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ORIGINAL ARTICLE

Antinociceptive action of *Achillea biebersteinii* methanolic flower extract is mediated by interaction with cholinergic receptor in mouse pain models

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Abstract
Achillea biebersteinii is a perennial aromatic herb that grows in the Mediterranean area. The leaves of this plant are used in foods as bittering and appetizing agents. In folk medicine, it is used for the treatment of stomachache and abdominal pain. In this study, the analgesic effect of *A. biebersteinii* methanolic flower extract was tested in three pain models, namely: writhing, tail-flick and paw-licking (formalin) tests. *A. biebersteinii* extract inhibited abdominal cramps produced by acetic acid. The effect of *A. biebersteinii* was better than that of 70 mg/kg indomethacin. In tail flick, *A. biebersteinii* extract increased latency at 30 min and was as effective as 100 mg/kg diclofenac sodium. In formalin test, *A. biebersteinii* extracts decreased paw-licking and flinching response in early and late phases. Atropine blocked the action of *A. biebersteinii* extract (300 mg/kg) in the late phase of formalin test as well as in writhing and tail-flick tests. GC–MS analysis revealed that ascaridole and iso-ascaridole were the main constituents of *A. biebersteinii* flower extract. In conclusion, this study shows for the first time that the antinociceptive effect of *A. biebersteinii* is mediated by the cholinergic receptor.

Keywords *Achillea biebersteinii* · Antinociceptive effect · Cholinergic receptor · Writhing test · Paw-licking test · Tail-flick test · GC–MS

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Introduction

Achillea was named after Achilles who used yarrow to heal the wounds of the soldiers in the Trojan War of the Iliad (Saeidnia et al. 2011). *Achillea* species are distributed in the Mediterranean area (Al-Jaber et al. 2014). *Achillea biebersteinii* Afan. (Asteraceae) is a non-woody perennial, aromatic, herbaceous plant, 20–50 cm long. The plant usually grows in patches. The leaves of this plant are up to 10 cm long, dissected to a feathery structure into narrow segments. Heads appear as dense flat topped inflorescence yellow aggregation (Al-Eisawi 1998).

Achillea biebersteinii Afan has many biological activities including wound healing (Akkol et al. 2011), anti-ulcer (Abd-Alla et al. 2016), anti-bacterial (Hammad et al. 2013), anti-oxidant (Mirahmadi and Norouzi 2017; Mazandarani et al. 2015), anti-gout (Hudaib et al. 2011), anti-cancer (Ghavami et al. 2010), anti-epimastigote (Saeidnia et al. 2005), anti-cholinesterase (Sevindik et al. 2015), anti-inflammatory (Mohamed et al. 2016), hypoglycemic (Ahmadi et al. 2017), neuroprotective (Alikhanzade et al. 2014) and anti-platelet effects (Al-Jaber et al. 2014), In addition, it is effective in treating endometriosis (Demirel et al. 2014).

Many *Achillea* species were reported to have antinociceptive effects such as *A. nobilis* subsp. *neilreichii* (Karabay-Yavasoglu et al. 2007), *Achillea umbellate* (Radulovic et al. 2012), *A. wilhelmsii, A. Phrygia, A. vermicularis, A. setacea* (Kupeli et al. 2007) and *A. aleppica* DC. subsp. aleppica (Iscan et al. 2006). In folk medicine, *A. biebersteinii* is taken as tea for abdominal pain and stomachache (Erbay et al. 2017). Also, a combination of this plant and mint leaves was used in bioactive formulations for the same purpose (Zengin et al. 2017). To the best of our knowledge, there is no previous comprehensive study that has evaluated the effectiveness of *A. biebersteinii* in reducing pain and its mechanism of action. The present study investigates the antinociceptive effect of flower extract of this plant and the possible mechanism involved in its action.

Methods

Plant collection, identification and extraction

Achillea biebersteinii flowers were collected from Al-Bukaan (Salt region, Jordan) during May 2015 and authenticated by Prof. Barakat Abu-Irmaileh, Faculty of Agriculture at the University of Jordan.

A flower extract of *A. biebersteinii* was prepared by soaking coarsely grinded, dried flowers in 96% methanol. The solvent was evaporated in a rotary evaporator using reduced pressure at 45 °C as a maximum temperature. Then, it was kept at − 20 °C. The extract was dissolved in sterile distilled water immediately before use.

