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**Original Research Article**

# Evidence of anti-inflammatory and anti-ulcer properties of aerial parts of *Centaurea tougourensis* Boiss. and Reut.

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## Abstract

***Purpose:*** *To determine the anti-inflammatory and anti-ulcer properties of the aerial parts of Centaurea tougourensis Boiss. & Reut.*
***Methods:*** *The effects of n-butanol (n-BuOH) extract of the aerial part of Centaurea tougourensis on carrageenan-induced paw edema and ethanol-induced gastric mucosal damage were determined at 2 doses (200 and 400 mg/kg, po) in a mouse model. For each test, the animals were randomly divided into negative and positive control groups, as well as extract-treated groups. The mice were observed for any sign of inflammation for a period of 24h.*
***Results:*** *Reduction of paw edema by C. tougourensis extract was highly significant ($p < 0.001$) at a dose of 400 mg/kg 24 h after carrageenan injection, with 55.26 % inhibition, followed very closely by 53.15 % inhibition at the dose of 200 mg/kg; indomethacin group showed an inhibition of 60 %. Histological examination supported the inhibition results. A significant reduction in inflammation by the extract at a dose of 400 mg/kg was also observed. No sign of ulcer was observed with C. tougourensis at the two doses (200 and 400 mg/kg). The total polyphenol content of the n-BuOH extract was 85.44 µg gallic acid equivalent/mg of extract. Tannins were the most abundant fraction (51.87 µg tannic acid equivalent/mg of extract), followed by flavonoids (25.55 µg quercetin equivalent/mg of extract).*
***Conclusion:*** *The results indicate that C. tougourensis may have potential beneficial effects in the treatment of diseases associated with inflammation and pain, besides its protective effect on the gastrointestinal tract.*

***Keywords:*** *Anti-inflammatory, Anti-ulcer, Centaurea tougourensis, Flavonoids, Phenolics, Tannin*



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## INTRODUCTION

It is well known that chronic inflammation is associated with several physiological disorders, especially those that interfere with the normal functions of the immune system, leading to serious and chronic diseases. As a result, inflammatory pain is generated by these stimuli

due to peripheral tissue damage [1]. Unfortunately, the use of synthetic anti-inflammatory treatments is often associated with undesirable side effects. Therefore, there is urgent need for identification and use of more natural and more efficient medications for chronic inflammation.

The inflammatory response is a complex process that involves key coordination mediators such as prostaglandins (PGs), nitric oxide (NO), tumor necrosis factor-α (TNF-α), interferon γ (IFNγ), interleukin-1 (IL-1) and IL-6 [2]. These factors ensure good response and the maintenance of internal homeostasis, especially against infections and microbial pathologies which are responsible for some serious chronic inflammations.

In the long term, these inflammatory reactions may damage the gastrointestinal tract, leading to the formation of ulcers [3]. One of the ulcer diseases most frequently seen is peptic ulcer which occurs in the stomach, the first portion of the intestine.

The pharmaceutical industry has considerably evolved during the last decade, allowing for the treatment of several diseases. Thus, the world population now benefits from the contributions of folk medicine, recognizing the empirical knowledge of ancestors [4]. The therapeutic use of medicinal plants is practiced in many countries of the world, especially in developing countries. One of the reasons for resort to medicinal plants is the lack of a modern medical system.

*Centaurea* is one of the largest genus of herbaceous plants, with more than 500 species mainly distributed in the Mediterranean and West Asia regions. Over the years, studies on *Centaurea* species have demonstrated their pharmacological potential such as antioxidant, anti-microbial, anti-diabetic, cytotoxic, anti-hemolytic and anti-inflammatory properties. These effects are due to their richness in bioactive secondary metabolites, especially flavonoids, terpenes, polyphenols, alkaloids, tannins and saponins [5,6].

## EXPERIMENTAL

### Plant material

A sample of *C. tougourensis* was collected in spring 2019 from Belezma National Park in the municipality of Fesdis, Algeria (GPS coordinates: latitude 35.621975; longitude 6.241327). The sample was identified by taxonomists in the Agronomy Department of the Batna-1 University (Algeria), where a voucher specimen (no. CT/2019/LPTPCMB) was deposited.

