

Original Article

***In vitro* and *in vivo* bioactivities of *Ambrosia maritima* and *Bituminaria bituminosa* organic extracts from Algeria**

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Abstract

Introduction: Two medicinal plants, *Ambrosia maritima* and *Bituminaria bituminosa*, growing in Algeria were investigated for their flavonoids content and their biological activities.

Methodology: Different organic extracts were prepared from the aerial parts of each plant using maceration method followed by liquid/liquid type extractions. The anti-antioxidant activity was tested using the β -carotene bleaching method. The anti-inflammatory activity was tested by performing the protein anti-denaturation assay. Acute toxicity and immunostimulatory effect were tested in mice, while the antimicrobial activity was tested according to the minimal inhibition concentration technique.

Results: In term of flavonoids content, ethyl acetate extract of *B. bituminosa* was the highest ($193.39 \pm 24.1 \mu\text{g QE/mg}$). Ethyl acetate extract of *A. maritima* showed antioxidant activity with IC_{50} value of $11.72 \pm 0.79 \mu\text{g/mL}$. The hydroethanolic extract of *A. maritima* showed the best anti-denaturation effect in a dose-dependent manner with the IC_{50} value of $131.07 \pm 0.027 \mu\text{g/mL}$. The studied plants showed no toxicity or mortality *in vivo*. Both plants showed a significant immunostimulatory effect; while the Butanolic extract of *B. bituminosa* demonstrated the best antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* strains.

Conclusions: We recommend *A. maritima* and *B. bituminosa* as potent sources of antioxidants and as antimicrobial agents for further assays to better elucidate their actions on the immune system.

Key words: medicinal plants, bioactivities, flavonoids, Algeria.

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Introduction

Immunity constitutes the natural defense system of organisms against damaging stimuli [1]. Based on how it functions, immune system can be classified into the innate immune system (non-specific) and adaptive immune system [2]. Inflammation is a defense mechanism of the immune system against tissue damage caused by a variety of stimuli. It is known that oxidative stress; an imbalance in the generation and elimination of cellular reactive oxygen species (ROS) [3]; and inflammation are interrelated processes [4], and were found to be tightly linked to many disorders, such as autoimmune diseases, stroke, diabetes mellitus, and cancer [5]. Interactions in the immune system are a systemic defense mechanism, but when overly deployed can be harmful to the host physiology and

may contribute to the development of several non-immune and immune chronic inflammatory disorders [1].

Agents that are able to activate or induce the immune system mediators or components are recognized as ‘immunostimulants’. These agents are capable of enhancing the immune system against autoimmunity, cancer, allergy, and infection. Furthermore, it is recognized that lipid peroxidation is associated with inflammation and oxidative stress, both of which have been acknowledged to play an important role in the initiation and progression of chronic diseases [6]. Moreover, protein denaturation is another well-documented aspect of inflammation, and the role of ROS to stimulate this process is established [4].

Natural antioxidants may prevent many diseases and could be a promising remedy if taken together with conventional drugs [5]. There is increasing interest in the use of herbal therapies as multi-component agents to modulate the complexity of the immune system in the prevention of related disorders instead of treating them. Interestingly, a broad range of immunomodulatory and/or anti-inflammatory effects have been attributed to natural components due to their related human immune system bioactivities. Herbal therapies known for their efficiency in reducing inflammation have been used in traditional medicines since ancient times. These herbal-derived molecules have been shown to possess immunomodulating properties [1,7]. These bioactive compounds include flavonoids, secondary metabolites found essentially in plants, vegetables and fruits. Flavonoids are known for their potent antioxidant ability and have considerable capacity to attenuate tissue damage or fibrosis related to macromolecules degradation and several studies *in vitro* and in animal models have demonstrated their potential to inhibit the onset and development of inflammatory disorders [7]. According to the World Health Organization (WHO), nearly 80% of the population around the world use herbal medicines as standard therapeutic modalities along with conventional drugs, and in Africa, this number is 85% [7]. Despite their promising potential and their proven efficiency, many medicinal plants are poorly investigated for their bioactivities, while others have not been studied yet [8], and their safety has been critically discussed in scientific studies [7].

On the other hand, the antimicrobial activities of a few plant species have been investigated. Understanding the antimicrobial mechanism of action of medicinal plant extracts is the first step in optimizing their use as natural antimicrobial agents, especially against pathogenic microbial strains that cause severe infections and are a public health concern [9].

Ambrosia maritima L. (Asteraceae) is a perennial herbaceous plant, widely distributed in the Mediterranean basin and Africa. This plant has antispasmodic effects and is able to relieve bronchial impairments and urinary tract issues [10,11]. The phytochemical composition of *A. maritima* includes a variety of secondary metabolites, such as pseudoguaianolides, sesquiterpene lactones, coumarins, triterpenes, and sterols. Sesquiterpene lactones have diverse bioactivities, including anti-inflammatory, antimicrobial, antitumor and antispasmodic properties [12].

