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Anti-inflammatory and antioxidant properties of Algerian *Santolina chamaecyparissus*

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ABSTRACT

The present study aimed to investigate the anti-inflammatory and the antioxidant properties of Algerian Santolina chamaecyparissus (S. chamaecyparissus) ethanol (SCE) and aqueous (SCA) extracts. The local treatment of ear mice by 2 mg S.chamaecyparissus decreased significantly ($p < 0.001$) the croton oil-induced ear edema. The inhibition exerted by SCA (31%) was less than that exerted by SCE (41%). Furthermore, S.chamaecyparissus extracts inhibited significantly ($P < 0.001$) the acetic acid-induced abdominal constrictions in mice. At 400 mg/kg, SCE was more potent (38.05%) than SCA (34.86%). The treatment with 1 mg/pouch of SCE and SCA reduced significantly ($P < 0.001$) the number of migrated leukocytes to $5.12 \pm 0.48 \times 10^6$ cells/mL and $5.60 \pm 0.56 \times 10^6$ cells/mL of exudates, respectively, compared to the control group. On the other hand, SCE and SCA showed a significant $DPPH^{\bullet}$ scavenging activity. The best scavenging activity was exerted by SCA with $IC_{50} = 35.27 \pm 3.24$ µg/mL flowed by SCE with $IC_{50} = 41.06 \pm 0.94$ µg/mL. Moreover, both extracts were able to chelate ferrous ions. However, SCA was more potent in chelating ferrous ions ($IC_{50} = 73.51 \pm 2.94$ µg/mL) than SCE (507.43 ± 12.44 µg/mL). The reducing power exerted by SCE was stronger ($IC_{50} = 49.12$ µg/mL) than that of SCA ($IC_{50} = 57.03$ µg/mL). Taken together, S. chamaecyparissus extracts exhibit antioxidant and anti-inflammatory activities. So, this plant could be exploited as a potential source of natural compounds to treat inflammatory and oxidative stress disorders.

Keywords: anti-inflammatory activity, antioxidant activity, *Santolina chamaecyparissus*, polyphenols, flavonoids.

INTRODUCTION

Steroidal and non-steroidal anti-inflammatory drugs are known to treat inflammation and pain. However, their prolonged use often leads to serious side-effects such as gastrointestinal tract dyspepsia, peptic ulceration, hemorrhage and perforation, leading to death in some patients [1]. On the other hand, synthetic antioxidants such as butylated hydroxyanisol and butylated hydroxytoluene, commonly used, are very effective, but they might be mutagenic [2] carcinogenic [3] and even toxic [4]. Therefore, the development and the utilization of safer and more effective anti-inflammatory and antioxidants from natural origin, which have a higher bioavailability and higher protective efficacy than synthetic ones, are desired. Medicinal plants may offer an alternative source for the anti-inflammatory and antioxidant drugs with significant effect against several pathologies [5]. Generally, natural antioxidants from the plant kingdom have been identified as major health beneficial compounds, and medicinal plants are considered as natural sources for alternative medicines. Usually, phytochemicals possess strong antioxidant ability as well as anti-inflammatory action, which are also the basis of other bioactivities and health benefits. In fact, various bioactive compounds from plants were discovered as a new medicinal drug [6].

Santolina chamaecyparissus L. is an aromatic plant belongs to Asteraceae family, widespread in the Mediterranean region. The aerial part of this plant is used in folk medicine for their analgesic, anti-inflammatory, antiseptic, antispasmodic, bactericidal, digestive, and vulnerary properties [7, 8]. The essential oil of this plant is used in perfumery and cosmetics. Phytochemical studies of S. *chamaecyparissus* yielding a number of secondary metabolites such as essential oils [9], flavonoids [10] and coumarins [11]. However, few studies on biological activities of *S. chamaecyparissus* have been reported [12-14]. Therefore, the current study was designed to evaluate the anti-inflammatory and antioxidant activities of aqueous and ethanol extract of the aerial part of this plant by using *in vitro* and *in vivo* tests.

