 ± 0.5 and 82.45 ± 0.13 mg/mL) and the ability to inhibit β-carotene oxidation (76.13 ± 0.93, 69.18 ± .95 %). All extracts have the ability to protect red blood cells from AAPH peroxyl radicals and significantly increase hemolysis time compared to vitamin C. Tested on normal human plasma at a concentration of 50 and 100 mg/mL, these extract prolonged prothrombin (PT) and partial thromboplastin (aPTT) times. In addition, CrE tested at doses of 150 and 300 mg/kg, showed potent antinociceptive effect (40.68 ± 3.84 and 66.61 ± 4.71%) and anti-inflammatory activity (65.71 ± 1.41 and 63.89 ± 2.17%). The results support the use of this plant in traditional medicine and suggest that it may contain phytochemicals that have the potential to be active agents as antioxidants, anticoagulants and analgesics.

***Keywords*:** Analgesic, antihemolytic activity, anticoagulant, anti-inflammatory activity, antioxidant, *Aristolochia clematitis*, flavonoids, polyphenols

Introduction

Medicinal plants have been used to treat many diseases around the world for decades. Since they are biodegradable and have fewer side effects, most of them are considered to be more useful and beneficial than traditional medicines.[1] Interest in the development of innovative natural remedies based on medicinal plants to protect human health is growing. The beneficial effects of bioactive molecules are attributed to secondary compounds such as alkaloids, terpenoids and polyphenolic compounds, particularly flavonoids.[2] These phenolic compounds are powerful antioxidants that fight free radicals that occur naturally through respiration and other metabolic processes. Free radicals are very reactive and can oxidize other nearby molecules. Overproduction of these harmful chemicals can lead to oxidative stress, which has been linked to inflammation, cancer, aging, cardiovascular and neurodegenerative diseases. In fact, natural antioxidants from plants can be used as dietary supplements to prevent and treat many diseases, as well as to maintain food quality and safety.[3] This ovoid the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tetrabutylhydroquinone (TBHQ), which have been associated with health risks such as carcinogenesis and genotoxicity.[4] Moreover, phenolic compounds have been reported to have various biological properties such as anti-inflammatory, anticoagulant effects[5], analgesics and anti-cancer.[6]

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In Algeria, medicinal plants are widely used in herbal medicine to treat diseases such as hypertension, diabetes, inflammation, stomach disorders and cancer. *Aristolochia clematitis*, also called "birth worth", is one of the oldest medicinal plants. It is known for its toxicity as it contains aristolochic acid, which has nephrotoxic and genotoxic effects.[7] This herb has shown many therapeutic effects, particularly in the treatment of cancer[8,9], toothache, fever, rheumatism, and snakebite.[10] This plant has also been used to heal skin infections, wounds, and induce labor.[11]

The aim of this research is to study biological properties including antioxidant and antihemolytic, anticoagulant activities and analgesic effect of *A. clematitis* extracts.

Materials and Methods

Plant material

The leaves of *Aristolochia clematitis* (Voucher N° 83777) were collected in May 2019 on Mount Babor at an altitude of 1600 meters, 70 km North of Setif. The samples were cleaned manually and dried in the shade and then ground. The plant was identified by Professor Chermat Sabah from the Department of Pharmacy, Faculty of Medicine, Ferhat Abbas University of Setif.

Animals

The Ethics Committee of Setif 1 University (N° 991 du 10 décembre 2020) approved the experimental protocol. All procedures were conducted in accordance to guidelines of the Organization for Economic Cooperation and Development for assessing the safety and efficacy of plants based medicines (OECD, 2001) and to the Committee of the "Association Algérienne des Sciences en Experimentation Animale" under law No. 88-08/1988. In this study, Constantine's National Center for Biotechnology Research (CRBT) provided the male Swiss mice weighing 22-25 g. The animals were housed in the animal house in a controlled environment that included a

12-hours light/dark cycle, a temperature of 25°C, standard mouse feed and water.