Drugs

Methanol was obtained from Tedia (USA). Na_2SO_4, Alkane standard (C8–C30) and atropine were purchased from Sigma-Aldrich (USA). Indomethacin was obtained from DAD (Jordan) and diclofenac sodium was from Diclopan (Taiwan). All drugs were prepared immediately before use by being dissolved in sterile normal saline and administered intraperitoneally (i.p).

Experimental animals

Female BALB/c mice (20–25 g) were obtained from Al-Ahliyya Amman University. Experimental animals were maintained at 23 ± 2 °C, 12 h light/12 h dark cycle. Food pellets and water were available ad libitum. At least 2 h before the experiment, the animals were kept in the laboratory for adaptation. Each mouse was only used once. In all experiments, mice were treated i.p with vehicle (control), standard drug 300 mg/kg, 400 mg/kg or 500 mg/kg *A. biebersteinii* extract 30 min prior to test performance. All followed procedures comply with The Jordanian Animal Welfare By-Law No. (11) of the year 2010 and the International Association for the Study of Pain (IASP) Guidelines for the Use of Animals in Research and were approved on 5 July 2015 by the ethical

committee for research on animals at Al-Ahliyya Amman University (No. AAU-2015,7/5).

Writhing test

Writhing test was performed by injecting 1% acetic acid i.p. The animals were immediately placed in transparent cages. Mouse behavior was video recorded. The number of writhes was counted for 20 min starting after 10 min from acetic acid injection. Percentage inhibition was calculated using the formula:

$$\%\text{Inhibition} = \frac{\text{no. of writhings in control} - \text{no. in extract-treated mice}}{\text{no. in control mice}} \times 100.$$

Animals were randomly divided into six groups each consisting of eight mice. Indomethacin (70 mg/kg) was used as a standard drug in the writhing test. Atropine (5 mg/kg), a cholinergic antagonist, was administered 15 min before the plant extract.

Tail-flick test

The tail-flick test was assessed by immersing the tail in a water bath at 55 ± 1 °C. Each group consisted of 14 mice. The time from immersing the tail till producing the first flick was recorded. A cutoff time was considered 10 s as in Abul-Husn et al. (2007). Diclofenac sodium (100 mg/kg) was used as a standard drug in the tail-flick test. Atropine (5 mg/kg) was administered 15 min before the plant extract.

Paw-licking test (formalin test)

The formalin test was carried out by injecting 20 µl of 2.5% formalin intraplantarly. The total time spent in licking, shaking, lifting and flinching of the injected paw was recorded in phase I (0–5 min) and phase II (25–30 min) after formalin injection. The percentage inhibition was calculated using the formula:

$$\%\text{Inhibition} = \frac{\text{average time of paw licking in control} - \text{average time of paw licking in extract-treated animals}}{\text{average time of paw licking in control}} \times 100.$$

Animals were randomly divided into six groups, each consisting of nine mice. Indomethacin (50 mg/kg) was used as a standard drug in the paw-licking test. Atropine (5 mg/kg) was administered 15 min before the plant extract.

Gas chromatography–mass spectrometric (GC–MS) analysis

The methanolic extract of *A. biebersteinii* was analyzed using a Varian CP-3800 GC/MS/MS-220 (Saturn, the Netherlands) system, equipped with a DB-5 GC capillary column (95% dimethyl polysiloxane, 5% diphenyl, 30 m × 0.25 mm i.d., 0.25 µm film 1 thicknesses). Using an automatic injector in the split mode, an aliquot of 1 µL of methanolic extract was injected into the GC. The column temperature was kept constant (isothermal) at 60 °C for 1 min, ramped to a final temperature of 246 °C at 3 °C min^{-1} and held isothermal constantly for a further 3 min. The ionization voltage was 70 eV, while the ionization source was 180 °C. Helium, 1.0 mL min^{-1}, was used as a carrier gas. Quantitative analysis was performed on Thermos Focus GC equipped with a split–splitless injector (split ratio 1:50), a flame ionization detector, and an optima-5 fused silica capillary column (5% diphenyl, 95% dimethyl polysiloxane, 30 m × 0.25 mm, 0.25 film thickness) and under the same conditions described for the GC/MS analysis part. Identification of the chemical constituents in the analyzed methanolic extract was performed by built-in libraries including Notational Institution of Standards and Technology Co. and Wiley Registry of Mass Spectral Data (USA) and by comparing their calculated retention indices relative to (C8–C30) n-alkanes with literature values measured on columns of identical polarity and/or co-injection of pure authentic compounds.