MATERIALS AND METHODS

Chemicals

Croton oil, Lambda-carrageenan, indomethacin, ferrozine [3-(2-pyridyl)-5, 6-bis (4-phenylsulfonicacid)-1,2,4-triazine)], iron(II) chloride ($FeCl_2$), iron(III) chloride ($FeCl_3$), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), potassium ferricyanide [K3Fe(CN)6], ethylenediamine tetra acetic acid (EDTA) were purchased from Sigma (Germany). 1,1-diphenyl-2- picrylhydrazyl (DPPH) was purchased from Fluka (Germany). All other reagents were from Sigma and Fluka (Germany) and were of analytical grade.

Plant material

The aerial part of *S. chamaecyparissus* was harvested during the flowering season in mid-May 2012, from Hammam Essoukhna, Sétif, region in eastern of Algeria. The plant was identified, authenticated taxonomically by Pr. H. Laouer (Laboratory of Botany, University of Setif 1, Algeria) and a voucher specimen (No. S.c. 2009-1) was preserved at the local Herbarium of Botany, Department of Botany, University of Sétif for future reference. The plant was air dried at room temperature and then reduced to powder.

Animals

Swiss Albino mice weighing 20-25g of either sex were obtained from Pasteur Institute of Algiers, Algeria. All animals were kept to acclimatize under the laboratory conditions for one week and were provided with standard rodent diet and water *ad libitum*. Aanimals were randomly selected for different experimental groups (6 animal/group) and fasted overnight prior the experiments. All procedures were performed in accordance with european Union Guidelines for Animals Experimentation (2007/526 /EC).

Preparation of *S. chamaecyparissus* extracts

S. chamaecyparissus aqueous extract (SCA) was prepared according to the traditional method. Briefly, 100 g of the powdered aerial part of the plant was boiled in 1 L of distilled water for 20 min. After filtration, the filtrate collected was centrifugated at 3000 rpm for 10 min. The obtained supernatant was lyophilized to give a pale brown powder (yield: 15.77%).

S. chamaecyparissus ethanol extract (SCE) was prepared by maceration of 100 g of the powdered aerial part of the plant with 80% ethanol at room temperature for 24h, under continuous shaking. After filtration, the filtrate was concentrated under reduced pressure at 40°C. The residue was lyophilized using a lyophilizator (PHYWE chrisa) to give a bright brown powder (yield: 15.07%). Extracts were stored at -32 °C until use.

Croton oil-induced ear edema

Croton-oil induced ear edema was performed according to Manga *et al.* [15] to evaluate the effect of *S. chamaecyparissus* extracts on acute inflammation. Cutaneous inflammation was induced in the inner surface of the right ear of mice (6 mice/group) by application of 15 µl of acetone containing 80 µg of croton oil as irritant. Treated animals received topically 2 mg/ear of *S. chamaecyparissus* extracts or 0.5 mg/ear of indomethacin, used as reference drug. The thickness of ears was measured before and 4 h after induction of inflammation using a dial calliper. The edema was expressed as an increase in the ear thickness due to croton oil application.

Analgesic activity (writhing test)

Analgesic activity of *S. chamaecyparissus* was tested according to Delporte *et al.* [16]. One hour after receiving orally 200 or 400 mg/kg of *S. chamaecyparissus* ethanol and aqueous extracts and indomethacin (10 mg/kg) as reference, mice were intra-peritoneal injected with 0.6% of acetic acid (10 mL/kg body weight). Immediately after the acetic acid injection, the number of abdominal writhes of each mouse was counted for 30 min. The percentage inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \left(\frac{Wc\text{-}Ws}{Wc}\right) \times 100$$

Where Wc is the number of writhes in control mice and Ws is number of writhes in mice treated by test samples.

Air pouch

The air pouches were raised on the dorsum by subcutaneous injection of 3 mL of sterile air, as previously described by Colville-Nash and Lawrence [17]. After 4 days, the pouches were re-inflated with 1,5 mL of sterile air. On the 7th day, mice were injected with 1 mL of extract (1 mg/pouch), indomethacin (0.1 mg/pouch) or sterile saline solution (control) into the pouch 1 h prior to the injection of 0.1 mL carrageenan (1%) under light chloroform anesthesia. The control group received only 0.1 mL of carrageenan suspension. Four hours after the treatment, the mice were sacrificed by cervical dislocation. The pouches were flushed by 1 mL of PBS, pH=7.4, and vigorously massage for 30 sec. The pouches were opened with a small incision and the exudates were collected. The leukocytes in the fluid were counted using hemocytometer coulter (MINDRAY Auto Hematology Analyser).