Extraction process

The extraction process was carried out by maceration with few modifications.[12] The powdered leaves (78 g) were extracted with pure methanol (780 mL) at room temperature for 5 days. This suspension was filtered through filter paper, and the filtrate was evaporated with a rotary evaporator at 45°C. Crude methanolic extract (CrE) was poured into plates, allowed dry in the oven at 40°C, and then scraped off. 11.5 g of dry extract were dissolved in boiling distilled water and left to settle in the refrigerator for 24 hours. This aqueous solution was filtered through filter paper and the filtrate subjected to fractionation using hexane first to remove lipids and chlorophyll, then ethyl acetate. By this treatment, two organic extracts (HeE: hexane fraction and EaE: ethyl acetate fraction) and a residual aqueous fraction (AqE) were obtained. Each fraction was evaporated with a rotary evaporator at 45°C. The various extracts were poured onto plate to dry in an oven at 40°C, then scraped off and stored in the freezer until use.

Determination of total phenol content (TPC)

Total phenol content was quantified using Folin-Ciocalteu method.[13] 1 mL of $1/10^{th}$ diluted of this reagent was mixed with 200 µl of each diluted extract and standard. After 4 min, 800 µl of aqueous sodium carbonate solution (7.5%) was added. The samples were shaken vigorously before being left in the dark at room temperature for 120 min. The absorbance of all samples was measured at 765 nm. The amount of total phenols in different extracts was calculated using a gallic acid standard curve and the results were expressed in milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g extract).

Determination of total flavonoid content (TFC)

The total flavonoid content of different extracts was determined by a colorimetric method using aluminum chloride ($AlCl_3$).[14]1 mL of each extract or standard (quercetin) was mixed with 1mL of aluminum chloride ($AlCl_3$) solution (2% in methanol), and the mixtures were kept in the dark at ambient temperature for 10 min.[14] The absorbance was measured at 430 nm. The concentration of flavonoids was calculated using a quercetin standard curve and expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g extract).

DPPH radical scavenging activity

The principle of this method is based on the reduction of the DPPH radical (DPPH·, purple) to a non-radical (DPPH-H) by recovering electrons and hydrogen atoms from the antioxidants, which decrease the absorbance at 517 nm.

The test was performed by adding 50 µl of different extract dilutions or standard (BHT) to 1250 µl of solution DPPH solution dissolved in methanol (0.004%). This setup was left in the dark at room temperature for 30 minutes. The absorbance of solutions was measured at 517 nm against a blank represented by the methanolic solution of DPPH.[15] The radical scavenging activity (RSA) expressed as a percentage (percentage) was calculated using the following formula:

$$RSA\ (\%) = [(A_{control} - A_{sample})/A_{control})] \times 100$$

RSA (%): percentage of free radical DPPH inhibition
A control: Absorbance of DPPH (t= 0 min).
A sample: Absorbance of the sample (t = 30 min).

The IC_{50} value is the concentration of antioxidant that inhibits 50% of DPPH. It was determined by plotting of the inhibition percentage of extracts and standard.

Hydroxyl radical scavenging assay

In this assay, 1 mL of different concentrations of each extract or the standard (ascorbic acid) was added to 1 mL of a reaction mixture containing $FeSO_4$ (9 mM), 1 mL H_2O_2 (0.3%) and 0.5 mL of salicylic acid (20 mM). The solutions were shaken vigorously before incubating for in a 37 °C water bath at 30 min. The absorbance was measured at 562 nm against a blank containing only the mixture ($FeSO_4/H_2O_2$), the extract or the standard and distilled water. The negative control was prepared by adding 150 µL of distilled water to the 300 µL of the reaction mixture water. The hydroxyl radical scavenging activity of each solution was calculated in percent inhibition using the following equation:

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

A_1: the absorbance of the reaction in the presence of the extract.
A_2: the absorbance of the reaction without salicylic acid, in the presence of the extract.
A_0: the absorbance of the negative control.

β-carotene bleaching assay

0.5 mg of β-carotene dissolved in 1 mL of chloroform, 25 µl of linoleic acid and 200 mg of Twin 40 were placed in a flask. This emulsion was evaporated on a rotary evaporator at 40°C to remove chloroform. Then 100 mL of oxygenated distilled water was added and the emulsion shaken vigorously. 2500 µl of this emulsion were mixed with 350 µl of extracts or synthetic antioxidant standard (BHT) at concentration of 2 mg/mL in distilled.[16] In the bleaching β-carotene assay, peroxidation of linoleic acid leads to the formation of hydroperoxydes, which induce discoloration of β-carotene. And the absorbance of each sample was determined at 490nm, after t = 0, 1, 2, 4, 6 and 24 hours. The Relative antioxidant activity (RAA%) of the extracts and the standard was calculated utilizing the following formula:

$$RAA\% = A_{sample}/A_{BHT} \times 100$$

RAA%: percentage of the antioxidant activity.
A sample: absorbance of the extract.
A BHT: absorbance of positive control (BHT).

