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Tarantula cubensis extract (Theranekron®) ınhibits ınflammation in carrageenan-ınduced acute paw edema in rats

[*Extrato de tarântula cubensis (Theranekron®) inibe a inflamação no edema induzido por algas vermelhas na pata de ratos*]

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ABSTRACT

The aim of this study was to investigate the anti-inflammatory effect of alcoholic extract of *Tarantula cubensis* alcoholic extract (TCAE) in experimentally induced inflammation in rats. Fifty-four adult Sprague-Dawley male rats were randomly divided into nine groups. Paw edema was induced by 0.2mL subplantar (s.p.) injection of 1% carrageenan (CAR) into the right hind paw. Rats were treated with the nonsteroidal anti-inflammatory drug (NSAID) indomethacin (INDO) (10mg/kg, p.o.) or TCAE at different doses (1, 10 or 100µg/kg) injected s.c. for systemic or s.p. for local anti-inflammatory effect. Saline was used as control. Changes in paw thickness, volume, and weight were calculated as percentages. Formalin-fixed paws were used for histopathological examination. We detected that TCAE applied s.c. at 10µg/kg and 100µg/kg doses resulted in thinner paw thickness, lower paw volume, and lower paw weights four hours after the induction of inflammation when compared with the INDO group ($p<0.05$). The paw edema inhibitory effect of TCAE applied at a dose of 10µg/kg, s.c. was 68% when compared with the INDO which had an inhibitory effect of 56%. These results were verified with similar histopathological findings. The anti-inflammatory feature of 10µg/kg of TCAE given systematically was similar to the effects of INDO. Our results suggest that TCAE has anti-inflammatory effects by reducing edema and decreasing inflammatory reaction. These results may be attributed to the inhibition of the production of proinflammatory mediators. Thus, TCAE may be considered as a potential anti-inflammatory agent for treating acute inflammatory conditions.

Keywords: inflammation; paw edema; rat; Tarantula cubensis

RESUMO

O objetivo deste estudo foi investigar o efeito anti-inflamatório do extrato alcoólico de Tarantula cubensis (TCAE) na inflamação induzida experimentalmente em ratos. Cinqüenta e quatro ratos Sprague-Dawley adultos machos foram divididos aleatoriamente em nove grupos. O edema da pata foi induzido pela injeção de 0,2mL de subplantar (s.p.) de 1% de carragena (CAR) na pata traseira direita. Ratos foram tratados com o medicamento antiinflamatório não esteróide (NSAID) indometacina (INDO) (10mg/kg, p.o.) ou TCAE em doses diferentes (1, 10 ou 100µg/kg) injetado s.c. para efeito sistêmico ou s.p. para efeito antiinflamatório local. A soro fisiológico foi usado como controle. As mudanças na espessura da pata, volume e peso foram calculadas como porcentagens. As patas fixadas com fórmalina foram usadas para exame histopatológico. Detectamos que o TCAE aplicado s.c. em doses de 10µg/kg e 100µg/kg resultou em menor espessura da pata, menor volume da pata e menor peso da pata quatro horas após a indução da inflamação quando comparado com o grupo INDO ($p<0,05$). O efeito inibidor do edema da pata de TCAE aplicado na dose de 10µg/kg, s.c. foi de 68% quando comparado com o INDO que teve um efeito inibidor de 56%. Estes resultados foram verificados com resultados histopatológicos semelhantes. A característica anti-inflamatória de 10µg/kg de TCAE dada sistematicamente foi semelhante aos efeitos do INDO. Nossos resultados sugerem que o TCAE tem efeitos anti-inflamatórios reduzindo o edema e diminuindo a reação inflamatória. Estes resultados podem ser atribuídos à inibição da produção de mediadores pró-inflamatórios. Assim, o TCAE pode ser considerado como um agente antiinflamatório potencial para o tratamento de condições inflamatórias agudas.

Palavras-chave: inflamação; edema de pata; rato; Tarântula cubensis.

