



BJP

Bangladesh Journal of Pharmacology

Research Article

**Analgesic and anti-inflammatory effects
of hydroalcoholic extract of *Astragalus
ibrahimianus***

Analgesic and anti-inflammatory effects of hydroalcoholic extract of *Astragalus ibrahimianus*

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Article Info

Received: 23 March 2023
Accepted: 15 May 2023
Available Online: 8 June 2023
DOI: 10.3329/bjp.v18i2.65135

Cite this article:

Aitbaba A, Sokar Z, Chait A. Analgesic and anti-inflammatory effects of hydroalcoholic extract of *Astragalus ibrahimianus*. Bangladesh J Pharmacol. 2023; 18: 41-48.

Abstract

This study aims to investigate the analgesic, anti-inflammatory, and antioxidant effects of the hydroalcoholic extract of *Astragalus ibrahimianus*, an endemic plant of Morocco. The analgesic effect was tested using the hot plate, writhing, and formalin tests. The anti-inflammatory activity was assessed by carrageenan and xylene-induced paw/ear edema respectively. The antioxidant activity was evaluated by three tests, namely DPPH, reducing power, and iron chelation. *A. ibrahimianus* treatment resulted in a significant reduction in carrageenan-induced paw edema and xylene-induced ear edema. Acute pretreatment with the extract reduced the number of abdominal cramps induced by acetic acid injection, increased paw withdrawal latency in the hot plate test, and suppressed both phases of the formalin test. The extract showed significant antioxidant activity regarding the standard molecules. In conclusion, *A. ibrahimianus* has analgesic, anti-inflammatory, and antioxidant properties.

Introduction

According to the World Health Organization, pain affects one in five adults, and it is estimated that one in ten people suffer from pain each year (Belfihadj, 2018).

Inflammation and pain have become the most important topic of many scientific works. Although the current therapies used to relieve pain are well-known for their side effects, opioids and non-steroidal anti-inflammatory drugs are among the most commonly used drugs in clinical practice (Ferrante et al., 2017). The opioid drugs pose problems of dependence (Meriem and Rania, 2022). Thus, traditional medications remain a crucial strategy to palliate these undesirable effects (Cao and Li, 2022).

Phytotherapy is, therefore, one of the main priorities against these pathologies. An interesting option in this field could be an extract of *Astragalus ibrahimianus*, an

endemic plant of Morocco never been studied. *Astragalus L.* is an annual, perennial, and vascular plant, that belongs to the Fabaceae family with nearly 2900 species (Kızıldağ et al., 2021). In Morocco, The *Astragalus* species are traditionally used for the treatment of cough and asthma (Bellakhdar et al., 1991). Further pharmacological and biological activities of other *Astragalus* species were recently discovered such as hepatoprotective activity (Kondeva et al., 2022) and anti-cancer properties (Sheik et al., 2021) and also the anti-diabesity, antifungal, antimicrobial, antioxidant, cytoprotective, antiparasitic and antiviral activities (Salehi et al., 2021).

The study aimed to investigate whether the hydroalcoholic extract of the aerial part of *A. ibrahimianus*, which has never been investigated, possesses potential biological activities. For this purpose, we studied the analgesic, anti-inflammatory, and antioxidant effects of the extract.



Materials and Methods

Plant material

Aerial parts of *A. ibrahimianus* were collected in June 2021 at Oukaimeden Station in the High-Atlas Mountains (74 km from Marrakesh, Morocco) at an elevation of 2600 m. The plant was identified by Prof A. Ouhammou, a taxonomist at the Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University. A copy of the specimen has been deposited in the Herbarium (Mark 14369).

Extract preparation

The hydroalcoholic extract was prepared by drying and grinding 50 g of aerial parts of *A. ibrahimianus* which were macerated in 80% ethanol for 24 hours. The extract was further filtered and vacuum-concentrated, and the yield was 8.8%.

