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Original Article

Antioxidant, cytotoxic and anti-inflammatory properties of *Anthemis tricolor* Boiss. through a series of cellular assays and inhibition of Turkish *Macrovipera lebetina obtusa* venom induced inflammation in rat

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ABSTRACT

Background and Aims: Venom of *Macrovipera lebetina* obtusa (Viperidae), Turkey's biggest viper, is highly toxic to humans and causes inflammation. In Anatolian traditional medicine, many *Anthemis* L. species are used in wound healing and against inflammatory diseases. In the present study, antioxidant, cytotoxic and anti-inflammatory activities of different endemic *A. tricolor* Boiss. extracts were evaluated by *in vitro* assays. Besides, the anti-inflammatory activities of chloroform extract against carrageenan and snake venom induced-edema were investigated in rats.
Methods: Antioxidant and anti-inflammatory activities were evaluated by DCFH-DA, iNOS, NF-κB inhibitions and NAG-1 gene activation, while WST-1 assay was preferred for cytotoxic activity. Besides, the hind paw edema test was used for *in vivo* studies.
Results: Chloroform extract exhibited the strongest NAG-1-inducing activity. In addition, this extract showed potent iNOS and NF-κB inhibition (IC_{50}:14.0 and 10.75 µg/mL, respectively) and cytotoxic effect against human osteosarcoma (U2OS; IC_{50}: 15.18 µg/mL) and human cervical cancer (HeLa; IC_{50}: 18.3 µg/mL) cell lines. Moreover, chloroform extract had stronger anti-inflammatory effects against both carrageenan and snake venom induced-edema formation than indomethacin. Fifty and 100 mg/kg extracts reduced the paw edema to 1-3% at 4 h after the snake venom injection. On the other hand, 50, 25 and 12.5 mg/kg of the extract completely inhibited inflammation induced by carrageenan.
Conclusion: This is the first report on the antioxidant, cytotoxic and anti-inflammatory effects of endemic *A. tricolor* by *in vitro* studies and snake venom-induced paw edema in rats. The plant exhibits strong potential for treating local tissue damage in snake bites.
Keywords: *Macrovipera lebetina obtusa, Anthemis tricolor,* cytotoxicity, antioxidant, anti-inflammatory, *in vivo*

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INTRODUCTION

Snake bite is a worldwide problem that can lead to serious injuries and even death (Chippaux, Williams, & White, 1991). In these cases, some local effects, such as necrosis, bleeding, pain and edema, which develop rapidly after injury, and usually cause a wound have been reported (Ohsaka, 1979; Otero et al., 2002; Rosenfeld, 1971; Sawai, 1980). Anti-venom treatment is the primary medical application in the therapy of bitten people by venomous snakes. However, studies have shown that anti-venom therapy is not effective in symptomatic treatment (Melo, Habermehl, Oliveira, & Nascimento, 2005; Morais, De Freitas, Yamaguchi, Dos-Santos, & DaSilva, 1994; Picolo, G., Chacur, Gutierrez, Teixeira, & Cury, 2002).

Most snake venom cases in Turkey are caused by viper bites. The blunt-nosed viper *Macrovipera lebetina* (Linnaeus, 1758) (Viperidae) is quite a poisonous species and is spread widely from the west and central Asia to northern Africa (Nilson & Andrén, 1988). In Turkey, *M. l. obtusa* (Dwigubsky, 1832) is distributed in East, Southeast and Northeast Anatolia. Venom of this species has cytotoxic, antitumoral and antimicrobial activities (Ghazaryan et al., 2015; Ozen, Igci, Yalcin, Gocmen, & Nalbantsoy, 2015; Suzergoz et al., 2016). Also, anti-inflammatory effects of *Centaurea calolepis* Boiss. and its major compound cnicin against *M. l. obtusa* venom have been investigated by our team previously (Demiroz, Albayrak, Nalbantsoy, Gocmen, & Baykan, 2018).

The genus *Anthemis* L. (Asteraceae) comprises of nearly 210 species distributed widely across South-West Asia, North and North-East Africa and Europe. Fifty-one species are present in Turkish flora (Boukhary, Aboul-ElA, & El-Lakany, 2019; Ghafoor, 2010). Generally, *Anthemis* species are known as "papatya, Alman papatyası" in Anatolia and capitulums (flowers) are used to treat urinary inflammation, dysmenorrhea, gastrointestinal disorders, hemorrhoids, hypertension and wounds in Anatolian traditional medicine (Baytop, 1999; Boukhary et al., 2019; Cakilcioglu, Khatun, Turkoglu, & Hayta, 2011; Tetik, Civelek, & Cakilcioglu, 2013). Cytotoxic, antimicrobial, anti-inflammatory, enzyme inhibition (elastase, α-amylase, α-glucosidase, anticholinesterase and tyrosinase inhibition), antidepressant, sedative, anti-diuretic and antioxidant activities of different *Anthemis* species have been reported previously (Acquaviva et al., 2012; Baltaci, Kolatan, Yilmaz, & Kivçak, 2011; Boukhary et al., 2019; Chemsa et al., 2018; Conforti et al., 2012; Hajdú, Zupkó, Réthy, Forgo, & Hohmann, 2010; Quaerenghi, Tereschuk, Baigori, & Abdala, 2000; Rossi, Melegari, Bianchi, Albasini, & Vampa, 1988; Sarikurkcu, 2020; Vučković, Vujisić, Klaas, Merfort, & Milosavljević, 2011). Phytochemical investigations have shown sesquiterpene lactones (nobilin, tatridin A, 1-epi-tatridin B), phenolics (caffeoylquinic acid derivatives, ferulic, gallic and 3-hydroxybenzoic acids) and flavonoids (salvigenin, apigenin, quercetin, naringenin, pectolinarigenin, eupatilin, rutin) are mainly secondary metabolites of this genus (Emir & Emir, 2020; Gönenç, Akkol, Süntar, Erdoğan, & Kıvçak, 2014; Pavlovic, Kovacevic, Couladis, & Tzakou, 2006; Staneva, Todorova, & Evstatieva, 2008; Sut et al., 2019; Todorova, Trendafilova, Danova, & Dimitrov, 2011).