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INTRODUCTION

Inflammation is a normal response to disturbed homeostasis caused by infection, injury, and trauma (Gurun *et al*., 2009). Inflammation and pain are two local defence reactions of living mammalian tissues in response to any injurious agent (Karakus *et al*., 2013). There are various components to an inflammatory reaction, which can contribute to the associated symptoms and tissue injury. Inflammation is essentially a protective process preserving the integrity of organisms (Heeba *et al*., 2014). It is a complex biological response of the body to cell damage and vascularized tissue, which can be classified as acute or chronic depending on the time of onset (Ferrero-Miliani *et al*., 2007). Acute inflammation is the body's primary response to injurious stimuli, and is characterized by pain, heat, redness, swelling, and loss of function (Hussein *et al*., 2012; Nathan, 2002).

From ancient times until now, several natural products and drugs of animal origin have been used in therapeutic applications for inflammatory disorders and related diseases. Today research focuses on finding anti-inflammatory agents with selective pharmacological effects and less toxicity (Lee *et al*., 2001; Picolo *et al*., 2000; Sadeghi *et al*., 2013; Viji and Helen, 2008). Theranekron® (TCAE) is an alcoholic extract of the venom of *Tarantula cubensis* which remains active in pharmaceutical compounds for a considerable time and has been used to treat a wide variety of conditions ranging from tumors to abscesses, septicemia, and toxemic injuries (Albay *et al*., 2010; Biricik *et al*., 2008; Cam *et al*., 2007; Gultiken and Vural, 2007; Kaçar *et al*., 2007; Lotfollahzadeh *et al*., 2012; Oryan *et al*., 2012; Sardari *et al*., 2007; Stampa, 1986). Sardari *et al*. (2007) claimed that TCAE reduces inflammation and stimulates re-epithelialization of the full-thickness cutaneous wounds in cows during the first 14 days after injury.

Several biological effects such as antiphlogistic, demarcative, and necrotizing have been ascribed to TCAE, but its effect on classical models of inflammation has not been fully investigated yet. In the present study, we investigated the anti-inflammatory activity of TCAE in a carrageenan (CAR)-induced paw edema model in rats and compared its efficiency with indomethacin (INDO), a commonly used anti-inflammatory agent.

MATERIALS AND METHODS

The study was performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and the study plan was approved by the Animal Care Committee of Bursa Uludag University (approval no 2013/02-05).

In this study, we used a total of 54 male Sprague-Dawley rats obtained from the Laboratory Animal Breeding and Research Center (DEHYUAM) of Bursa Uludag University. The animals weighed between 200-220g, kept at 22°C in separate cages and fed *ad libitum* throughout the study. To keep a stable balance of body fluids, the animals were not allowed any food or water during the 20 hours before the experiment but were only given water (1.5mL/100g body weight) four hours before the experiment.

Carrageenan (Lambda, C1013) was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany) and dissolved in isotonic saline. Indomethacin (Endol®, 25mg capsule) was obtained from Deva (Istanbul, Turkey) and TCAE (1:100/D2, Richter Pharma AG, Wels, Austria) was from Interhas (Ankara, Turkey). Xylazine hydrochloride (Rompun®, Bayer AG, Leverkusen, Germany) and ketamine hydrochloride (Alfamine®, Alfasan International BV, Woerden, Netherlands) were provided by Ata Fen (Izmir, Turkey).

The rats were divided into nine groups (n=6, each) for the experimental procedure. Briefly, group I received 0.2 ml of saline applied s.c. at the plantar surface of the right hind paw and served as control. To induce inflammation, animals in groups II-IX were given 0.2 ml CAR (1.0 % solution in isotonic saline) on the plantar surface of the right hind paw as described previously by Gurun *et al*. (2009), Karakus *et al.* (2013), Hussein *et al.* (2012), and Morais-Zani *et al.* (2013). While group II received CAR only, group III (the positive control group) was injected also with the nonsteroidal anti-inflammatory drug (NSAID) INDO (10 mg/kg of body weight, per os). Groups IV, VI, and VIII received TCAE s.c. for observation of the systemic effect, and groups V, VII, and IX received TCAE subplantarly (s.p.) for investigating the local effect.

Before and for the first four hours after the CAR injection, the dorsoventral thickness of each hind paw was measured hourly using a dial caliper (Aesculap AA845 caliper, B. Braun Melsungen AG, Melsungen, Germany) placed at the border of the phalanges and metatarsals. The measurement was taken when each edge of the caliper was just touching the dorsal and ventral surface of the hind paw (the caliper was not squeezed onto the hind paw).