Bituminaria bituminosa (L.) C. H. Stirton is a perennial wild legume plant, widely distributed

throughout the Mediterranean basin, traditionally used in the treatment of spasms, fever, and epilepsy [13]. It is also considered a source of pharmaceutically active compounds with relevant bioactivities such as furanocoumarins (psoralen, angelicin), pterocarpanes (erybraedin C, bitucarpin A), and flavonoids (daidzin, isoorientin) [13,15,16,14]. Its phytoconstituents have been found to have an antimicrobial effect against different strains like *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli*, among other anti-HIV activity, and were able to prevent and treat solid organ transplantation rejection, and has anti-proliferative, estrogenic, hepatic-protective, anti-allergy, anti-inflammatory, apoptotic, and anti-tumor properties [15,17–23].

In this study, we evaluated the beneficial activities of various extracts from *B. bituminosa* and *A. maritima*, sampled from the east of Algeria, by testing *in vitro* peroxidation inhibition and the protein anti denaturation, and the preliminary antimicrobial properties against the following clinical strains: *Staphylococcus aureus*, *Candida albicans*, *Escherichia coli*, *Klebsiella pneumonia*, and *Enterococcus faecalis*; along with the *in vivo* immunostimulatory and acute toxicity effects.

Methodology

Ethics statement

The ethical approval of experimental animal use was obtained from the ethics committee of institutional animals and conducted according to the Executive Decree number: 10–90 completing the Executive Decree number: 04–82 of the Algerian Government, and the ethical principles and the experiments were conducted in strict compliance according to ethical principles and guidelines for the ‘Purpose of Control and Supervision of Experiments on Animals’ OECD guidelines [24].

Chemical reagents

Unless stated in the text, all the reagents used were purchased from Sigma (Sigma, St Louis, MO, USA).

Plant material

The aerial parts of *A. maritima* were collected in October 2015 in Béjaïa region in the North-East of Algeria Ighzer Amokrane, Ouzellaguen- Bejaia (36°33'4.51"N - 4°36'29.19"E), while *B. bituminosa* aerial parts were collected in May 2016 from Taza National Park in the region of Ziama Mansouria, Jijel in the North-East of Algeria (36°42'17.37"N - 5°33'23.70"E). Both plants were identified by Pr. K.

Rebbas from Nature and Life Department (University of M'sila, Algeria), and samples were deposited in his personal herbarium. The plant materials were air-dried at room temperature for three weeks in the shade, then ground into a fine powder using an electric mill, and stored until use.

Animals

Adult male albino mice (*Mus musculus*, 2-2.5 months old) with a weight range between 20–33 g were selected to carry out *in vivo* experiments. The animals were randomly selected, marked for individual identification, and kept in cages before dosing for at least 5 days (to allow adaptation to the lab's conditions). The temperature of the animal room was about 22 °C (\pm 3 °C), with a relative humidity of 30%, and artificial lighting (12h light/12h dark). Water was available *ad libitum* [24], and food was provided in the form of dry pellets (Bouzaréah, Algeria).

Liquid-liquid extraction

The extraction process was performed according to Lemoui *et al* [25]. Briefly, air-dried and powdered aerial parts (1 kg) from each plant were macerated at room temperature in ethanol and water (EtOH/H₂O) (80/20: v/v) mixture and repeated 3 times every 24h. The hydroalcoholic solution of each plant was concentrated and dried. The crude extract was treated with distilled water, and the aqueous solution was used for liquid-liquid extractions using solvents with increasing polarity (petroleum ether, chloroform, ethyl acetate and n-butanol, respectively). The organic phases were concentrated until they were dry to obtain the petroleum ether, chloroform, ethyl acetate and n-butanol extracts, respectively. The extracts were referred to as AMH for hydroethanolic extract of *A. maritima*, and BBH for hydroethanolic extract of *B. bituminosa*; AMC, AMA and AMB were the chloroform, ethyl acetate and n-butanol extracts of *A. maritima*; BBC, BBA and BBB were the chloroform, ethyl acetate and n-butanol extracts of *B. bituminosa*. The extracts were stored at 4 °C until next use.

Flavonoid content (TFC)

Total flavonoid content (TFC) was evaluated using aluminum chloride (AlCl₃) [26], with minor modifications to adapt for use in a 96-well microplate. Briefly, an aliquot of 50 μ L of each extract (0.25 mg/mL) was added to 10 μ L of aluminum nitrate (Al(NO₃)₃) solution (10%), 10 mL of 1M potassium acetate (CH₃COOK), and 130 μ L of methanol. The mixture was thoroughly stirred and incubated for 10

min at room temperature. The absorbance was recorded at 430 nm. All samples were analyzed 3 times, and the flavonoid content was determined using a quercetin calibration curve. TFC was calculated as μ g of quercetin equivalent/mg of dry plant extract weight (μ g QE/mg).