Chemicals products

Indomethacin, morphine and aspirin were purchased from Laprophan (Morocco). Acetic acid, formalin, xylene, and carrageenan were obtained from the Sigma-Aldrich (France). All drugs, solutions and chemicals were prepared freshly before use and administered at 10 mL/kg.

Determination of total phenolic content

The quantity of total phenolic content of the extract was quantified according to the Folin-Ciocalteu method with some modifications (Singleton et al., 1999). For

this, 0.4 mL of diluted extract and 1.5 mL of Folin-Ciocalteu reagent were mixed. Five minutes later, 1.6 mL of sodium carbonate (7.5%) was added. Then, the solution was incubated for 2 hours at room temperature before measuring the absorbance at 765 nm on a spectrophotometer. Gallic acid was used as a standard and results were reported as mg EAG/g.

Determination of total flavonoid content

The total flavonoid content amount in the extract was estimated using the method described elsewhere (Zhishen et al., 1999). Shortly, 200 μ L of extract was diluted with 1 mL of distilled water and 60 μ L of 5% NaNO₂ and 60 μ L of 10% AlCl₃. After 5 min, 400 μ L of 1M NaOH was added to the medium. The absorbance was measured at 510 nm. Quercetin was used as a standard and results were reported as mg EQ/g.

Quantification of condensed tannins

The total condensed tannins were measured using the method described elsewhere (Heimler et al., 2005). 400 μ L of the diluted sample was added to 3 mL of 4% methanol vanillin solution and 1.5 mL of concentrated HCL. The mixture was incubated for 15 min, and the absorbance was measured at 500 nm. The quantity of total condensed tannins was expressed in mg equivalent of catechin/g.

Determination of DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was used to measure the antioxidant activity of the extract,

Box 1: FRAP Antioxidant Assay

Principle

The ferric reducing ability power (FRAP) assay is a method for detecting antioxidant capacity. It consists of preventing the development of Fe (II)-ferrozine complexes when samples were incubated with ferrous iron.

Requirements

Ferric chloride solution; Phosphate buffer (0.2M, pH=6.6); Potassium ferricyanide (1%); Trichloroacetic acid (10%); Butylated hydroxytoluene; Quercetin; Spectrophotometer (model: VR-2000, No.: 4120026)

Procedure

Step 1: A half milliliter of the extract at different concentrations (from 0.25 to 2 mg/mL) is mixed with 2.5 mL of a 0.2 M phosphate buffer solution (pH 6.6) and 2.5 mL of potassium ferricyanide solution at 1%.

Step 2: The whole set is incubated in a water bath at 50°C for 30 min then 2.5 mL of 10% trichloroacetic acid is added to stop the reaction and the tubes are centrifuged at 3,000 rpm for 10 min.

Step 3: An aliquot (2.5 mL) of supernatant is combined with 2.5 mL of distilled water and 0.5 mL of 0.1% aqueous ferric

chloride solution.

Step 4: The absorbance of the reaction medium was read at 700 nm against a similarly prepared blank, replacing the extract with distilled water to calibrate the apparatus (spectrophotometer).

Step 5: The positive control is represented by a solution of standard antioxidants, quercetin and butylated hydroxytoluene whose absorbance was measured under the same conditions as the samples. An increase in absorbance corresponds to an increase in the reducing power of the tested extracts

Calculation

The FRAP value was calculated using the following equation:

$$\text{FRAP value} = [(A_1 - A_0)/(A_c - A_0)] \times 2$$

Where A_c is the absorbance of the positive control, A₁ is the absorbance of the sample, and A₀ is the absorbance of the blank

Advantages

The FRAP assay is a relatively simple, quick, and inexpensive direct method of measuring the total antioxidant activity