The paw volume was measured with a plethysmometer (Ugo Basile, Gemonio, Italy) immediately before s.p. injection of CAR and at the end of the study, and the paw volumes (ml) were recorded.

At the end of the experiment, rats were anesthetized with 3mg/kg xylazine hydrochloride + 50mg/kg ketamine hydrochloride, i.p., and then sacrificed. Both hind paws were cut from the proximal of the lateral malleolus and weighed by a digital scale (Radwag®, AS 3Y, Radom, Poland).

The swelling degree of paw and inhibition rate of edema was calculated as follows: % edema inhibition = (Vc-Vt) x 100/Vc, where Vc and Vt depict average edema volume of the control (Group II) and test group, respectively. The percentage of inhibition of paw volume for treated groups was calculated by comparing the value with that of mean paw volume of the control group (Group II).

Paw edema was successfully induced by the s.c. administration of CAR and the experiment was terminated 4 h after the CAR treatment, as the prominent clinical and pathological signs of acute inflammation peaked. Right hind paws were collected and after fixation in 10% neutral buffered formalin and processing routinely, 5µm sections were cut and stained with hematoxylin-eosin (H&E). In the light microscopic examination hyperemia of blood vessels, tissue edema, and infiltration of neutrophils and of blood vessels were evaluated. Microscopically, ten areas were randomly selected and evaluated under x400 magnification in a semiquantitative system. As there was no sign of inflammation in paws of the saline group (Group 1) animals, that group was considered as 0.

The results were expressed as the mean ± SEM. Data were analyzed with one-way ANOVA using SPSS version 16.0 software (SPSS Science, Chicago, IL, USA) and the differences at level $p<0.05$ were considered statistically significant.

RESULTS

Subcutaneous injection of CAR into the hind paw of rats caused an acute local inflammatory response as manifested by a peaked edema at 4 h after application of the phlogistic agent (Fig. 1).

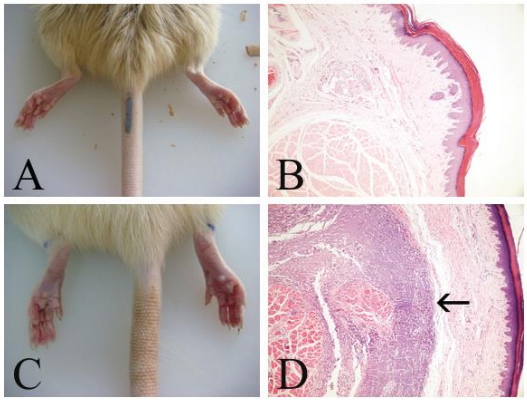

Figure 1. Gross and microscopical images (A and B, respectively) of the saline injected control animal (Group 1) and carrageenan (CAR)-injected animal (Group II)(C and D, respectively) at 4 hours. Carrageenaan induced prominent edema (arrow) of the right hind paw. Microscopical slides are stained with H&E.

Gross and microscopic views of the paws in experimental groups are shown in Fig. 2. When the difference between hours 0 and 4 was compared, we detected that 10µg/kg and 100µg/kg s.c. TCAE groups (groups VI and VIII, respectively) had thinner paw thickness when compared with the CAR+INDO group (group III) ($p<0.05$) (Fig. 3).

Gross and microscopic views of the paws in experimental groups are shown in Fig. 2. When the difference between hours 0 and 4 was compared, we detected that 10µg/kg and 100µg/kg s.c. TCAE groups (groups VI and VIII, respectively) had thinner paw thickness when compared with the CAR+INDO group (group III) ($p<0.05$) (Fig. 3).

The measured paw volumes at each hour are shown in Fig. 4 and percentages of edema inhibition are summarized in Table 1. In saline group due to the absorbtion and absence of inflammatory reaction, percentage of edema inhibition on paw volume in that group was not mentioned. We detected that 10µg/kg (Group VI) and 100µg/kg (Group VIII) s.c. TCAE groups had lower paw volume than in CAR+INDO group (Group III) according to difference between hour 0 and 4th hour (p<0.05) (Figure 4). Among the three different TCAE concentrations after s.c. administration, 10µg/kg and 100µg/kg showed significant edema decreasing effect (68% and 63%, respectively) in paw volume (Table 1) (p<0.05).

