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Fatima Zohra Belbachir, Lamia Hamdan Ramdani, Caroline Decombat, Mael Gainche, Pierre Chalard, et al.. Chemical Profile and Assessment of Antioxidant, Anti-Inflammatory, and Antiproliferative Effects of *Ammodaucus leucotrichus* Coss. & Dur. Fruit Extracts on Human Breast Cancer. *Natural Product Communications*, 2024, 19 (3), pp.1934578X241239827. 10.1177/1934578x241239827. hal-04582794

HAL Id: hal-04582794

<https://hal.science/hal-04582794v1>

Submitted on 22 May 2024

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Chemical Profile and Assessment of Antioxidant, Anti-Inflammatory, and Antiproliferative Effects of *Ammodaucus leucotrichus* Coss. & Dur. Fruit Extracts on Human Breast Cancer

Natural Product Communications
Volume 19(3): 1–9
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sagepub.com/journals-permissions
DOI: 10.1177/1934578X241239827
journals.sagepub.com/home/npx



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Abstract

Objective: In this study, we analyzed and investigated the antioxidant, anti-inflammatory, and antitumoral properties of methanolic (Met), dichloromethane (Dic), ethyl acetate (Ac), and diethyl ether (Et) fruit extracts of *Ammodaucus leucotrichus* on the MCF-7 breast cancer cell line. **Methods:** The extracts of *Ammodaucus leucotrichus* fruits, explored in this study, were obtained by cold maceration and analyzed by LC-MS. 1,1-diphenyl-2-picrylhydrazyl (DPPH), β -Carotene and Bovine Serum Albumin (BSA) assays were used to assess antioxidant and anti-inflammatory activities. The antiproliferative and proapoptotic effects of extracts were determined by the Resazurin test and quantitative real-time polymerase chain reaction (qRT-PCR) analysis. **Results:** Phytochemical screening of *Ammodaucus leucotrichus* fruit extracts revealed the presence of flavonoids (luteolin and cynaroside), iridoid derivatives, phenolic acids, and identified Rhodojaponin—a major cytotoxic compound. The DPPH and BSA assays revealed the most potent radical-scavenging activity ($IC_{50} = 0.16$ mg/mL) and anti-inflammatory effect ($IC_{50} = 35.87$ μ g/mL) in Met extract. Met, Dic, Ac, and Et extracts, at 100 μ g/mL, exhibited significant antiproliferative activity against MCF-7 reaching inhibition percentage of 34%, 31%, 28%, and 13%, respectively. qRT-PCR analysis was marked by the inhibition of proliferative and inflammatory markers Proliferating Cell Nuclear Antigen (*PCNA*) and *IL-6* and the up-regulation of antioxidant and proapoptotic genes *Nrf2*, *Bax*, and *Caspase 3* in Met and Dic extracts, without significant changes to anti-apoptotic gene *Bcl-2* expression. **Conclusion:** The study revealed the potential therapeutic effects of *Ammodaucus leucotrichus* fruit extracts, suggesting they could be used as an adjuvant to breast cancer therapies.

Keywords

Ammodaucus leucotrichus, chemical composition, anti-inflammatory, oxidative stress, cell proliferation, apoptosis, breast cancer

Received: September 2nd, 2023; Accepted: February 29th, 2024.

Introduction

Breast cancer is the leading cause of cancer death for women worldwide.¹ Despite recent advances, breast cancer treatments are known to have devastating side effects and to harm the patients' health.² *Ammodaucus leucotrichus* Coss. & Dur. is a plant native to the Algerian Sahara, belonging to the *Apiaceae* family.³ It is widely used in traditional medicine as a flavoring—especially in Arabic-African countries.⁴ Its fruits and leaves are commonly consumed in infusion to combat various infantile diseases of the digestive system, including indigestion, vomiting, spasms, colic, and constipation.⁵ The seeds have been reported to be used for the treatment of cough, pulmonary diseases, allergy and tachycardia.^{6,7} *Ammodaucus leucotrichus* fruits have

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also recently been shown to modulate different processes involved in various phases of cancer development.⁸

The main medicinal properties of *Ammodaucus leucotrichus* are attributable to its extracts and essential oils. In previous studies, several extracts and isolated compounds of *Ammodaucus leucotrichus* have shown beneficial biological properties, including antimicrobial, anti-inflammatory, antioxidant, and antitumoral activities.^{9,10} The major compound of its essential oil is perillaldehyde¹¹—a monoterpenoid known for its significant impacts on stress and cancer.^{12,13} According to recent findings, also, *Ammodaucus leucotrichus* extracts induced cytotoxic effects in various human tumor cell lines.¹⁴

