Toxicon 65 (2013) 34–40

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Viper venom induced inflammation with *Montivipera xanthina* (Gray, 1849) and the anti-snake venom activities of *Artemisia absinthium* L. in rat

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ARTICLE INFO

Article history:
Received 23 July 2012
Received in revised form 12 December 2012
Accepted 18 December 2012
Available online 17 January 2013

Keywords:
Artemisia absinthium
Anti-inflammatory activity
Montivipera xanthina
Snake venom
Carreganen

ABSTRACT

The present study was conducted to explore the characterization of *Montivipera xanthina* crude venom partially by *in vitro* and *in vivo* and the anti-snake venom activities of *Artemisia absinthium* L. in comparison with carrageenan-induced acute inflammation model in rats. The LD_{50} value was estimated as 8.78 mg/kg within 24 h by different venom doses administrated intraperitoneally in mice. The IC_{50} value was 0.43 ± 0.18 μg/ml after 48 h treatment while the calculated value was 0.73 ± 0.10 μg/ml for the culture media totally refreshed after 2 h treatment with venom. Wistar rats were treated intraperitoneally with *A. absinthium* extract, 30 min before venom or carrageenan was injected subplantarly into the left hind paw. Intraperitoneal administration of 25 and 50 mg/kg extract was inhibited venom induced paw swelling at 0.5, 1, 2 and 3 h ($p < 0.05$) while 12.5, 25 and 50 mg/kg extract treatment was inhibited carrageenan-induced paw swelling at 2, 3, 4 and 5 h ($p < 0.05$). In conclusion, the *in vivo* toxicity and inflammatory actions and *in vitro* cytotoxic actions of crude *M. xanthina* venom were performed as a first report and inhibition of venom-induced inflammation by methanolic extract of *A. absinthium* was described.

1. Introduction

Snake envenoming is a major public health problem and the snakebites cause significant morbidity and mortality worldwide. There are nearly 3000 different species of snakes found in the world of which 10–14% is considered venomous (Chippaux, 1998; Chotwiwatthanakun et al., 2001; Ertem, 2004; Ismail et al., 2007; Meenatchisundaram et al., 2009). Cesaretli and Ozkan (2010) reported that the analysis of the National Poison Information Center records showed a total number of 550 snakebite cases between 1995 and 2004 in Turkey. Specifically, the most abundant species in Turkey belong to the following families: Viperidae, Elapidae and Colubridae. The Ottoman Viper, *Montivipera xanthina* (Gray, 1849), an almost endemic species to Turkey, is known from the Central, Southern and Western Anatolia as well as some adjacent islands of Greece with vertical distribution to 2000 m (Budak and Göçmen, 2005; Arıkan et al., 2005).

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0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2012.12.017

Inflammation is a major characteristic of envenomation by snakes from viperine species (Rosenfeld, 1971; Sawai, 1980) and prominent local edema is a general clinical finding in snake-bitten victims (Ohsaka, 1979; Otero et al., 2002). A common suggested treatment for snakebites involves the administration of antivenoms as standard of care; however, subsequent studies have revealed that antivenom therapy is not very effective in neutralizing the local effects of the venom of snakes (Melo et al., 2005; Morais et al., 1994; Picolo et al., 2002). New propensity in envenomation therapy suggests that it may be probable to interfere with the local tissue lesion and systemic effects of the venom not only by using serum therapy but also by modulating some inflammatory mediators produced by the patient following envenomation (Gutierrez et al., 2007; Magalhaesa et al., 2011). Thus, new therapeutic agents to reduce inflammation might be considered as an alternative treatment for the envenomations.