DPPH free radical scavenging assay

DPPH free radical scavenging activity was determined according to Que *et al.* [18] with some modifications. An aliquot of 1 mL at different concentrations (5-500 µg/mL) of SCA, SCE or BHT was mixed with 1 mL of DPPH$^{\bullet}$ (0.1 mM). The mixture was shaken vigorously and then incubated at room temperature for 30 min in the dark. The absorbance was measured at 517 nm against the blank prepared with methanol. The DPPH$^{\bullet}$ scavenging activity was calculated using the following formula:

$$\text{Scavenging activity (\%)} = \left(\frac{\text{Ac-As}}{\text{Ac}}\right) \times 100$$

Where Ac is the absorbance of control, and As is the absorbance of the samples. The concentration of samples required to reduce 50% of the initial DPPH radicals (IC_{50}) was calculated from linear regression analysis.

Ferrous ions chelating activity

The chelating of ferrous ions by the ethanol and aqueous extracts of *S. chamaecyparissus* was estimated by the method of Le *et al.* [19]. Briefly, the extracts samples (25 - 1500 µg/mL) were added to 50 µl of $FeCl_2$ (0.6 mM). The reaction was initiated by the addition of 50 µl of ferrozine (5 mM), and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the solution was then measured spectrophotometrically at 562 nm against a control without extract. EDTA was used as a reference and the chelating activity was then calculated as follows:

$$\text{Chelating activity (\%)} = \left(\frac{\text{Ac-As}}{\text{Ac}}\right) \times 100$$

Where Ac is the absorbance of the control and As is the absorbance the sample. The concentration of samples required to reach 50% of the chelating activity (IC_{50}) was calculated from linear regression analysis.

Reducing power activity

Reducing power of *S. chamaecyparissus* ethanol and aqueous extracts was determined according to Bougatef *et al.* [20]. In this experiment, all chemical and plant extract were prepared in 0.2 M phosphate buffer (pH 6.6). A volume of 625 µl SCE and SCA, at different concentrations (20-200 µg/mL) was mixed with 625 µl of 1% K_2FeCN_6. The mixture was then incubated at 50°C for 20 min, and the reaction was terminated by the addition of 625 µl of 10% TCA. The reaction medium was centrifugated for 10 min at 2500 rpm. An aliquot of 625 µl of the supernatant was mixed with 625 µl of distilled water and 125 µl of $FeCl_3$ (0.1%). The absorbance was measured at 700 nm. BHT was used as a standard antioxidant. Higher absorbance indicates higher reducing power.

Statistical analysis

Results are expressed as mean ± SEM. Differences between the control and the treatments in these experiments were tested for significance using one way ANOVA followed by Tukey's multiple comparison tests. The differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of *S. chamaecyparissus* extracts on croton oil-induced ear edema

Four hours after the topical application of the irritant agent, the mice in the control group developed an edema at their right ears with a thickness of 137 ± 13 µm. The local treatment of mice by 2 mg of ethanol extract of *S. chamaecyparissus* decreased significantly ($p < 0.001$) the edema compared to the control group mice. The inhibition

exerted by SCA 31% was less than that exerted by SCE (41%). Effect of both extracts was less than that of indomethacin 81% (Figure 1).