A number of studies have been performed on non-volatile compounds of this plant and revealed the presence of common polyphenols derived from luteolin, as well as phenolic acid such as di-O-caffeoyl-malonylquinic acid.^{15,16} Further findings highlighted the presence of other families of compounds such as flavonoids, alkaloids, coumarins, and terpenoids.^{14,17}

This general study considers the chemical profile of *Ammodaucus leucotrichus* fruit extracts and reveals their antioxidant, anti-inflammatory, antiproliferative, and proapoptotic properties in human breast cancer cells. The specimens of *Ammodaucus leucotrichus* were collected in Algeria and chemical studies were carried out using cold maceration, followed by LC-MS analysis. The antioxidant activity of the extracts of *Ammodaucus leucotrichus* was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH), β -Carotene assays, and analysis of the Nrf2 marker. Furthermore, their anti-inflammatory properties were explored by means of the Bovine Serum Albumin (BSA) and quantitative polymerase chain reaction (qPCR) methods. Both antiproliferative and proapoptotic effects of extracts were investigated in MCF-7 human breast cancer cells.

Results and Discussion

Chemical Composition of Ammodaucus leucotrichus Fruit Extracts

The yield of Met, Dic, AC, and Et extraction from *Ammodaucus leucotrichus* fruits were 11.92%, 7.1%, 6.6%, and 7.07% respectively. Of all the extracts, Met extract was the one with the highest number of metabolites in solution (Figure 1a). This result was not surprising, as methanol is a non-selective solvent compared to others like dichloromethane. In the Met extract (Table 1), we found common polyphenols such as 1,5-dicaffeoylquinic acid, flavonoids (luteolin and cynaroside), and iridoids derived from loganin. Numerous compounds showed a common fragmentation pattern, like loganin with fragments in negative mode at $[M-H]^- = 179.0555$, characteristic of a hexose moiety fragmentation (glucose, for example), $[M-H]^- = 101.0231$, characteristic of the methyl ester and ether cycle fragmentation in iridoids, and $[M-H]^- = 59.0123$, characteristic of the methyl ester group. An example of loganin fragmentation is shown in Figure 1b. In view of all those fragments, we suspected the presence of iridoids in *Ammodaucus leucotrichus* Met extract. The apolar part of the spectra

showed the presence of luteolin as one of the major compounds in all extracts. We also found two common fatty acids derived from trihydroxy-octadecadienic acid. Finally, in the Met and Dic extracts, the fragmentation pattern of the compound at $[M-H]^- = 367.2129$ showed two major fragments at $[M-H]^- = 265.1445$ and $[M-H]^- = 101.0608$, which are characteristic of Rhodojaponin VI. Fragmentation of Rhodojaponin VI is shown in Figure 1b. Those diterpenes are well known for their cytotoxic activities and have been reported as potential insecticides.¹⁸ The diterpenes also appeared in Ac and Et extracts but in lower quantities.

Antioxidant Activity of Ammodaucus leucotrichus Fruit Extracts

The antioxidant activity of Met, Dic, Ac, and Et extracts of *Ammodaucus leucotrichus* fruits was determined by DPPH radical and β -Carotene assays. The hydrogen-donating ability of the DPPH radical reflects its antioxidant power. The antioxidant effect of the four extracts was evaluated at different concentrations ranging from 0.093 to 1.5 mg/mL, compared with butylated hydroxytoluene (BHT) as a positive control. The DPPH-scavenging test on Dic, Ac, and Et extracts presented antioxidant activity with values of IC_{50} , of the order of 0.49, 0.76 and 0.86 mg/mL, respectively. Met extract showed more potent activity, with $IC_{50} = 0.16$ mg/mL (Table 2) and revealed a significant increase of the expression of the antioxidant gene Nrf2 (nuclear factor erythroid 2-related factor 2) in MCF-7 (2.4 ± 0.4 fold, $P \leq 0.05$) (Figure 2). Furthermore, the data from the β -Carotene assay supported the DPPH results, demonstrating similar IC_{50} for each extract (Table 2). Nevertheless, Met extract remained the more active compared to the positive control (BHT). The antioxidant effects of *Ammodaucus leucotrichus* fruit extracts have been reported previously¹⁹ and are likely due to the extracts' richness in antioxidant compounds. Indeed, the previous report demonstrated that luteolin, the major compound in all extracts, was highly effective against oxidative stress in different systems, which is probably related to the antioxidative effect observed with fruit extracts of *Ammodaucus leucotrichus*.²⁰ A recent study also determined the antioxidant capacity of extracts containing diterpenes—especially Rhodojaponin VI derivative.²¹ Moreover, the activation of Nrf2 is reported to be one of the most efficient mechanisms in the regulation of ROS production in MCF-7.²² Indeed, Nrf2 controls the expression of enzymes involved in ROS detoxification, such as glutathione peroxidase 2 and several glutathione S-transferases.²³ Thus, the activation of Nrf2 could potentially be the molecular pathway by which extracts reduce oxidative stress in MCF-7.