The genus *Artemisia* L. is one of the largest and most widely distributed genera of the family Asteraceae. Most of the species have been used worldwide in folk medicine since ancient times. The genus *Artemisia* is represented by 23 species in the flora of Turkey (Cullen, 1975; Davis et al., 1988). *Artemisia absinthium* L., commonly known as "wormwood", is a perennial aromatic herb distributed generally in middle and south Anatolia. It is known as "pelinotu" and has been used as stomachic, tonic, antihelmintic, antipyretic and diuretic in Turkish folk medicine (Baytop, 1999). Phytochemical studies on *A. absinthium* have revealed mainly sesquiterpene lactones (artabsin, absinthin, anabsinthine) and essential oils (thujone as main constituent). Also flavonoids, coumarines and lignans have been reported previously (ESCOP, 1997; Wright, 2002; Aberham et al., 2010). *Artemisia campestris* L. is extensively used in folk medicine for their antivenom, anti-inflammatory, anti-rheumatic and antimicrobial properties in Tunisia (Le Floc'h, 1983; Memmi et al., 2007). On the other hand, there are no reports to indicate that the *A. absinthium* extracts may have antivenom or *in vivo* anti-inflammatory effects.

The main purpose of this study was to investigate *in vitro* cytotoxicity, *in vivo* lethality, the characteristics of the edema induced by *M. xanthina* venom, anti-inflammatory activity and the therapeutic efficacy of the methanolic extract of *A. absinthium* on venom and carrageenan-induced models of acute rat hind paw inflammations.

2. Materials and methods

2.1. Plant material and extraction

The aerial part of the *A. absinthium* were collected from Antalya, Alanya 1514 m (N:37 47 10, E:28 56 05) in flowering period. Voucher specimens (5657) were deposited in Herbarium of Ege University, Faculty of Pharmacy (www.izef.ege.edu.tr). Aerial parts of the plants were dried under shade and powdered using an electric blender. Powdered plant material was extracted with methanol (Merck) in a Soxhlet extractor in 64 °C for 3 days. Solvent was evaporated to dryness under reduced pressure on rotary evaporator (Buchi Re 111, Switzerland). The extracts were solved in distilled water and lyophilized (Christ, Germany).

2.2. Venom and experimental animals

The experimental protocol was approved by the Ege University, Local Ethical Committee of Animal Experiment (Date: 23.12.2011, number 2011/210). All tests were performed with pooled venom from *M. xanthina* which is a venomous snake almost endemic to Turkey, Fig. 1. The *M. xanthina* specimens used in the study were collected in the Cambel, Kemalpasa (Vilayet Izmir) from Turkey and had already reached sexual maturity. The specimens were taken to the Reptile Biology and Ecology Research Laboratory (Ege University, Faculty of Science, Department of Biology, Zoology Section) alive and kept in the terrarium and their venoms were extracted without applying any pressure on their venom glands, as described by Tare et al. (1986). Due to the fact that the venom extracts contained some dead cells, pooled venom was diluted in physiologic saline and centrifuged for 5 min at 600 × *g* and stored at −20 °C.

Male/Female Wistar albino rats weighing 150–200 ± 20 g and the male Swiss albino mice (7–8 weeks-old) weighing 28–32 g were purchased from the Experimental Animal Center of Ege University (Izmir, Turkey). Mice and rats were maintained under standard conditions of temperature 22 ± 1 °C with regular 12 h light: 12 h dark cycle and were allowed free access to standard laboratory food and water.

2.3. Protein content determination

Protein content was assayed triplicate for each diluted venom sample in saline, using bovine serum albumin as a standard by Bradford method (22) at 595 nm (Molecular Devices, Versamax, USA).

2.4. Cell culture and in vitro cytotoxicity assay

Mouse fibroblastic (L929) cell-lines were purchased from the HUKUK (Animal Cell Culture Collections) in

Fig. 1. The Ottoman Viper, *Montivipera xanthina* (Gray, 1849).

Foot-and-Mouth Disease Institute (Ankara) of Ministry of Agriculture & Rural Affairs of Turkey. The cell lines were maintained in RPMI 1640 (Gibco, UK) medium supplemented with 4% heat-inactivated fetal bovine serum, 1% L-glutamine (Biochrome, Germany) and 1% gentamycine (Biochrome, Germany) in a humidified atmosphere with 5% CO_2, at 37 °C. The cells were subcultured twice a week.