Antioxidant activity (β -carotene/linoleic acid assay)

β -carotene-linoleic acid system was used to evaluate the capacity of plants to inhibit lipid peroxidation as described by [27,28]. Briefly, an emulsifying agent was obtained by mixing 0.5 mg of β -carotene in 1 mL of chloroform to which 25 μ L of linoleic acid and 200 mg of Tween 40 were added. The chloroform was then evaporated under vacuum (R215, BÜCHI Labortechnik, Flawil, Switzerland) and the residue was taken in 50 mL of hydrogen peroxide (H₂O₂). Next, 40 μ L of extracts with different concentrations ranging from 12.5 to 800 μ g/mL were mixed with 160 μ L of the previous emulsion. The microplate was incubated for 2 h at 50 °C. Ethanol was used as a negative control, while butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as antioxidant standards. The absorbance was read at 470 nm, at t = 0 min and t = 120 min. The results were expressed as the inhibitory concentration at 50% (IC₅₀).

The inhibition percentage of β -carotene bleaching was calculated using the following formula:

$$I(\%) = \left(\frac{As[t = 0] - As[t = 120]}{Ac[t = 0] - Ac[t = 120]} \right) \times 100$$

Where: I (%) = percentage of inhibition.

As [t = 0] = the absorbance at 470 nm of the sample at 0 min.

As [t = 120] = are the absorbance at 470 nm of the sample at 120 min.

Ac [t = 0] = the absorbance at 470 nm of the positive control at 0 min.

Ac [t = 120] = the absorbance at 470 nm of the positive control at 120 min.

Anti-inflammatory activity (protein anti-denaturation assay)

The anti-inflammatory potential of the studied plants' extracts was assessed *in vitro* by measuring their capacity to counteract heat-induced denaturation of bovine albumin serum (BSA) as described by [29]. Briefly, a solution of BSA (0.2% w/v) was prepared in a Tris-HCl buffer with pH adjusted to 6.8. Solutions of standards and extracts were prepared in distilled water, with concentrations ranging from 32.5 μ g/mL to 250 μ g/mL. 500 μ L of each extract was added to 500 μ L of

0.2% w/v BSA. Ketoprofen® and Diclofenac sodium (Voltaren®) were used as positive controls. The test tubes were incubated for 15 min at 37 °C and then heated at 72 °C for 5 minutes. The absorbance at 660 nm was taken after cooling to the room temperature using a UV-VIS spectrophotometer (U-2810 Spectrophotometer, Digilab Hitachi, AJN. Scientific, Stoughton, MA. USA) Each experiment was performed in triplicate, and the results were expressed as mean ± SD. The percentage of denaturation inhibition was expressed as the % basis relative to control, using the following formula:

$$\text{Inhibition Percentage of Denaturation} = \frac{\text{Absorbance of control} \times \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$$

Acute toxicity test

The acute toxicity of AMH and BBH extracts was assessed as described in [24] with slight modifications. Briefly, groups of 5 animals were dosed using the fixed concentrations of 10, 100, 1000, and 2000 mg/Kg of body weight by injection via intraperitoneal route. Each animal was examined every 30 min with special consideration during the first 4 hours for clinical signs of mortality or toxicity including pain, suffering, and impending directly after the administration of the doses. Thereafter, the animals were examined daily, for a total of 14 days (except where they needed to be removed from the study, and humanely killed for animal welfare reasons; or were found dead). Body weight of each individual was recorded on a daily basis for 14 days.

Immunostimulatory effect of plant extracts (phagocytic activity)

The immunostimulatory capacity was tested *in vivo* on mice (*Mus musculus*) using the carbon clearance method from the institutional phagocytic activity of reticuloendothelial systems (RES) as reported in [30]. Briefly, concentrations range of 25, 50,100, and 200 mg/Kg of AMH and BBH extracts were prepared in 10 mL 0.9% NaCl. The mice were divided into five groups: I, II, III, IV, and V; and received different treatments. Group I (control) was administered (via intraperitoneal route) 0.5 mL of 0.9% NaCl solution. Groups II, III, IV, and V received plant extracts of various concentrations: 25, 50, 100, and 200 mg/Kg of body weight respectively. 48h later, 0.1 mL/10g of carbon ink solution was injected into the tail vein of each animal. This mixture was prepared with 4 mL of saline, 3 mL of black carbon ink, and 4 mL of 3% gelatin solution.

Blood samples (14 drops ≈ 25µL) were subsequently withdrawn from the retro-orbital plexus using heparin glass capillaries at time 5 and 15 min, then lysed with 4 mL of 0.1% sodium carbonate solution. The absorbance of mixture was measured at 676 nm. The phagocytic activity is expressed by the phagocytic index K: the rate of reticuloendothelial clearance of the antigen (the carbon ink), which measures all the reticuloendothelial system function in contact with the circulating blood; and by corrected phagocytic index α: unit of active weight organs (liver and spleen), while the clearance rate is expressed as the half-lifetime of the antigen in the blood (t_{1/2}) expressed in minutes (min).