References

Oyaizu, 1986 ; Liaqat et al., 2021

according to Mansouri et al. (2005) modified by Benmeddour et al. (2013). Briefly, 1.5 mL of a methanolic solution of DPPH (6×10^{-5} M) was mixed with 60 μ L of the extract at different concentrations of the extract (2, 4, 6, and 8 mg/mL). The mixture obtained was kept away from light at room temperature for 30 min. Then, the absorbance was measured at 515 nm against a control composed of 1.5 mL of the DPPH solution and 60 μ L of methanol. Trolox was used as a positive control. The percentage of inhibition was calculated according to the formula below:

$$\% \text{ Inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

The activity was expressed in mg/mL

Iron chelating ability

The iron chelation of the extract was determined by the method described elsewhere (Liyana-Pathirana and Shahidi, 2007). 0.4 mL of extracts or standard chelator (EDTA), 0.285 mL of distilled water, and 0.275 mL of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.2 mM) were mixed. After 5 min of incubation, 40 μ L of ferrozine (5 mM) was added, the mixture was stirred and allowed to react for 10 min and the absorbance was measured at 562 nm. The chelating activity was expressed as a percentage using the equation below:

$$\text{Chelating activity (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

The ferrous ion chelation capacity was expressed in mg/mL

Animals

Adult male Swiss mice (25–35 g) and Wistar albino rats (150–200 g) of both sexes were provided by the animal care unit of the Faculty of Science Semlalia, Cadi Ayad University, Morocco's Marrakech. The animals were kept at constant ambient temperature ($22 \pm 2^\circ\text{C}$) conditions with a 12 hours light/12 hours dark cycle. They had free access to food and drink.

Acute toxicity study

Four groups of mice (five animals per group) were equally divided. The extract was administered orally to three groups in doses of 1000, 2000, and 5000 mg/kg at a rate of 10 mL/kg. As a negative control, distilled water was administered to one group. The first two hours following extract administration were used to observe mice for indicators of toxicity and death. After treatment, the mice were weighed every day and monitored for 14 days.

Acetic acid-induced abdominal writhing

This test was performed using the method described elsewhere (Ferreira et al., 2000). Animals (5 mice per group) were treated with plant extract (250, 500 or 1000 mg/kg), control (10 mL/kg) or acetylsalicylic acid (200 mg/kg) followed after 45 min by intraperitoneal injection of 0.6% acetic acid solution (10 mL/kg). The resulting abdominal constrictions or writhes were

observed and counted within 30 min after the acetic acid injection.

Formalin-induced paw licking

According to the method described elsewhere (Hunskar and Hole, 1987), this test was conducted. Various doses of the extract (250, 500, and 1000 mg/kg) were given orally to groups of mice ($n=5$) 45 min before a 20 μ L injection of 2% formalin (v/v in 0.9% saline) was given into the subplantar area of the right hind paw. The vehicle was given to the control group (10 mL/kg of saline). The preferred analgesic medication was morphine (10 mg/kg, intraperitoneal injection) and acetylsalicylic acid (200 mg/kg, intraperitoneal injection). After injecting each mouse with formalin, the duration of paw licking was measured over 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase).

Hot plate test

The hot plate test was done as described elsewhere (Okolo et al., 1995). Animals are tested in a glass cylinder on a heated metal plate that was kept at a temperature of $55 \pm 1^\circ\text{C}$. Reaction time was measured as the latency to unpleasant reactions such as licking, shaking one paw, and jumping. Three oral dosages of the extract (250, 500, and 1000 mg/kg) were administered to treated mice. 10 mL/kg of water was administered orally to the control group. 10 mg/kg of morphine was injected intraperitoneally. After treatment, latencies to nociceptive responses were measured 30, 60, 90, and 120 min later.

Xylene-induced ear edema

The ear edema induced by xylene was carried out as previously described (Tang et al., 1984). Briefly, 45 min before administering 20 μ L of xylene inside and outside the right ear, each mouse received the extract (250, 500, or 1000 mg/kg), indomethacin (10 mg/kg), or vehicle (10 mL/kg). The control ear was the left ear. Mice (five per group) were sacrificed 30 min after being exposed to xylene by being injected with a lethal dose of chloral hydrate. Slices with a diameter of 7 mm were cut and weighed. We evaluated the weight differential between the identical animal's left and right ears to determine the extent of ear edema.

