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Phenolic content, anti-inflammatory and antioxidant activities of *Anacyclus clavatus* extracts

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Abstract

The anti-inflammatory and the antioxidant activities of methanol (ME) and aqueous (AE) extracts of *Anacyclus clavatus* were evaluated. Phenolic constituents in the extracts were screened. Croton oil-induced ear edema in mice, carrageenan-induced paw edema and pleurisy in rats were evoked. The antioxidant effect was tested by 1,1-diphenyl-2-picrylhydrazyl, ion chelating, lipid peroxidation tests. Both extracts are rich in phenolic compounds. The application of 2 mg per ear of ME or AE inhibited ear edema by 84 and 83 %, respectively. The oral treatment of rats with 200 or 400 mg kg^{-1} of ME reduced paw edema by 64 and 74%, respectively, whereas the inhibition by AE was by 65 and 80%, respectively. At 400 mg kg^{-1}, the extracts decreased exudation and neutrophil migration into the pleural cavity by 64 and 66%, respectively, while the inhibition by AE was 42 and 55%, respectively. On the other hand, ME exerted scavenging activity higher than AE, while the AE chelating activity was more than that of ME. However, both extracts had similar inhibitory effect on lipid peroxidation. In general, *A. clavatus* may be used as a source of anti-inflammatory and anti-oxidant agents.

Key words: *Anacyclus clavatus*, anti-inflammatory activity, antioxidant activity, phenolic compounds.
Abbreviations: AE, aqueous extract; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ME, methanol extract.

Introduction

An imbalance between oxidants and antioxidants leads to oxidative damage to biological molecules, including lipids, proteins and nucleic acids. This damage is responsible for the pathogenesis of several human diseases, such as neurodegenerative diseases, lung diseases, cardiovascular diseases, diabetes, and atherosclerosis (Rahman et al. 2012). Moreover, when overproduced, reactive oxygen species attack tissue and then provoke an inflammatory response by production of pro-inflammatory mediators and chemotactic factors, which amplify the inflammation, resulting in chronic inflammation (Mittal et al. 2014). In fact, chronic inflammation is another important factor that may cause or contribute in the pathogenesis of many diseases. Thus, scavenging reactive oxygen species and suppressing their formation, either by inhibiting enzymes or by chelating trace elements involved in free radical production, are thought to be an effective means to depress the level of oxidative stress of organisms.

Usually, phytochemicals possess strong antioxidant ability as well as anti-inflammatory action, which are also the basis of other bioactivities and health benefits. Antioxidants can reduce inflammation via the inhibition of pro-inflammatory mediators, as well as the increase of the anti-inflammatory mediator production (Costa et al. 2013; Moura et al. 2015).

Medicinal plants may offer an alternative source for the anti-inflammatory and antioxidant drugs, and have significant effect against several pathologies. Various bioactive compounds from plants were discovered as a new medicinal drug (Lahlou 2013).

Anacyclus clavatus Pers. is an aromatic medicinal plant belonging to the Asteraceae family. It is widely distributed in the Mediterranean countries and known for its medicinal properties. In Algerian traditional medicine, the aerial parts of this plant are used to dispel gas and prevent bloating. Its leaves and stems are used as digestive herbal tea and as a traditional remedy against digestive disorders (Benitez, Gonzalez-Tejero 2010).

Phytochemical analysis revealed the presence of essential oils in the leaves/stems and flower extracts of *A. clavatus* (Aliboudhar, Tigrine-Kordjani 2013). Some of these compounds exhibit antibacterial activity (Hammami et al. 2013), and antitumour activity (Yi-Qun et al. 2008). Few studies have been reported that have shown the anti-inflammatory activity of *A. clavatus*. The current study was designed to evaluate anti-inflammatory activity and antioxidative capacity of different extracts of *A. clavatus* by using a series of *in vivo* and *in vitro* tests.

Materials and methods

Chemicals

Indomethacin, ferrosine [3-(2-pyridyl)-5, 6-bis(4-phenyl-sulfonicacid)-1,2,4-triazine)], $FeCl_2$, $FeCl_3$, EDTA, trichloroacetic acid, potassium thiocyanate, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), folin-ciocalteu, gallic acid, tannic acid, quercetin, $K_3Fe(CN)_6$], Na_2CO_3, Tween 20, λ-carrageenin, carboxymethyl cellulose, aspirin and croton oil were purchased from Sigma (Germany). Linoleic acid and 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene; BHT) was obtained from Fluka (France). All other reagents were of analytical grade and supplied from Panreac (Spain), Riedel-de Haen, Prolabo and Sigma (Germany).

Plant material

The plant *Anacyclus clavatus* was collected in May 2012 from Bougaa area in Algeria. The plant was identified and authenticated taxonomically. A voucher specimen (No. A.C. 2012-1) was preserved for future reference at the local Herbarium of Botany, Department of Botany, University of Sétif 1. The aerial part was air-dried at room temperature, then reduced to powder and stored away from light until use.