In vitro cytotoxicity of venoms was determined, based on the procedure described for the screening of cytotoxic agents in general. Screening of the venom cytotoxicity, based on metabolic cell viability, was done using a modified MTT [3-(4, 5- Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] assay (Mosmann, 1983) that affects the mitochondrial reductase activity of viable cells. After treatment with venom, the survivals of viable cells in monolayer culture were determined. L929 cell line was cultivated for 24 h in 96 well microplates with 8×10^4 cells/ml as initial concentration. Then, the cultured cells were treated with different amounts of the venom and incubated for 48 h at 37 °C. The same experiment was performed following 2 h of incubation with different concentrations of venom and then the culture medium was refreshed and incubated for 48 h. The growth inhibition was compared with the untreated controls and it was found that the venom concentration inhibits growth by 50% (IC_{50}).

The assay is based on cleavage of the yellow tetrazolium salt, MTT, which forms water-insoluble, dark blue formazan crystals. This cleavage only takes place in living cells by the mitochondrial enzyme succinate-dehydrogenase. The water-insoluble, dark blue formazan crystals are solubilized by using dimethyl sulfoxide. The optical density of the dissolved material is measured at 570 nm (reference filter, 690 nm) with U.V. visible spectrophotometer (Molecular Devices, U.K.).

2.5. Determination of IC_{50}

Cytotoxicity was expressed as mean percentage increase relative to the unexposed control ± SD. Control values were set at 0% cytotoxicity. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a four parameters logistic model was used to calculate the IC_{50}, which is the concentration of nanomaterial causing 50% inhibition in comparison to untreated controls. The mean IC_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent measurements that were reproducible and statistically significant. The IC_{50} values were reported at ±95% confidence intervals (±95% CI). This analysis was performed with Graph Pad Prism (San Diego, USA).

2.6. Determination of LD_{50}

For that purpose Swiss albino mice weighing 28–32 g were used ($n = 10$ for each group). The LD_{50} value (dose of venom required to kill 50% of the animals studied) was determined by a Probit test using 50% death within 24 h after intraperitoneal (ip) administration of physiologic saline (control group) and venom at different doses.

2.7. Evaluation of M. xanthina venom-induced edema

In order to evaluate the edema induced by *M. xanthina* and establish the challenge-dose, groups of six male rats were injected subplantar into the left hind paw with 25, 37.5 and 50 μg/paw of *M. xanthina* venom dissolved in isotonic saline, whereas the contralateral paw was injected with isotonic saline. The paw volume was measured plethysmographically (Lettica, LE 7500, Barcelona, Spain) at ½, 1, 2, and 4 h following *M. xanthina* venom injection. In addition, groups of six male rats were injected intraperitoneal (ip) with indomethacin (10 mg/kg). Half an hour later, animals were injected subplantar into the left hind paw with 37.5 μg/paw of *M. xanthina* venom, while the contralateral paw was injected with isotonic saline. The paw volume was measured as described above.

2.8. Assessment of anti-inflammatory activity

Anti-inflammatory activity was evaluated by inducing paw edema using venom and carrageenan in rats. Rats were deprived of food overnight and treated ip with *A. absinthium* extracts (12.5, 25 and 50 mg/kg) 30 min before 37.5 μg/paw *M. xanthina* venom in isotonic saline or *A. absinthium* extracts (25, 50 and 100 mg/kg) 30 min before 0.1 ml 1% carrageenan in isotonic saline was injected subplantar into the left hind paw (Winter et al., 1962). The contralateral paw was injected with saline and used as a control. The anti-inflammatory test was repeated with 10 mg/kg indomethacin (Sigma Chemical Co., St. Louis, USA) administration for both models. The volume difference between the inflammatory agents and saline injected paws was used to evaluate the inflammatory response. Paw volume (V) was measured by water plethysmometer (Lettica, LE 7500, Barcelona, Spain) immediately before and 1, 2, 3 and 4 h after the injection of venom or 1, 2, 3, 4, 5 and 6 h after the injection of carrageenan into the plantar region of the left hind paw ($n = 6$ for each group). The percent inhibition of edema induced by venom or carrageenan was calculated for each group using Eq. (1).

$$\text{Inhibition of edema}(\%) = V_{control} - V_{treated}/V_{control} \quad (1)$$

2.9. Acute toxicity of A. absinthium extracts

Animals used in the venom and carrageenan-induced paw edema experiments were observed for the duration of 48 h and cases of morbidity or mortality were recorded for each group at the end of surveillance period.