### Experimental animals

Female Swiss Albino mice weighing 25 – 30 g were purchased from *Pasteur Institute, Algiers,* and maintained at ambient temperature of 22 ± 1 °C under 12 h light/12 h dark cycle, with free access to feed and water for two weeks, to acclimatize them to laboratory conditions. The experiments were performed in line with the guidelines of the National Research Council [7]. The study was approved by the Biology Animal Ethics Committee of University of Batna-2, Algeria (approval no. was 14/DBO/FSNV/ UB2/2017).

### Preparation of plant extract

The aerial parts of *C. tougourensis* were dried in a dry, ventilated place away from sun rays, and then ground to obtain 300 g of fine powder. Maceration was carried out three times with 3 L of EtOH-$H_20$ at a volume ratio of 70: 30 at room temperature for 3 days. Thereafter, liquid-liquid extraction was carried out with the following solvents: hexane, ethyl acetate and n-butanol.

### Preliminary phytochemical screening

Qualitative analysis of the phyto-constituents present in *C. tougourensis* was carried out using standard procedures based on precipitation and colorimetric reactions. The main secondary metabolite classes investigated in phytochemical screening were tannins, steroids, flavonoids, anthocyanins, triterpenes, alkaloids, quinones, saponins, mucilages, cyanogenic compounds and cardiotonic glycosides.

### Determination of total phenolic (TPC), flavonoid (TFC) and tannin (TTC) contents

These tests were performed according to the method of Makkar *et al* [8]. To estimate total phenolic content (TPC), a mixture consisting of 1 mL of extract or standard (gallic acid), 1.8 mL of 10 % Folin–Ciocalteu reagent and 1.2 mL of 7.5 % sodium carbonate was prepared. Absorbance was measured 1 h later at 765 nm. Results were expressed as µg gallic acid equivalent (GAE) per mg extract (µg/mg) and as mg/g of plant powder.

In the determination of flavonoid content (TFC), a solution consisting of 500 µL of extract or standard (quercetin) and 150 µL of 5 % sodium nitrite was vortexed and allowed to stand for 5 min. Then, it was incubated again for 6 min after the addition of 150 µL of aluminum trichloride (10

%). Thereafter, 2 mL of sodium hydroxide (5%) was added to the solution and the final volume was adjusted to 5 mL with distilled water. The absorbance at 510 nm was measured 15 min later. The total flavonoids content was expressed as µg of quercetin equivalent (QE) per mg extract, and as mg/g of plant powder.

Total tannin content (TTC) was estimated as follows: for every replicate; 500 µL of plant extract was added to 100 mg of polyvinylpolypyrrolidone (PVPP) and incubated for 4 h at 4 °C. The tubes were then vortexed and centrifuged at 3000 rpm for 10 min at 4 ° C. Then, 100 µL of the resultant supernatant containing non-tannin phenolics was adjusted to 1 mL with distilled water. Folin-Ciocalteu reagent (1 N, 0.5 mL) was added to each test tube, followed by addition of 2.5 mL of 5 % sodium carbonate. The resultant solution was vortexed and incubated in the dark at room temperature for 40 min. The amount of TTC was expressed as µgof tannic acid equivalent (TAE) per mg of plant extract, and as mg/g of plant powder.

### Evaluation of acute toxicity

Oral acute toxicity test was carried out using female Wistar mice (n = 6). A single dose of 2000 mg/kg was tested in accordance with the Organization for Economic Development (OECD) guideline no. 425 [9]. The animals were observed individually during 14 days for any clinical signs of toxicity.

### *In vivo* anti-inflammation test

This test was performed to evaluate anti-inflammatory potential of the plant extract. The mice were divided into four groups (n = 6), and pre-treated orally as follows: group 1 (negative control) received 0.9 % NaCl at a dose of 10 ml/kg, p.o.; group 2 (reference) was given indomethacin at a dose of 20 mg/kg, i.p., while groups 3 and 4 received n-butanol extract of *Centaurea tougourensis* at doses of 200 and 400 mg/kg, respectively, p.o. After 30 min of the pre-treatment, right hind paw edema was induced in each mouse using sub-plantar injection of 0.1 mL of 1 % carrageenan. The diameter of the edema was measured using a digital caliper before, and at 1, 2, 3, 4, 5, 6 and 24 h after the injection of carrageenan [10].

In addition, the percentage inhibition of inflammation was calculated using Eq 1.