Animals

Swiss albino mice weighing 25 to 30 g and Albino Wistar rats weighing 170 to 210 g of either sex were obtained from the 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. Animals were randomly selected for different experimental groups (six animals per group) and fasted overnight prior to the experiments. All procedures were performed in accordance with European Union Guidlines for Animal Experimentation (2007/526 /EC).

Preparation of Anacyclus clavatus *extracts*

Methanol extract (ME) of *A. clavatus* was obtained by maceration of the powdered plant (10 g 100 mL^{-1}) with 80% methanol for 24 h under continuous shaking at room temperature. After filtration, the filtrate was concentrated under reduced pressure at 40 °C. The residue was lyophilized to give a dark brown powder (yield 19%).

Aqueous extract (AE) of *A. clavatus* was prepared by boiling 50 g of powdered plant in 500 mL distilled water for 20 min, followed by filtration and centrifugation for 10 min. The supernatant obtained was lyophilized to give a dark brown powder (yield 17%). Both extracts were stored at –32 °C until use.

Determination of total polyphenol concentration

The concentration of total phenolics in the extracts was determined according to a modified method of Li et al. (2007). Briefly, a volume of 100 mL of various concentrations of extracts solutions was added to 500 mL of Folin-Ciolcalteu (10%). After 4 min, 400 mL of 7.5% Na_2CO_3 was added. The mixture was shaken for 2 h at room temperature and the absorbance was recorded at 765 nm. Gallic acid was used as a standard. The concentration of total phenolic compounds in the extracts was determined as mg of gallic acid equivalent per 1 g of extract (mg GAE g^{-1} extract).

Determination of flavonoid concentration

Total flavonoid concentration in the extracts was determined according to Bahorun et al. (1996). Briefly, 1 mL of 2% $AlCl_3$ in ethanol was added to 1 mL of the extracts (2 mg mL^{-1}). After 10 min of incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used as a standard. Total flavonoid content was expressed as mg of quercetin equivalent per 1 g of extract (mg QE g^{-1} extract).

Determination of tannin concentration

Tannin concentration was determined using the hemoglobin precipitation assay according to Hagerman, Butler (1989). Tannic acid was used as standard. An aliquot of 0.5 mL of each extract was mixed with 0.5 mL of hemolyzed bovine blood. The mixture was reacted for 20 min at room temperature, and then subjected to centrifugation at 4000 rpm for 10 min at 4 °C. The absorbance was measured at 576 nm and tannic content was expressed as mg tannic acid equivalent per g of extract (TAE g^{-1} extract).

HPLC-TOF/MS analysis

HPLC-TOF/MS analysis of *A. clavatus* extracts was carried out as described elsewhere (Abay et al. 2015). This HPLC method was developed and validated to analyze phenolic acids and flavonoids in the plant extracts. An Agilent Technology of 1260 Infinity HPLC System was coupled with a 6210 Time of Flight (TOF) LC/MS detector and ZORBAX SB-C18 (4.6 × 100 mm, 3.5 µm) column. Mobile phases A and B were ultra-pure water with 0.1% formic acid and acetonitrile, respectively. Flow rate was 0.6 mL min^{-1} and column temperature was 35 °C. Injection volume was 10 µL. The solvent program was as follow: 0 to 1 min 10% B; 1 to 20 min 50% B; 20 to 23 min 80% B; 23 to 25 min 10% B; 25 to 30 min 10% B. Ionization mode of the HPLC-TOF/MS instrument was negative and operated with a nitrogen gas temperature of 325 °C, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi, capillary voltage of 4000 V and finally, fragmentor voltage of 175 V. For sample analysis, dried crude extracts (200 ppm) were dissolved in methanol at room temperature. Samples were filtered passing through a PTFE (0.45 µm) filter by an injector to remove particulates.

Croton oil induced ear edema in mice

Croton-oil induced ear edema was evoked according to

Manga et al. (2004). Cutaneous inflammation was induced in the inner surface of the right ear of mice (6 mice per group) by application of 15 µL acetone containing 80 µg croton oil as an irritant. Treated animals received topically 2 mg per ear of methanol, aqueous extract of *A. clavatus* (disolved in acetone/water) or 0.5 mg per ear of indomethacin, used as reference drug. The thickness of ears was measured before and 6 h after the induction of inflammation using a dial calliper. The edema was expressed as an increase in the ear thickness due to croton oil application.

