



# *Astragalus fasciculifolius* manna; antinociceptive, anti-inflammatory and antioxidant properties in mice

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## Abstract

**Introduction:** Inflammation is one of the body's natural defence mechanisms alongside the cells or tissues damage due to various stimuli or infections. Pain is one of the greatest common symbols associated with different diseases. Therefore, researchers are trying to find new medications that bring patients relief with fewer side effects. Herbal medicine has been used for periods to treat acute and chronic pains.

**Objectives:** This research evaluated the anti-inflammatory, antinociceptive and antioxidant properties of *Astragalus fasciculifolius* manna extract.

**Materials and Methods:** The anti-inflammatory and antioxidant potency of the extract were determined by xylene test and ferric reducing antioxidant power assay.

**Results:** *Astragalus fasciculifolius* manna extract at doses of 400, 800 and 1200 mg/kg significantly reduced acetic acid-induced writhing and formalin-induced paw licking (late and early phase) but did not affect mechanical hyperalgesia caused by heat. Moreover, naloxone failed to inhibit the antinociceptive activity of the extract. The extract of *A. fasciculifolius* manna also exhibited high anti-inflammatory activity against xylene induced ear edema. Treatment of mice with the extract did not improve serum antioxidant capacity.

**Conclusion:** Results of the present research showed that *A. fasciculifolius* manna can prevent pain and inflammation because of the presence of high amount of saponin.

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## Introduction

Pain is one of the most common signs associated with different diseases and represents a major health and economic burden for society. It may lead to disability and reduction of the person's quality of life. The estimates of chronic pain prevalence range from 8 to over 60% depending on the population. There are several different types of drugs available to treat and alleviate pain (1). Present analgesic drugs, despite their confirmed pain-relieving effects, have significant side effects containing gastrointestinal complications, kidney damage, respiratory depression and emesis. They can also lead to physical and mental dependence (2). Researchers are therefore trying to find new medications that bring patients relief with fewer side effects. Herbal medicine has been used for centuries to treat acute and chronic pains (3).

Inflammation is one of the body's natural defence mechanisms alongside cells or tissues damage either because of various stimuli (chemical, mechanical and thermal) or infections. Prostaglandins and pro-

## Key point

Herbal medicine has been used for centuries to treat acute and chronic pains. *Astragalus membranaceus* has been reported in previous pharmacological studies such as antioxidant, immunoregulatory with anti-tumour effects. Results of the present study showed that *Astragalus fasciculifolius* manna can inhibit pain and inflammation because of the presence of high amount of saponin.

inflammatory cytokine play important roles as local messengers in the generation of the inflammatory response (4). Pain, redness, heat, swelling and edema are key symptoms of inflammation. It has been shown that many anti-inflammatory drugs such as a non-steroidal anti-inflammatory drug (NSAID) can alleviate inflammation and therefore relieves pain (5). Long-term use of these drugs can lead to gastrointestinal tract ulceration, renal impairment and bleeding. Therefore, it is necessary to find new analgesic and anti-inflammatory drugs which do not cause NSAIDs like side effects. Some herbal medicine such as white willow bark,

curcumin (turmeric), green tea, pycnogenol (maritime pine bark), *Uncaria tomentosa* (cat's claw) and capsaicin (chili pepper) have been reported to alleviate inflammation and associated pain by suppressing inflammatory cytokine and prostaglandin synthesis (6).

*Astragalus* is a large genus of flowering plant, with more than 3000 species belonging to the family Fabaceae. The plants of this genus are broadly distributed throughout the temperate and arid region of the world (7). They have a long history of use as herbal medicine in traditional and folk medicines (8). Antioxidant, anti-inflammatory, analgesic, immunoregulatory and anti-tumour effects of the extract and phytochemical components of some species such as *Astragalus membranaceus* have been reported in previous pharmacological studies (8-10). *A. fasciculifolius* is a species in the *Astragalus* genus that grows wild in Iran. There is no previous study on the analgesic, anti-inflammatory and antioxidants effects of this plant.

### Objectives

The key points of this research were; 1) to evaluate the analgesic activity of *A. fasciculifolius* manna extract against chemical (acetic acid and formalin) and thermal (hot plate) induced pain in mice; 2) to determine anti-inflammatory effects of *A. fasciculifolius* manna extract against xylene induced inflammation in mice; 3) to evaluate the properties of *A. fasciculifolius* manna extract on serum total antioxidant potency in mice.