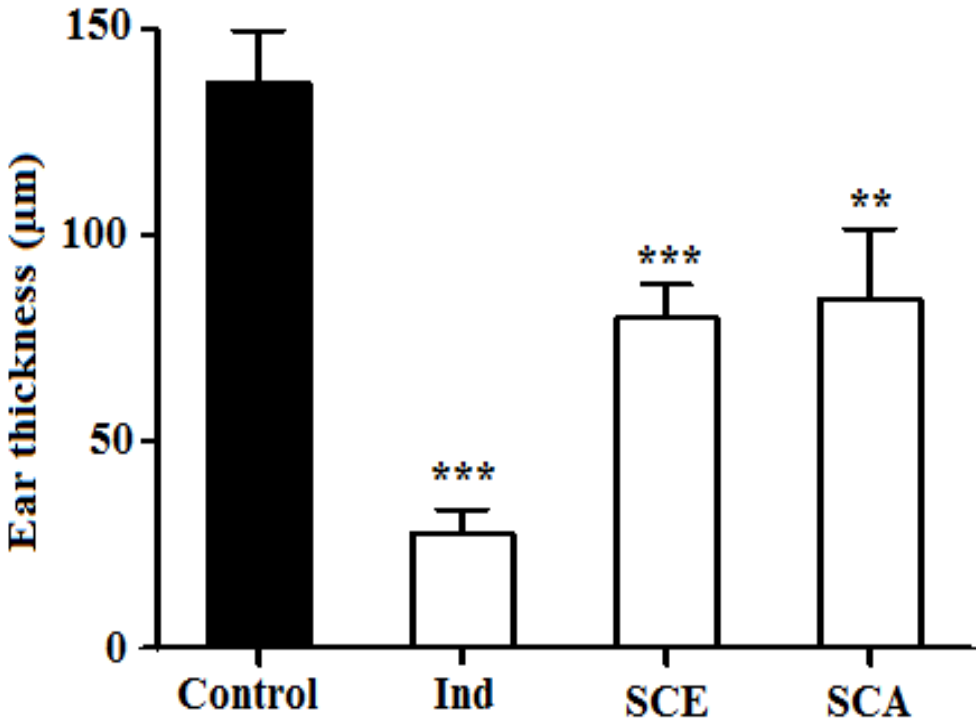

Figure 1. Effect of aqueous (SCA), ethanol (SCE) extracts of *S. chamaecyparissus* and indomethacin (Ind) on croton oil induced-ear edema in mice. Mice were treated with 2 mg/ear of ethanol extract (SCE), aqueous extract (SCA) or 0.5 mg/ear of indomethacin (Ind). Control group received saline solution only. Edema is expressed as mean thickness of ears before and 4 h after croton oil application. Values are expressed as mean ± SEM (n = 6). **: $p < 0.01$, ***: $p < 0.001$ compared to the control.

Effect of *S. chamaecyparissus* extracts on acetic acid-induced writhing

Both extracts of *S.chamaecyparissus* induced a significant ($P < 0.001$) inhibition of acetic acid-induced abdominal constrictions in mice. At 400 mg /kg, ethanol extract was more potent than aqueous extract and indomethacin (10 mg/kg), used as reference (Table 1).

Table 1. Effect of ethanol (SCE), aqueous (SCA) extracts of *S. chamaecyparissus* and indomethacin (Ind) on acetic acid-induced writhing in mice.

Group	Number of writhing	Inhibition (%)
Control	82.13 ± 2.82	/
Ind 10 mg/kg	58.80 ± 4.91 ***	28.40 ± 5.98 ***
SCA 200 mg/kg	67.00 ± 5.44 *	18.42 ± 5.23 *
SCA 400 mg/kg	53.50 ± 2.75 ***	34.86 ± 3.34 ***
SCE 200 mg/kg	61.11 ± 3.34 **	25.59 ± 4.07 ***
SCE 400 mg/kg	50.88 ± 2.98 ***	38.05 ± 3.63 ***

*Data are expressed as mean ±SEM (n = 6). *P < 0.05 , **P <0.01, *** P < 0.001 compared to the control.*

Effect of *S. chamaecyparissus* extracts on air pouch

After 4 h of the injection of 0.1 mL of carrageenan, mice of the control group (untreated) developed an inflammation characterized with infiltration of 8.84 ± 0.54 × 10^6 leukocytes/mL into air pouch exudate. Treatment with 1 mg/pouch of ethanol extract and aqueous extracts of *S. chamaecyparissus* reduced significantly ($P < 0.001$) the number of infiltrating leukocytes to 5.12 ± 0.48 × 10^6 cells/mL and 5.60 ± 0.56 × 10^6 cells/mL of exudates, respectively. These values correspond to 42.08% and 36.67% of inhibition, respectively compared to the control group. The Inhibition value of ethanol extract was close to that obtained with 0.1 mg/pouch of indomethacin (46.45%) (Figure 2).