Carrageenan-induced paw edema

Anti-inflammatory activity was determined by measuring carrageenan-induced paw edema in rats according to the method described elsewhere (Winter et al., 1962). The extract was administered to Wistar rats divided into 4 groups (Control; extract 250, 500, 1000 mg/kg and indomethacin 10 mg/kg), 45 min before carrageenan injection (100 μ L of 1% in normal saline). Paw volume was measured using a digital caliper before carrageenan injection and 1, 2, 3, and 4 hours after injection. The results obtained were compared

with those obtained in rats given oral indomethacin (10 mg/kg).

The percentage of inhibition of edema was determined using the formula:

$$\% \text{Inhibition} = [(V \text{ control} - V \text{ sample}) / V \text{ control}] \times 100$$

where V sample is the volume (mL) of mice paw given test drug or extract at corresponding time and V control is the paw mice volume of the control group at the same time

Statistical analysis

For each measurement, the data were reported as the mean values \pm SEM. SigmaPlot 12.0 software was used to assess and perform the results using a one-way analysis of variance (one-way ANOVA). For differences that were significant ($p < 0.05$), the post hoc technique was utilized.

Results

Phytochemical study

A phytochemical study shows that *A. ibrahimianus* aerial component extract contained significant and intriguing amounts of total phenol, flavonoid, and condensed tannins (11.2 ± 1.6 mg GAE/g, 8.4 ± 0.4 mg QE/g, and 1.3 ± 0.2 mg CE/g, respectively).

Acute toxicity

All evaluated doses of 1000, 2000, and 5000 mg/kg of *A. ibrahimianus* were proven to be safe. At 14 days following *A. ibrahimianus* administration, no mortality and no significant changes in body weights or organ weights ($p > 0.05$) were noticed (data not shown). As a result, the extract's oral LD₅₀ was greater than 5000 mg/kg. This finding points to the extract's low toxicity (OECD, 2001). Three-treatment doses of 250, 500, and 1000 mg/kg body weight were chosen for additional *in vivo*

research on the results of this toxicity test.

Writhing test

All doses of the extract reduced significantly the number of writhing dose-dependently ($p < 0.001$) (Figure 1A). Therefore, the lower dose showed relatively moderate analgesic activity with 56.6% inhibition of acetic acid-induced writhing compared to the control, and the dose 500 and 1000 mg/kg groups showed 74.0% and 79.2% respectively. Also, the acetylsalicylic acid used as the standard agent elicited a significant reduction of 87.0% ($p < 0.001$) of the writhing number.

Formalin test

The extract had significant analgesic effects by reducing paw licking time in the neurogenic and inflammatory phases tested, but the reduction was most pronounced in the second phase (Figure 1B). Administration of *A. ibrahimianus* at 250, 500, and 1000 mg/kg reduced paw licking time by 57.2, 54.1, and 41.0% and 86.2, 85.1, and 88.7% in the first and second phases of the formalin trial, respectively. The reference drug acetylsalicylic acid was more effective in the second stage (88.7%). Morphine shortened the licking time in both phases.

Hot plate test

The data from the antinociceptive activity indicated that *A. ibrahimianus* increased significantly ($p < 0.001$) the time latency at 30, 60, and 90 min compared to the control (Figure 1C). The standard drug (morphine 10 mg/kg) also increased the time significantly in all time intervals compared to the control group.