Carrageenan induced paw edema in rats

Paw edema was induced by injecting 0.1 mL of 1% λ-carrageenan into the subplantar region of the right hind paw of rats (Winter et al., 1962). One hour before carrageenan injection, rats received orally 200 and 400 mg kg^{-1} of *A. clavatus* methanol, aqueous extract (prepared in saline solution) or 200 mg kg^{-1} aspirin (suspended in CMC 1%). Rats of control group were injected with 0.1 mL λ-carrageenan and received orally only the vehicle before the injection. To assess the edema the injected paw was measured using a plethysmometer (UGO Basile, Varese, Italy) initially (V_0) and 1, 2, 3, 4, 5, and 6 h after carrageenan injection (V_t). Inflammation was calculated as the increase in volume of the paw after treatment subtracted from the basal volume. Results were expressed as percentage of inhibition of edema, calculated according to the following equation:

$$\% \text{ inhibition} = [(V_t - V_0) \text{ control} - (V_t - V_0) \text{ treated}] / (V_t - V_0) \text{ control}] \times 100.$$

Carrageenan-induced pleurisy in rat

The carrageenan-induced pleurisy in rats was assessed according to Cuzzocrea et al. (1998). Treated rats (six rats per group) were administered orally 2 mL (400 mg kg^{-1}) of methanol or aqueous extract of *A. clavatus* (prepared in saline solution), one hour before the intra-pleural injection of 0.2 mL of the λ-carrageenan 1%. Rats of the untreated control group were treated orally with 2 mL of saline solution. Animals were lightly anaesthetized with chloroform and submitted to a skin incision at the level of the left sixth inter-costal space. The underlying muscle was dissected and saline solution (0.2 mL) containing 1% λ -carrageenan (0.2 mL) was injected into the pleural cavity. For rats of the negative control group, 0.2 mL of sterile 0.9% NaCl instead of the λ-carrageenan solution was injected in their pleural cavity and they were not treated with any other substance. The skin incision was closed with a suture and the animals were allowed to recover. Four hours after the injection of λ-carrageenan, rats were killed and their chests were carefully opened, and the pleural cavity is subsequently washed with 2 mL of heparinized saline solution. The exudate and washing solution were removed by aspiration and the total volume was measured. Any exudate, contaminated with blood was discarded. The amount of exudates was calculated by subtracting the volume injected (2 mL) from the total volume recovered. The leukocytes in the exudate were suspended in PBS and counted with an optical microscope after vital Trypan blue staining.

DPPH radical scavenging assay

The free radical scavenging activity of *A. clavatus* methanol and aqueous extracts was measured using 1,1-diphenyl-2-picryl-hydrazil (DPPH), according to the method described by Que et al. (2006) with slight modifications. A volume of 500 mL of the DPPH solution (0.1 mM) was added to 500 mL of extract solutions or standard (BHT) at different concentrations, the mixture was incubated in the dark for 30 min at room temperature, and then the absorbance was recorded at 517 nm. The percentage of DPPH scavenging activity was calculated by the following equation:

$$\text{Scavenging activity (\%)} = [(A_c - A_t) / A_c] \times 100,$$

where A_c is the absorbance of the control (in the absence of the abstracts) and A_t is the absorbance of the test sample (in the presence of the extracts).

Metal chelating activity

The chelating of ferrous ions by methanol and aqueous extracts of *A. clavatus* was estimated by the method of Li et al. (2007). Briefly, extract samples at different concentrations were added to a solution of 0.6 mmol L^{-1} $FeCl_2$ (50 µL). 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. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated. EDTA was used as a reference. The percentage of chelating activity was calculated using the following equation:

$$\text{Chelating activity (\%)} = [(A_c - A_t) / A_c] \times 100,$$

where A_c is the absorbance of control (in the absence of the abstracts) and A_t is the absorbance of the test sample.

Total antioxidant activity

The total antioxidant activity of methanolic and aqueous extracts of *A. clavatus* was determined according to the thiocyanate method using a linoleic acid system (Gulcin et al. 2005) with slight modifications. The linoleic acid emulsion was prepared by mixing 0.028 g linoleic acid, 0.028 g Tween 20 and 10 mL phosphate buffer (0.04 M, pH 7.0). The mixture was then homogenized, and 0.6 mL phosphate buffer containing 0.6 µL plant extract and BHT (50 µg mL^{-1}) were mixed with 0.6 ml of linoleic acid emulsion. The mixed solutions were incubated at 25 °C in the dark for 96 h. The peroxide level was determined after reaction of 20 µL of sample solution with 20 µL of $FeCl_2$ (20 mM in 3.5% HCl) and 20 µL thiocyanate (30%). The absorbance of each mixture was determined at 500 nm after 3 min of reaction and every 24 h for 96 h. During linoleic acid oxidation, the peroxides formed oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} form a complex with SCN^- and this complex

Table 1. Polyhenols, tannins and flavonoids content of *A. clavatus* methanol extract (ME) and aqueous extract (AE). Values are mean ± SD (n = 3). GAE, gallic acid equivalents; QE, quercetin equivalents; TAE, tannic acid equivalents

Extract	Polyphenols (mg GAE g^{-1} extract)	Flavonoids (mg QE g^{-1} extract)	Tannins (mg TAE g^{-1} extract)
ME	131.30 ± 6.88	9.96 ± 0.43	39.21 ± 6.55
AE	79.06 ± 3.24	16.39 ± 1.38	31.14 ± 2.27

has a maximum absorbance at 500 nm. Therefore, high absorbance indicated high linoleic acid oxidation. The percent inhibition of lipid peroxidation was calculated according to the following equation:

Peroxidation inhibition (%) = $[(A_c - A_t) / A_c] \times 100$,

where A_c is the absorbance of control (in the absence of the abstracts) and A_t is the absorbance of the test sample.