2.10. Data analysis

Results are reported as means ± SEM (n), with n indicating the number of animals. LD_{50} value was determined using by a Probit test while curve fits and IC_{50} calculation were performed with Graph Pad Prism (San Diego, USA). Data were analyzed using the Student t-test, ANOVA, or nonparametric tests. Differences between extract or drug treated and control groups were also evaluated using Dunnetts t-test. The mean and SD of $n = 6$ were calculated. A probability value of $p \leq 0.05$ was considered statistically significant.

3. Results

Protein content of *M. xanthina* venom was determined to adjust venom dose in all tests. The diluted (1:2000) raw venom protein concentration was found to be 8.28 mg/ml. LD_{50} value of venom was calculated in order to find out immunization dose in Swiss albino mice. The estimated 24 h LD_{50} value after administration of a single dose of venom in the different amounts (2.5, 5, 7.5, 10 and 15 mg/kg) was found as 8.78 mg/kg, Table 1. The LD_{50} values demonstrated that the venom was highly toxic for Swiss albino mice. The animals, independent from each other, died within 24 h after ip injection. Half of the mice (50%) died as a result of the injections at the 7.5 and 10 mg/kg. On the other hand, only one mouse died after administration of 5 mg/kg of venom. However, all mice died after ip injection of 15 mg/kg venom which is the highest concentration.

The cytotoxic effect and IC_{50} value of venom on L929 cells were investigated by using different concentrations of the venom. The MTT assay results showed that venom inhibits cell proliferation in a dose-dependent manner. While concentrations of 0.1562 and 0.3125 μg/ml were not toxic during 48 h of continuous exposure to the venom, all concentrations ≥0.625 μg/ml showed marked cytotoxicity; there was an increased number of rounded cells and growth inhibition and the cells showed various morphological abnormalities such as the increased organelles and large vacuoles in the cytoplasm, surface ruffles or morphological damages (Fig. 2). The IC_{50} value was found to be 0.43 ± 0.12 μg/ml for 48 h treatment whereas IC_{50} value was found 0.73 ± 0.10 μg/ml once the culture media totally refreshed after 2 h treatment with venom (Fig. 3).

The inflammatory effect of *M. xanthina* venom was assessed by rat paw edema. Wistar albino rats were treated with increasing doses of *M. xanthina* venom. *M. xanthina* venom injection produced significant inflammation in rat paws showing significant dose-related effect. Edema formation peaked 1 h after administration of *M. xanthina*. Highest effect was obtained with 50 μg/rat. The dose of 37.5 μg was preferred as challenge dose for the evaluation of the anti-inflammatory activity of *A. absinthium* extracts, Fig. 4. *M. xanthina* venom injection did not produce any behavioral changes in rats.

A. absinthium extract at a dose of 50 mg/kg (ip) was found to generate highest inhibition (at ½, 1, 2 and 3 h) of venom-induced inflammation whereas the dose of 25 mg/kg and indomethacin (10 mg/kg) also showed considerable inhibition at ½ h after venom treatment ($p < 0.05$). On the other hand, the dose of 100 mg/kg showed slight decrease of edema, ($p < 0.05$), Fig. 5.

Carrageenan injection induced significant edema formation in rat paws. Pretreatment with *A. absinthium* extract before carrageenan injection resulted in significantly reduced edema formation with a dose of 50 mg/kg at 1 h ($p < 0.05$). However, treatment with indomethacin (10 mg/kg) and *A. absinthium* extracts inhibited the edema responses at all doses 1 h after venom injection, ($p < 0.05$), Fig. 6. On the contrary, *A. absinthium* extract showed significantly enhanced anti-inflammatory effect compared to indomethacin drug half an hour after venom or carrageenan treatment ($p < 0.05$). Notably the *A. absinthium* extract showed anti-inflammatory effect without inducing any noticeable acute toxicity at all exposure doses.