Reducing power method

This test was used to determine the ability of plant extracts to reduce the ferric iron of the ferricyanide complex -Fe3+ to ferrous iron -Fe2+. 2.5 mL of the extract or standard was mixed with 2.5 mL phosphate buffer (pH 6.6) and 2.5 mL potassium ferricyanide (1%). The mixture was incubated in water bath at 50°C for 20 min, then 2.5 mL (10%) of trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 mL of the upper solution layer was mixed with 2.5 mL of distilled water and 0.5 mL of freshly prepared ferric chloride solution (0.1%). The absorbance was measured at 700 nm against a blank. Ascorbic acid was used as a positive control.

Antihemolytic activity

The antihemolytic activity of *A. clematitis* extracts was assessed by the method used by Guemmaz *et al.*[17] In this assay, hemolysis was induced by peroxyl radicals of 2, 2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), which damage red blood cell (RBC) membranes.

Ethylenediamimetetraacetic acid (EDTA) tubes were used to collect blood from mice and were centrifuged at 3000 rpm and 4°C for 10 min. The supernatant was removed and the pellet washed three times with phosphate buffered saline PBS (310 mM, pH 7.4). 20 µl of extract or standard (vitamin C) at a concentration 1 mg/mL and 80 µL of erythrocyte suspension (2% in PBS) were distributed in a microplate. 136 µL of AAPH (300 mM) were then added to the mixture and incubated at 37°C. The absorbance was measured every 15 minutes at 630 nm. The negative control containing RBCs and AAPH was considered to have 100 % hemolytic activity and the hemolysis in the presence of the extracts or vitamin C was calculated as follows:

$$\%\ hemolysis = [(Ac-As)/Ac] \times 100$$

Were Ac is the absorbance of the control (AAPH+RBC) and As the absorbance in the presence of the extract or vitamin C.

The results were expressed as the halftime (HT_{50} in min) required for 50% hemolysis of the initial erythrocytes. A high halftime indicates good resistance of RBCs to free radical attack.

Effect of A. clematitis extracts on plasma clotting parameters

The various extracts were tested on human plasma to verify their ability to inhibit its clotting in PT and aPTT assays. In PT test, the clotting time was determined according to the method of QI et al.[18] A test tube containing citrate (blood: anticoagulant, 9:1) was used to collect human blood from healthy volunteers and centrifuged at 3000 rpm for 5 min. The platelet-poor plasma (50 µl) was mixed with 50 µl of each plant extract at a concentration of 50 and 100 mg/mL, and the samples were incubated at 37°C for 5 min. 200 µl of reagent (thromboplastin) was added and the eppendorfs were left at room temperature checking for clot formation in the plasma. The measured clotting time was compared to the PT of a normal control.

To determine the activated partial thromboplastin time (aPTT), 100 µl of test plasma (50 µl of platelet poor plasma + 50 µl extract) was mixed with 100 µl of reagent. After incubation at 37ºC for 5 min, 100 µl $CaCl_2$ was added and the clotting time was recorded as the activated partial thromboplastin time and compared to that of a normal control.

Analgesic effect of A. clematitis crude extract using writing test

The analgesic activity of *A. clematitis* crude extract was studied using acetic acid-induced writing in mouse model.[19] The experiment was performed on male Swiss mice, which had free access to food and water in the animal room and alternated a light/dark cycle every 12 hours. After a week of adjustment, the animals were divided into four groups of five mice weighing 23 and 25 g, tagged and fasted for 16 hours before testing. CE was administered orally to groups 1 and 2 at 150 and 300 mg/kg body weight, respectively. Groups 3 (negative control group) and 4 (positive control group) each received distilled water and aspirin (100 mg/kg body weight), respectively. After 1 hour of administration, all mice were injected intraperitoneally with 0.3 mL of acetic acid (0.9%) to induce pain, and the number of writings per mouse was counted every 5 minutes for 30 minutes. The percentage of protection against abdominal constrictions was used to evaluate the rate of analgesia and was determined using the following formula:

Inhibition (%) = (control mean-test mean) x 100/control mean

Anti-inflammatory activity

The anti-inflammatory activity of CE and EAE extracts was assessed using xylene-induced ear edema in mice according to Kou et al.[20], with some modifications. Male Swiss mice weighing between 26 and 30g were divided into six groups of six animals each. Groups 1 and 2 received CE at 150 and 300 mg/kg. Distilled water and indomethacin (50 mg/kg) were administered to groups 3 and 4. One hour after oral treatment of the mice, edema was induced by topical application of xylene (50 µL) on the dorsal surface of the right earlobe. 45 minutes later, the thickness of the right ear was measured with a digital caliper. The size of the right ear edema was calculated by measuring the difference in ear thickness before and after xylene application. The reduction in edema was expressed as a percentage in the treated group compared to the control group.

Statistical analysis

Results from *in vitro* experiments were expressed as mean ±error and results from *in vivo* tests as mean± SEM. Statistical analysis was performed using Graph Pad Prism 5. The analysis of variance was determined by One-way ANOVA using Dunnett testto make simple distinctions. At a significance level of $p < 0.05$, the difference was considered statistically significant.

Results and Discussion

The yield of *A. clematitis* extracts varied from 4.54 to 26.03 (Table 1). High TPC values of 93.62 ± 1.63 and 87.61 ± 1.65 mg GAE/g were recorded for the crude extract (CrE) and the ethyl acetate fraction (EaE), and moderate level 61.45±1.08 mg GAE/g for aqueous fraction (AqE). The TFC content was higher for CrE (13.53±0.15 µg QE/mg), but moderate for EaE (6.50±0.2 µg QE/mg) and AqE (5.71±0.1 µg QE/mg).

The scavenger DPPH radical assay is a commonly used model to assess the ability of plant extracts to scavenge free radicals. The antioxidant activity of different extracts was significantly different at $P<0.001$(Table 2). CrE and EaE exhibited the highest antioxidant capacity to neutralize DPPH radicals with a minimum IC_{50} value of 50.66 ± 0.14 and 69.57 ± 0.38 µg/mL, respectively, followed by moderate activity for AqE (119.3 ± 0.5 µg/mL) and HeE (138.37 ± 0.66 µg/mL). These results were slightly higher than that of Benmehdi et al.[21], root extract of the same species collected at Bechar (southern Algeria), and *Aristolochia longa L*.[22]

Hydroxyl radicals are formed from hydrogen peroxide in the presence of iron ions and due to their high reactivity. They can damage important macromolecules including lipids, proteins and nucleic acids.[23,24] Table 2 showed that all *A. clematitis* extracts have the ability to scavenge the generated hydroxyl radicals. The strongest extracts were CrE and EaE with similar IC_{50} (0.894 ± 0.042 and 0.905 ± 0.890 mg/mL, respectively), slightly higher than the standard (BHT) whose IC_{50} was 0.573 ± 0.003 mg/mL. However, HeE and AqE showed the lowest activities (4.135 ± 0.080 and 4.950 ± 0.046 mg/mL, respectively).

The results also showed that all *A. clematitis* extracts were able to prevent the degradation of β-carotene and reduce ferric ion to ferrous ion in reducing power assay (Table 2).The order of decrease in relative antioxidant activity was EaE>CrE>AqE>HeE, corresponding to 76.13 ± 0.93, 69.2 ± 4.95, 61.92 ± 2.62 and 55.65 ± 2.9 %, respectively.

Table 1: Yield (%), total phenolic and flavonoids contents of *A. clematitis* extracts.

Extracts	Yield (%)	TPC (mg GAE/g)	TFC (µg QE/mg)
CrE	26.03	93.62 ± 1.63[a]	13.53 ± 0.15[a]
HeE	5.54	15.77 ± 0.28	2.61 ± 0.44[d]
EaE	5.38	87.61 ± 1.65[b]	6.50 ± 0.2[b]
AqE	4.54	61.45 ± 1.08[c]	5.71 ± 0.1[c]

Values were expressed as mean ± SD (n = 3).Letters in same column indicate a significant difference ($p< 0.05$).