Statistical analysis

Results were expressed as mean ± SD *in vitro* and as mean ± SE *in vivo*. The significance of differences between control and the various tests was determined by an ANOVA test followed by a Dunnett/Tukey test for multiple comparisons. The differences were considered statistically significant at $p \leq 0.05$.

Results

Polyphenol, flavonoid and tannin content

Table 1 shows that methanol extract of *A. clavatus* was richer in polyphenols and tannins than the aqueous extract, while the aqueous extract was richer in flavonoids than methanol extract.

HPLC-TOF/MS analysis

HPLC-TOF/MS analysis revealed the presence of phenolic acids and flavonoids in the *A. clavatus* extracts (Table 2 and Fig. 1). However, methanol extract was richer than the aqueous one. Chlorogenic acid was detected as major constituent (1839.77 mg kg^{-1} of plant) of ME, while apigetrin was detected as a major flavonoid (505.74 mg kg^{-1} of plant) in this extract. Moreover this extract contained high amounts of apegenin, diosmin and quercetin-3-β-D-glucoside. Several compounds (caffeic acid, rutin, quercetin-3-β-D-glucoside, sinapic acid, polydatine and apigenin) were detected as traces in AE.

Effect of Anacyclus clavatus *extracts on croton oil-induced ear edema in mice*

The mice in the control group that received only the croton oil solution developed after 6 hours an edema characterized by an increased thickness (94 ± 9.7 μm). The treatment with 2 mg per ear of the methanolic extract induced a very significant reduction ($p < 0.001$) of inflammation compared to the control mice group. The increase in thickness after 6 hours was 20 ± 8.3 μM, which corresponded to an inhibition of 84%. The aqueous extract exerted almost the same effect as the methanol extract (83%) (Fig. 2). This inhibition was better than that exerted by 0.5 mg per ear of indomethacin (70%), used as a reference anti-inflammatory agent.

Effect of Anacyclus clavatus *extracts on carrageenan-induced paw edema*

The subplantar injection of carrageenan in the control group caused edema with a maximum volume (78%) after 4 to 6 h of injection. The oral pretreatment of rats by extracts of

Table 2. Phenolic compounds in methanol and aqueous extracts of *A. clavatus* determined by HPLC-TOF/MS. RT, retention time; –, trace

Coumpound	RT	ME mg phenolic kg^{-1} plant	AE mg phenolic kg^{-1} plant
Gentisic acid	4.39	24.60	23.69
Chlorogenic acid	6.03	1839.77	180.17
4-hydroxybenzoic acid	6.59	41.75	17.65
Protocatechuic acid	6.96	26.64	22.34
Caffeic acid	7.68	21.41	–
Syringic acid	8.04	28.06	24.45
Vanillic acid	8.69	12.99	10.30
Rutin	9.51	0.92	–
Quercetin-3-β-D-glucoside	10.03	51.98	–
Naringin	10.60	4.59	0.07
Sinapic acid	10.61	18.46	–
Polydatine	10.76	4,29	–
Diosmin	11.04	128.99	43.05
Apigetrin	11.31	505.74	26.50
Cinnamic acid	15.40	–	12.21
Apigenin	16.48	173.75	–