These three parameters are calculated using the equations 1, 2 and 3:

$$K = \frac{\log OD_1 - \log OD_2}{t_1 - t_2} \tag{1}$$

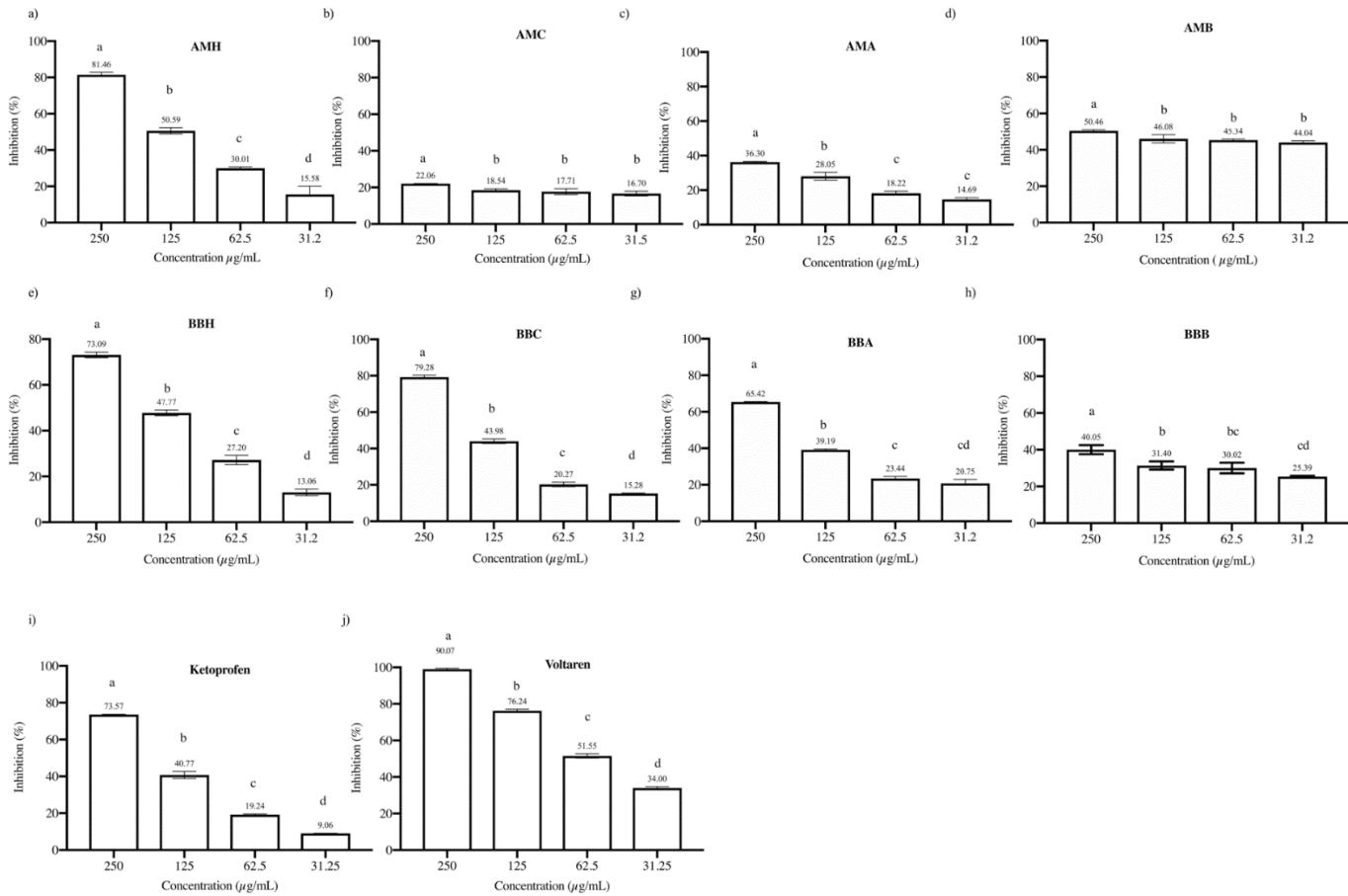
$$t_{1/2} = \frac{0.693}{K} \tag{2}$$

$$\alpha = \sqrt[3]{K} \times \frac{\text{Body weight}}{\text{liver wt} + \text{spleen wt}} \tag{3}$$

Antimicrobial activity

The antimicrobial activity of AMH and BBH extracts was assessed according to the broth microdilution method that was performed to determine the minimum inhibitory concentration (MIC) following the protocol of the Clinical and Laboratory Standard Institute [31] against the following clinical strains: *S. aureus*, *C. albicans*, *E. coli*, *K. pneumonia* and *E. faecalis*. The tested extracts were dissolved in 10% DMSO and diluted to prepare a stock solution of 20 mg/L. Then, 12 serial two-fold dilutions of extracts were set directly in a microtiter plate containing broth to obtain a range of concentrations from 10 to 0.0048 mg/mL. Previously, the organic solvents used in the extraction were tested for their antimicrobial effect with a final concentration of 1%, and had no significant effect on the tested strains. To prepare the inoculum, colonies were diluted in salt solution at a final concentration of 0.5 McFarland, and confirmed by reading their optical density at a wavelength of 530 nm. The experiment was performed in both LB broth and RPMI-1640 media on sterile 96-well microplates. 100 µL of each extract dilution and 100 µL of bacterial/yeast suspension at a concentration of 10⁶ CFU/mL were added to each well and incubated at 37 °C for 24 to 48 h. The MIC was determined to be the lowest concentration of the extract that inhibits the bacterial growth.