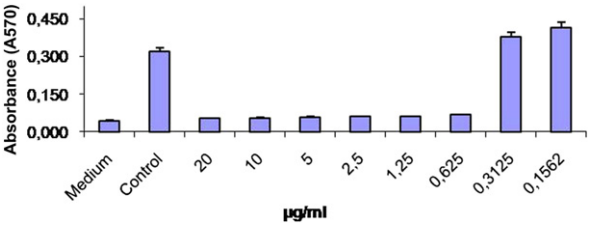

Fig. 2. Cytotoxic effect of *M. xanthina* crude venom on L929 cells after exposed to different concentrations of venom for 48 h. Cell viability was determined using by MTT assay, control was exposed to vehicle only which was taken as 100% viability. Data are expressed as the mean ± S.D.

4. Discussion

Presently snakebite (*M. xanthina*) envenoming is a considerable health emergency in Turkey and in these geographical areas (Arıkan et al., 2005; Budak and Göçmen, 2005; Cesaretli and Ozkan, 2010). Viper snake *venom* is *highly toxic to humans and this snake is* considered to be one of the most dangerous snakes in the world (Aggarwal and Thavaraj, 1994; Balija et al., 2005; Kresánek et al., 2004; Warrell and Fenner, 1993). Envenomation by snakes of the family Viperidae is characterized by prominent local effects, including necrosis, hemorrhage, edema, and pain, which develops rapidly after the snakebite and often results in permanent sequelae (Gutiérrez and Lomonte, 1989, 1997; Dart et al., 1992; Warrell, 1995).

Table 1
Lethality of *Montivipera xanthina* venom injected intraperitoneally into Swiss albino mice and estimated value of LD_{50}.

Conc. mg/kg	No of mice	24 h	
		Dead	Live
Control	10	0	10
2.5	10	0	10
5	10	1	9
7.5	10	5	5
10	10	5	5
15	10	10	10
Estimated LD_{50} (mg/kg)		8.78	

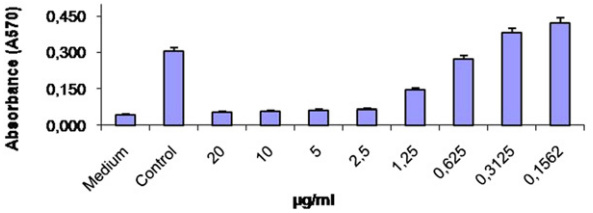

Fig. 3. Cytotoxic effect of *M. xanthina* crude venom on L929 cells, after 2 h of incubation with different concentrations of venom medium was refreshed and the incubation was continued for 48 h. Cell viability was determined using by MTT assay, control was exposed to vehicle only which was taken as 100% viability. Data are expressed as the mean ± S.D.

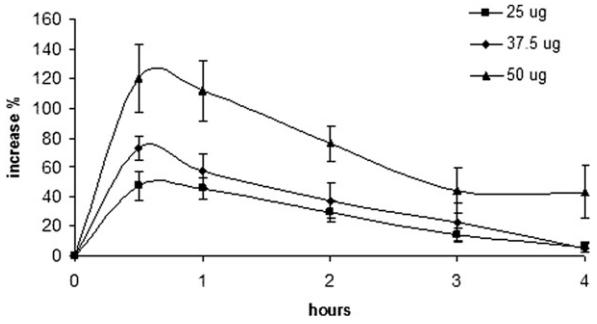

Fig. 4. Dose–response curve of *M. xanthina* venom (25, 37.5, 50 mg/kg) induced inflammation in the rat hind paw model. Data are represented as means ± SEM of duplicate measurements of six rats.