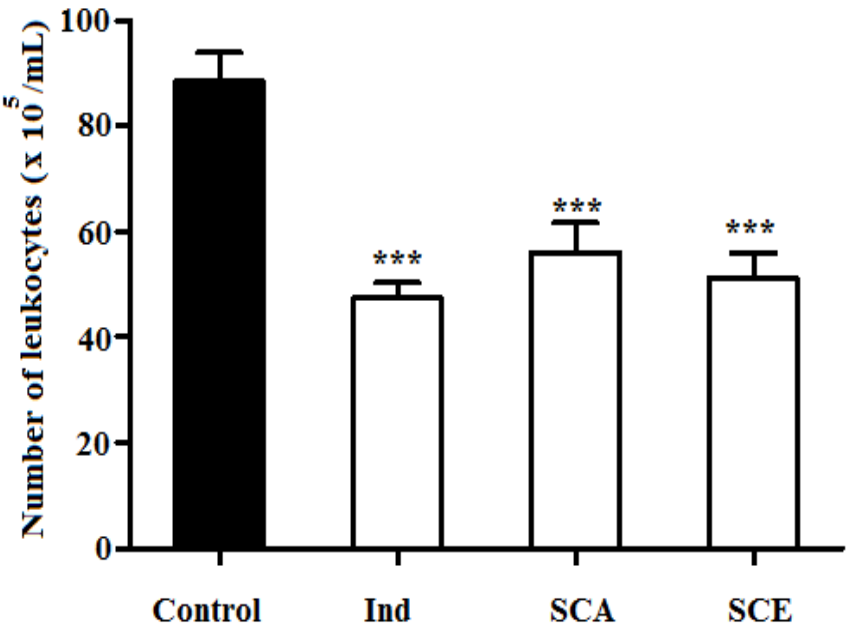

Figure 2. Effect of aqueous extract (SCA) and ethanol extract (SCE) of *S. chamaecyparissus* (1 mg/pouch) on leukocyte migrated into air pouch exudate. The pouch inflammation was induced by 0.1 mL of carrageenan (1%). One hour before the induction of inflammation, mice were treated by 1mg/pouch of the extract or 0.1 mg/pouch of indomethacin (Ind). Values are mean ± SEM (n = 6). *** P <0.001 compared to the control (without treatment).

DPPH free radical scavenging activity

At 80 µg/mL, SCE and SCA extracts showed a significant free radical scavenging activity with 85.71% and 89.95%, respectively (Figure 3). The best free radical scavenging activity was exerted by SCA with IC_{50} = 35.27 ± 3.24 µg/mL flowed by SCE with IC_{50} = 41.06 ± 0.94 µg/mL. These values are better than that obtained with BHT (IC_{50} = 61.69 ± 5.02 µg/mL), used as standard antioxidant.

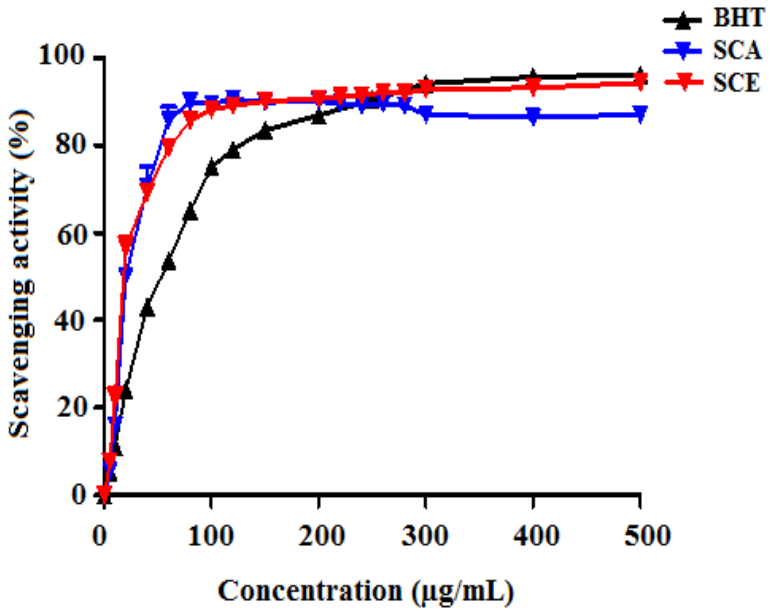

Figure 3. DPPH scavenging activity of aqueous (SCA) and ethanol (SCE) extracts of *S. chamaecyparissus* and butylated hydroxytoluene (BHT). Values are means ± SD (n=3).

Ferrous ions chelating activity

Both extracts of *S. chamaecyparissus* showed concentration-dependent metal ion chelating activity. However, SCA was more potent in chelating ferrous ions (IC_{50} = 73.51 ± 2.94 µg/mL) than SCE (507.43 ± 12.44 µg/mL). The EDTA used as a reference exhibited a potent chelating activity with an IC_{50} value of 5.97 µg/mL (Figure 4).