Inhibition (%) = [((VC – VT))/VC]x 100 …. (1)

where *VC* represents the mean edema volume in the control group at a given time, while *VT* is the mean edema volume in the group treated with the vehicle or extract at the same time.

### Anti-ulcer studies

Ethanol-induced ulcer model was used to determine the effect of the extract on gastric mucosal damage. After a fasting period of 12 h, the mice were divided into five experimental groups (n = 6) and pre-treated orally as follows: Groups 1 and 2 served as negative control and received 0.9 % NaCl at a dose of 10 ml/kg, p.o.; group 3 (positive control) received omeprazole at a dose of 20 mg/kg, i.p., while groups 4 and 5 received n-butanol extract of *C. tougourensis* at doses of 200 and 400 mg/kg, respectively, p.o. After 1 h of ulcer induction with administration of 90 % ethanol at a dose of 10 ml/kg, p.o., the animals were sacrificed and their stomachs were removed and examined, first macroscopically, and later microscopically [11].

### Histological examination

Paws and stomachs were fixed in formaldehyde solution (10 %) and dehydrated with a gradient mixture of ethanol and xylene, followed by paraffin embedding. Tissue sections were stained with hematoxylin and eosin, and examined under a light microscope.

### Statistical analysis

Data are expressed as mean ± SEM, and were statistically analyzed with one-way ANOVA for comparison amongst groups. The GraphPad Prism (version 8) software was used for data analysis. Values of $p < 0.05$ were considered indicative of statistical significance.

## RESULTS

### Phytochemical profile and phenolic contents

Different fractions were obtained after extractions with 1.58 % n-butanol, 1.03 % ethyl acetate and 0.42 % n-hexane. Phytochemical screening of *C. tougourensis* revealed the presence of several classes of secondary metabolites namely tannins, flavonoids, anthocyanins, triterpenes, quinones, saponins and mucilages. Alkaloids were also present but at low levels. These results are presented in Table 1. The quantitative results presented in Table 2 indicate that n-BuOH extract of *C. tougourensis* contained high amounts of polyphenols (1.34 ± 0.06 mg/g of plant powder). However, the tannin content in this fraction (51.87 ± 0.02 µg tannic acid

**Table 1:** Phytochemical profile of *C. tougourensis* aerial extract

| Phytochemical | Test | Result |
|---|---|---|
| Tannins | Ferric chloride test | +++ |
| Steroids | Liebermann Burchard test | - |
| Flavonoids | Shinoda's test | +++ |
| Anthocyanins | Bornträger test | ++ |
| Triterpenes | Liebermann Burchard test | +++ |
| Alkaloids | Dragendroff test | + |
| Quinones | NaOH test | ++ |
| Saponins | Foam test | +++ |
| Mucilages | Ethanol test | +++ |
| Cyanogenic compounds | Guignard sodium picrate test | - |
| Cardiotonic glycosides | Keller Kiliani test | - |

***Key:*** - = absent; + = low level; ++ = moderate level; +++ = high level

**Table 2:** Phenolic contents of n-BuOH extract of *C. tougourensis*

| Total polyphenol content | | Flavonoid content | | Total tannin content | |
|---|---|---|---|---|---|
| (µg GAE/mg) extract | (mg/g plant powder) | (µg QE/mg extract) | (mg/g plant powder) | (µg TAE/mg extract) | (mg/g plant powder) |
| 85.44 ± 3.89 | 1.34 ± 0.06 | 25.55 ± 1.11 | 0.40 ± 0.01 | 51.87 ± 0.02 | 0.81 ± 0.01 |

All values are expressed as mean ± SD (n = 3)

equivalent/mg of extract) was higher than the flavonoid content (25.55 ± 1.11 µg quercetin equivalent/mg of extract). In terms of mg/g of plant powder, the tannin and flavonoid contents were 0.81 ± 0.01 and 0.40 ± 0.01, respectively.

## Acute toxicity

During the two weeks of observation, no signs of toxicity or death were recorded. The locomotor activity, and feed and water intake per day of each mouse were regular. There were no allergic reactions or loss of fur. These results suggest the non-toxic nature of *C. tougourensis.*

## *In vivo* anti-inflammation activity

Table 3 showed that during the early phase and precisely 1 h after carrageenan injection, there was a significant increase in mouse paw edema in all treated groups. This was followed immediately by a gradual decrease in paw edema. This decrease was highly significant for almost all treated groups, when compared to control group ($p < 0.001$).