Table 2: Antioxidant activity of *A. clematitis* extracts and standards (BHT and ascorbic acid) in scavenging DPPH and OH radicals, reducing power and β-carotene bleaching assays

Extracts	RSA of DPPH IC_{50} (µg/mL)	RSA of OH IC_{50} (mg/mL)	RP EC_{50} (mg/mL)	RAA (%)
CrE	50.66 ± 0.14[a]	0.894 ± 0.042[a]	113.85 ± 0.5[b]	69.2 ± 4.95[b]
HeE	69.57 ± 0.38[b]	4.135 ± 0.080[c]	915.12 ± 40[d]	55.65 ± 2.9[d]
EAE	119.3 ± 0.5[c]	0.905 ± 0.890[b]	82.45 ± 0.13[a]	76.13 ± 0.93[a]
AqE	138.37 ± 0.66[d]	4.950 ± 0.046[d]	214.3 ± 14.84[c]	61.92 ± 2.62[c]
BHT	13.44 ± 0.3	-	-	100 ± 1.19
Ascorbic acid	-	0.573 ± 0.003	11.93 ± 0.17	-

Values were expressed as mean ± SD (n = 3).Letters in same column indicate a significant difference ($p< 0.05$).

Table 3: Effect of *A. clematitis* extracts on PT and aPTT clotting time.

Extracts	Dose 50 mg/mL		Dose 100 mg/mL	
	PT (sec)	aPTT (sec)	PT (sec)	aPTT (sec)
CE	116.67 ± 0.57	45 ± 2.82	No clotting	No clotting
HeE	302.5 ± 2.12	90 ± 0.7	No clotting	No clotting
EAE	83 ± 1.41	40 ± 0.7	No clotting	60 ± 1.41
AqE	62.5 ± 0.70	07 ± 0.5	25 ± 1	45 ± 0.7
Control	11 ± 2.82	27 ± 1.41	11 ± 2.82	27 ± 1.41

Values were expressed as mean ± SD (n = 3).

Human erythrocytes hemolysis is often used as a model to study free radical-induced damage to biological membranes. These free radicals are generated by the thermal degradation of AAPH, which attack red blood cell membrane to induce lipid peroxidation and cause hemolysis.[25] The results of inhibition of erythrocyte hemolysis by the plant extracts showed potent activity by prolonging the time required for hemolysis of 50% of RBCs (Figure 1). Therefore, EAE has the strongest antihemolytic activity, and the percentage of hemolysis was very low (<9%) after 225 minutes at the concentrations tested. At a concentration of 1 and 0.5 mg/mL, the HT_{50} was not reached after 225 minutes for CrE, AqE and the standard (vitamin C). At a concentration of 1mg/mL, CrE and AqE have HT_{50} of 116.25 ± 2.32 and 208.67 ± 5.5 min, respectively, which was more significant than vitamin C. The lowest activity was obtained by HeE with a HT_{50} was 74.8 ± 4.06 min.

Antioxidant activity can be monitored by several methods with different mechanism such as free radical scavenging, chelating metals and inhibition of lipid peroxidation. In this study, the results showed the potential antioxidant of the crude extract (CrE) and the ethyl acetate fraction (EAE) in all antioxidant model systems. It is known that many bioactive compounds have higher affinity to organic solvents than to water, so their recovery with ethanol and ethyl acetate is better. These activities can be linked to the content of phenolic compounds in the extracts[26,27] and/or to the individual active phytoconstituents or their synergistic effect.[28] Polyphenols, especially flavonoids, are powerful antioxidants that help stabilize free radicals, thereby preventing chronic diseases such as cancer and cardiovascular disease.

In addition, *A. clematitis* extracts had significant anticoagulant activity and can inhibit thrombin by prolonging both PT and aPTT. Thus, all extracts showed a strong prolongation of the PT clotting time at a concentration of 50 mg/mL by 5- to 27- fold compared to the normal clotting time (11 ± 2.82 seconds). In contrast, no coagulation was observed with CrE, HeE and EaE at a concentration of 100 mg/mL. In the aPTT test, CrE, HeE and EaE showed the highest anticoagulant activity at a dose of 50 mg/mL compared to the control (27 ± 1.41 seconds). However, in the presence of CrE and HeE at a concentration of 100 mg/mL, human serum does not clot (Table 3).