### Materials and Methods

#### Extraction of plant

Dried Manna of *A. fasciculifolius* manna was acquired from a local herbal market located in Kerman, Iran. Then, it was scientifically confirmed by an expert botanist (Dr. H.A. Shirmardi), a voucher specimen (no.: 721) was registered for it at the Medicinal Plants Research Center. Powdered leaves were extracted with ethanol (50%) by maceration method. Then, after 72 h, the mixture was filtered by using Whatman filter paper No. 1 and concentrated with a rotary evaporator under vacuum at 40°C (11).

#### Determination of total phenolic content

Total phenolic content was assayed agreeing with the method of Maisuthisakul et al (12). Briefly, 0.1 mL of diluted extract (60°C methanol) was mixed with 0.5 mL of Folin-Ciocalteu as a reagent and 0.4 mL of 7.5% sodium carbonate solution. The mixture was incubated for 30 minutes in the dark place at laboratory temperature, and then the absorbance was read at 765 nm against blank. The total phenolic contentments were presented as milligrams equivalent of gallic acid per gram of dried extract.

#### Determination of total flavonoid and flavonol contents of the extract

Total flavonoid content in *A. fasciculifolius* manna extract was determined using the colourimetric assay. Concisely,

0.5 mL of the extract solution in 60°C methanol was mixed with 1 mL of 2% aluminium chloride and 6 mL of 5% potassium acetate. After 40 minutes of incubation at room laboratory, the absorbance of the reaction mixtures was measured at 415 nm. The aluminium chloride colourimetric method was used for flavonol content, after 150 min incubation the absorbance of the reaction mixture was read at 440 nm. Total flavonoids and flavonols were presented as milligram of equivalent rutin per gram of dried extract (12).

#### Determination of antioxidant capacity by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method

The antioxidant potency of *A. fasciculifolius* manna extract was determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. Two milliliters of the extract was added to 2 mL of 0.1 mM DPPH- ethanol solution and mixed. After incubation for 15 minutes at laboratory temperature, the absorbance was determined against blank at 517 nm. The percent inhibition of DPPH free radicals was computed from  $(A_{\text{control}} - A_{\text{sample}} / C_{\text{control}}) \times 100$ , where  $A_{\text{blank}}$  is the absorbance of the control reaction, and  $A_{\text{sample}}$  is the absorbance of the solution in the presence of herbal extract. The concentration causing 50% inhibition of DPPH free radicals ( $IC_{50}$ ) was computed by the regression equation for the concentration of extract and percentage inhibition (12).

#### Determination of total saponin content of the extract

At first, the powdered plant was mixed with distilled water and stirred vigorously for 30 seconds. The formation of persistent foam after 30 minutes confirmed the presence of saponins. The powdered plant was extracted with ethanol (50%) under reflux for 3 hours in order to determine saponin content. The mixture was filtered by using Whatman filter paper No.1 and concentrated under vacuum by rotary evaporator. The resulted aqueous extract was extracted three times with equal volumes of n-butanol. n-Butanol phase was then decanted and then concentrated by rotary evaporation under vacuum. The residues were freeze-dried and weighed as total saponin (13).

#### Experimental animals

Male mice, weighing 25-30 g were kept under standard laboratory circumstances (12 h light/12 h dark cycle at  $21 \pm 2^\circ\text{C}$ ) with free access to water and standard laboratory food (2).

#### Hot-plate test

This section of the investigation, 48 animals were accidentally divided into six groups. Each group included 8 mice. The groups included distilled water (1 mg/kg), plant extract (400, 800, 1200 mg/kg), morphine (0.5 mg/kg), and naloxone (0.5 mg/kg) + extract at the most effective dose. One day before the hot plate tests, animals were allowed to acclimate to the turn-off hot plate apparatus for

3-5 minutes. Fifteen minutes after the drug injection, hot-plate test was done. The mice were placed on the hot plate with constant temperature of 48°C. The time between the placing of the animal and the first response (paw-licking, lifting or shaking and animal jumping) was measured as latency to reach pain threshold. Animals were tested on a hot plate every 15 minutes for 1 hour. To prevention of tissue damage, the time of test was less than 60. In addition, the experiment was performed only once and the mice were removed from the study (2).