Anthemis has 10 taxa in Cyprus flora and *A. tricolor* Boiss. is one of the two endemic species of this genus in Cyprus. The major compounds of *A. tricolor* essential oil have been reported as germacrene D (5.5%), β-caryophyllene (9.1%) and muurola-4,11-diene (20.2%) (Karaalp et al., 2014). There are also two studies on the anti-inflammatory and antibacterial effects of this species (Demirkan, Özçınar, & Kıvçak, 2019; Gulsoy Toplan, Tuysuz, Mat, & Sariyar, 2017). In this study, cytotoxic and antioxidant activities of *A. tricolor* were reported in detail. Moreover, the anti-inflammatory effects of this species against the standard inductive agent carrageenan and snake venom have been investigated.

Edema is a clear sign of inflammatory response and is due to mediators that cause local vasodilatation, such as histamine, bradykinin, serotonin, prostaglandin E2 and I2 (De Toni et al., 2015). Inflammation is one of the major symptoms not only for snake bite but also cancer, obesity and metabolic disorders. The NAG-1 gene, which is responsible for the apoptotic elimination of cancer cells, is related to the anti-inflammatory action of NSAIDs and the induction of NAG-1 is a COX-independent mechanism (Baek, Wilson, Lee, & Eling, 2002). Another important gene associated with the expression of several proinflammatory genes, such as cytokines and inducible enzymes, is nuclear factor kappa B (NF-κB). Activation of NF-κB plays a central role in initiating and promoting the inflammatory response. Nitric oxide (NO) is a pleiotypic inflammatory mediator, which is produced by inducible nitric oxide synthase (iNOS). Under pathological conditions, macrophages can significantly increase NO production. Thus, iNOS and NO inhibition are important goals of anti-inflammatory drug discovery (Gosslau, Li, Chi-Tang, Chen, & Rawson, 2001).

Reactive oxygen species (ROS) are mediators of oxidative stress that can damage all cell structures. It has a major role in several diseases such as cancer, impaired immune system and increased risk of infectious diseases, diabetes, cardiovascular disorders, etc. Exogenous antioxidants (natural products) can prevent, or repair injuries caused by ROS and enhance the immune defense against cancer and degenerative diseases (Alexieva, Markova, Nikolova, Aragane, & Higashino, 2010). It is still urgent to identify new anticancer agents with selective toxicity, although there have been advances in cancer research. Natural products, mainly originated from plants, are used clinically as anti-cancer agents (Cragg & Newman, 2005). Plant extracts are the starting point of investigation of leading anticancer compounds from natural sources.

In this study, we aimed to understand the antioxidant, cytotoxic and anti-inflammatory activities of *A. tricolor* and to show the effects of its different extracts on the substances involved in the inflammation mechanism both *in vitro* and *in vivo*. For this purpose, the anti-inflammatory effects of the extracts were investigated by *in vitro* cell-based tests and *in vivo* in rats against different inflammation inducers such as *Macrovipera lebetina obtusa* venom and carrageenan.

MATERIALS AND METHODS

Reagents

The cells were obtained from ATCC (Rockville, MD, USA). DCFH-DA, RPMI-1640 and DMEM/F12 media were purchased from Invitrogen (Carlsbad, CA). The NF-κB reporter construct contained two copies of the element from the immunoglobulin

K promoter (p BIIXLUC) and was a gift from Dr. Riccardo Dalla-Favera (Columbia University, New York, USA). The Sp-1 reporter plasmid (pGL3-promoter) was from Promega. The luciferase constructs containing NAG-1 promoter were a gift from Dr. Elling, NIH, North Carolina. The Luciferase Assay kit was from Promega (California, USA). All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

Snake venom

The lyophilized venom has obtained from pooled venom extracted for previous studies from two adult *M. l. obtusa* males which were from Osmaniye province in Turkey (Ozen et al., 2015). The lyophilized venom was dissolved in isotonic saline and sterilized by filtration through a 0.22 µm filter. The protein concentration of crude venom was determined by the BCA assay (Thermo Scientific, USA). Measurements were performed at 562 nm by a spectrophotometer (Thermo Scientific, USA).

Plant material and extraction

From our previous study, *A. tricolor* was collected from Taşkent Village, Cyprus during the flowering period (April 2010) (NEUN Herbarium no. 01337) (Karaalp et al., 2014).

For extraction, aerial parts of the *A. tricolor* (20 g) were dried and powdered. Then, *n*-hexane, chloroform and methanol extracts were prepared with an ultrasonic bath (3x 200 ml, 4 h for each) and evaporated to dryness under reduced pressure at 40°C (Demiroz, Nalbantsoy, Kose, & Baykan, 2020).

***In vitro* experiments**

To prepare a stock solution, lyophilized extracts were dissolved in DMSO at a concentration of 10 mg/mL. The concentrations of the extracts were 0.5, 5 and 50 µg/ml in DCFH and iNOS inhibition and NAG-1 activation assays. The final concentration of DMSO was adjusted to be less than 0.5%.