Anti-Inflammatory Effects of Ammodaucus leucotrichus Fruit Extracts

The *in-vitro* anti-inflammatory activity of *Ammodaucus leucotrichus* fruit extracts was measured using the BSA protein denaturation

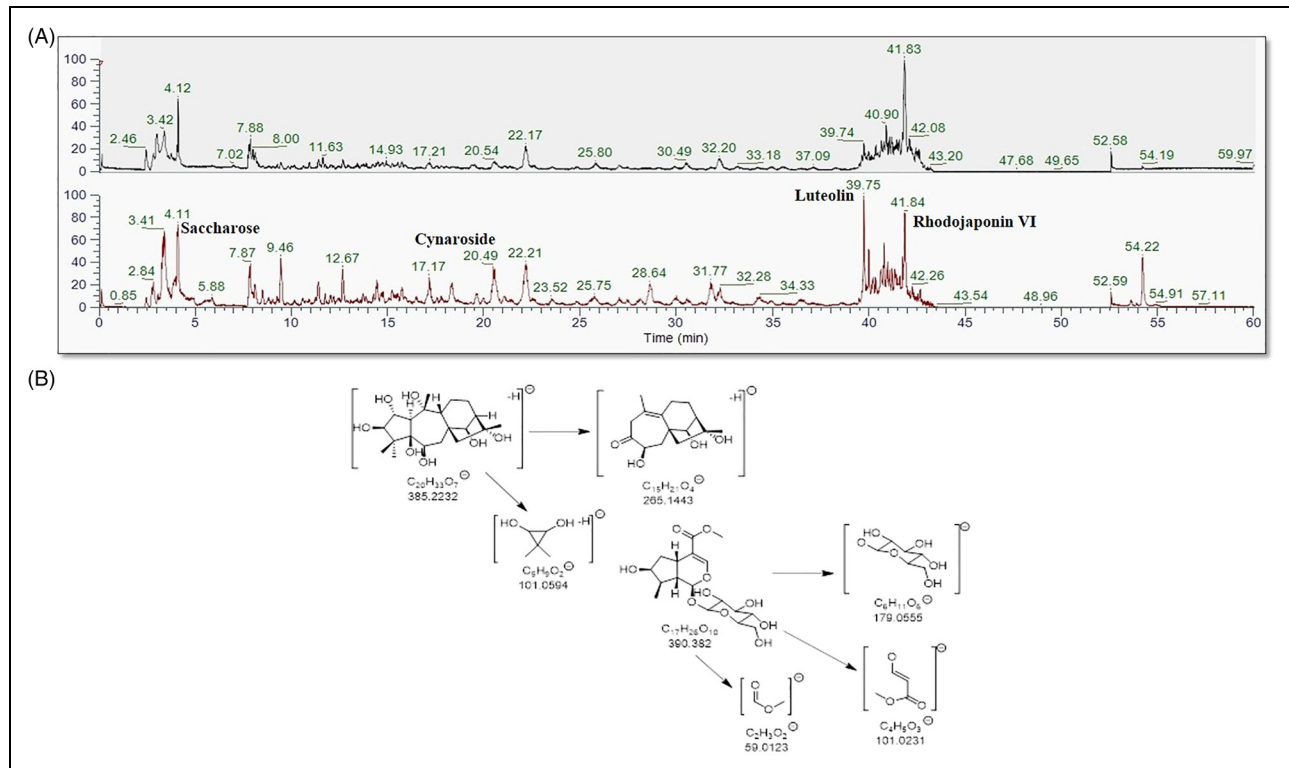


Figure 1. (A) Chromatogram of met fruit extract of *Ammodaucus leucotrichus*. (B) Fragmentation pattern of Rhodojaponin VI and Logenin in negative mode.

Table 1. Retention Time (Rt), Tentative Identification of Compounds in Met Fruit Extract of *Ammodaucus leucotrichus*.