Figure 1. Anti-inflammatory effect of *A. maritima* and *B. bituminosa*.



a, b, c, d, e, f, g, h, i, j: represent the anti-inflammatory activity of AMH, AMC, AMA, AMB, BBH, BBC, BBA, BBB, Voltaren, and Ketoprofen, respectively. AMH: Hydroethanolic extract of *A. maritima*. BBH: Hydroethanolic extract of *B. bituminosa*. BBC: Chloroform extract, BBA: Acetate ethyl extract, BBB: Butanolic extract for *B. bituminosa* plant; and AMC: Chloroform extract, AMA: Acetate ethyl extract, AMB: Butanolic extract. Values (expressed as means ± SD of three parallel measurements) that do not share a common letter are significantly different ($p < 0.05$ for one way ANOVA and Tukey's multiple comparison tests).

Table 1. Total flavonoids content, antioxidant activity and anti-inflammatory effect of *A. maritima* and *B. bituminosa*.

Extract	Total flavonoids content (µg QE/mg)	Antioxidant activity (IC ₅₀ : µg/mL)	Anti-inflammatory activity (IC ₅₀ : µg/mL)
BHT	/	1.05 ± 0.01 ^a	/
BHA	/	0.90 ± 0.02 ^a	/
Voltaren®	/	/	63.87 ± 1.2 ^a
Ketoprofen®	/	/	165.96 ± 1.39 ^b
AM	18.83 ± 2.34 ^a	43.02 ± 3.44 ^b	131.07 ± 0.027 ^c
AMC	117.54 ± 10.33 ^b	14.73 ± 0.59 ^c	> 250
AMA	61.89 ± 1.93 ^c	11.72 ± 0.79 ^{c,d}	> 250
AMB	26.28 ± 1.38 ^{a,d}	19.90 ± 0.01 ^{c,e}	241.7 ± 0.038 ^d
BB	56.31 ± 3.58 ^{c,e}	43.87 ± 2.19 ^{b,f}	148.5 ± 0.034 ^e
BBC	17.94 ± 0.83 ^{a,d,f}	28.52 ± 0.68 ^g	151 ± 0.024 ^f
BBA	193.39 ± 24.15 ^g	39.08 ± 6.91 ^{b,f,h}	177.4 ± 0.011 ^g
BBB	158.15 ± 3.8 ^h	50.44 ± 2.88 ^{b,f,i}	> 250

Statistical analysis

Recorded results (expressed as mean ± SD of triplicate measurements) were analyzed using two-way ANOVA for the body-weight measurement (results of the acute toxicity test). A one-way ANOVA test followed by a post-hoc Tukey’s multiple comparisons test were carried-out for the remaining assays. The significance level was determined at $p < 0.05$. GraphPad Prism software version 8 (GraphPad Software Inc, California, USA) was used to perform the statistical analyses.

Results and discussion

Flavonoids content

Evaluation of total flavonoids demonstrated that *B. bituminosa* was the richest in terms of flavonoids content, with the highest amounts of BBA and BBC extracts (193.39 ± 24.15 and $158.15 \pm 3.8 \mu\text{g QE/mg}$, respectively; $p < 0.0001$), while the AMC extract was the richest in *A. maritima*, with an IC_{50} value of $117.54 \pm 10.33 \mu\text{g QE/mg}$ (Table 1).

β-carotene/linoleic acid assay

The assessment of lipid peroxidation inhibition revealed that the three AMA, AMC, and AMB extracts of *A. maritima* recorded the lowest IC_{50} values, with 19.90 ± 0.01 , 11.72 ± 0.79 , and $19.90 \pm 0.01 \mu\text{g/mL}$ respectively; indicating strong antioxidant activity relative to the standards ($p < 0.0034$). The lowest IC_{50} observed in *B. bituminosa* was recorded by the BBC extract, with a concentration of $28.52 \pm 0.68 \mu\text{g/mL}$ ($p < 0.0001$) (Table 1).

Anti-inflammatory activity

An overall anti-inflammatory effect was observed with both plants’ extracts in a dose-dependent manner. Interestingly, AMH, BBH, and BBC extracts had denaturation inhibition ability with the lowest IC_{50} values of 131.07 ± 0.027 , 148.5 ± 0.034 , and $151 \pm 0.024 \mu\text{g/mL}$ respectively, whereas the concentration of $250 \mu\text{g/mL}$ was the most effective for these extracts with inhibition percentages of $81.4\% \pm 1.38$, $73.09\% \pm 1.24$, and $79.27\% \pm 0.96$ respectively (Figure 1a, 1b, and 1f). These values represent two-fold of the Voltaren® IC_{50} ($63.87 \pm 1.2 \mu\text{g/mL}$, $p < 0.0001$) with percentage of inhibition at $250 \mu\text{g/mL}$ of $99.07\% \pm 0.44$, but remained close to the Ketoprofen® IC_{50} ($165.96 \pm 1.39 \mu\text{g/mL}$) with $73.57\% \pm 0.11$ ($p > 0.05$), with the Voltaren® being more effective than the Ketoprofen® ($p < 0.0001$) (Figure 1i, and 1j). Moreover, a mild inhibition was observed with BBA and AMB extracts

with respective values of 241.7 ± 0.038 and $177.4 \pm 0.011 \mu\text{g/mL}$ (Figure 1d, and 1g) whereas no significant activity was induced by AMC, AMA, and BBB extracts (Figure 1b, 1c, and 1h) (Table 1).