Inhibition of Reactive Oxygen Species (ROS) generation

Inhibition of cellular oxidative stress was measured by the DCFH method according to Reedy et al. (Reddy, Gupta, Jacob, Khan, & Ferreira, 2007). Briefly, myelomonocytic HL-60 cells (1 x 10^6 cells/mL) cultured in RPMI 1640 medium were treated with test compounds (0.5, 5, 50 µg/mL) for 30 min. Then, cells were stimulated with 100 ng/mL phorbol 12-myristate-13-acetate (PMA, Sigma) for 30 min as previously described by our team (Demiroz et. al., 2020). DCFH-DA (Molecular Probes, 5 µg/mL) was added and cells were further incubated for 15 min. Levels of DCF were measured on a PolarStar plate reader with excitation wavelength at 485 nm and emission at 530 nm. The antioxidant activity of test samples was determined in terms of % decrease in DCF production compared to the vehicle control. Trolox was used as the positive control.

Activation of nonsteroidal anti-inflammatory drug (NSAID) activated gene-1 (NAG-1)

Human chondrosarcoma cells (SW1353) cultured in DMEM/F12 were transfected with 25 µg of luciferase plasmid construct (pcDNA3.1-NAG-1) containing a full-length NAG-1 cDNA by electroporation at 160 V and one 70-ms pulse using a BTX Electro Square Porator T 820 8BTX IN (San Diego, CA). Transfected cells were plated in 96 well plates at 1x10^5 cells/200 µl/well in DMEM/F12 supplemented with 10% FBS. After 24 h, the cells were treated with different concentrations of test compounds (0.5, 5, 50 µg/mL) for 24 h (Nalbantsoy et al., 2012). Luciferase activity was measured using the Luciferase Assay kit (Promega, USA). Light output was detected on a SpectraMax plate reader and fold activation of NAG-1 activity was calculated in comparison to the vehicle control (Nalbantsoy et al., 2012). Diclofenac was used as the positive control.

Inhibition of iNOS (Inducible Nitric Oxide Synthase) activity

Mouse macrophages (RAW264.7) were cultured in phenol red-free RPMI medium with 10% bovine calf serum. The assay cells were seeded in 96-well plates (50,000 cells/well) and incubated for 24 h. The assay was performed as previously described (Demiroz et al., 2020). The cultured cells were treated with different concentrations of the extracts (0.5, 5, 50 µg/mL). The level of nitrite in the medium was measured by using Griess reagent. The percent inhibition of nitrite production by the samples was calculated in comparison to the vehicle control. IC_{50} values were obtained from dose curves.

Cytotoxicity assay

In vitro cytotoxic activities of extracts of *A. tricolor* were evaluated using WST1 reagent (Roche, Mannheim, Germany) by analyzing the number of viable cells by the cleavage of tetrazolium salts added to the culture medium. Three cancer cell lines, U2OS (human osteosarcoma cell line), A549 (human lung cancer cell line), HeLa (human cervical cancer cell line) and one non-cancer cell line, 293 HEK (human embryonic kidney cell line) were cultured in DMEM supplemented with L-glutamine (2 mmol/L), 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum. Cells in the exponential growth phase were seeded in 96-well plates to make 5,000 cells/wells and 24 h later, samples were added at various concentrations (100, 50, 25, 10, 5 and 2 µg/mL). After 48 h drug exposure, cell viability was measured using WST-1 cell proliferation reagent. Absorbance at 450 nm and a reference wavelength at 620 nm were determined by microplate reader (Versamax, Molecular Devices, Sunnyvale, California, United States). The absorbance of the culture medium with supplemented WST-1 in the absence of cells was used as the background control. All measurements were performed in triplicate (Parlar et al., 2018).

***In vivo* experiments**

Acute toxicity of A. tricolor extracts

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

Experimental animals

The experimental protocol was approved by Ege University's Local Ethical Committee of Animal Experiment (Date: 21.10.2015, number: 2015-078). Male/Female Wistar albino rats weighing 150– 200 ± 20 g were purchased from the Experimental Animal Center of Ege University (Izmir, Turkey). Rats were maintained under standard conditions of temperature 22 ± 1°C with a regular 12 h light: 12 h dark cycle and were allowed free access to standard laboratory food and water.

***M. l. obtusa* venom and carrageenan-induced inflammation**

M. l. obtusa venom (75 µg/paw) and carrageenan 1% (10 mg/kg) were used as challenge doses to create edema, as stated in our previous study (Demiroz et al., 2018). The venom was dissolved in isotonic saline and injected sub-plantarly into the left hind paw of the rats. Control and indomethacin groups (against carrageenan and venom) were tested at the same time as a previous study performed by our team to reduce the use of animals due to ethical regulations. Therefore, the results in Table 4, Figure 1 and Figure 2 include the values determined in the previous study.

Assessment of anti-inflammatory activity

Rats were deprived of food overnight. Male and female rats were divided randomly into 11 groups (n=6). Twenty-five, 50 and 100 mg/mL of *A. tricolor* extracts against *M. l. obtusa* venom including 75 µg/paw *M. l. obtusa* venom+Tween 20 (5%) as a negative control group and 2.5, 12.5, 25 and 50 mg/mL *A. tricolor* extracts against carrageenan including carrageenan+Tween 20 (5%) as a negative control group were tested to compare (n=6). Chloroform extracts were dissolved in Tween 20 (5%) and administrated orally. Thirty minutes after administration, 0.1 mL of inductive agent (*M. l. obtusa* venom