Reducing power activity

Figure 5 showed that the reducing power exerted by both extracts of *S. chamaecyparissus* was concentration-dependent manner. Ethanol extract exhibited the stronger reducing power (IC_{50} = 49.12 µg/mL) than the aqueous extract (IC_{50} = 57.03 µg/mL). However, BHT used as reference exhibited a potent reducing power with IC_{50} value of 16.10 µg/mL.

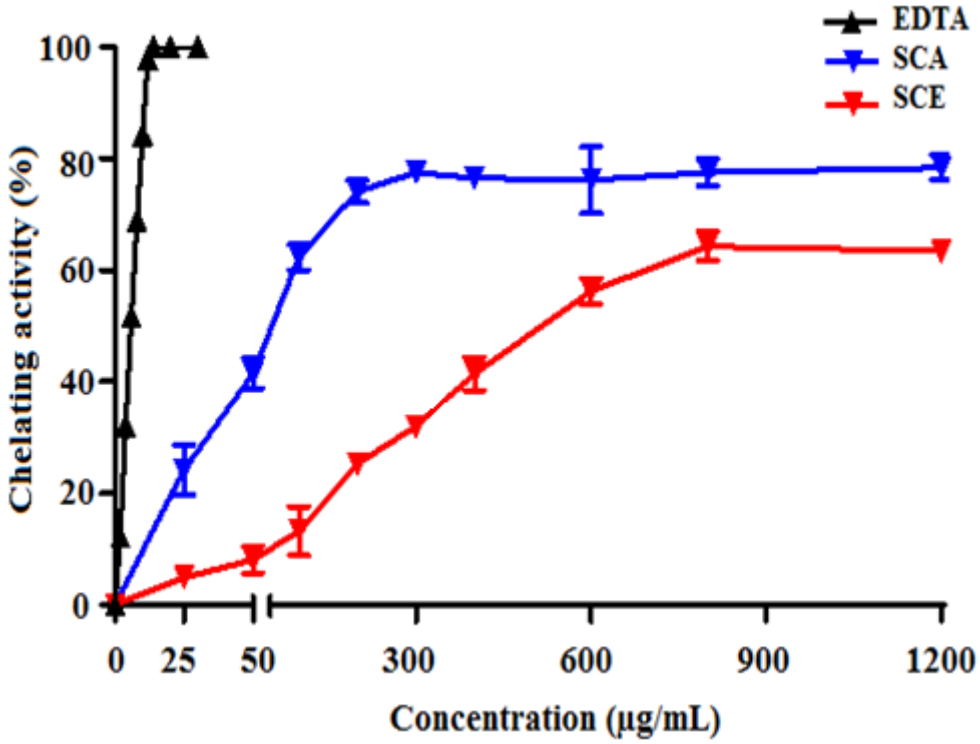

Figure 4. Ferrous iron chelating activity of aqueous (SCA), ethanol (SCE) extracts of *S. chamaecyparissus* and EDTA. Values are mean ± SD (n = 3).