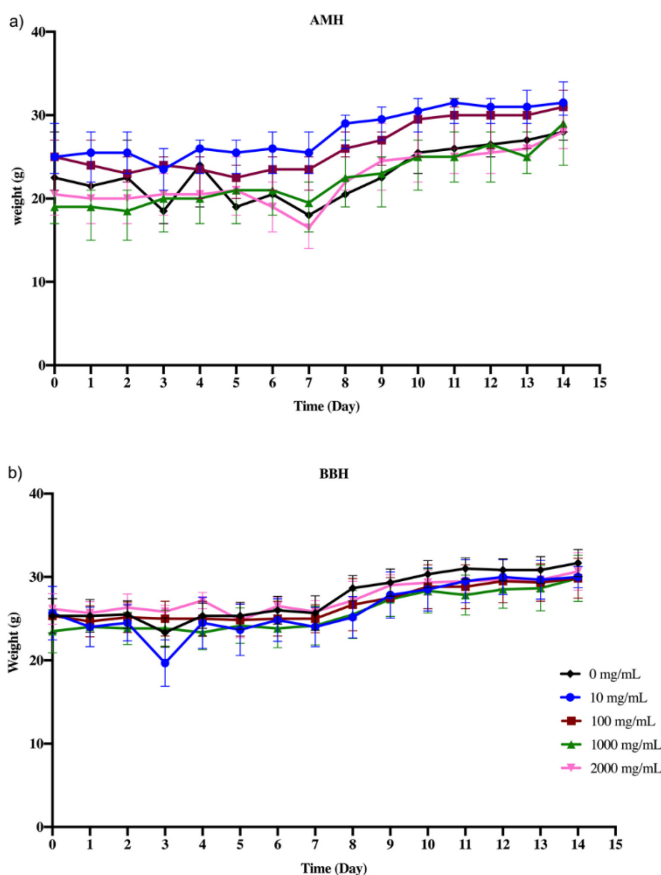
Acute toxicity

No mortality was recorded in the acute toxicity assay with any of the tested groups, in both AMH and BBH extracts up to 2000 mg/Kg , during the 14 day-assessment times. The animals showed no abnormal behavior or clinical signs, including toxicity, pain, suffering or impeding. Parallely, no significant body weight fluctuation was registered during the period of experimentation ($p = 0.8543$). This result indicates that LD_{50} is higher than 2000 mg/Kg (Figure 2a, and 2b).

Immunostimulatory activity

As shown in Figure 3, an overall dose-dependent immunostimulatory effect was exerted by both tested extracts: AMH and BBH. A significant increase in the

Figure 2. Body weight kinetics (Acute toxicity test).



a: Body weight variation of animals in presence of AMH extract. **b:** Body weight variation of animals in presence of BBH extract. Values are expressed as means ± SD of three parallel measurements.

reticuloendothelial clearance rate of the antigen (K index) was recorded in *B. bituminosa*, with the highest rates observed with high doses of 100 mg/Kg and 200 mg/Kg, respectively (100 mg/Kg: 0.066 ± 0.01 and 200 mg/Kg: 0.059 ± 0.01) ($p < 0.001$), while AMH showed no significant difference compared to the control group ($p > 0.05$) (Figure 3a). This finding may be interpreted as of BBH to enhance the RES activity. Parallely, a significant increase of active organ weight expressed by the phagocytic index α alpha was also induced by both extracts. Compared to the control group that recorded a value of $\alpha = 4.63 \pm 0.50$, the highest dose of AMH (200 mg/Kg) showed a higher value of $\alpha = 7.00 \pm 0.84$, while BBH lowest doses of 25 and 50 mg/Kg registered increased values of 6.52 ± 0.20 and 6.92 ± 0.12 respectively ($p < 0.0001$) (Figure 3b). Our plants reduced significantly the $t_{1/2}$ parameter in a dose-dependent manner, with the shortest duration recorded for the BBH high doses of 100 and 200 mg/mL, with the corresponding values of 11.098 ± 3.36 min and 12.96 ± 4.53 min, while in AMH extract the shortest duration was also recorded by the high dose of 200 mg/Kg (19.76 ± 3.22 min). These values were significantly different from those recorded for the control group (37.64 ± 2.68 min with $p < 0.0001$) (Figure 3c).