#### Acetic acid test

In this part, 48 mice were classified into 6 groups. Group 1 as the control group, received distilled water, groups 2, 3 and 4 received doses of 400, 800 and 1200 mg/kg of *A. fasciculifolius* manna extract, group 5 received morphine (1 mg/kg) as a standard analgesic drug and group 6 received naloxone (1 mg/kg as an opioid antagonist) + effective dose of the extract. Then, 30 minutes after intraperitoneal (IP) injection of the drugs, 100 mg/kg of %1 acetic acid was injected into each mouse to induce abdominal writhing. Then, the total number of abdominal writhing was recorded during 30 minutes (2).

#### Formalin test

Animals were divided into 6 groups like the acetic acid test groups. Fifteen minutes after drugs injection, 20 µL of formalin (2%) was administrated (into the right hind paw) and behavioural response to pain (lifting and licking of the same paw) was checked and recorded for 30 minutes (every 5 minutes). The first 5 minutes was considered as the acute phase and the last 5 minutes was considered as the chronic phase (2).

#### Xylene test

Anti-inflammatory effect of the extract was investigated by the xylene test based on the method of Torres-Rêgo et al (14). 6 groups of 8 individuals were treated as follows; group 1 received intraperitoneal injections of saline, group 2 received dexamethasone (13 mg/kg, IP), groups 3, 4 and 5 received doses of 0.5, 1 and 2 mg/kg of extract by an intraperitoneal injection. Inflammation was created by intradermal injection of xylene (0.03 mL) into the frontal and dorsal sides of the right ear. All the drugs were injected 15 min before the xylene injection. After 2 hours, mice were sacrificed and their ears were cut. Circular pieces (7 mm) of both the left (untreated) and right (treated) ears were prepared and weighed. Inflammation was determined as the difference of weight between the two earpieces.

#### Serum antioxidant capacity

After behavioural test, by cardiac puncture, blood samples were taken from each animal, and the serum was separated by centrifugation. The ferric reducing ability of plasma (FRAP) assay was applied to measure the total antioxidant capacity of the serum. This technique is based on the

ability of the serum to reduce ferric-tripyridyltriazine ( $\text{Fe}^{3+}$ -TPTZ) to a ferrous form ( $\text{Fe}^{2+}$ ) which gives a complex ( $\text{Fe}^{2+}$ -TPTZ) with blue colour and a maximum absorption at 593 nm (15).

## Results

### Antioxidant properties of the plant

Table 1 displays the antioxidant capacity of extract of *A. fasciculifolius* manna in DPPH method. The  $\text{IC}_{50}$  value of this extract was found to be 223.57 µg/mL.

### Phytochemical contents of the extract

The content of phenolic compounds in the *A. fasciculifolius* manna was  $36.35 \pm \text{mg GAE/g}$  extract while the contents of flavonoids and flavonols were negligible. The saponin content of the dried plant (25 g) was approximately 32% w/w.

### Hot-plate test

The latency time of pain reactions to thermal stimuli in the hot plate test is displayed in Figure 1. Morphine as a standard analgesic drug significantly increased the response latency to pain at both 15 and 60 min of the test period ( $P < 0.05$ ). Doses of 400, 800 and 1200 mg/kg of the extract of *A. fasciculifolius* manna did not prolong the latency to reach pain threshold. At 0 and 60 minutes, there was a significant decrease in response latency to pain in extract treated groups ( $P < 0.05$ ).

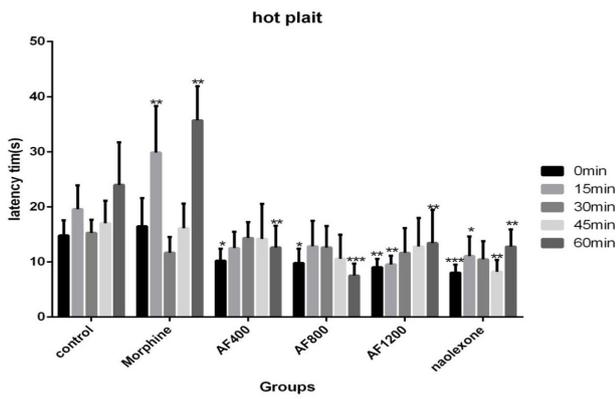
### Formalin test

Figure 2 shows the influence of *A. fasciculifolius* manna extract on formalin-induced nociceptive behaviour during phase I and phase II. In the first phase and final phase, the number of paw licking/lifting behaviours in the group of control was significantly higher than those in the group of morphine-treated ( $P < 0.05$ ). *A. fasciculifolius* manna extract significantly decreased both first and final phase formalin-induced pain behaviour ( $P < 0.05$ ). During the final phase, *A. fasciculifolius* manna extract reduces the paw licking/lifting behaviours in a dose-dependent mode. Moreover, the IP injection of naloxone (1 mg/kg) inhibited the analgesia induced by *A. fasciculifolius* manna extract only during the late phase.