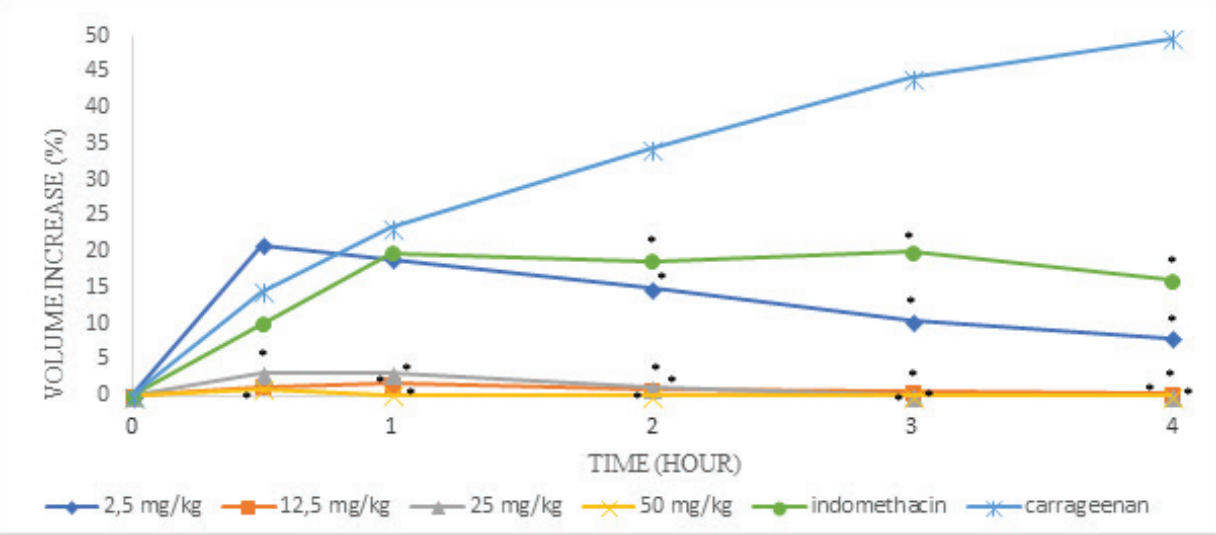

Figure 1. Anti-inflammatory potential effect of *A. tricolor* extract (2.5, 12.5, 25, 50 mg/kg) and indomethacin (10 mg/kg) against carrageenan-induced edema in rats. (*p<0.001 compared to carrageenan) carrageenan-induced edema.

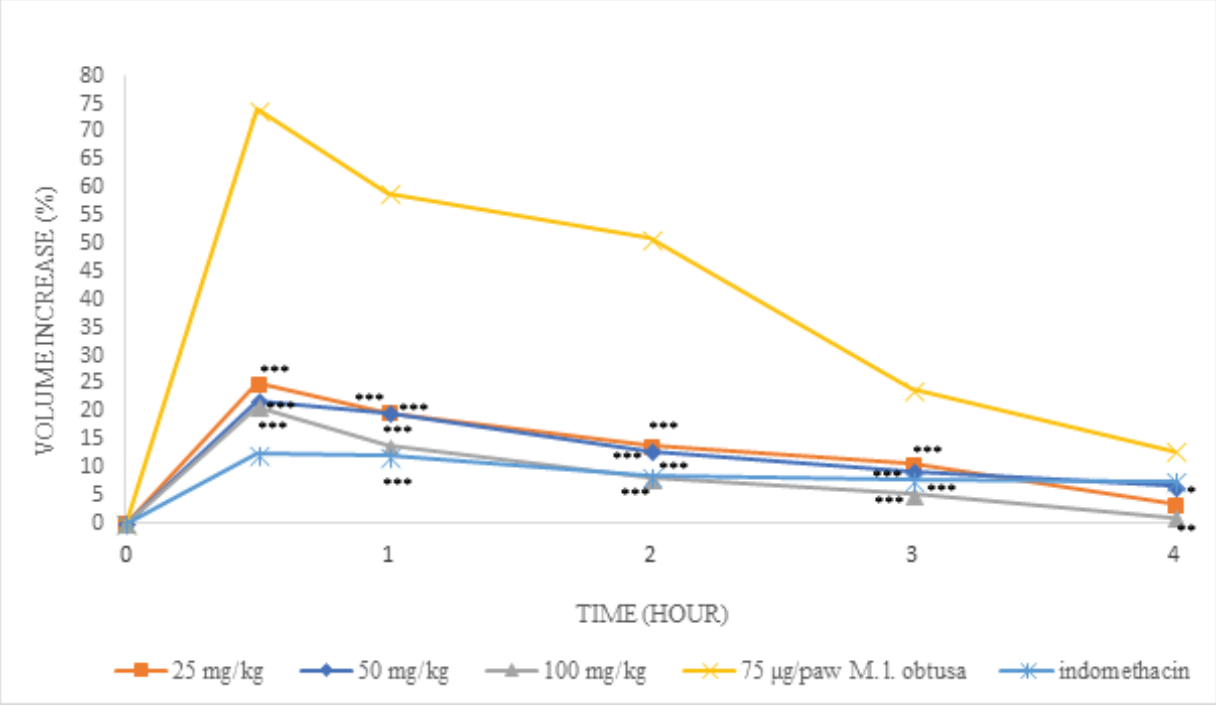

Figure 2. Anti-inflammatory potential effect of *A. tricolor* extract (25, 50, 100 mg/kg) and indomethacin (10 mg/kg) against 75 µg/paw of *M. l. obtusa* venom (*p < 0.05, **p < 0.01, ***p < 0.001 compared to *M. l. obtusa* venom).