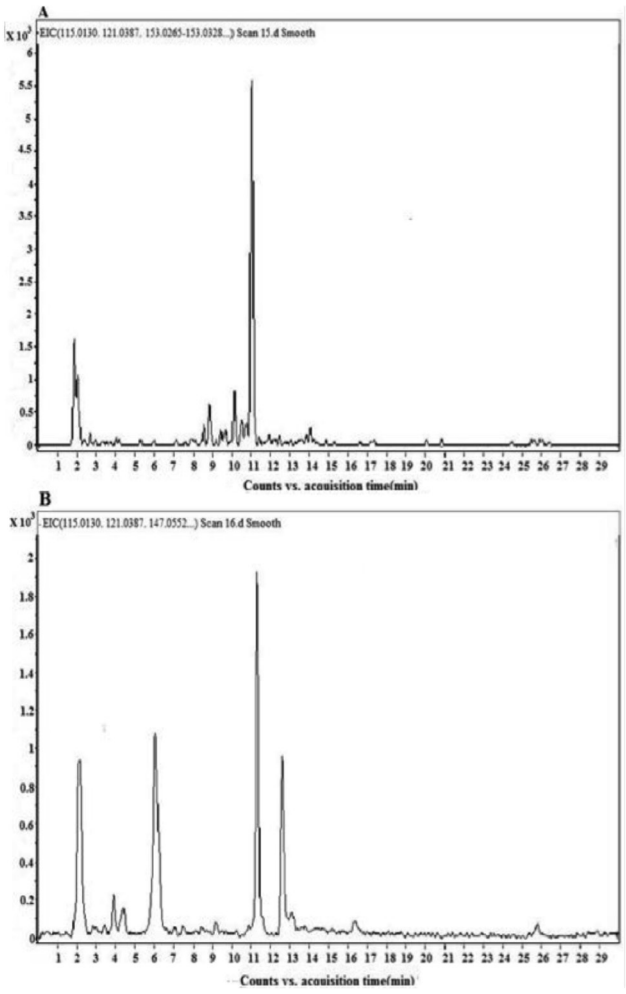

Fig. 1. HPLC chromatograms of phenolic compounds in *A. clavatus* methanol (A) and aqueous (B) extracts..

A. clavatus or aspirin significantly prevented the formation of edema. Indeed, the treatment of rats with 200 or 400 mg kg^{-1} of methanol extract inhibited the edema after 6 h by 64 and 74%, respectively. At 400 mg kg^{-1} the inhibition was close to that of aspirin, which was used as a standard anti-inflammatory agent. At the same doses, the aqueous extract exerted an anti-edematous effect with inhibition rates of 65 and 80%, respectively (Fig. 3).

Effect of Anacyclus clavatus *extracts on carrageenan-induced pleurisy in rats*

The rats of the control group, which received orally saline solution, have developed after 4 h an acute pleurisy characterized by an exudate volume of 0.55 ± 0.07 mL (Fig. 4A). This exudate contained 26.70 ± 1.13 × 10^6 PMNs (Fig. 4B). The pretreatment with 400 mg kg^{-1} of ME and AE decreased significantly the development of the pleurisy. Indeed, the methanol extract inhibited the exudation and the polymorphonuclear leukocyte migration by 64 and 66%, respectively, compared with rats of the control group. At the same dose, aqueous extract inhibited the exudation

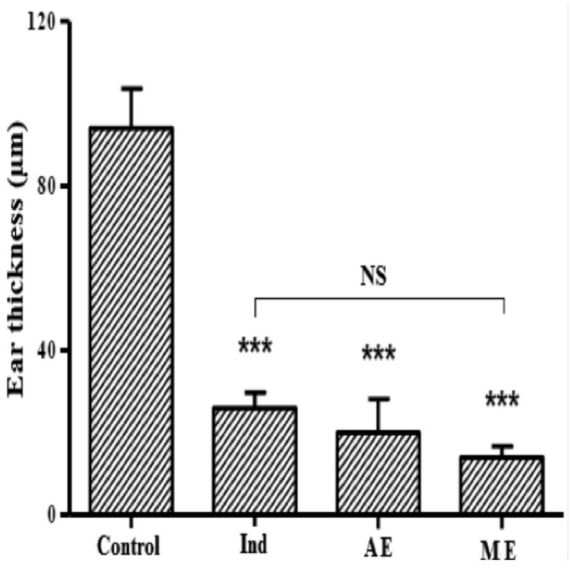

Fig. 2. Effect of *A. clavatus* extracts on croton oil-induced ear edema in mice. Mice were treated with 2 mg per ear of methanolic extract (ME), aqueous extract (AE) or 0.5 mg per ear of indomethacin (Ind). Control group received only sterile saline solution. Edema is expressed as mean thickness of ears before and 6 h after croton oil application. Values are expressed as means ± SE ($n = 6$). ***, $p < 0.001$ vs control, NS, not significant.

by 42% and the number of migrating polymorphonuclear leukocytes into the exudates by 55%. This inhibitory activity was less than that observed with aspirin at 200 mg kg^{-1},