During the late phase (4 – 24 h), the inhibition of paw edema by the plant extract was good at both doses (200 and 400 mg/kg), but was slightly higher at the dose of 400 mg/kg. The n-BuOH extract of *C. tougourensis* at doses of 200 and 400 mg/kg showed the highest percentage inhibition of paw edema 24 h after carrageenan injection (53.15 % inhibition for the dose of 200 mg/kg, and 55.26 % inhibition for the dose of 400 mg/kg), while indomethacin group had 60 % reduction in paw edema within the same period. The indomethacin group showed a slight increase in paw circumference (0.1 mm) 24 h after carrageenan injection, while non-negligible decreases in paw edema were produced by the two doses of n-BuOH extract (1.78 ± 0.04 mm and 1.7 ± 0.03 mm for 200 mg/kg and 400 mg/kg, respectively).

The histopathological investigation using hematoxylin & eosin staining showed that the groups of mice pre-treated with indomethacin or n-BuOH extract of *C. tougourensis* at a dose of 400 mg/kg did not present any signs of inflammation, as shown in Figure 1 C and Figure 1 E, respectively. Several sections showed keratinized skin with squamous epithelium resting on dermis composed of annexal structures with normal morphology. In addition, there were bundles of striated muscles and bone trabeculae surrounding the medullary spaces which contained hematopoietic elements of normal richness. Hyperkeratosis was observed in the group treated with n-BuOH extract of *C. tougourensis* at a dose 200 mg/kg (Figure 1 D), probably due to inflammatory response. In contrast, vascular congestion was observed in the carrageenan-control group (Figure 1 B), which suggested onset of inflammatory response reaction. However, this congestion could also have resulted from trauma due to cutting of the paw.

**Table 3:** Effect of oral administration of n-BuOH extract of *C. tougourensis* and indomethacin on mice paw thickness

| Treatment | Dose (mg/kg) | Paw diameter (mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Before treatment | After treatment | | | | | |
| | | | 1h | 2h | 3h | 4h | 5h | 6h | 24h |
| Control | - | 1.75 ± 0.06 | 3.35 ± 0.14 | 3.78 ± 0.25 | 3.99 ± 0.19 | 4.29 ± 0.24 | 4.68 ± 0.09 | 4.02 ± 0.12 | 3.8 ± 0.13 |
| Indomethacin | 20 mg/kg | 1.69 ± 0.03 $^{ns}$ | 2.12 ± 0.03$^{***}$ (36.71%) | 1.91 ± 0.05$^{***}$ (49.47%) | 1.7 ± 0.04$^{***}$ (57.39%) | 1.59 ± 0.1$^{***}$ (62.93%) | 1.4 ± 0.02$^{***}$ (70.08%) | 1.42 ± 0.06$^{***}$ (64.67%) | 1.52 ± 0.02$^{***}$ (60%) |
| n-BuOH extract | 200 mg/kg | 1.74 ± 0.02 $^{ns}$ | 3.1 ± 0.14$^{ns}$ (7.46%) | 2.96 ± 0.09$^{**}$ (21.69%) | 2.66 ± 0.06$^{***}$ (33.33%) | 2.46 ± 0.08$^{***}$ (42.65%) | 2.29 ± 0.05$^{***}$ (51.06%) | 2.13 ± 0.05$^{***}$ (47.01%) | 1.78 ± 0.04$^{***}$ (53.15%) |
| n-BuOH extract | 400 mg/kg | 1.72 ± 0.03 $^{ns}$ | 2.79 ± 0.1$^{**}$ (16.71%) | 2.73 ± 0.12$^{***}$ (27.77%) | 2.53 ± 0.04$^{***}$ (36.59%) | 2.39 ± 0.05$^{***}$ (44.28%) | 2.27 ± 0.04$^{***}$ (51.49%) | 2.09 ± 0.04$^{***}$ (48%) | 1.7 ± 0.03$^{***}$ (55.26%) |

Values are mean ± SEM (n = 6). One-way ANOVA, followed by multiple Dunnet's test. *$P$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001, compared with control group. $^{ns}$ Not significant. Values given in parentheses represent percentage inhibition of paw edema