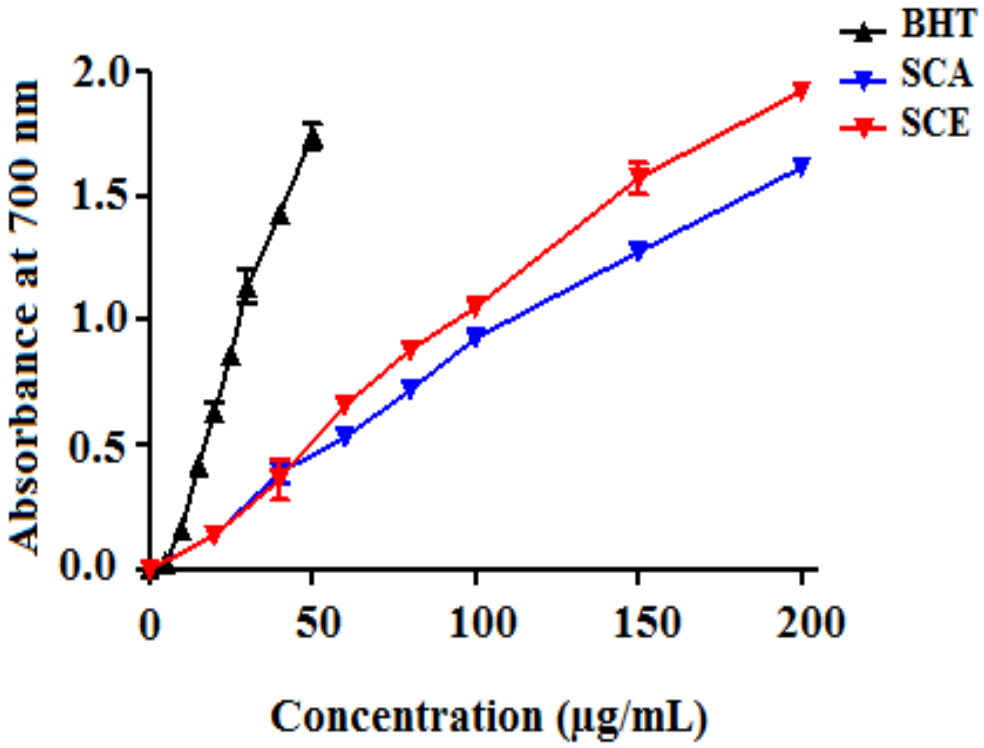

Figure 5. Reducing power of aqueous (SCA) and ethanolic (SCE) extracts of *S. chamaecyparissus* and BHT. Values are mean ± SD (n=3).

DISCUSSION

Despite the progress that has occurred in the development of therapy, there is still a need of effective and potent antioxidant, anti-inflammatory and analgesic drugs. In this regard, it has been widely shown that many plant-derived substances play a relevant role in the process of development of new drugs to treat complaints related with pain [21]. In the present study, the anti-inflammatory and the antioxidant properties of *S. chamaecyparissus* were evaluated.
The overall results showed that *S. chamaecyparissus* ethanol and aqueous extracts exerted a significant anti-inflammatory activity in different models of inflammation used in this study. In croton oil-induced ear edema in mice, edema events are triggered by protein kinase C (PKC), which leads to PLA_2 activation and then the release of a variety of bioactive eicosanoids, which are implicated in the development of inflammatory events [22]. Croton oil contains 12-o-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents. This agent is able to activate PKC, which in turn activates other enzymatic cascades such as cyclooxygenase 2 and inducible nitric oxide synthase [23]. This cascade of events stimulates vascular permeability, vasodilation, leukocyte migration, histamine and serotonin release and activate synthesis of eicosanoids by cyclooxygenase and 5-lipoxygenase enzymes [22]. Moreover, Protein kinase C promotes various immune mediators that increase and maintain the inflammatory response [24]. Local pre-treatment of ear mice with *S. chamaecyparissus* ethanol and aqueous extracts decreased significantly the edema compared to the control group of mice. The inhibition exerted by the ethanol extract was better than that exerted by the aqueous extract, while the effect of both extracts was less than that of the standard anti-inflammatory drug (indomethacin). Indomethacin inhibits cyclooxygenase 1 and 2, therefore the edema formation and the production of the pro-inflammatory mediators such as TNF α, IL-6 and PGE_2 [25]. The activity observed with the two studied extracts is probably due to the presence of active substances, which can cross the skin barrier and exerted their anti-inflammatory effect [15]. Flavonoids and polyphenols are likely candidate for this effect [26, 27].

According to our findings, both extracts of *S. chamaecyparissus* can be considered to be safe and nontoxic. So, these extracts were administered orally to mice, in order to confirm the peripheral mechanism of action and establish any possible central involvement by checking their analgesic activity. Acetic acid has been reported to cause hyperalgesia by liberating endogenous substances such as prostaglandins, leukotrienes, histamine and kinins, which have been implicated in the mediation of pain perception [28]. Both extracts of *S.chamaecyparissus* extracts exhibited a significant analgesic effect as shown by the inhibition of acetic acid induced writhing in mice. At 400 mg/kg, ethanol extract was more potent than aqueous extract and indomethacin, used as reference. The inhibition of acetic acid-induced abdominal constrictions in mice by S. *chamaecyparissus* suggest a peripherally mediated analgesic activity based on the association of the model with stimulation of peripheral receptors, especially the local peritoneal receptors at the surface of cells lining the peritoneal cavity [29, 30].