or carrageenan) dissolved in isotonic saline was injected subplantar into the left hind paw. Isotonic saline (0.1 mL) was injected in the same way into the contralateral hind paw as a negative control group (n=6, for each group). Both paw volumes were measured by using a hydroplethysmometer (UGO BASILE, 21020 Monvalle VA, Italy) at 0.5, 1, 2, 3 and 4 h following inflammatory agent injection. Indomethacin (10 mg/kg) was used as the positive control anti-inflammatory agent (Sigma Chemical Co, St, Louis, USA) (Demiroz et al., 2018). The data obtained from the different groups was reported as the mean ± SD and % edema increase was calculated by the following formula:

$$Inhibition\ of\ edema\ (\%) = (Vcontrol - Vtreated)/Vcontrol$$

Data analysis

Each experiment was performed in triplicate for cytotoxic activities and anti-inflammatory experiments. Values were presented as mean ± standard error of the mean (SEM). IC50 calculation, and variance analysis (standard deviation calculation) were performed with Graph Pad Prism. Data were analyzed using the Student t-test, ANOVA, or nonparametric tests. Differences between extract or drug treated and control groups in vivo tests 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.

RESULTS

In present study, aerial parts of *A. tricolor* were extracted with *n*-hexane, chloroform and methanol. Then, antioxidant, cytotoxic and anti-inflammatory activities of the extracts were evaluated in *in vitro* and *in vivo* conditions. Yields of *n*-hexane, chloroform and methanol extracts were found as 1.44, 0.72 and 7.24% (288, 143 and 1447 mg), respectively.

***In vitro* experiments**

In vitro anti-inflammatory activities were evaluated over cellular mechanisms such as NF-κB, iNOS and ROS inhibition and induction of NAG-1. Results are shown in Table 1. While hexane and chloroform extracts showed moderate inhibition of iNOS (IC_{50}:16.5 ± 2.1 µg/mL and 14.0 ± 1.4 µg/mL respectively) and NF-κB (IC_{50}:15.0 ± 4.2 µg/mL and 10.75 ± 1.7 µg/mL respectively), methanol extract of the plant was inactive towards both targets. Moreover, hexane and chloroform extracts exhibited a weak inhibitory effect on Sp-1 dependent luciferase expression. Inhibition of cellular oxidative stress was measured by the DCFH method. The chloroform extract demonstrated the highest effect (IC_{50}: 4.45 ± 0.88 µg/mL), followed by methanol extract (IC_{50}: 36.0 ± 9.90 µg/mL) and finally hexane extract was found to be inactive in the inhibition of the ROS generation mechanism.

Dose response of NAG-1 induction by the extracts is given in Table 2. All extracts exhibited significant activity. The chloroform and hexane extracts showed the highest activity with about 7-8-fold induction at 50 µg/mL, followed by the methanolic extract (approximately 5-fold). At the doses of 5 µg/mL and 0.5 µg/mL, activation by all extracts was lower than the standard drug diclofenac (4.7-fold at 20 µM).

The cytotoxicity of the extracts against cancerous U2OS (human osteosarcoma), A549 (human lung cancer), HeLa (human cervical cancer) and non-cancerous HEK293 (human embryonic kidney) cell lines were evaluated and results are summarized in Table 3. Both hexane and chloroform extracts inhibited proliferation of the non-cancerous HEK293 cells. However, the strongest cytotoxic effect was observed in the chloroform extract against U2OS (IC_{50}:15.18 µg/mL) and HeLa (IC_{50}: 18.3 µg/mL). The hexane extract showed less toxicity against U2OS cell

Table 1. ROS, NOS, NF-κB and Sp-1 inhibition activities of *A. tricolor* extracts (IC_{50}, µg/mL).

	ROS	iNOS	NF-κB	Sp-1
Hexane extract	-	16.5 ± 2.1	15.0 ± 4.2	36.5 ±1.6
Chloroform extract*	4.45 ± 0.88	14.0 ± 1.4	10.75 ± 1.7	45.5 ± 4.9
Methanol extract	36.0 ± 9.9	-	-	-
Trolox	0.22 ± 0.02	N.A.	N.A.	N.A.
Parthenolide	N.A.	3.2 ± 0.7	0.9 ± 0.1	6.25 ± 1

IC_{50} values were presented as mean ± SEM. Dash means no activity; N.A.: Not applicable. (*p< 0.5 for Chloroform extract compared to other extracts in all of activities).

Table 2. *A. tricolor* extracts on NAG-1 fold induction.

	50 µg/mL	5 µg/mL	0.5 µg/mL	20 µg/mL
Hexane extract	6.8 ± 0.8	3.4 ± 1.0	2.5 ± 0.2	N.A.
Chloroform extract*	7.9 ± 2.1	4.1 ± 0.1	4.4 ± 0.9	N.A.
Methanol extract	5.2 ± 0.6	4.1 ± 1.1	2.9 ± 0	N.A.
Diclofenac	N.A.	N.A.	N.A.	4.7 ± 0.1

Values were presented as mean ± SEM. N.A.: Not applicable. (*p < 0.5 for Chloroform extract compared to other extracts).

line than chloroform extract (IC_{50}: 33.25 µg/mL), it was ineffective against HeLa and A549. In addition, the methanol extract had no activity against any of the cell lines tested.

In vivo experiments

In vivo studies were performed with chloroform extract, which had the highest effect in the *in vitro* anti-inflammatory studies. Also, the extract had no effect, inducing no obvious acute toxicity at all, in the exposure doses in the rats.