Xylene-induced ear edema

The results show that after the topical application of xylene to the ear, the difference in ear weight was significantly greater in the control group compared to the treatment groups (Table I). *A. ibrahimianus* at doses

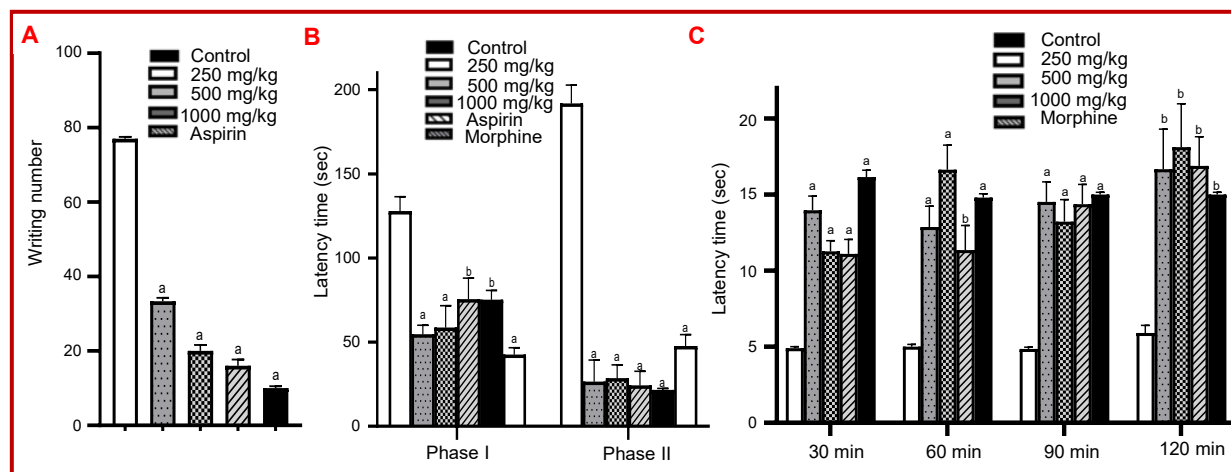


Figure 1: Effect of *A. ibrahimianus* on nociception tests: writhing test (A); hot plate test (B); Formalin test (C). Results are presented as mean \pm SEM. Superscript 'a' means p value < 0.05 ; 'b' means p value < 0.01 ; 'c' means p value < 0.001 compared to the control group

Table I		
Effect of <i>A. ibrahimianus</i> on xylene-induced ear edema in mice		
Treatment	Ear edema (mg)	Inhibition (%)
Control	59.9 ± 11.7	-
Indomethacin (10 mg/kg)	10.4 ± 1.1 ^a	82.6
Extract (250 mg/kg)	15.1 ± 2.4 ^b	74.8
Extract (500 mg/kg)	18.0 ± 0.9 ^b	69.8
Extract (1000 mg/kg)	16.1 ± 2.8 ^b	73.0

Data are mean ± SEM; n=5; Superscript 'a' means p value <0.001; 'b' means p value <0.01

of 250 (74.8%), 500 (39.8%), and 1000 (73.0%) mg/kg inhibited significantly xylene-induced ear edema in mice of all treated groups cases compared to the control ($p < 0.01$). Indomethacin (10 mg/kg) had a significant anti-inflammatory effect, reducing ear edema by 82.6% ($p < 0.001$). The data demonstrate that *A. ibrahimianus* can significantly inhibit xylene-induced ear edema.

Carrageenan-induced paw edema

The anti-inflammatory effect of *A. ibrahimianus* on carrageenan-induced paw edema was evaluated (Table II). After treatment with carrageenan, the paw volume of the control group increased in a clear time-dependent manner. However, paw volume in animals treated with *A. ibrahimianus* 250 (20.4%), 500 (18.5%), and 1000 (23.4%) mg/kg was significantly increased from 2 to 4 hours after carrageenan treatment ($p < 0.001$). The treatment with indomethacin (10 mg/kg) had a significant same effect as that exerted by the extract.