Statistical analysis

The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using GraphPad prism version 6 for all measured parameters. A p value less than 5% was considered significant.

Results

In writhing test, 300, 400 and 500 mg/kg *A. biebersteinii* methanolic extracts inhibited abdominal cramps by 77.6%, 63.4%, and 55.0%, respectively. The lowest dose (300 mg/kg) of the extract was more efficient than 70 mg/

kg indomethacin (59.4% inhibition) in reducing abdominal cramps. Also, the lowest dose (300 mg/kg) had more effect than the highest dose (500 mg/kg). Atropine blocked the action of *A. biebersteinii* (300 mg/kg) partially and significantly (Fig. 1).

In the tail-flick test, *A. biebersteinii* extracts (300 and 400 mg/kg) increased latency at 30 min; but not after 1.5 h of treatment. Both doses were not statistically different from 100 mg/kg diclofenac sodium. Atropine blocked the action of *A. biebersteinii* (300 mg/kg) significantly (Fig. 2).

In paw-licking test (formalin test), *A. biebersteinii* extracts decreased flinching and paw licking in early and late phases of the test. The percentage inhibition of paw licking in the early phase was 50.2, 49.6, 45.1 and 41.0% for 300, 400 and 500 mg/kg *A. biebersteinii* extract and 50 mg/kg indomethacin, respectively (Fig. 3). In the late phase of formalin test, the percentage inhibition of paw licking was 68.1, 68.1, 46.8 and 55.9% for 300, 400 and 500 mg/kg *A. biebersteinii* extract and 50 mg/kg indomethacin, respectively (Fig. 4). The cholinergic receptor antagonist atropine reversed the antinociceptive action of this plant in the late phase only (Figs. 3, 4).

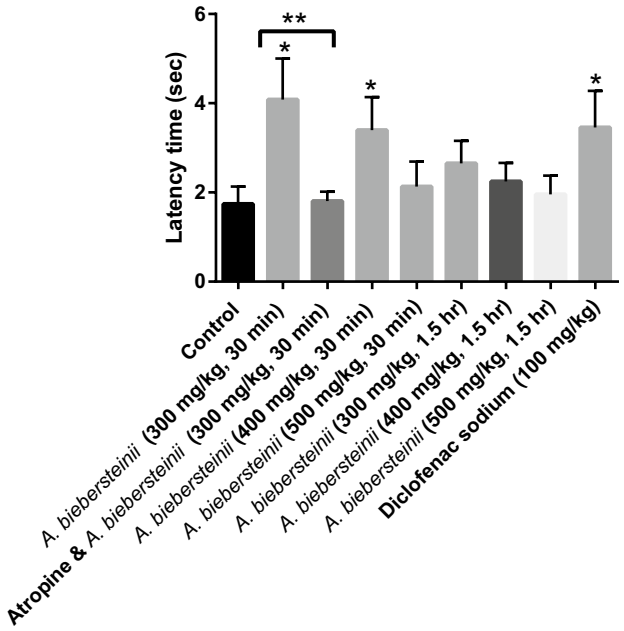

Fig. 2 Results of tail-flick test. *Significant difference from the control, $p < 0.05$. **Significant difference between *A. biebersteinii* (300 mg/kg) and *A. biebersteinii* (300 mg/kg) with atropine (5 mg/kg), $p < 0.05$