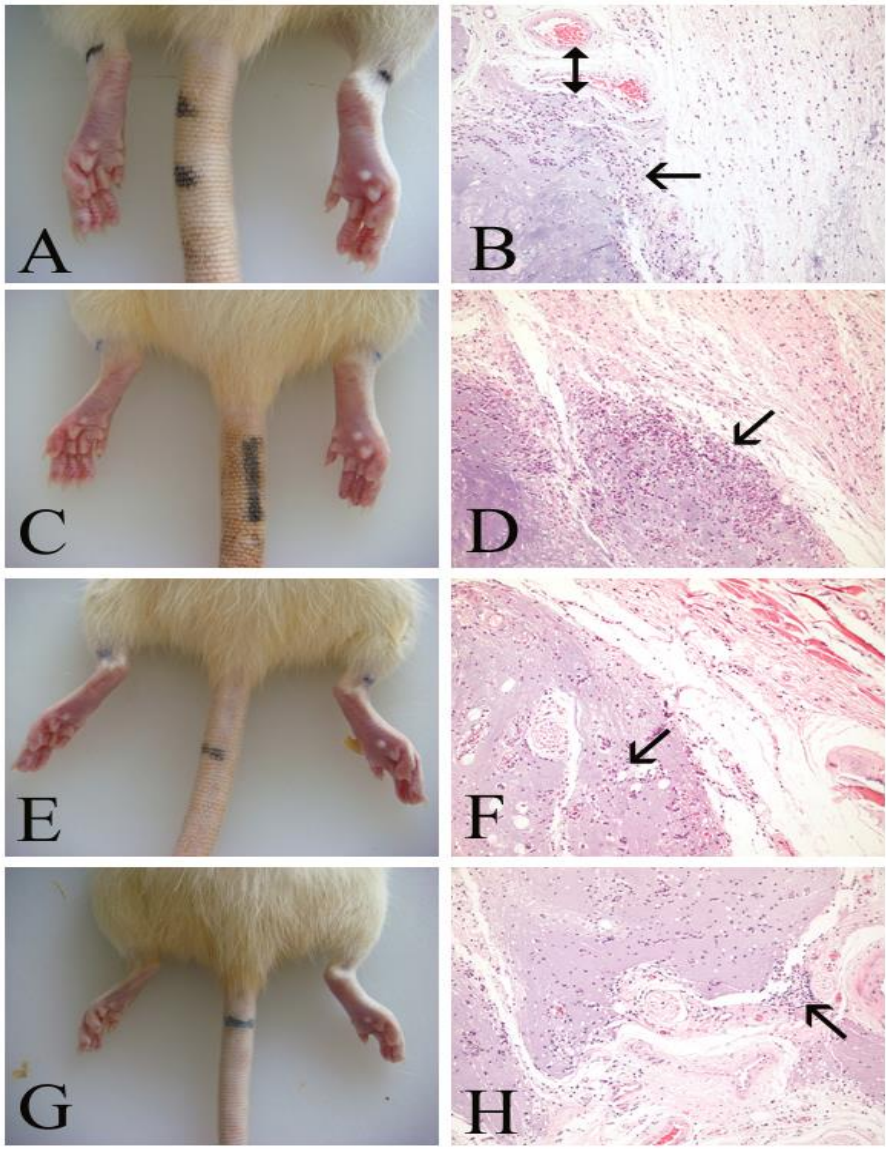

Figure 2. Gross (left column) and microscopical (right column) images at 4 hours after CAR administration in CAR+INDO (Group III) (A and B), and subcutaneously administered 1 µg/kg CAR+TCAE (Group IV) (C and D), 10 µg/kg CAR+TCAE (Group VI) (E and F), and 100 µg/kg CAR+TCAE (Group VIII) (G and H) groups (CAR: Carrageenan, INDO: Indomethacin, TCAE: Alcoholic extract of *Tarantula cubensis*). Inflammation scores were 1, 3, 2, and 1 in photos B, D, F, and H, respectively. Arrows depict inflammatory cells (neutrophils). Double-headed arrow demonstrates hyperemic blood vessels in Fig-2b Microscopical slides are stained with H&E.