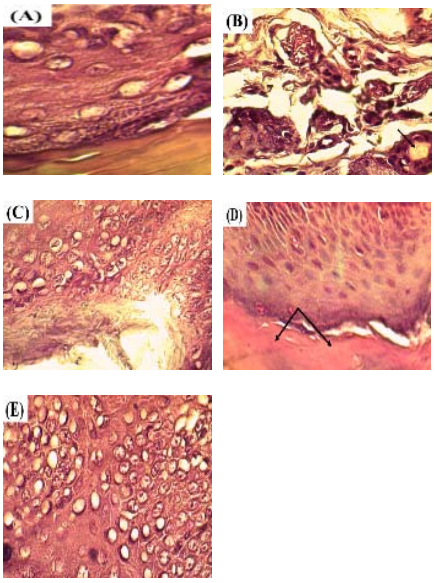


**Figure 1:** Histopathologic features of mouse paw tissues after sub-plantar injection of carrageenan (H&E stain, ×100). (A) Normal control, (B) carrageenan control, (C) indomethacin-treated group (20 mg/kg), (D) n- BuOH extract-treated group (200 mg/kg), (E) n-BuOH extract-treated group (400 mg/kg)

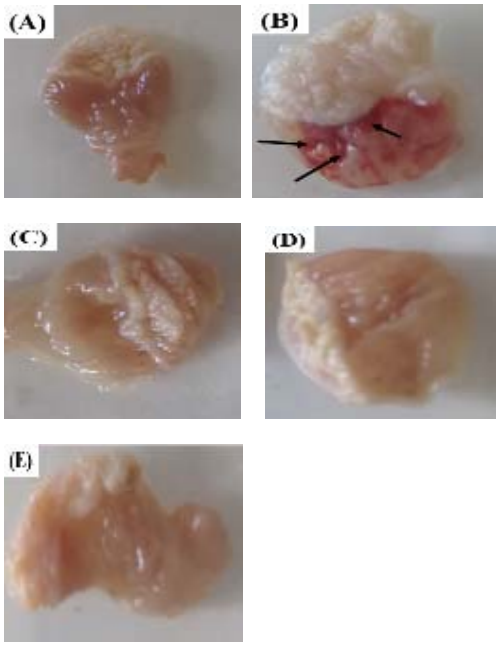


**Figure 2:** Effect of n-BuOH extract of *Centaurea tougourensis* on the macroscopic appearance of gastric mucosa in EtOH-induced ulcer in mice; (A) normal control, (B) ulcer-control, (C) omeprazole-treated group (20 mg/kg), (D) n-BuOH extract-treated group (200 mg/kg), (E) n-BuOH extract-treated group (400 mg/kg)

### Anti-ulcer effect of *Centaurea tougourensis*

Macroscopic (Figure 2) and histopathological (Figure 3) results revealed that except for the control group (Figure 3B) which showed visible vascular congestion and tissues lesions, almost all treated groups did not present any sign of ulcer.

## DISCUSSION

Previous phytochemical studies on *Centaurea* species reported high levels of tannins, flavonoids, polyphenols and terpenes in these species. This finding is consistent with the results obtained in this study. Similar results were reported by Zengin *et al* [12].

These researchers correlated the pharmacological potential of eight *Centaurea* species with their high contents of polyphenols, flavonoids

and tannins, especially in *C. triumfettii, C. patula* and *C. pulchella*. Qualitative and quantitative analyses of n-BuOH fraction of *Centaurea choulettiana* Pomel revealed high levels of phenolic compounds and flavonoids [13]. On the other hand, Joujeh *et al* [14] demonstrated dose-effect relationship between phenolic content of *Centaurea iberica* and anti-hemolytic effect.

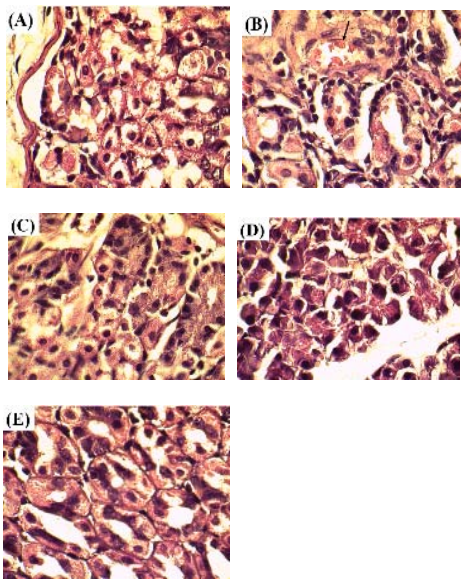


**Figure 3:** Histopathologic examination of mice stomach tissues after EtOH-induced ulcer (H&E stain, × 100); (A) normal control, (B) ulcer-control, (C) omeprazole-treated group (20 mg/kg), (D) n-BuOH extract-treated group (200 mg/kg), (E) n-BuOH extract-treated group (400 mg/kg)