Carrageenan-induced inflammation

Percentages of paw volume increase were tested for the 2.5, 12.5, 25 and 50 mg/kg doses of the *A. tricolor* extract against carrageenan-induced edema. Percent edema (Edema %) increases at all doses can be seen in Table 4. The results were compared with the carrageenan and indomethacin results in our previous study, which was performed by our group concurrently with the current study. According to the previous study, at 4 h post induction of carrageenan, the percentage paw volume peaked around 49.64%. After indomethacin application, a plateau was observed between 1 and 3 h (81-82% inhibition) and finally a 16.01% edema increase was determined at 4 h (Demiroz et al., 2018). The current study showed all doses of extracts inhibited edemas at 0.5 h after the carrageenan injection. Moreover, *A. tricolor* extracts had much stronger anti-inflammatory activity than indomethacin against carrageenan-induced edema. At the first half hour, 1-3% volume increases were observed with 12.5, 25 and 50 mg/kg extracts and then the edemas were inhibited approximately 100% at end of 4 h. As an exception, an approximate 21% increase was observed 0.5 h after 2.5 mg/kg extract application. However, it had a similar effect to indomethacin at the end of 4 h (92.04 % inhibition) (Figure 1).

Snake venom-induced inflammation

In the previous study, 75 µg/paw of the venom was chosen as the challenge dose. The percentage volume of the paw measured peaked at 73.5% at 0.5 h after venom injection and reduced to 13.5%, the lowest percentage of paw volume was at 4 h. After 10 mg/kg indomethacin application, the percentage paw volume increase induced by *M. l. obtusa* venom were

Table 3. Cytotoxic activities of *A. tricolor* extracts on different cell lines (IC_{50}, µg/mL).

Extract/Cell Line	A549	U2OS	HeLa	HEK293
Hexane	-	33.25 ± 1.72	82.10 ± 5.65	34.38 ± 1.06
Chloroform*	40.85 ± 2.23	15.18 ± 0.84	18.30 ± 0.98	12.26 ± 0.71
Methanol	-	-	-	-

IC_{50} values were presented as mean ± SEM. Dash means no activity. (*p < 0.5 for Chloroform extract compared to other extracts

Table 4. Anti-inflammatory effect of *A. tricolor* extract against *M. l. obtusa* venom and carrageenan-induced edema in rats.

Group	Agent	Dose (mg/kg)	Edema Increase (%)				
			0.5 h	1 h	2 h	3 h	4 h
Control	Car.*	100 µL (%1 Car.)/paw	14.43±3.42	23.34±2.57	34.35±3.93	44.2±4.9	49.64±4.33
	*M. l. obtusa**	75 µg /100 µl/paw	73.5±3.39	58.67±3.39	51.17±1.35	23.5±1.45	13.5±1.11
A. tricolor	Car.	2.5	20.91±0.05	19.02±0.09	**14.84±0.13**	**10.39±0.11**	**7.96±0.09**
		12.5	**1.18±0.02**	**1.65±0.04**	**0.82±0.03**	**0.47±0.02**	**0.23±0.01**
		25	**3.12±0.05**	**3.03±0.05**	**1.12±0.02**	**0**	**0**
		50	**1.0±0.02**	**0.12±0**	**0**	**0**	**0**
A. tricolor	*M. l. obtusa*	25**	22.0±0.17	19.86±0.18	12.94±0.17	9.4±0.12	6.73±0.1
		50**	25±0.09	19.85±0.08	13.98±0.05	10.69±0.04	3.4±0.07
		100***	20.93±0.08	13.83±0.07	8.2±0.04	5.26±0.05	1.22±0.01
Ind. (10 mg/kg)	Car.*	100 µL (%1 Car.)/paw	10.03±3.01	19.87±4.03	18.7±3.45	19.99±2.14	16.01±2.59
	*M. l. obtusa**	75 µg /100 µL/paw	12.67±6.78	12.49±5.67	8.92±5.24	8.06±4.37	7.96±3.72

Values are represented as mean ± SD. Carrageenan (Car.), Indomethacin (Ind.). *Based on the previous study (Demiroz et al., 2018) (*A. tricolor* and indomethacin **p < 0.05, ***p < 0.01 compared to *M. l. obtusa* venom) (For the bold written values, p < 0.001 compared to carrageenan control group data)

12.67% and 7.96% at 0.5 and 4 h respectively (Demiroz et al., 2018). In the current study, the volume of the paw increase (%) calculated for 25, 50 and 100 mg/kg doses of *A. tricolor* chloroform extract against venom induced-edema is given in Figure 2. The most effective dose was the 100 mg/kg extract, which inhibited the edema at 79.07% at the first half hour. On the other hand, all of the extracts which reduced the edema to 1.22-6.73% had a very strong effect at the end of 4h. While the 100 mg/kg dose of the extract inhibited the edema approximately 99% at 4 h, the anti-inflammatory effect of indomethacin was 92% (Figure 2).