Antioxidant activity

Regarding the outcomes of standard antioxidant, *A. ibrahimianus* demonstrated an interesting antioxidant impact with the three methods used (DPPH, iron chelation, and reducing power). With an IC_{50} value of

Table III				
Antioxidant activity of <i>A. ibrahimianus</i>				
Antioxidant assay	Plant extract (IC_{50})	Standard antioxidant (IC_{50} mg/mL)		
		Quercetin	BHT	EDTA
DPPH	4.4 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	ND
FRAP	1.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ND
Iron chelation	1.6 ± 0.0	ND	ND	0.05 ± 0.0

Data are mean ± SEM; n=5

4.2 ± 0.1 mg/mL, the extract was able to transform the stable, purple-colored radical DPPH into yellow-colored DPPH-H (Table III). The information obtained additionally showed that the extract had a strong ability to scavenge iron chelation ($IC_{50} = 1.6 ± 0.0$ mg/mL). A good lowering power was also demonstrated by *A. ibrahimianus* ($IC_{50} = 1.1 ± 0.0$ mg/mL).

Discussion

In the present work, the extract of *A. ibrahimianus* in mice shows no evidence of acute toxicity or death. This is classified as the lowest toxicity class (OECD, 2001).

A. ibrahimianus exhibits significant analgesic activity in multiple pain models, including the writhing test, the formalin test and the hot plate test. First, the effect of *A. ibrahimianus* on peripheral pain perception was investigated using the acetic acid-induced writhing model in mice, which is commonly used to assess central and peripheral analgesia (Oguejiofor et al., 2013). Endogenous chemicals (bradykinin, serotonin, histamine, substance P) are released by acetic acid, which causes pain (Bhukya et al., 2009). When acetic acid is injected, it causes hyperalgesia, which is characterized by abdominal muscle contractions, forelimb extension, and lengthening of the body. Both

Table II					
Effect of <i>A. ibrahimianus</i> on the carrageenan-induced paw edema model					
Treatment	Mean paw volume (mL)				
	0 hour	1 hour	2 hours	3 hours	4 hours
Control	1.6 ± 0.0 (-)	1.7 ± 0.0 (-)	2.1 ± 0.1 (-)	3.1 ± 0.2 (-)	3.6 ± 0.2 (-)
Indomethacin (10 mg/kg)	1.5 ± 0.0 (4.8)	1.4 ± 0.0 ^b (17.8)	1.6 ± 0.1 ^c (25)	1.6 ± 0.0 ^c (46.1)	1.84 ± 0.0 ^c (49.1)
Extract (250 mg/kg)	1.4 ± 0.0 (9.7)	1.7 ± 0.0 (-1.7)	1.9 ± 0.1 ^a (10.1)	2.5 ± 0.0 ^c (17.1)	2.8 ± 0.0 ^c (20.4)
Extract (500 mg/kg)	1.7 ± 0.0 (-3.6)	1.7 ± 0.0 (-0.5)	1.9 ± 0.0 ^a (10.6)	2.6 ± 0.1 ^c (14.1)	2.9 ± 0.1 ^c (18.5)
Extract (1000 mg/kg)	1.6 ± 0.0 (-2.4)	1.7 ± 0.0 (-1.7)	1.8 ± 0.0 ^a (12.5)	2.4 ± 0.2 ^c (19.6)	2.7 ± 0.1 ^c (23.4)

Data are mean ± SEM; n=5; Data within parenthesis means %inhibition; Superscript 'a' means p value <0.05; 'b' means p value <0.01; 'c' means p value <0.001

analgesics (such as morphine) and non-steroidal anti-inflammatory medicines (such as aspirin) affect these peripheral nociceptive fibers (Riditid et al, 2008). In this study, a dose-dependent reduction of the acetic acid-induced abdominal contraction response was achieved with *A. ibrahimianus*. As a result, the pharmacological activity of *A. ibrahimianus* may be the basis for the potential mechanism of action of its analgesic effects by inhibiting or releasing endogenous chemicals (arachidonic acid metabolites) that excite pain nerve terminals.