Carrageenan-induced paw edema in a rodent experimental model is a well-known test used to determine the anti-inflammatory potential of plant extracts. The test occurs in two phases. The increase in paw edema during the first phase could be explained by the huge release of histamine and serotonin, resulting in release of bradykinin and increase in vascular permeability [15]. The results obtained in this study indicate a possible anti-inflammatory effect of *C. tougourensis,* and they are in agreement with the established fact that reduction in edema take place in the second and third hours after carrageenan injection [16]. The decrease in paw edema in *C. tougourensis* groups during the late phase could be due to a strong inhibition exerted by the n-BuOH extract on prostaglandin E2 (PGE2) and nitric oxide (NO) which are the principle pro-inflammatory mediators, by inhibiting the expressions of nitric oxide synthetase (iNOS) and cyclooxygenases (COX-1 and COX-2) [2]. This could also imply blockage of the recruitment of key cytokine (IL-1β) which is normally involved in the second phase, thereby blocking the appearance of this phase [17]. The indomethacin group showed a slight increase in paw diameter 24 h after carrageenan injection, probably due to slight reactivation of inflammatory process [10].

Saponins exert remarkable anti-inflammatory effects by directly blocking inflammation [18]. They decrease neutrophil infiltration, thereby lowering inflammatory response based on immunity. Recent research involving triterpenes suggested that these compounds have significant anti-inflammatory properties e.g., wound healing by accelerating the involvement of several mediators called ‘cascade of healing’ at cellular and molecular levels. These events result in reduction of wound closure time.

The results of this study suggest that a high dose of n-BuOH extract of *C. tougourensis* may block the inflammatory process due to its appreciable phenolic and tannin contents. Indeed, previous work showed that high tannin content may be responsible for pharmacological effects such as significant disabling of macro- and micro-vascular complications [20].

The anti-ulcer effects seen in this study are in agreement with results of other research works involving murine models which showed significant anti-ulcer effects of some *Centaurea* species via prevention of formation of gastric mucosa lesions due to their high phenolic and flavonoid contents [21]. These compounds reduce the risk of developing inflammatory bowel diseases such as ulcerative colitis (UC) and Crohn's disease, by suppressing the JAK2/STAT3 and NF-κB signaling pathways [22]. A study by Pirvu *et al* demonstrated that polysaccharides may act in synergy with polyphenols to enhance the gastroprotective effects of *Centaurea cyanus* [23]. This could be explained by the fact that polysaccharides provide a protective layer that consolidates the gastric mucous layer.

Mucilages have been shown to be useful in the treatment of several gastrointestinal diseases. A study on ulcerated rat groups showed that mucilages significantly reduced the acidity in the gastric juice and increased the gastric pH [23]. This suggests that mucilages may act as barriers that protect the digestive tract from the various digestive enzymes and excess gastrointestinal acidity [24]. In this study, it was found that *C. tougourensis* contained high levels of mucilage,

and the procedure used in extract preparation favored the extraction of these molecules.

## CONCLUSION

This study has demonstrated the phytochemical and pharmacological potentials of *Centaurea* species. Further investigations are required for better understanding of the chemical composition and the full potentials of *Centaurea tougourensis.*

## DECLARATIONS

### *Acknowledgement*

The authors wish to express thanks to Prof. Bachir Oudjehih of the Agronomy Department of the University of Batna-1 (Algeria) for identifying the plant, and to Dr Souad Bensaci from Anapathology unit of the University Hospital Center of Batna (Algeria) for histological analysis. the authors also thank DGRSDT of the Algerian Ministry of Higher Education and Scientific Research (MESRS, DGRSDT).

### *Conflicts of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Mohamed Sabri Bensaad performed the in vivo experiments and wrote the manuscript. Saliha Dassamiour wrote the manuscript and conducted data analysis. Leila Hambaba supervised the work. Mohamed Amine Kahoul and Mohammed Benhoula carried out the phytoscreening aspect of the work.



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