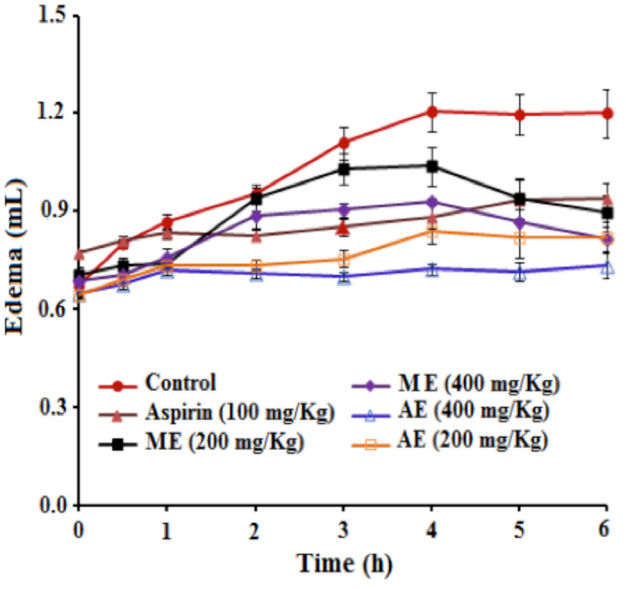

Fig. 3. Effect of *A. clavatus* extracts on carrageenan-induced paw edema in rat. The edema was induced by sub-plantar injection of 0.1 mL of carrageenan 1% in the rat pre-treated orally with 200 mg kg^{-1} and 400 mg kg^{-1} of methanolic extract (ME), aqueous extract (AE) and 100 mg kg^{-1} body weight of aspirin. The control received only the saline solution. Each value represents the increase in volume of the injected paw at different times after injection of carrageenan. Values are means ± SE ($n = 6$).

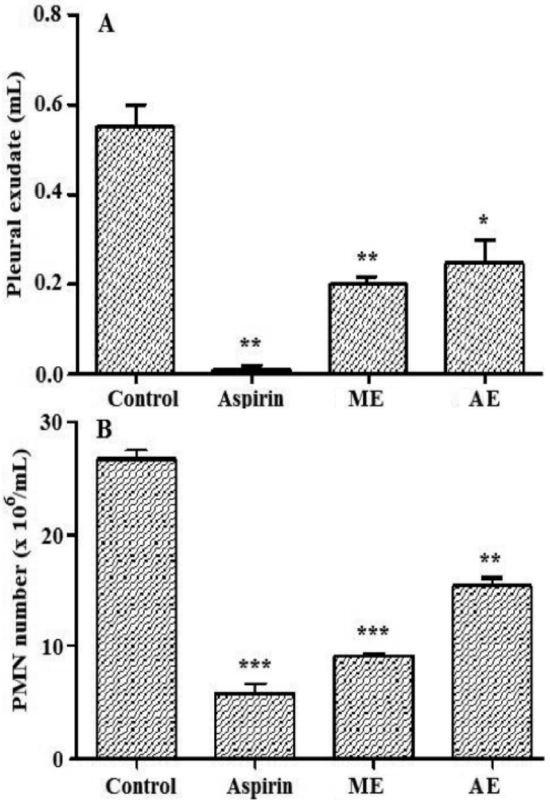

Fig. 4. Effect of *A. clavatus* extraxts on λ-carrageenan-induced pleurisy in rats. Rats were pretreated orally with 400 mg kg^{-1} methanol (ME), aqueous (AE) extracts or 200 mg kg^{-1} aspirin. The control was pretreated with normal saline solution and then injected by λ-carrageenan. A, volume of exudate aspirated from the pleural cavity 4 h after the injection of λ-carrageenan. B, number of polymorphonuclear leukocytes (PMNs) migrated into exudates 4 h after the injection of λ-carrageenan. Results are expressed as mean ± SE (n = 6). ***p < 0.001; **p < 0.01 vs the control. .

which decreased the exudation by 98% and the number of polymorphonuclear leukocytes by 78% (Fig. 4 A,B).

DPPH free radical scavenging activity

A. clavatus aqueous and methanol extracts showed a concentration-dependent anti-radical activity (Fig 5). At 100 μg mL^{-1}, methanol extract exerted maximum activity (90%). This effect was better than that of the standard BHT, which in turn was better than that of aqueous extract. The IC_{50} of ME, AE and BHT was 28.30 ± 3.45, 68.98 ± 1.64 and 44.36 ± 3.10, respectively.

Metal chelating activity

Results showed that both extracts of *A. clavatus* exerted chelating activity. However, the chelating activity of aqueous extract was better than that of the methanol extract. Indeed, at 300 μg mL^{-1}, AE exerted 88% inhibition, whereas ME reached this percentage of inhibition only at 600 μg mL^{-1} (Fig. 6). EDTA used as a standard chelator exerted high chelation activity (99%) at only 14 μg mL^{-1}.

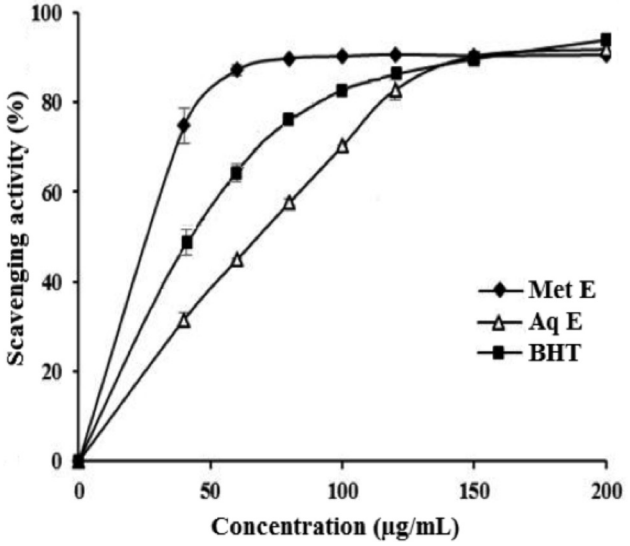

Fig. 5. Free radical scavenging activity of methanolic extract (ME), aqueous extract (AE) of *A. clavatus* and butylated hydroxytoluene (BHT). Values are means ± SD (n = 3)..