DISCUSSION

In vitro experiments

In the present study, the model cells were used for each method. While HL60 cells are often preferred for ROS generation (Teufelhofer et al., 2003), in the NAG-1 activation assay the SW1353 cell line has been established as an inflammatory model (Pang et al., 2021). Using the same cell lines, Zhao's team evaluated the inhibition of ROS, iNOS and NF-κB and the activation of NAG-1 of six octulosonic acid derivatives isolated from *A. nobilis*. Although none of the molecules had significant activity against NF-κB or iNOS, all of them enhanced NAG-1 activity 2-3-fold at 50 μM and decreased ROS generation (Zhao et al., 2014). In our case, all extracts at 50 ug/mL had a stronger activity in the activation of NAG-1 than the standard drug. Also, 50 ug/mL of the chloroform extract (7.9-fold) was approximately twice that of the 5 ug/mL extract (4.1-fold), whereas the efficacy of the hexane and methanol extracts was similarly increased in a dose-dependent manner. In addition to NAG-1 activation, strong inhibition of iNOS and NF-κB of the chloroform and hexane extracts may be due to the synergistic effect of the molecules in the extracts. Likewise, the essential oil of *A. weidmanniana*, rich in terpene molecules and collected from Turkey, also exhibited a strong inhibitory effect on NO production (IC_{50}:41.2 μg/mL) (Conforti et al., 2012). There are many reports on *in vitro* antioxidant and anti-inflammatory effects of *Anthemis* species, such as *A. nobilis, A. aetnensis, A. arvensis* and *A. cotula* (Acquaviva et al., 2012; Al-Snafi, 2016; Boukhary et al., 2019; Mantle, Eddeb, & Pickering, 2000; Vučković et al., 2011; Zhao et al., 2014). However, antioxidant and anti-inflammatory effects and NAG-1 activation of *A. tricolor* extracts have been for the first time in this study.

To our knowledge, there is no report on the cytotoxic effect of *A. tricolor* and interestingly, there are also limited studies on extracts, essential oils or isolated compounds from *Anthemis* genus (Conforti et al., 2012; Radulović et al., 2013). However, it is well known, that biological activities of the plants are associated with the polarity of extraction solvent and the chemical composition of extracts. Thus, while the polar (aqueous) extract of *A. atropatana* showed negligible toxicity against HEK293 cells, dichloromethane extract of *A. mirheydari* had more toxicity against LS180 (human Caucasian colon adenocarcinoma), MCF-7 (breast cancer) and MOLT-4 (human T lymphoblast) cells than methanol extract (Jassbi et al., 2016; Khosravi, Mirzaie, Kashtali, & Noorbazargan, 2020). In our case, non-polar extracts (hexane and chloroform) were more effective than the polar (methanol) extract, concordant with the literature.

Sesquiterpene lactones are the chemical markers of the *Anthemis* genus (Staneva et al., 2008) and their cytotoxic and anti-inflammatory activities have been well demonstrated like many terpenoids (Rüngeler et al., 1999; Wong & Menendez, 1999). Non-polar fractions are rich in these compounds. Thus, the non-polar (chloroform) extract of *A. ruthenica* and isolated sesquiterpenes and flavonoids from this extract had strong inhibition of HeLa, MCF7 and A431 (human squamous carcinoma) cells proliferation (Hajdú et al., 2010; Réthy et al., 2007). Additionally, NF-κB DNA binding activity of sesquiterpene lactones from *A. arvensis* and *A. cotula* have been reported (Vučković et al., 2011). In the present study, biological activities of *A. tricolor* extracts in different polarities were tested against seven different cell lines (HL-60, SW1353, RAW 264-7, U205, A549, HeLa, HEK293) and the highest effects were observed in chloroform and hexane extracts in general. From this point of view, the effects of apolar fractions of *A. tricolor* (hexane and chloroform extracts) on cytotoxicity, NO generation and NF-κB may be attributed to sesquiterpenic compounds. In a previous study, sesquiterpenes of *A. plutonia*, an endemic species to Cyprus and related to *A. tricolor* have been identified (Bruno, Maggio, Arnold, Diaz, & Herz, 1998; Oberprieler & Vogt, 1999). However, further detailed phytochemical investigations on *A. tricolor* are needed.

In vivo experiments

According to the literature survey, hexane and sesquiterpene lactone extracts (SLE) of *A. tricolor* have been more effective than methanol extract against erythema in rats (Demirkan et al., 2019). In addition, there are many studies on the anti-inflammatory activities of other *Anthemis* species. For example, 500 mg/kg of methanol extract of *A. scrobicularis* had 63.06% inhibition at the end of 2 h (Yusufoglu, Alam, Salkini, & Zaghloul, 2014). Gonenc's team reported SLE, hexane and diethyl ether extracts of *A. wiedemanniana* inhibited carrageenan-induced edema in mice. While 200 mg/kg of hexane and diethyl ether extracts shrank the swelling at 180 min (25.5 and 23.8% inhibition), the same dose of SLE was effective for three hours (26.7-30.7% inhibitions) (Gönenç et al., 2014). In another study, while 200 mg/kg of SLE of *A. aciphylla* var. *aciphylla* inhibited 78% of carrageenan-induced paw edema at 5 h, the same dose of ethanol extract was effective at 1 h (68% inhibition) (Baltaci et al., 2011). Based on these studies, non-polar extracts of *Anthemis* genus such as SLE, hexane, and diethyl ether are more effective than polar fractions against inflammation and our results were confirmed by the literature. Chloroform extract of *A. tricolor* was the most effective fraction among all of extracts in the *in vitro* studies. Similarly, the *in vivo* studies showed low doses of chloroform extract of *A. tricolor* had the strongest activity against inflammation induced by carrageenan compared to the literature.