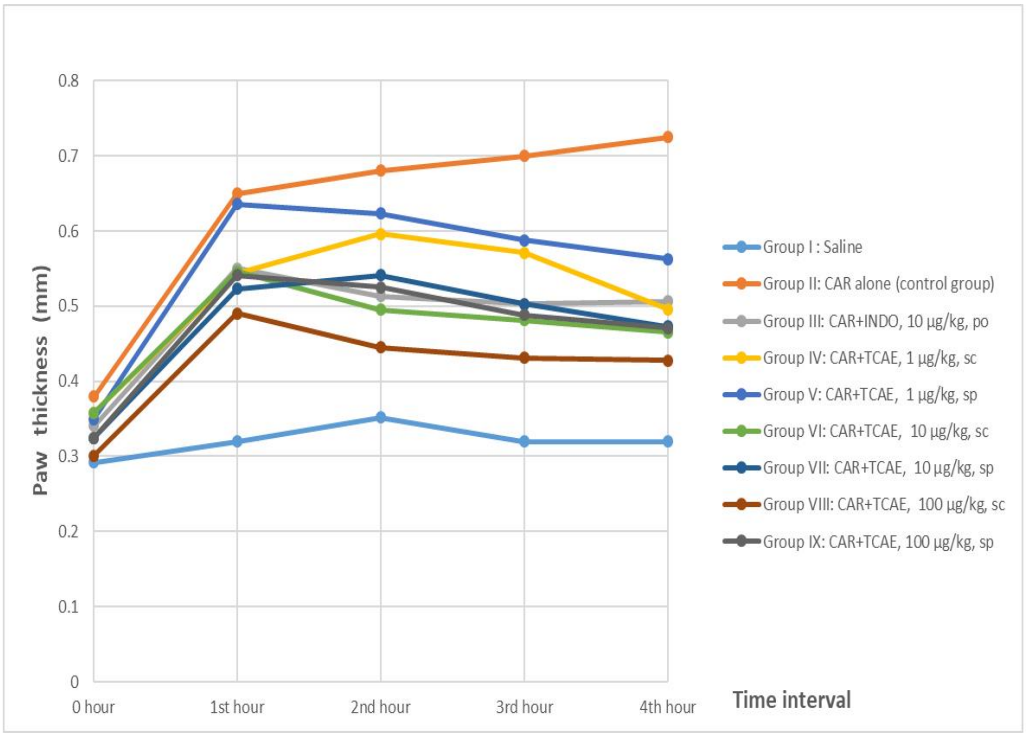

Figure 3. The effect of TCAE on the paw thickness in CAR-induced paw edema in rats. Rats were treated with INDO, or subcutaneously (s.c.) or subplantarly (s.p.) with three different doses of TCAE after CAR injection (TCAE: Alcoholic extract of *Tarantula cubensis*, CAR: Carrageenan, INDO: Indomethacin). Data are presented as the mean±SEM (n=6).