Antivenoms can prevent or reverse most of life-threatening systemic effects associated with the snakebite envenoming and play a vital role in minimizing mortality and morbidity. On the other hand, antivenoms are not able to efficiently neutralize the local toxic effects of snake venom (Melo et al., 2005; Magalhaesa et al., 2011; Morais et al., 1994; Picolo et al., 2002). The investigation of new compounds from plant extracts to accompaniment serum therapy has attracted the interest of various researchers (Ode and Asuzu, 2006; Magalhaesa et al., 2011; Martz, 1992; Morais et al., 1994; Magalhaesa et al., 2011; Pithayanukul et al., 2004). *A. absinthium* is a well known herb that has been used in traditional medicine in various health disorders. Also there are a lot of *in vitro* and *in vivo* studies that have been conducted evaluating its antibacterial, antimalarial, antihelmintic, hepatoprotective, gastroprotective and stomachic properties (Escop, 1997; Gilani and Janbaz, 1995; Hose, 2002, Caner et al., 2008; Bora and Sharma, 2010). Accordingly, this study investigates the toxicity, cytotoxicity, the induction of inflammation of *M. xanthina* snake venom and the anti-inflammatory potential of the methanolic extract from *A. absinthium* against the edema induced by venom.

The high protein content was determined in crude venom of *M. xanthina* as expected according to the literature. The pharmacological study of snake venoms and toxins often involves the use of animals or animal tissues (Broad et al., 1979). Recently researchers advocate that cell-based assays

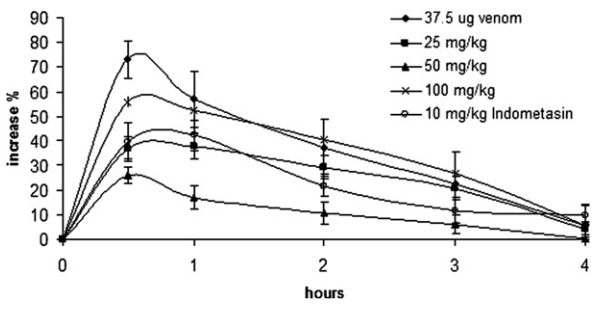

Fig. 5. Inhibition of inflammatory effect of *M. xanthina* venom (37.5 mg/kg) by *A. absinthium* extracts (25, 50 and 75 mg/kg). Data are represented as means ± SEM of duplicate measurements of six rats. Also shown are the percentage inhibitions of edema compared with the negative control formed by venom.

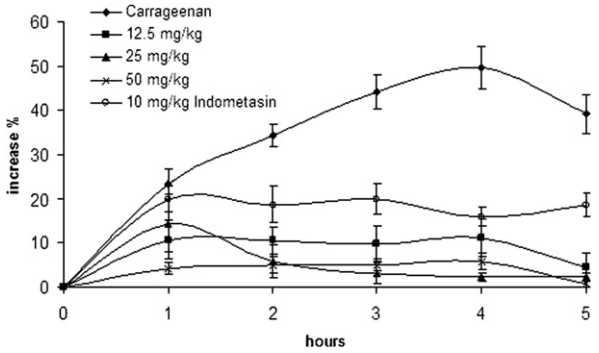

Fig. 6. Inhibition of inflammatory effect of carrageenan (0.1 ml 1% carrageenan/rat) by *A. absinthium* extracts (12.5, 25 and 50 mg/kg). Data are represented as means ± SEM of duplicate measurements of six rats. Also shown are the percentage inhibitions of edema compared with the negative control formed by carrageenan.

to examine the cytotoxicity of venom are an alternative to animal testing (Bustillo et al., 2009; Kalam et al., 2011; Konstantakopoulos et al., 2009; Nalbantsoy et al., 2012; Oliveira et al., 2002; Omran et al., 2004). The *in vitro* results reported here show that crude venom of *Montivipera xanthine*, highly cytotoxic for cultured fibroblasts, cause viability decrease, the disappearance of normal morphological characteristics, rounding up, detachment and fatality at the maximum concentration. The cells treated with venom showed potential to enhance significant observable deterioration and deformation of their cellular contents in a *dose-dependent* manner. The obtained results in this study agree with the other authors' reports (Bustillo et al., 2009; Kalam et al., 2011; Nalbantsoy et al., 2012; Omran et al., 2004).