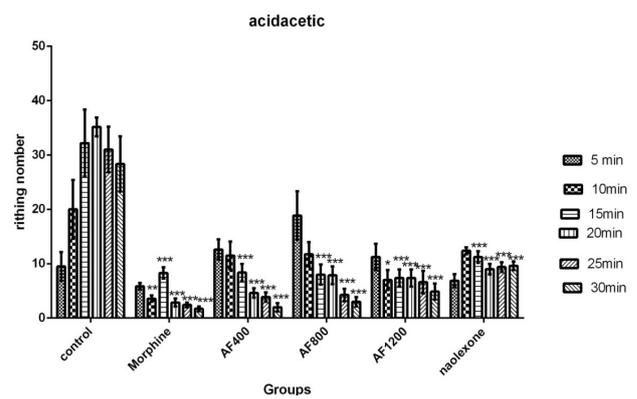
### Acetic acid test

Table 1. The antioxidant capacity of *A. fasciculifolius* extract in DPPH method

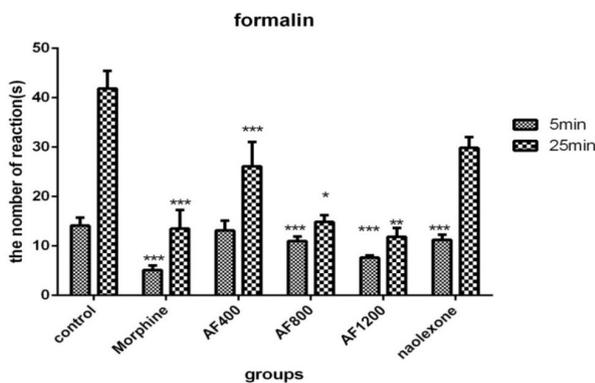
| Sample                    | Concentration (µg/mL) | DPPH radical scavenging activity (%) | IC50 (µg/mL) |
|---------------------------|-----------------------|--------------------------------------|--------------|
| <i>A. fasciculifolius</i> | 100                   | 23.38                                | 223.57       |
|                           | 150                   | 36.73                                |              |
|                           | 200                   | 51.24                                |              |
|                           | 250                   | 52.96                                |              |
|                           | 300                   | 59.90                                |              |
|                           | 350                   | 67.91                                |              |



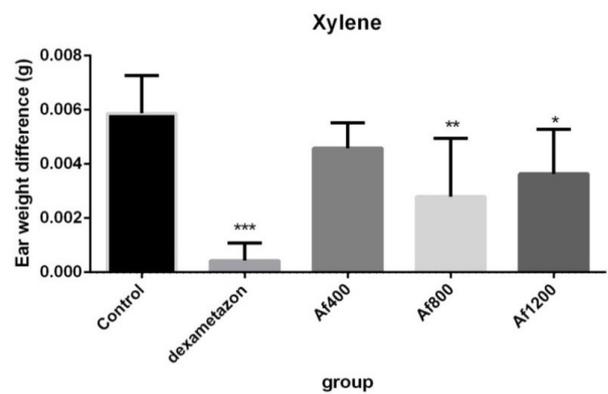
**Figure 1.** Effect of *A. fasciculifolius* extract at doses of 400, 800 and 1200 mg/kg on latency of pain response in hot plate test. Data showed as mean  $\pm$  SEM, \* $P < 0.05$ , \*\* $P = 0.05-0.01$ , \*\*\* $P < 0.01$



**Figure 3.** Effects of *A. fasciculifolius* extract at doses of 400, 800 and 1200 mg/kg on acetic acid-induced abdominal writhing in mice. Data showed as mean  $\pm$  SEM, \* $P < 0.05$ , \*\* $P = 0.05-0.01$ , \*\*\* $P < 0.01$ .