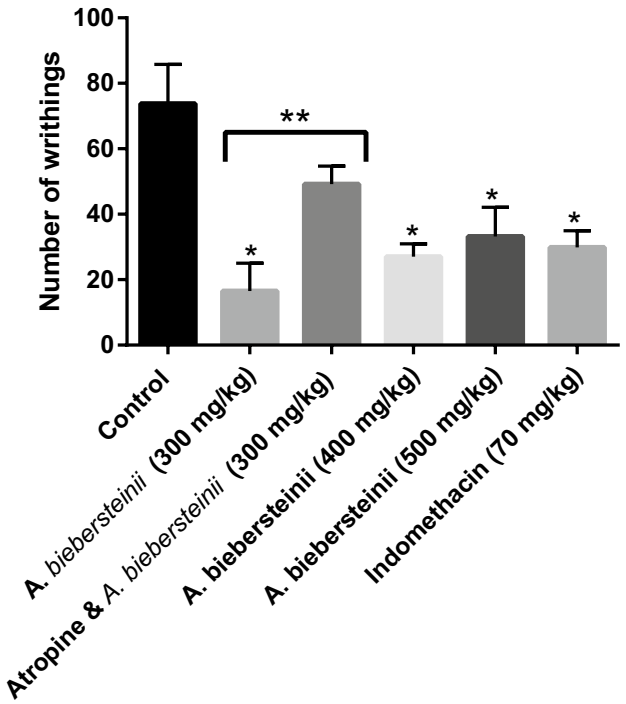

Fig. 1 Results of writhing test. *Significant difference from the control, $p < 0.05$. **Significant difference between *A. biebersteinii* (300 mg/kg) and *A. biebersteinii* (300 mg/kg) with atropine (5 mg/kg), $p < 0.05$

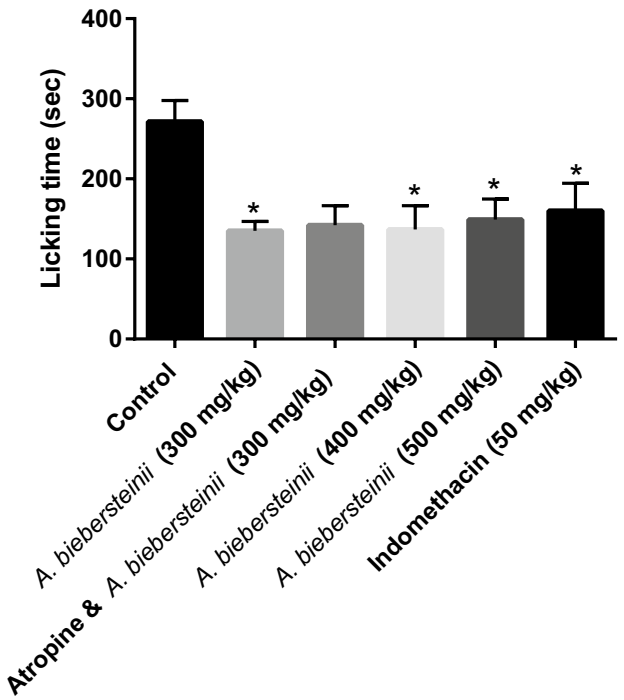

Fig. 3 Results of phase I of paw-licking test. *Significant difference from the control, $p < 0.05$

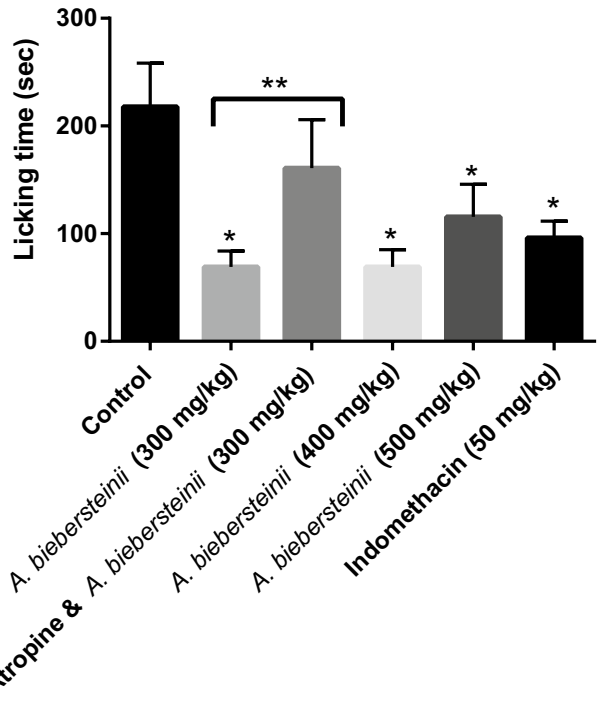

Fig. 4 Results of phase II of paw-licking test. *Significant difference from the control ($p < 0.05$). **Significant difference between *A. biebersteinii* (300 mg/kg) and *A. biebersteinii* (300 mg/kg) with atropine (5 mg/kg), $p < 0.05$

Table 1 Chemical composition of *A. biebersteinii* methanolic flower extract collected from Jordan