M. l. obtusa, blunt-nosed viper, the largest snake from the Viperidae family in Turkey, causes a significant percentage of snake bite cases (Mermer, Gocmen, & Cicek, 2012). It is known that *Vipera lebetina* (*Macrovipera lebetina*) venom triggers the

inflammation mechanism via their metalloproteases, which can stimulate proinflammatory factors such as TNF-α, IL-1, IL-6, IL-10 and IFNγ (De Toni et al., 2015; Farsky et al., 2000; Gutierrez, Rucavado, Escalante, & Diaz, 2005; Moura-da-Silva, Butera, & Tanjoni, 2007; Rucavado, Nunez, & Gutierrez, 1998; Teixeira, Cury, Moreira, Picolob, & Chaves, 2009; Trummal et al., 2005). PLA2 homologs, a kind of metalloprotease, exhibit severe myotoxic activity, affect plasma membrane integrity, cause toxicities such as hyperalgesia, edema and the release of proinflammatory cytokines (Kang et al., 2011; Lomonte & Rangel, 2012; Teixeira et al., 2009). Proinflammatory cytokines are usually produced by macrophages and affect COXs expression, and increase the level of iNOS production via activating the nuclear factor (NF)-κB signaling pathway (Hanada & Yoshimura, 2002; Hung, Hsu, Chung, & Huang, 2016; Zhang & An, 2007). Active COXs are involved in the synthesis of prostaglandin E2s, vasodilatation and vascular permeability increase in the area where snake venom is injected (Harris, Padilla, Koumas, Ray, & Phipps, 2002; Hirata & Narumiya, 2012; Moreiraa, Teixeiraa, Silva, D'Império Limab, & Dos-Santos, 2016; Teixeira et al., 2009).

The inflammatory effect of snake venom can vary according to the amount of venom exposure, protein concentration and the substances which are involved in the inflammatory mechanism in the protein content (Chippaux et al., 1991). Determining the amount of protein in venom is important to understand the toxicity and inflammatory potential of the venom. The content and protein concentration of venom varies according to species and subspecies of the snake, location, gender, age, nutrition, season and even captivity (Chippaux, 1998; Chippaux et al., 1991; Creer, Chou, Malhotra, & Thorpe, 2002; Daltry, Wüster, & Thorpe, 1996; Durban et al., 2011; Gubensek, Sket, Turk, & Lebez, 1974; Igci & Demiralp, 2012; McCleary, Sridharan, Dunstan, Mirtschin, & Kini, 2016; Menezes, Furtado, Travaglia-Cardoso, Camargo, & Serrano, 2006; Sarhan, Mostafa, Elbehiry, & Saber, 2017). In our previous study, the protein concentration of *M. l. obtusa* venom was found to be quite high (1999.5 µg/mL) (Demiroz et al., 2018). According to Igci and Demiralp (2012), *M. l. obtusa* venom contains 24% metalloprotease and 34% PLA2 (Igci & Demiralp, 2012). Additionally, characterization studies show *M. l. obtusa* venom contains bradykinin-activating peptide, disintegrin fragment, C-natriuretic peptide A, dimeric disintegrin, PLA2 PIII-metalloproteinase, serine proteinase- Factor V serine proteinase, thrombin-like serine proteinase, and L-amino acid oxidase protein families (Igci & Demiralp, 2012; Sanz, Ayvazyan, & Calvete, 2008).

Because of the venom's content, edema peak times and amount aren't clearly known. However, in general, the first hour is defined as the golden hour and is considered critical for these cases (Forgey, 2008; Isbister et al., 2013). In some case procedures, it is recommended to check the patient for the first three hours, hourly, then every few hours (India Ministry of Health & Family Welfare, 2006). According to the values we found in our study, although the amount of edema at the 4th hour still has a significant difference, the critical time for *M. l. obtusa* venom seems to be the first three hours. In the previous study, it was seen that there was a similar timeline for edema caused by another viper species, *Montivipera xanthina* venom (Demiroz et al., 2018).

Chloroform extract of the plant, which showed strong anti-inflammatory effect in the *in vitro* experiments, had similar effect as the *in vivo* studies against carrageenan-induced and *M. l. obtusa* venom inflammation model in rats. Snake venom reached its peak in 0.5 h and caused a rapid inflammatory effect, while carrageenan produced inflammation for a longer time. Concentrations at 12.5, 25 and 50 mg/kg of *A. tricolor* chloroform extract were more effective on carrageenan-induced inflammation compared to venom. However, 75 µg/paw of *M. l. obtusa* venom caused a 73.5% increase in paw volume at 0.5 h, while three doses of *A. tricolor* chloroform extract (25, 50 and 100 mg/kg) reduced paw volume nearly to 20-25% at the same time. It has been shown that *A. tricolor*, which has strong anti-inflammatory effect in the *in vitro* tests performed, showed similar results in *in vivo* studies. Compared to the standard drug, indomethacin, 2.5-100 mg/kg of *A. tricolor* chloroform extracts had more effect against the inflammation caused by carrageenan and *M. l. obtusa* venom. Based on the literature survey, there is only one study on the inhibition of edema caused by *M. l. obtusa* venom. It was performed with *Centaurea calolepis* and cnicin by our team. Fifty mg/kg of the extract was chosen as the most effective dose against venom-induced inflammation (22.55% inhibition), while 2.5 mg/kg of cnicin shrank the edema to 19.10% at 4 h (Demiroz et al., 2018). Our results showed all of the doses of *A. tricolor* (25, 50 and 100 mg/kg) had stronger effect than *C. calolepis*. The anti-inflammatory effect in rats may be due to sesquiterpene molecules such as other *Anthemis* species or *Centaurea*, another member of Asteraceae (Baltaci et al., 2011; Demirkan et al., 2019; Demiroz et al., 2018; Gonenc et al., 2011).

CONCLUSION

To our knowledge, there has been no study on the antioxidant, cytotoxic and anti-inflammatory effects of endemic *A. tricolor* by *in vitro* studies up to now. Moreover, by this study, anti-inflammatory activity of *A. tricolor* against carrageenan and snake venom-induced paw edema have been evaluated for the first time.

A. tricolor has enormous potential for the treatment of inflammation and local viper bites. Further studies should focus on purification of bioactive compounds as lead compounds for anticancer and anti-inflammatory drugs.

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