The IC_{50} obtained with AE, ME and EDTA was 74.64 ± 11.68, 152.93 ± 1.67, and 5.97 ± 0.20, respectively.

Total antioxidant activity

Fig. 7 shows the kinetics of linoleic acid peroxidation in the presence and absence of ME, AE and BHT. The absorbance of the samples was stable throughout the 96 h of incubation, indicating strong antioxidant activity compared to the control. At 50 μg mL^{-1}, ME and AE inhibited lipid peroxidation by 75%. This value is statistically similar to that of BHT. At the same concentration, BHT exerted inhibition of 77% (Fig. 7).

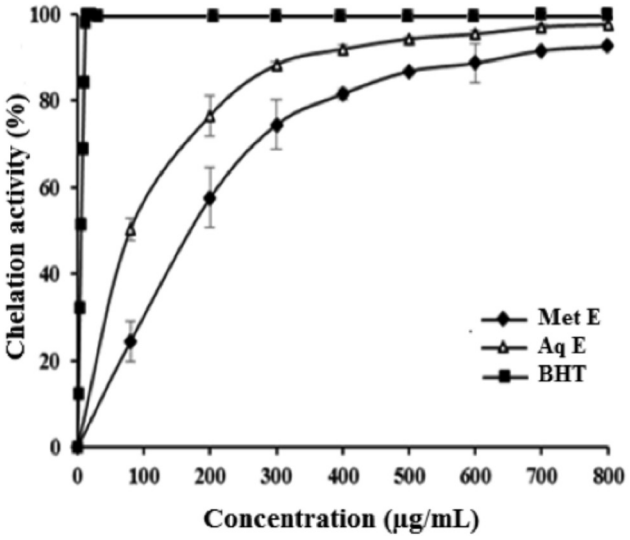

Fig. 6. Ferrous ion chelating activity of methanolic extract (ME), aqueous extract (AE) of *A. clavatus* and EDTA. Values are means ± SD (n = 3)..

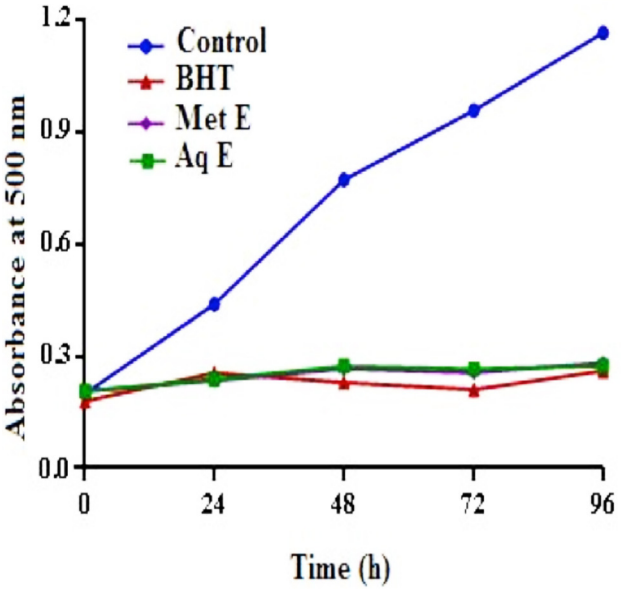

Fig. 7. Kinetics of the inhibition of lipid peroxidation by methanolic extract (ME), aqueous extract (AE) of *A. clavatus* and butylated hydroxytoluene (BHT). Values are means ± SD (n = 3).

Discussion

Anti-inflammatory drugs and synthetic antioxidants are often associated with several adverse effects on human health, such as gastrointestinal ulcers and cardiovascular risk (Al-Saeed 2011), and liver damage and carcinogenesis (Gulcin et al. 2005). Therefore, the development of alternative anti-inflammatory agents and antioxidants mainly from natural sources has attracted considerable attention. Medicinal plants may offer a safer and an effective alternative treatment for inflammatory and oxidative-stress related diseases. In this context, the anti-inflammatory and the antioxidant properties of *Anacyclus clavatus* were investigated.