Biochemically, snake venoms are complex mixtures of pharmacologically active proteins and polypeptides and characteristically include from 30 to over 100 protein toxins. Some of these proteins exhibit enzymatic activities, while several others are non-enzymatic proteins and polypeptides (Kang et al., 2011; Menez, 1998; Teixeira et al., 2009). The enzymes that exist in the venom, mainly phospholipases A2 and metalloproteinases, appear to play an important role in inflammation (Koh et al., 2006; Samy et al., 2012; Teixeira et al., 2009). The increased levels of inflammatory cytokines such as IFN-γ, IL-6 were related to expression of iNOS and augmented levels of prostaglandin E2 and expression of COX-2 in occurrence of inflammatory processes induced by venom (Samy et al., 2012; Teixeira et al., 2009). On the other hand, paw edema induced by carrageenans characterizes a classical inflammation model that has been comprehensively used in anti-inflammatory drug development. Correspondingly, the inflammatory process is characterized by production of inflammatory cytokines, prostaglandins, leukotrienes, histamine, bradykinin, nitric oxide, platelet-activating factor and by the release of cells from other chemical compounds in carrageenan induced inflammation model as well (Cuzzocrea et al., 2000; Silva et al., 2010).

In this study we verified the characteristics of the edema induced by *M. xanthina* venom in rats. According to the results of the dose–response experiment, a dose of 37.5 μg/kg was preferred as the challenge-dose, due to its effective

inflammatory response without causing any deleterious effect on animal's overall physical integrity, and enabling to demonstrate the inhibitory effect of the plant extract. The significant reduction of the edema was observed by indomethacin in venom induced edema which is known a cyclooxygenase inhibitor suggesting a role for cyclooxygenase-pathway metabolites such as prostaglandins.

The methanolic extract of *A. absinthium* was demonstrated as being able to significantly inhibit the *M. xanthina* venom induced edema formation. In comparison to both inflammation models, the *A. absinthium* extract doses of 50 mg/kg showed significant anti-inflammatory effect in both models ($p < 0.05$) whereas the doses of 12.5 and 25 mg/kg also showed comparable effect in carrageenan-induced edema, ($p < 0.05$). On the other hand, methanolic extracts of *A. absinthium* at the highest dose (100 mg/kg) used seemed to be devoid of anti-inflammatory activity in venom induced inflammation model since immunomodulator properties of *A. absinthium* are well known and highest dose response could be attributed to explain ineffectiveness of the dose (Lee et al., 2004; Omer et al., 2007). Several sesquiterpene type molecules have been isolated from *Artemisia* species and reported to have anti-inflammatory properties (Hui et al., 2004; Reddy et al., 2006; Emami et al., 2010; Ruikar et al., 2011). Moreover, flavonoids isolated from *Artemisia* species have been shown to exhibit anti-inflammatory activities (Moscatelli et al., 2006; Yin et al., 2008). Lee et al. (2004) demonstrated that the tetramethoxy hydroxyl flavon that has been isolated from *A. absinthium* was shown to have inhibitory effect on inflammatory mediators via the suppression of Nf-κB. Furthermore a chalcone cardomonin isolated from wormwood has been shown to exhibit anti-inflammatory activity in cellular models of inflammation (Hatziieremia et al., 2006). Since the major compounds of *A. absinthium* are sesquiterpenes and flavonoids our results could be attributed to these compounds. However further chemical investigations and detailed bioactivity studies on the preliminary mechanisms of these compounds are needed to better understand the action of *A. absinthium*.

In conclusion, this is the first report on the inflammatory effects of *M. xanthina* venom. Furthermore, the results of this research would be useful i) for further toxicological studies with this species to investigators ii) in developing topical anti-snake venom preparations from *A. absinthium* extracts to treat local wound effects, inflammatory reactions and muscle necrosis including antivenom treatment which is valuable for the supportive treatment of viper bites.

Conflict of interest

There is no conflict of interest.

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