These findings showed that CrE, HeE and EaE may contain phytochemicals that interfere with hemostasis systems. Triterpenes, flavonoid compounds such as caffeic acid, rosmarinic acid, quercetin, rutin, hyperoside, and luteolin-7-O-glucoside have been shown to inhibit clot formation in PT and aPPT tests.[29,30]

The analgesic activity was assessed by writhing test, which has been reported to be useful for the investigation of peripheral antinociceptive activity and performed as a chemical model pain. Administration of CrE with 150 and 300 mg/kg caused a significant decrease in abdominal constrictions and the pain inhibition was 40.68 ± 3.84 and 66.61 ± 4.71%, respectively (Figure 2). The analgesic effect was comparable to that of Aspirin. In the other hand, this extract showed a potent anti-inflammatory activity of 65.71 ± 1.41 and 63.89 ± 2.17%, respectively in both doses and was similar to that of indomethacin (66.03 ± 4%).

The inflammatory response results from the release of arachidonic acid from phospholipids via cyclooxygenase (COX) and prostaglandin biosynthesis.[31] The significant reduction in pain by the extract may be due to the presence of analgesic principles that act on the prostaglandin pathways. The results confirm previous reports suggesting that alkaloids, flavonoids, steroids and tannins possess significant analgesic activity.

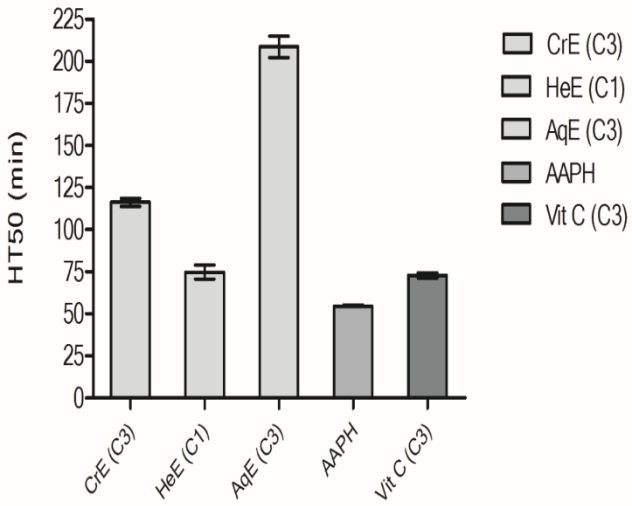

Figure 1: Halftime (min) of *A. clematitis* extracts at the concentrations tested C1 (1mg/mL), C2 (0.5 mg/mL) and C3 (0.1 mg/mL). Values were expressed as mean ± SD (n = 3).

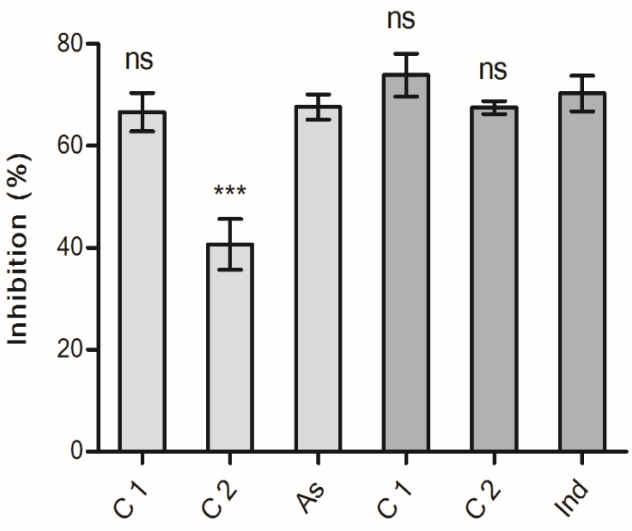

Figure 2: Analgesic effect and anti-inflammatory activity of crude extract (CrE) of *A. clematitis* at doses of 300 mg/kg (C1) and 150 mg/kg (C2), and positive controls (As: aspirin and Ind: indomethacin). Data are represented as Means ± SEM (n=5). ns: P<0.05, ***: significantly different at P<0.001.

Conclusion

The results demonstrated that the extracts of *A. clematitis* exhibited antioxidant activity and ability to protect erythrocyte membrane from AAPH induced hemolysis. The extract may contain bioactive compounds that can be used as an alternative and/or complementary medicine in the treatment of pain. Studies are needed to identify active compounds from this medicinal plant.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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