In order to assess the efficacy of *S. chamaecyparissus* ethanol and aqueous extracts against proliferative phase of inflammation, carrageenan-induced air-pouch was carried out. In this acute inflammatory model, the proliferation of macrophages, neutrophils, and fibroplasts are the sources of inflammation [31]. The results of this study revealed that the treatment with 1 mg/pouch of ethanol extract and aqueous extracts of *S. chamaecyparissus* reduced significantly ($P < 0.001$) the number of infiltrating leukocytes into the air pouch cavity. The Inhibition value of ethanol extract was close to that obtained with 0.1 mg/pouch of indomethacin. This result indicates that the studied extracts may alter the action of endogenous factors that are involved in the migration of neutrophils into inflated site. Moreover, the observed anti-inflammatory effects of *S. chamaecyparissus* extracts may be due also to the presence of antioxidant compounds. In fact, reactive oxygen species generated during inflammation by phagocytic cells and during the metabolism of arachidonic acid can activate the phospholipase A_2, which release more arachidonic acid from phospholipids membrane that subsequently transformed into pro-inflammatory mediators [32]. Antioxidants can reduce inflammation via the inhibition of pro-inflammatory mediators, as well as the increase of the anti-inflammatory mediator production [33, 34]. This suggestion is supported by the obtained antioxidant results. Indeed, both extracts of extracts of *S. chamaecyparissus* exhibited a significant anti-radical, iron chelating and reducing activities.

The scavenging effect of antioxidants on DPPH radical is thought to be due to their hydrogen donating ability [18]. At 80 µg/mL, the two extracts of *S. chamaecyparissus* exhibited a very significant free radical scavenging activity with 85.71% and 89.95%, respectively. This scavenging effect is better than that obtained with BHT, used as standard antioxidant. This antiradical activity of *S. chamaecyparissus* extracts is probably due to polyphenols components known for their antioxidant activity [35, 36]. In fact, there is a close relationship and positive correlation between the phenolic content and antioxidant activity [37]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals by their hydrogen donating ability [38].

Ferrous ions (Fe^{2+}) are able to generate free radicals from peroxides by Fenton reaction and may be involved in the progression of related diseases. Thus, antioxidants capable of chelating Fe^{2+} will minimize the ion's concentration and inhibit its capacity to catalyze free radical formation, resulting in protection against oxidative damages [39]. In this study, the chelating of ferrous ions by *S. chamaecyparissus* extracts was estimated. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted and the red color of the complex is decreased. Measurement of the rate of the color reduction therefore allows estimation of the chelating activity of the coexisting chelators [40]. Both extracts of *S. chamaecyparissus* exhibited a considerable Fe^{2+}-chelating activity at the tested concentration ranges. However, S. *chamaecyparissus* aqueous extract was more potent in chelating ferrous ions than *S. chamaecyparissus* ethanol extract. This result indicates that the aqueous constituents are more able to inhibit the formation of ferrous complex with the reagent ferrozine, suggesting the chelating activity of these extract and the capture of the ferrous ions before ferrozine. It has been reported that chelating agents are effective as secondary antioxidants as they reduce the redox potential, thereby stabilizing the oxidized form of the metal ions [41]. The standard chelator EDTA exhibited a potent chelating activity. This secondary antioxidant activity prevent the generation of OH radicals via the Fenton reaction. Metal ions are largely sequestered *in vivo* but high ferrous ion chelating ability would prevent compounds from aggravating certain metal overload diseases [42].

The reduction of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) is measured by the strength of the green-blue color of solution which absorbs at 700 nm. Ethanol extract exhibited stronger reducing power than the aqueous extract. However, BHT used as reference exhibited the strongest reducing power. The capacity of reducing observed in the extracts is due to the presence of reductones and may serve as an indicator of its potent antioxidant activity. The antioxidant effect of reductones is based on the destruction of the free radical chain by donating a hydrogen atom [38]. Polyphenols which may act in a similar way as reductones react with free radicals to turns them into more stable products and abort free radical chain reactions [43].

CONCLUSION

Results of this study showed that *Santolina chamaecyparissus* extracts exhibit a good anti-inflammatory and antioxidant activities. These properties support the use of this plant to treat inflammatory disorders and oxidative-stress related diseases.

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