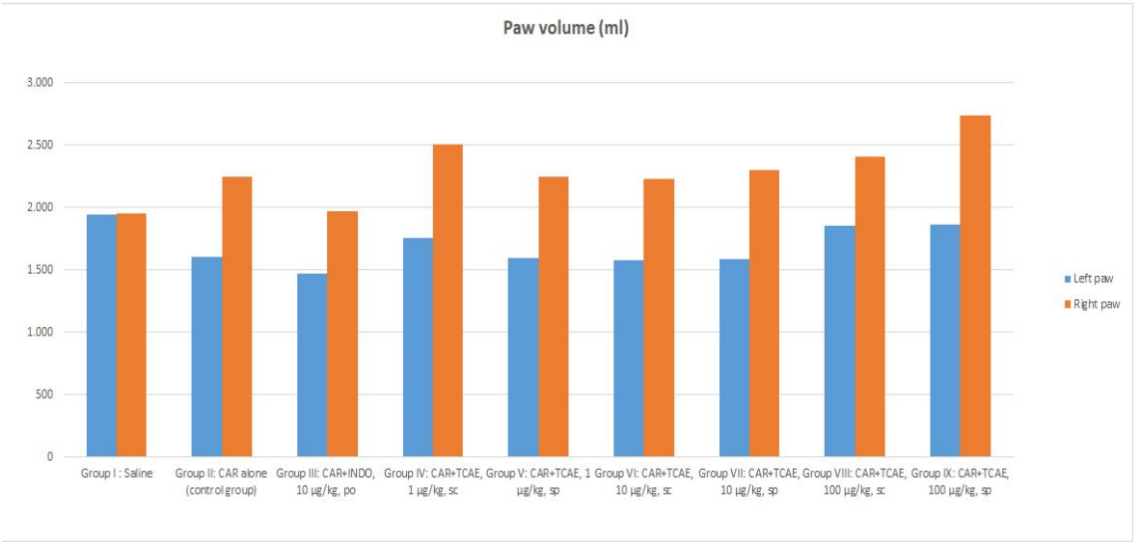

Figure 4. The effect of TCAE on the paw volume in CAR-induced paw edema in rats. Rats were treated with INDO, or subcutaneously (s.c.) or subplantarly (s.p.) with three different doses of TCAE after CAR injection (TCAE: *Tarantula cubensis* alcoholic extract, CAR: Carrageenan, INDO: Indomethacin). Data are presented as the mean±SEM (n=6).

Table 1. Comparison of the percentages of paw edema inhibition (volume) among the treatment groups of rats (n=6, each)

	The percentage of edema inhibition			
	1st hour	2nd hour	3rd hour	4th hour
Group III (positive control): CAR + INDO (10µg/kg, p.o.)	39*	50*,a	53*	56*,a
Group IV: CAR+TCAE (1µg/kg, s.c.)	30	38*	44*	50*,a
Group V: CAR+TCAE (1µg/kg, s.p.)	28	33	40*	50*,a
Group VI: CAR+TCAE (10µg/kg, s.c.)	31	44*	56*	68*,b
Group VII: CAR+TCAE (10µg/kg, s.p.)	29	40*	43*	55*,a
Group VIII: CAR+TCAE (100µg/kg, s.c.)	30	42*	48*	63*,b
Group IX: CAR+TCAE (100µg/kg, s.p.)	27	38*	46*	52*,a

*Significant difference from the control (Group II) (p<0.05).

[a,b] Different superscripts within the same column indicate significant difference (p<0.05).

CAR: Carrageenan, INDO: Indomethacin (Endol®), TCAE: *Tarantula cubensis* alcoholic extract (Theranekron®)

p.o. per os, s.c. subcutaneous, s.p. subplantarly

When the difference between hour 0 and 4 was compared, we detected that 10µg/kg and 100µg/kg s.c. TCAE groups had lower paw weights than in CAR+INDO group (p<0.05) (Fig. 5).

Comparison of the histopathological changes is summarized in Table 2. When the overall histopathological findings were evaluated among the experimental groups, INDO treatment clearly inhibited the neutrophil infiltration and alleviated edema formation (p<0.005). Injection with 10µg/kg and 100µg/kg TCAE doses resulted with similar histopathological findings with INDO when compared with control group (Group III). Subcutaneous administration of 10µg/kg of TCAE resulted with statistically significant decrease in the number of neutrophil infiltrations when compared with those of other doses of TCAE (1µg/kg, 100µg/kg, p<0.005 each). When the effects of INDO were compared with those of s.c. 10µg/kg dose of TCAE, both treatments significantly reduced the cellular infiltration. When we compared the effects of s.c. and s.p. administered 10µg/kg of TCAE, s.c. administration generated more favorable results.