**Figure 2.** Effects of *A. fasciculifolius* extract at doses of 400, 800 and 1200 mg/kg on formalin-induced nociceptive behavior during early phase and late phase. Data showed as mean  $\pm$  SEM, \* $P < 0.05$ , \*\* $P = 0.05-0.01$ , \*\*\* $P < 0.01$ .



**Figure 4.** Effect of *A. fasciculifolius* extract at doses of 400, 800 and 1200 mg/kg on ear inflammation induced by xylene in mice. Data showed as mean  $\pm$  SEM, \* $P < 0.05$ , \*\* $P = 0.05-0.01$ , \*\*\* $P < 0.01$ .

**Figure 3** displays the influences of *A. fasciculifolius* manna extract on abdominal writhing induced by acetic acid- in mice. Morphine as the reference drug (1 mg/kg), significantly reduced the number of writhing produced by acetic acid ( $P < 0.05$ ). The number of abdominal writhing was significantly lower in *A. fasciculifolius* manna extract treated groups compared with the acetic acid group ( $P < 0.05$ ). There was not any statistically significant change between groups received different doses of the extract ( $P > 0.05$ ). Doses of 400, 800 and 1200 mg/kg of *A. fasciculifolius* manna extract affected a time-dependent decrease in the number of writhing induced by acid acetic. As shown in Figure 3, pretreatment with naloxone (1 mg/kg, IP) did not inhibit the analgesia induced by *A. fasciculifolius* manna extract ( $P > 0.05$ ).

**Xylene test**

**Figure 4** shows the influence of *A. fasciculifolius* manna extract on the ear inflammation created by xylene in mice. Mouse ear edema was created by intradermal injection of xylene into the right ear. Dexamethasone (13 mg/kg, IP)

significantly decreased the xylene induced ear edema in mice ( $P < 0.05$ ). Intraperitoneal injection of the doses of 800 and 1200 mg/kg of *A. fasciculifolius* manna extract caused significant reductions in ear edema induced by xylene. Moreover, 1200 mg/kg of the extract displayed better activity of anti-inflammatory ( $P < 0.05$ ).

**Serum antioxidant capacity**

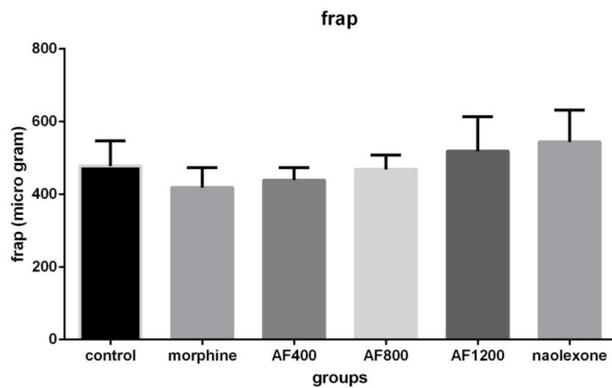
**Figure 5** shows the total serum antioxidant capacity of different groups. No significant difference observed between different experimental groups.

**Ethical issues**

All experimental and animal care procedures were performed in based on the guide for the care and use of laboratory animals and were accepted by research and ethics committee of Shahrekord University of Medical Sciences (ethical code; IR.SKUMS.REC.1394.74).

**Statistical analysis**

All information was shown as the mean  $\pm$  SEM. One-way ANOVA followed by Duncan Multiple range test was



**Figure 5.** Effect of *A. fasciculifolius* extract at doses of 400, 800 and 1200 mg/kg on serum antioxidant capacity in mice. Data showed as mean  $\pm$  SEM, \* $P < 0.05$ , \*\* $P = 0.05-0.01$ , \*\*\* $P < 0.01$

used to compare the means between experimental groups. Additionally,  $P < 0.05$  was considered statically significant.

## Discussion

We used tests of hot plate, acetic acid and formalin to study the *antinociceptive* activities of *A. fasciculifolius* manna extract. The anti-inflammatory and antioxidant actions of the extract were also determined by xylene test and FRAP.

The test of hot plate is suitable for estimation of the centrally acting analgesics which are recognized to increase the pain threshold of animal towards heat (16). This test is seemed to involve opioids and measures the compound reaction to an acute non-inflammatory nociceptive input (17). In this study, dose of 0.5 mg/kg of morphine considerably prolonged the reaction latency to thermally induced pain. Morphine is a centrally acting analgesic that binds to  $\mu$  opioid receptors and therefore relieves pain (18). This study showed that *A. fasciculifolius* manna extract did not prolong the reaction time of mice to noxious thermal pain. This reveals that the extract of *A. fasciculifolius* is not working through the central nervous system.