Antimicrobial activity

The antimicrobial activities of AMH and BBH extracts are shown in Table 2. The results demonstrated a potential antimicrobial activity of BBH extract against Gram-positive bacterium *Stacpyholcoccus. aureus* with MIC value of 0.625 mg/mL, Gram-negative bacterium *E. coli* with MIC value of 5 mg/mL, and against the yeast strain *C. albicans* with MIC with a value of 0.625.

Table 2. Antimicrobial activity of *Ambrosia maritima* and *Bituminaria bituminosa*.

Strains	Extracts MIC value (mg/mL)	
	AMH	BBH
<i>Staphylococcus aureus</i>	1.25	0.625
<i>Candida albicans</i>	10	0.625
<i>Klebsiella pneumonia</i>	> 20	> 20
<i>Enterococcus faecalis</i>	> 20	> 20
<i>Escherichia coli</i>	2.5	5

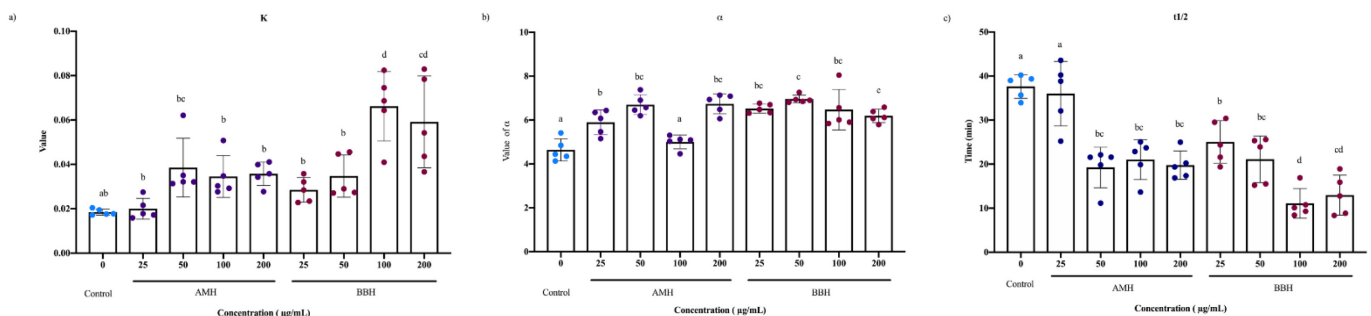
AMH extract had antimicrobial effect against the same strains with MIC values of 1.25, 2.5 and 10 mg/mL respectively. However no antimicrobial activity was observed against *K. pneumonia* and *E. faecalis* strains.

Discussion

In this paper, antioxidant and anti-inflammatory activities of extracts from *A. maritima* and *B. bituminosa* were investigated. Furthermore, the toxicity of these plants’ extracts and their immunostimulatory effects were observed *in vivo*.

The total flavonoid content of our extracts was measured using the Al_2Cl_3 method. BBH plant extract was the richest in term of flavonoids content, with BBA and BBC showing the highest amounts of flavonoids. Our findings are in concordance with those of Sarikurku *et al.* [32] who observed that the methanol extract of *B. bituminosa* is rich in flavonoids (5.29 μ mol rutin equivalent (REs)/g dry plant). It has been demonstrated that *B. bituminosa* phytochemical profile contains a variety of flavonoids and their derivatives, mainly isoflavones (daidzin) and flavones (Isoorientin, a Luteolin derivative). In addition, *B. bituminosa* has

Figure 3. Immunostimulatory activity of *A. maritima* and *B. bituminosa*.



a: The phagocytic index K measurement in presence of AMH and BBH extracts, **b:** corrected phagocytic index α measurement in presence of AMH and BBH. **c:** The half-time life of the antigen in the blood ($t_{1/2}$) expressed in minutes (min) measured in presence of AMH and BBH. AMH: Hydroethanolic extract of *A. maritima*. BBH: Hydroethanolic extract of *B. bituminosa*. Groups that do not share a common letter are significantly different ($p < 0.05$ for one way ANOVA and Tukey’s multiple comparison tests).

been reported to have considerable quantities of other phytochemicals which have pharmaceutical activities like furanocoumarins (Psoralen, Angelicin) and pterocarpanes (Erybraedin C, Bitucarpin A) [13,15,16].

On the other hand, AMC extract was the richest in the case of *A. maritima*. This finding is similar to the results of Said *et al.* [33], who demonstrated the richness of *A. maritima* in terms of flavonoids. In addition to these metabolites, *A. maritima* includes a variety of other secondary metabolites, such as pseudoguaianolide sesquiterpene lactones, coumarins, triterpenes, sterols, and sesquiterpene lactones [12]. Phenolic acids and flavonoids play a preventive role in the development of some cancers and chronic diseases. These components are commonly present in many species and herbs.