No.	Rt (min)	Chemical Formula	M-H _{exp}	MS ² fragment	Identification
1	4.11	C ₁₂ H ₂₂ O ₁₁	341,1076	89/59/341/179/71	Saccharose
2	7.87	C ₁₁ H ₁₇ O ₈ N	290,0875	128/200/290/170/230	L-pyrroglutamic acid-O-hexose ester
3	12.67	C ₁₆ H ₂₆ O ₉	361.1494	361/101/161/59/71	Iridoid derivative
4	15.76	C ₁₆ H ₂₄ O ₈	343.1388	59/343/71/101/89	Iridoid derivative
5	20.49	C ₂₁ H ₂₀ O ₁₁	447.0918	285/447/284/448/286	Cynaroside
6	22.21	C ₂₇ H ₃₈ O ₁₃	569.2225	523/361/59/101/165	Iridoid derivative
7	23.52	C ₂₅ H ₂₄ O ₁₂	515.1182	191/353/179/161/192	1,5-dicaffeoylquinic acid
8	32.24	C ₂₇ H ₄₂ O ₁₄	589.2487	543/89/149/59/101	Iridoid derivative
9	39.75	C ₁₅ H ₁₀ O ₆	285.0395	285/133/151/286/175	Luteolin
10	40.62	C ₁₈ H ₃₂ O ₅	327.2168	327/211/229/171/291	Trihydroxy-octadecadioneic acid
11	40.96	C ₁₈ H ₃₄ O ₅	329.2322	329/211/229/183/99	Trihydroxyoctadeca-10(E)-dioneic acid
12	41.84	C ₂₀ H ₃₂ O ₆	367.2112	101/265/367/203/113	Rhodojaponin VI

inhibition assay. The heating of BSA results in the loss of protein configuration and functionality, causing inflammatory mechanisms. Results showed that extracts and sodium diclofenac inhibited BSA denaturation in a concentration-dependent manner (Figure 3a). Met extract presented the highest protein denaturation inhibition (IC₅₀ = 35.87 µg/mL), followed by Dic (IC₅₀ = 59.18 µg/mL), Ac (IC₅₀ = 101.9 µg/mL), and Et (IC₅₀ = 131.27 µg/mL) extracts. Met extract also demonstrated a better anti-BSA denaturation than diclofenac sodium (IC₅₀ =

48.02 µg/mL). Moreover, both Met and Ac extracts significantly downregulated the pro-inflammatory gene expression *Il-6* (0.4 ± 0.1 and 0.6 ± 0.1 fold, $P \leq 0.05$, respectively) in MCF-7 cells, reflecting their potent anti-inflammatory activity (Figure 3b). These results are in accordance with previous studies, which revealed that hydroethanolic extract of *Ammodaucus leucotrichus* reduced the expression of pro-inflammatory enzymes¹⁶ and inhibited the inflammatory process in various *in vivo* models.²⁴ In addition, luteolin and

Table 2. DPPH Radical-Scavenging and β -Carotene Activities of Various *Ammodaucus leucotrichus* Fruit Extracts at Concentrations (0.093-1.5 mg/mL). BHT: Positive Control. Data are Presented as Mean \pm SEM, n = 6, * $P \leq 0.05$; ** $P \leq 0.001$ Compared to the Control.

Samples Concentrations	Inhibition percentage of radical DPPH (%)					IC ₅₀ (mg/mL)	IC ₅₀ (mg/mL)
	0.093	0.1875	0.375	0.75	1.5 (mg/mL)	DPPH• Scavenging	β -carotene
Met extract	35.62 \pm 3.08**	54 \pm 1.77**	64.7 \pm 0.83**	71.82 \pm 0.7**	90.79 \pm 0.49**	0.16 \pm 0.79	0.21 \pm 0.5
Dic extract	20.87 \pm 2.66*	39.66 \pm 1.82**	46.4 \pm 1.58**	55.47 \pm 1.26**	68.64 \pm 1.27**	0.49 \pm 0.30	0.50 \pm 0.29
Ac extract	14.61 \pm 3.7*	32.11 \pm 2.12**	40.62 \pm 1.94**	49.69 \pm 1.49**	60.37 \pm 1.51**	0.76 \pm 0.11	0.62 \pm 0.20
Et extract	9.05 \pm 2.55	30.16 \pm 2.56**	45.65 \pm 6.21**	48.01 \pm 1.94**	57.8 \pm 1.88**	0.86 \pm 0.06	0.74 \pm 0.12
BHT	4.99 \pm 2.69	8.34 \pm 3.24	34.32 \pm 2.12**	53.88 \pm 1.64**	70.9 \pm 1.2**	0.65 \pm 0.18	0.70 \pm 0.10

DPPH: 1,1-diphenyl-2-picrylhydrazyl; BHT: butylated hydroxytoluene.