No.	Retention index	Compound	%
1	974	Sabinene	0.2
2	1027	*p*-Cymene	1.11
3	1019	Alpha-terpenine	0.2
4	1034	1.8-Cineol	0.8
5	1102	Linalool	0.35
6	1105	Trans-sabinene	0.9
7	1184	Terpinen-4-ol	0.51
8	1230	Nerol	0.48
9	1245	Ascaridole	43.22
10	1260	Cis-piperitone epoxide	0.44
11	1262	Trans-piperitone epoxide	0.9
12	1299	Thymol	0.8
13	1306	Carvacrol	1.6
14	1311	Iso-ascaridole	37.87
15	1356	Eugenol	1.13
16	1505	Lavandulyl-2-methylbutanoate	1.01
17	1581	Trans-sesquisabinene hydrate	1.2
18	1588	Hexadecene	0.7
19	1637	Gamma-eudesmol	0.8
20	1789	Octadecene	0.38
21	1959	Hexadecenoic acid methyl ester	0.6
22	1959	Hexadecenoic acid	0.67
23	2300	Tricosane (N-)	1.8
24	2500	Pentacosane (N-)	1.78

GC–MS analysis

Twenty-four compounds were identified in the methanolic extract of *A. biebersteinii* flowers collected from Jordan (Table 1). Ascaridole 43.22% and iso-ascaridole 37.87% were the main constituents.

Discussion

The use of plants is gaining renewed interest as potential sources of lead compounds in the search for new medications to treat different diseases or as sources for new drugs. In our work, *A. biebersteinii* flower extracts reduced the number of writhes produced by 1% acetic acid significantly. The percentage inhibition of 300 mg/kg *A. biebersteinii* extract was (77.6% inhibition) better than that of 70 mg/kg indomethacin (59.4% inhibition). Similar results were obtained by Pires et al. (2009) in which the hydroalcoholic extracts of *A. millefolium* aerial parts (500 and 1000 mg/kg) decreased acetic acid-induced abdominal contractions by 65 and 23%, respectively. Polar extract of *A. fragrantissima* (produced by 400 mg/kg) was more effective, with 55% inhibition, than non-polar extract in reducing abdominal cramps (Abdel-Rahman et al. 2015). In *p*-benzoquinone writhing test, the ethanol extracts of *A. wilhelmsii, A. setacea, A. phrygia* and *A. vermicularis* showed antinociceptive activity at 500 mg/kg dose (Kupeli et al. 2007). Also, the essential oils of different species of Achillea such as *A.aleppica* DC. subsp. aleppica (Iscan et al. 2006) and *A. umbellata* (Radulovic et al. 2012) exhibited antinociceptive activity in writhing tests. However, these essential oils were less potent than 200 mg/kg acetylsalicylic acid.

In our study, 300 and 400 mg/kg doses of *A. biebersteinii* methanolic extracts increased latency of tail-flick test after 30 min, but not after 1.5 h. Similar results were obtained by the aqueous and methanolic extracts of *A. ageratum* that exhibited analgesic properties (Garcia et al. 1997). This effect was more potent with the methanolic extract in the tail-flick assay where the estimated ED_{50} was 187.62 ± 37.96 (Garcia et al. 1997). On the other hand, the ethanolic extract of *A. nobilis* subsp. *neilreichii* (Kerner) Formanek (400 mg/kg) had no significant activity in the tail-flick test (Karabay-Yavasoglu et al. 2007).

Formalin test is sensitive to various classes of analgesic drugs. Two distinct phases of high licking activity can be observed. Phase I (neurogenic pain) starts within seconds after injecting formalin in the paw. This early phase is due to direct chemical activation of nociceptive primary afferent

fibers. The late phase or phase II lasts from 20 to 30 min after the injection of formalin. Phase II is also known as inflammatory pain and reflects ongoing activity in primary afferents and central sensitization of spinal cord circuits secondary to the inputs that occurred during phase I. Opioids inhibit both phases of the formalin test equally, while many non-steroidal anti-inflammatory drugs and corticosteroids inhibit only the second phase (Abotsi et al. 2016). In the present work, all tested doses of *A. biebersteinii* extract decreased licking time significantly in both the early (neurogenic) and late (inflammatory) phases of formalin test. *A. nobilis* subsp. *neilreichii* flower heads extract exhibited an antinociceptive effect during the late phase of the formalin test (100, 200 and 400 mg/kg, i.p.), but not during the early phase (Karabay-Yavasoglu et al. 2007). The hydroalcohol extracts of *A. millefolium* (500 and 1000 mg/kg) aerial parts without flowers did not exhibit any effect on the early or late responses in formalin test (Pires et al. 2009).