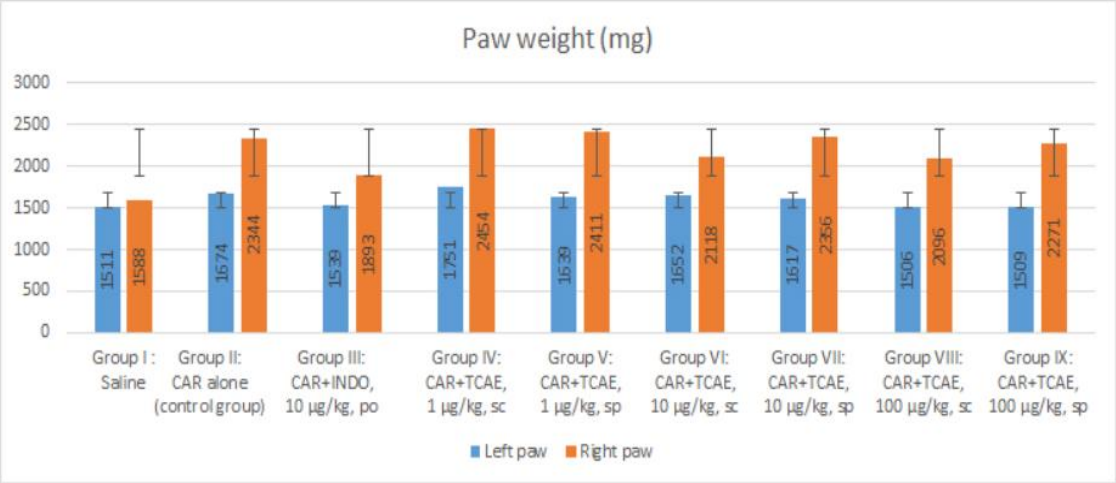

Figure 5. The effect of TCAE on the paw weight in CAR-induced paw edema in rats. Rats were treated with INDO, or subcutaneously (s.c.) or subplantarly (s.p.) with three different doses of TCAE after CAR injection (TCAE: *Tarantula cubensis* alcoholic extract, CAR: Carrageenan, INDO: Indomethacin). Data are presented as the mean ± SEM (n=6).

Table 2. Comparison of mean hyperemia, edema, neutrophil counts and scores among groups of rats

	Hyperemia[a]	Edema[a]	Neutrophil count[a]
Group I: Saline	0.20±0.45[c,d,e]	1.40±0.55[c]	-
Group II: CAR alone (control)	2.83±0.41[b,d,e]	2.83±0.41[b,d,e]	498.17±36.09
Group III: CAR+INDO (positive control)	1±0[b,c]	1.33±0.52[c]	253.83±32.43[c,d,e]
Group IV: CAR+TCAE (1µg/kg, s.c.)	2.33±0.52[b]	2.5±0.55[b,d,e]	490.50±17.42[d,e]
Group V: CAR+TCAE (1µg/kg, s.p.)	2.5±0.55[b]	2.83±0.41[b,d,e]	483.00±17.64[d,e]
Group VI: CAR+TCAE (10µg/kg, s.c.)	1.67±0.82[b,c]	1.33±0.52[c]	326.83±93.63[c]
Group VII: CAR+TCAE (10µg/kg, s.p.)	2.67±0.52[b]	2.50±0.55[b,d,e]	456.50±17.84[d,e]
Group VIII: CAR+TCAE (100µg/kg, s.c.)	1.83±0.41[b,c]	1.83±0.52[c]	380.66±44.45
Group IX: CAR+TCAE (100µg/kg, s.p.)	2.67±0.52[b]	2.66±0.52[b,d,e]	482.52±40.67[d,e]

[a]Data represent the mean + SEM of observations from six rats.
[b]Significantly different when compared with the saline group (Group I).
[c]Significantly different when compared with the CAR group (Group II).
[d]Significantly different when compared with the CAR+TCAE, 10µg/kg, s.c.-treated group (Group VI).
[e]Significantly different when compared with the CAR+TCAE, 100µg/kg, s.c.-treated group (Group VIII).
CAR: Carrageenan, INDO: Indomethacin (Endol®); TCAE: *Tarantula cubensis* alcoholic extract (Theranekron®)
p.o. per os, s.c. subcutaneous, s.p. subplantarly

DISCUSSION

In the present study, the anti-inflammatory effect of three different doses of Theranekron applied via two different routes was investigated in a CAR-induced rat paw edema model. The anti-inflammatory potency of Theranekron was compared with that of the INDO.