The acetic acid-induced writhing test is considered as an animal model of inflammatory pain. It is utilized as a screening method for assessing analgesic or anti-inflammatory effects of natural agents (18). It has been recommended that acetic acid works by discharging endogenous inflammatory mediators or irritation of the visceral surface, which causes to the release of inflammatory mediators including amines (e.g., histamine, serotonin), kinins (e.g., bradykinin), prostanoids (e.g., prostaglandins) and cytokines (tumor necrosis factor alpha, interleukin-1 $\beta$  and interleukin-8) (19,20). Results of this study showed that the hydro-alcoholic extract of *A. fasciculifolius* significantly reduces the number of acetic acid induced abdominal constriction or writhing in mice. In addition, its painkilling activity is not inhibited by naloxone which indicates that the opioid receptors are not involved in the analgesic effects of the extracts. The antinociceptive influence of the extract against acetic acid-

induced pain can because of either its action on visceral receptors sensitive to acetic acid or inhibition of the prostaglandin and pro-inflammatory cytokine synthesis in the gastrointestinal tract (21).

In our study, we used xylene test to assess the anti-inflammatory influence of *A. fasciculifolius* manna extract. Xylene induces the discharge of inflammatory mediators from sensory neurons. These mediators, in turn, work on peripheral target cells, for example mast cells and immune cells to produce inflammation characterized by warmth, redness and edema (22,23). In our study, *A. fasciculifolius* manna extract played a significant anti-inflammatory effect against xylene induced ear edema. Thus, the anti-inflammatory effect observed for *A. fasciculifolius* manna extract can be related to the inhibition of inflammatory mediators. Several medicinal plants were also reported to be effective in inhibiting xylene induced ear edema (24,25).

We also used test of formalin which is a valid and reliable test of nociception and inflammatory pain (2,4) to assess the analgesic influence of *A. fasciculifolius* manna extract. The first phase of formalin-induced nociception is because of the direct chemical stimulation of the nociceptors and the late phase is due to the inflammation of peripheral tissue. It was told that substance P and bradykinin contribute in the first phase, whereas histamine, serotonin, prostaglandins, nitric oxide and bradykinin are participated in the final phase. The results of our study exhibited that *A. fasciculifolius* hydro-alcoholic extract produces considerable analgesic activity in both first and final phase of the test of formalin. It can be concluded that the extract works by inhibiting the discharge and/or action of these substances because the extract showed significant inhibitory effects in both phases.

The phytochemical screening of *A. fasciculifolius* manna extract shown the presence of high amount of saponins. Many saponins isolated from medicinal plants have been informed to have antinociceptive and anti-inflammatory activities. For example, saponins separated from roots of *Chlorophytum borivilianum* significantly reduced histamine- and carrageenan-induced inflammation in rats (26). Saponin from *Quillaja saponaria* was also found to reduce the carrageenan induced inflammation in mice (27). The saponin fraction of *Zizyphus lotus* was reported to alleviate paw edema and pain by inhibition of the nitrite production (28). The anti-inflammatory properties of the saponins from *Entada phaseoloides* is attributed to the inhibition of the creation of nitric oxide and pro-inflammatory cytokines for example tumor necrosis factor alpha, interleukin-1 $\beta$ , interleukin-6 and interleukin-8 (29). Wang et al also found that pirostane-steroidal saponins from *Bletilla striata* show significant anti-inflammatory effects against cyclooxygenase-1 and cyclooxygenase-2 (30). The antinociceptive effect observed for *A. fasciculifolius* manna extract in our study

can be attributed to the presence of higher amounts of saponin which possess anti-inflammatory effects.

### Conclusion

In conclusion, this study exhibited that *A. fasciculifolius* manna extract attenuates acid acetic- and formalin-induced pain in mice. It also inhibits the xylene evoked inflammation.

### Authors' contribution

MSH and ZL designed the research. NA conducted experimental part of animal study. ShA and AT analyzed data and prepared the final draft of the manuscript. All authors read and signed the final manuscript.

### Conflicts of interest

The authors declare that they have no competing interest.

### Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission and redundancy) have been completely observed by the authors.

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