In our work, the action of *A. biebersteinii* extract in writhing test, tail-flick test and the late phase of formalin test was mediated by interaction with cholinergic receptor. This was evident by abolishing its effect when atropine, a non-specific muscarinic antagonist, was added. To our best knowledge, our study represents the first report on antinociceptive activity of *A. biebersteinii* and its interaction with cholinergic receptor.

In the writhing test, tail-flick test and phase II of paw-licking test, the activity of the lower dose (300 mg/kg) was more than the effect in higher doses (400 or 500 mg/kg). Plant extracts contain many phytochemicals having complex interactions such as synergistic, additive or antagonistic interactions. At high concentrations, some components of the extract may bind nonspecifically to receptors thereby decreasing the interaction of the active compound with the receptor involved in analgesic action. A similar trend was observed by Pires et al. (2009), in which the antinociceptive effect of *A. millefolium* high dose (1000 mg/kg) was less than that of the lower dose (500 mg/kg) in the writhing test.

In vitro, anti-cholinesterase activity of *A. biebersteinii* was reported (Sevindik et al. 2015). The inhibition of cholinesterase enzyme results in accumulation of acetylcholine which may exert an antinociceptive action. This analgesic mechanism of action was reported for several cholinesterase inhibitors such as physostigmine (Mojtahedin et al. 2009), amphetamine and its derivatives (Rezin et al. 2012), khat (Afify et al. 2017) and PhKv toxin (Rigo et al. 2017). However, the side effects of anti-cholinesterase agents may limit their usefulness as analgesic agents (Lauretti 2015). Further studies are needed to explore whether *A. biebersteinii* exerts anti-cholinesterase activity in vivo and if this mechanism contributes to the antinociceptive activity of this plant.

In our study, GC–MS analysis revealed that ascaridole (43.22%) and iso-ascaridole (37.87%) were the major constituents of *A. biebersteinii* flower extract. Our results agree with the findings of Hamad (2012) who reported that the major constituents in *A. biebersteinii* collected from Jordan were ascaridole (61.39%), trans-dihydro-H-terpinyl acetate (14.26%), *p*-cymene (8.1%) and H–terpinene (4.88%). Similarly, Bader et al. (2003) reported that *cis*-ascaridole, *p*-cymene, carvenone oxide and camphor were the major compounds in the oil of the flowering aerial parts of *A. biebersteinii* collected from Jordan. Using HPLC–MS, Hammad et al. (2013) found that quercetin 3-β-D-glucoside and ferulic acid were the major constituents in aqueous and hydro-ethanolic extract of Jordanian *A. biebersteinii*. Differences in extract preparation and analysis methods account for differences in the findings between Hammad et al. (2013) and the present study.

In folk medicine, *A. biebersteinii* is taken orally as herbal tea (Erbay et al. 2017). In our study, methanol was used for extraction of *A. biebersteinii* flowers by the cold method. Similar to the aqueous extract, the methanolic extract contains hydrophilic compounds. However, differences in composition between hot aqueous and alcoholic extracts are expected to exist. According to Hammad et al. (2013), the aqueous extract contained less phenolics compared to 70% ethanolic extract. Another difference between the present study and the ethnobotanical use of this plant is the route of administration. This study utilized i.p route which has differences in the pharmacokinetics of the tested extract from the oral route. Orally administered compounds are subjected to chemical modifications by gut bacteria. In addition, their absorption varies according to their chemical nature. Therefore, clinical studies are needed to test the pain-relieving efficiency of *A. biebersteinii* in humans.

The antinociceptive activity of *A. biebersteinii* can be attributed to several active constituents isolated from this plant such as its main constituent, the monoterpene ascaridole (Mohammadhosseini et al. 2017). In fact, ascaridole exhibited antagonistic effect on the *N*-methyl-D-aspartate (NMDA) receptor that is responsible for central sensitization and chronic pain (Calado et al. 2015). Other constituents in *A. biebersteinii* flower extract have been well documented for analgesic effects like eugenol (Park et al. 2011), *p*-cymene (Bonjardim et al. 2012) and carvacrol (Melo et al. 2012). Further work is needed to study the interaction of *A. biebersteinii* flower extract with NMDA receptors and to investigate the antinociceptive effect and mechanism of action of the essential oil of *A. biebersteinii*.

Conclusion

The results of our study indicate the involvement of cholinergic receptor in the antinociceptive action of *A. biebersteinii*. Further studies are needed to clarify the subtypes of

muscarinic receptors (M1–M5) involved in the antinociceptive activity of this plant.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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