Carrageenan-induced hind paw edema is a widely used experimental model for evaluating acute inflammation and effect of anti-inflammatory drugs. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects (Haider *et al*., 2011; Nunes *et al*., 2007). The acute CAR-induced paw inflammation has been characterized as a biphasic event and several mediators are involved in this inflammatory reaction (Haider *et al*., 2011; Viji and Helen, 2008). Histamine, serotonin, bradykinin, and substance P release in the first phase (0-1 h), whilst the delayed phase (after 1 h) mainly is sustained by infiltration of polymorphonuclear cells into the site of inflammation which induces secretion of several proinflammatory mediators such as nitric oxide, prostaglandins, and cytokines (Karakus *et al*., 2013; Sadeghi *et al*., 2013). The second phase of edema formation is sensitive to both clinically useful steroidal and non-steroidal agents (Gilligan *et al*., 1994; Sadeghi *et al*., 2013). Our results demonstrated that Theranekron suppressed neutrophil infiltration into the CAR-injected paws. This conclusion is in accordance with the following results: (1) Theranekron reduced the neutrophil migration and tissue destruction induced by CAR based on pathological assessment of paw biopsies, and (2) Theranekron significantly reduced the inflammatory changes in the CAR-induced inflammation model in paws.

Tarantula cubensis venom, as a homeopathic drug sold under the name Theranekron, is prepared by the alcohol extraction of venom from the spider *Tarantula cubensis* (Cam *et al*., 2007) and has been used in cattle, horses, sheep, goats, and dogs. The effects of Theranekron on a wide range of conditions such as tumors, abscesses, septicemia, and toxemic injuries (Albat *et al*., 2010; Biricik *et al*., 2008; Cam *et al*., 2007; Lotfollahzadeh *et al*., 2012; Oryan *et*

al., 2012; Sardari *et al*., 2007) have been extensively reviewed, but the effect of Theranekron on classical models of inflammation has not been investigated.

Edema is one of the fundamental actions of acute inflammation and is an essential parameter to be considered when evaluating compounds with potential anti-inflammatory activity (Hussein *et al.*, 2012). In our study, the anti-inflammatory activity of TCAE was clearly observed in CAR-induced rat paw edema, with a maximal effect at 4 hr after CAR injection. The anti-inflammatory effect of 10µg/kg and 100 µg/kg of subcutaneous TCAE that was observed in this study was like the anti-inflammatory effect of the drug INDO (10mg/kg of body weight), a well-known NSAID and a cyclooxygenase (COX-1 and 2) inhibitor. Perhaps the action of TCAE could be mediated by inhibiting COX-1 or -2 because the CAR inflammatory model basically reflects the actions of prostaglandins.

Indomethacin was able to prevent the development of edema at a rate of 56% at 4th hour after CAR administration. The TCAE at a s.c. dose of 10µg/kg and 100µg/kg could prevent the development of edema at a rate of 68% and 63% at 4th hour, respectively. Our study results showed that s.c. administration of 10µg/kg and 100µg/kg TCAE were more effective in inhibiting paw edema than INDO. In the first two hours percentage of edema inhibition with INDO was higher, but TCAE at s.c. dose of 10µg/kg and 100µg/kg considerably inhibited edema at the end of the 4 hour. The late term effects of 10µg/kg and 100µg/kg s.c. TCAE were more favorable than INDO group in our experimental model.

Edema is a characteristic sign of inflammation. Four hours after s.p. injection of CAR, ipsilateral paw volume was significantly increased in the CAR-TCAE groups, as compared with the pre-injection paw volume. Although low dose (1µg/kg) did not produce effective anti-inflammatory effects, treatment with 10µg/kg and 100µg/kg of s.c. TCAE significantly reduced the periferal edema evoked by CAR injection.

In our study, the decrease in the paw volume, thickness and weight indicated that the acute inflammatory phase was effectively suppressed by TCAE treatment. Results indicated that the 10µg/kg and 100µg/kg s.c. administration of TCAE produces a more significant anti-inflammatory effect in CAR-induced paw edema compared to INDO group.

We showed herein that a single administration of TCAE induces an anti-inflammatory effect both on paw edema and cell migration induced by CAR. The current study suggests that the anti-inflammatory activity of TCAE may be a promising therapeutic strategy in the management of various inflammatory diseases. In the meantime, it is important to emphasize that this study has some limitations. The mechanism of how TCAE inhibits inflammation is not clear. To reveal this process, proteomics analyses of edema fluid obtained in a similar study are being carried out to identify the molecules involved in this event and these results will be published elsewhere.

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and preparation of paper.

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