Many phenolic compounds with strong antioxidant activity have been characterized in plants. We evaluated their antioxidant effect by measuring their ability to inhibit lipid peroxidation. All our extracts showed a considerable ability to inhibit β -carotene peroxidation. AMA, AMC, and AMB extracts recorded the lowest IC₅₀ values; indicating a relevant antioxidant ability of *A. maritima*. This result is in agreement with those of [11,33,34] who confirmed that isolated leads from this species showed significant antioxidant activity when assessed with different methods. Interestingly, it is conceivable that modulation of the inflammatory responses represents the most important role of oxidized lipids, thus playing a major role in the development of a variety of chronic inflammatory disorders [6]. It has been proposed that polyphenolic flavonoids act as antioxidants by inhibiting lipid peroxidation; low-density lipoprotein (LDL) oxidation and scavenging oxygen radicals [35].

With our plants' extracts exhibiting a significant ability to counteract lipids peroxidation it can be promising in the early stages of drug screening and discovery to focus on the bioactivities of their phytoconstituents. Protein denaturation is a well-documented effect of alterations in the biological, chemical, and physical properties of cellular proteins by mild disruption of their structure. This is also known to be a net aspect of macromolecules damage during inflammation [36,37]. In this regard, we assessed the ability of our extracts to inhibit the thermal denaturation of serum bovine albumin protein (BSA). AMH, BBH, and BBC extracts showed the best anti-denaturation effect in a dose-dependent manner with the lowest IC₅₀ values. Accordingly, many flavonoid-rich plants demonstrate anti-inflammatory effect by exhibiting an anti-denaturation capacity [38–42]. Previous studies

have also discussed the anti-inflammatory effects of both *A. maritima* and *B. bituminosa*, in relation to their phytochemical profile [12,43].

The anti-inflammatory activity of our plants may be attributed to their rich phenolic contents which are known to exert antioxidative and anti-inflammatory properties, mainly due to their capacity to modulate key cellular protein functions [44]. Furthermore, we performed two *in vivo* tests on *M. musculus*, where the first test was carried out to evaluate the safety of our plant extract. Both of our plants showed no toxicity or mortality towards mice. Overall, herbal medicines are considered as nontoxic and effective if used rationally [45]. It has been estimated that 25% of modern drugs are based on molecules from plants that were initially used in traditional medicine, such as Aspirin (Ephedrine), and Paclitaxel (Artemisinin). However, inappropriate use of herbal medicines can cause deleterious and dangerous effects [46]. Clinical safety and monitored pharmacology testing of potential medicinal plants is effective in identifying therapeutically relevant safety issues. In this context, we tried to evaluate the immunostimulatory ability of both plants using the carbon clearance test *in vivo*. An overall immunostimulatory effect was induced in a dose-dependent manner by tested extracts of AMH and BBH. Our results are in line with other studies which reported that different plant extracts rich in polyphenols and flavonoids exerted a considerable immunostimulatory effect [47–51]. Secondary metabolites such as flavonoids, polysaccharides, lactones, alkaloids, diterpenoids, and glycosides are present in several plants, and have been reported to be responsible for the plants immunomodulating properties. Several pathologies can be alternatively counteracted by immunomodulators using medicinal herbs, instead of chemotherapy. The discovery and purification of more specific immunomodulatory components from plant origin is gaining prominence [1].

AMH and BBH extracts showed potential antimicrobial activity is in concordance with Ekor [9], reporting a potential activity exerted by the methanol extract of *A. maritima* leaves against both *E. coli* and *S. aureus*. Parallely, chloroform extracts showed maximum antibacterial activity with a high concentration of 2 mg/mL against *S. aureus*, *K. pneumonia* and, *E. coli* strains [13].

Interestingly, several phytoconstituents from medicinal plants commonly flavonoids, alkaloids, tannins, and terpenoids, are known for their antimicrobial properties.

In summary, our findings reinforce the reported antioxidant, anti-inflammatory, and immunomodulatory activities exerted by plant-based phytochemicals, which represent a promising tool to be exploited for treating and preventing immune system disorders, due to their varied biological effects, tolerance, and good patient compliance. However, clinical assays are considered as a challenging aspect due to limited specific studies. In addition, the assessment of pure compound identification, purification, their individual biological and pharmacological screening should be assessed in comparison with conventional treatments.

Conclusions

Since both inflammation and oxidative stress are the main self-defense strategies of our bodies to counteract pathogens, plants with anti-inflammatory and/or radical scavenger properties are considered as a potential source of new alternative drugs with fewer side effects and low cost. In this paper, antioxidant, anti-inflammatory, immunomodulatory, and antimicrobial effects have been demonstrated for two medicinal herbs growing in the North-East of Algeria, namely *A. maritima* and *B. bituminosa*, by elucidating lipids anti-peroxidation and protein anti-denaturation effects, and were shown to be associated with their flavonoids rich content. Along with their ability to stimulate the phagocytic system, their safety as a source of phytochemical components was proven *in vivo*. Further investigations should be conducted for adequate application of these plant-derived extracts as alternative drugs for conventional treatments of immune system disorders and related infectious pathologies.

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Authors' contributions

I.R. was the lead author who wrote the manuscript. S.Z. was the supervisor of this work and contributed to its technical and academic realization, and followed the revision of the manuscript. S.Z and I.R developed the main ideas discussed in the paper. L.G, L.A.S, D.U, F.Z.S and Z.K provided materials, help, and technical guidance during the realization of the experimental part.

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