The formalin-induced nociception test consists of two distinct phases to measure the behavioral effects of analgesics (Randolph and Peters, 1997). The first stage (neurogenic stage) is caused by direct chemical stimulation of nociceptors. Substance P, glutamate, and bradykinin are thought to be involved in this stage. The second phase (inflammatory phase) is associated with the inflammation of peripheral tissues (Nascimento et al., 2013), which is the result of the release of several inflammatory mediators such as histamine, serotonin, and prostaglandins (Rácz and Zimmer, 2006). The results of this test indicate the possibility of anti-inflammatory components in the extract. The formalin test's second phase's observed inhibition indicated that *A. ibrahimianus* might function as a prostaglandin production inhibitor. Mice with carrageenan-induced paw edema were used in the carrageenan-induced paw edema model to verify the potential anti-inflammatory action of *A. ibrahimianus*.

Carrageenan-induced paw edema is a well-known model of acute inflammation because the pathophysiological changes following carrageenan administration are similar to the clinical situation (Fulgenzi et al., 2005). In the present study, a significant increase in paw edema due to biphasic inflammation in rats injected with carrageenan was observed, which is consistent with previous results for different *Astragalus* species (Hakim et al., 2010). Experimental data showed that *A. ibrahimianus* extract dose-dependently suppressed edema volume 3 hours after carrageenan stimulation. Based on the above findings, the anti-edema effect of *A. ibrahimianus* extract may be due to blocking the release of all inflammatory mediators involved in the vascular and cellular phases.

According to the hot plate test results, *A. ibrahimianus* showed significant analgesic activity in animal models. *A. ibrahimianus* phytochemical screening revealed the presence of substantial amounts of polyphenols, flavonoids, and tannins. Numerous studies using various experimental animal models have shown that flavonoids, tannins, and other polyphenolic chemicals have analgesic and anti-inflammatory activities (Krasteva et al., 2007; Naseri et al., 2012). In addition, tannins and flavonoids are known to prevent the production of prostaglandins.

The current study demonstrated that extract of the aerial parts of *A. ibrahimianus* possesses significant antioxidant activity. A literature search on free radical scavenging in *A. ibrahimianus* has not been reported elsewhere, but previous studies on different *Astragalus* species have reported significant antioxidant activity from these plants (Butkutė et al., 2018; Teyeb et al., 2012). The antioxidant potential of *A. ibrahimianus* aerial parts extract may be due to its phytochemical composition. According to research by Khan et al. (2015), phenolic substances can actively encourage antioxidant effects. The ability to scavenge a range of ROS, including superoxide and nitric oxide radicals, is a property of flavonoids with strong antioxidant activity in vitro (Halliwell, 2008). Additionally, a prior study indicated that flavonoids extracted from *Astragalus mongholicus* Bunge, a potential adjuvant in the atherosclerotic spectrum and perhaps a reducer of cardiovascular disease, had strong antioxidant activity (Wang et al., 2012). Additionally, anti-radical investigations on *A. acmophyllus*, *A. talasseus*, *A. microcephalus*, and *A. gamnifer* found considerable antioxidant potential in their extracts and linked this impact to their elevated total phenolics and associated flavonoid content (Albayrak and Kaya, 2018).

Conclusion

A. ibrahimianus has analgesic and anti-inflammatory effects in various animal models. The extract displays antioxidant activity as well. Together, these findings imply that *A. ibrahimianus* may be effective in treating pain and inflammatory in people.

Financial Support

Self-funded

Ethical Issue

All experiments have been completed according with European Community guidelines (EEC Directive 86/609/EEC, 24 November 1986). Every attempt has been made to decrease animal struggling and to lessen the range of animals utilized in all experiments. The study was ethical and approved by the Council Committee of the Research Laboratory, Faculty of Science, Cadi Ayyad University of Marrakech.

Conflict of Interest

Authors declare no conflict of interest

Acknowledgement

Authors thank Mr. Regragui Abderazak for his assistance in providing drugs and animals.

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