To study the inflammatory processes, a croton oil-induced ear edema experimental model is wide used. In this model, edema events are triggered by protein kinase C, which leads to phospholipase A_2 activation and then the release of a variety of bioactive eicosanoids, which are implicated in the development of inflammatory events (Cuzzocrea 1998). Also, protein kinase C promotes various immune mediators such as cytokines and chemokines, which increase and maintain the inflammatory response (Kim et al. 2013). Croton oil is able to activate protein kinase C, which in turn activates other enzymatic cascades, such as cyclooxygenase 2 and inducible nitric oxide synthase (Aquila et al. 2009). This cascade of events stimulates vascular permeability, vasodilation, polymorphonuclear leukocyte migration, histamine and serotonin release and activates synthesis of eicosanoids by cyclooxygenase and 5-lipoxygenase enzymes (Cuzzocrea 1998). Local pre-treatment of ear mice with *A. clavatus* methanol and aqueous extracts reduced significantly the size of the ear edema (Fig. 2). This effect was better than that exerted by indomethacin, used as standard anti-inflammatory drug to inhibit cyclooxygenase 1 and 2, the formation of exudate and the production of the pro-inflammatory mediators such as TNF α, IL-6 and PGE2 (Bidaut-Russell 2008). The activity observed with the studied extracts is probably due to the presence of active substances that can cross the skin barrier and exert anti-inflammatory effect (Manga et al. 2004). Flavonoids and polyphenols are likely candidates for this effect (Gonzalez et al. 2011; Zhong et al., 2012).

In the carrageenan-induced paw edema model, the subcutaneous injection of carrageenan into the rat paw produced plasma exudation associated with the migration of neutrophils into the inflated site and increased arachidonic acid product release (Cuzocrea 1998). Oral administration of *A. clavatus* extracts elicited a significant reduction of paw edema formation at all assessment times (Fig. 3), which indicated that these extracts contain compounds that may act as anti-inflammatory agents by inhibiting the release the pro-inflammatory mediators. In fact, Romier-Crouzet et al. (2009) reported the inhibition of inflammatory mediators by polyphenolic plant extracts.

Moreover, oral administration of *A. clavatus* extracts significantly attenuated the development of pleurisy (Fig. 4 A,B), by inhibiting plasma exudation as well as leukocyte recruitment to the inflated site after 4 h following the induction of pleurisy by λ-carrageenan. This result may attribute to the phyto-constituents of the extracts that are able to reduce the production of mediators involved in the development of the acute inflammatory reaction. Indeed, phytochemical screening showed that both studied extracts are rich in flavonoids, polyphenols and tannins. These compounds are good inhibitors of serotonin, histamine and leukocyte migration (Middleton et al. 2000). Flavonoids have been found to have anti-inflammatory activity in both proliferative and exudative inflammation phases, and they inhibit histamine, cytokine, prostaglandin and leukotriene release (Park et al. 2008; Rathee et al. 2009). Furthermore, phenolic compounds are a very effective treatment against inflammatory disorders (Gonzalez 2011).

The observed anti-inflammatory effects of *A. clavatus* 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 releases more arachidonic acid from the phospholipid membrane, which is subsequently transformed into pro-inflammatory prostaglandins and leukotrienes (Geronikaki, Gavalas, 2006). This suggestion is supported by the obtained antioxidant results. Indeed, both extracts of extracts of *A. clavatus* exhibited a significant anti-radical, iron chelating, and anti-lipid peroxidation activity.

The DPPH radical scavenging activity of the two extracts

was concentration-dependent (Fig. 5). Methanolic extract, which showed the highest content of phenolic compounds exhibited the highest scavenging activity (90%). There is a close positive correlation between phenolic content and antioxidant activity (Zhao et al. 2014). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralising free radicals by their hydrogen donating ability (Prasad et al. 2010).

The metal chelating activity is based on chelating of Fe^{2+} ions by the reagent ferrozine, which leads to the formation of ferrozine-Fe^{2+} ions complex (Dinis et al. 1994). *A. clavatus* aqueous extract exhibited a chelating activity higher than the methanol extract (Fig. 6). 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 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 (Gulcin et al. 2007).

Lipid peroxidation is proceeded by radical-mediated abstraction of the hydrogen atom from a methylene carbon in a polyunsaturated fatty acid side chain (Yin et al. 2011), and the inhibitory effects on lipid peroxidation and autoxidation of linoleic acid have been attributed to the radical scavenging activity (Bajpa et al. 2014). Anacyclus clavatus extracts were similarly able to reduce linoleic acid peroxidation (Fig. 7). This ability to modify lipid peroxidation is linked not only to the structural characteristics of the antioxidants agents but also to their ability to interact with and penetrate the lipid bilayer (Salcedo et al. 2014). It has been shown that the structure and the lipophilicity of polyphenols are determinant factors of antioxidant properties of these compounds in the lipid layer of the membrane (Djeridane et al. 20010). Phenolic compounds are the main class of natural antioxidants and there is a close positive correlation between the phenolic content and antioxidant activity of plant extracts (Zhao et al. 2014).

In conclusion, *Anacyclus clavatus* extracts exhibit anti-inflammatory and antioxidant activities, and phenolic constituents could be responsible for these activities. This plant may be considered as new promising source for functional foods and pharmaceuticals.

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