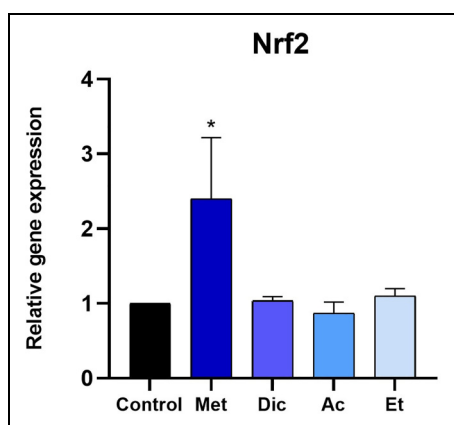


Figure 2. Relative expression of the antioxidant gene *Nrf2* in MCF-7 after treatment with 100 μ g/mL of Met, Dic, Ac, and Et extracts. Data are presented as mean \pm SEM, n = 3, * $P \leq 0.05$.

phenolic acids have been shown to act as anti-inflammatory compounds, through the regulation of transcription factors STAT3, NF- κ B, and AP-1, and inhibition of cyclooxygenase enzyme,^{25,26} while rhodajaponin significantly reduced levels of the pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α .²⁷

Antiproliferative Effects of *Ammodaucus leucotrichus* Fruit Extracts

In order to demonstrate the potential cytotoxic effects of fruit extracts on the MCF-7 human breast cancer cells, cells were treated with different concentrations of each extract (Met, Dic, Ac, and Et) ranging from 10 to 100 μ g/mL for 72 h. Results showed a decrease of cell proliferation in a dose-dependent manner reaching a percentage of inhibition of 34%, 31%, 28%, and 13% at 100 μ g/mL with Met, Dic, Ac, and Et extracts, respectively (Figure 4a). qPCR further supported the reduction of cell proliferation by inhibition of Proliferating Cell Nuclear Antigen (PCNA) gene expression in Met extract (0.5 \pm 0.1 fold, $P \leq 0.05$) (Figure 4b). Recent insights support our results, revealing a significant cell viability decrease with *Ammodaucus leucotrichus* ethanolic extract in various human cancer cell lines where IC₅₀ is greater than 200 μ g/mL.⁸ Referring to the composition of fruit extracts of *Ammodaucus*

leucotrichus, we could identify potentially cytotoxic constituents. Indeed, this is the first report revealing the presence of rhodajaponin, whose potent anticancer effects have been identified previously.¹⁸ In addition, luteolin has demonstrated antiproliferative effects through the regulation of PCNA, a key proliferating cell marker, and other cell cycle pathway genes in MCF-7 cells.²⁸ Indeed, PCNA is an essential protein that participates in a variety of processes of DNA metabolism. Luteolin also acts by activation of the JAK/STAT pathway and the inhibition of NLRP3 in the inflammasome.²⁹⁻³¹ Furthermore, Ndongwe et al.³² thoroughly explored the therapeutic potential of iridoid derivatives and reported their antitumor properties on breast cancer. These results indicated that the fruit extracts of *Ammodaucus leucotrichus* are rich in cytotoxic compounds potentially involved in antiproliferative activities observed in MCF-7 breast cancer cells.

Proapoptotic Effects of *Ammodaucus leucotrichus* Fruit Extracts

Our study revealed a significant increase of *Bax* (3.2 \pm 0.3; 1.9 \pm 0.4 fold, $P \leq 0.05$) and *Caspase 3* (2.1 \pm 0.6; 1.5 \pm 0.4 fold, $P \leq 0.05$) expression following treatment with Met and Dic extracts. On the contrary, no significant effect on the mRNA expression of *Bcl-2* was observed with any of the extracts (Figure 5). Our results support recent findings, where an apoptosis fold increase was observed with *Ammodaucus leucotrichus* at similar concentration in other cancer cells.⁸ Based on the reported studies, enhancement of proapoptotic gene expression could be attributed to the potential contribution of cynaroside in the inhibition of tumor growth and regulation of apoptosis.³³ Furthermore, most of the diterpenes, as Rhodajaponin demonstrated, act as anticancer agents and promote the induction of apoptosis.^{18,34,35} In addition, further findings demonstrated that iridoid derivatives strongly modulate apoptosis through the regulation of the Bax/Bcl-2 ratio.³⁶ Taken together, these results suggest that extracts of *Ammodaucus leucotrichus* would increase the Bax/Bcl-2 ratio, resulting in an up-regulation of *Caspase 3* expression and the promotion of apoptosis.

Although this report revealed the potential therapeutic effects of *Ammodaucus leucotrichus* fruit extracts, it has limitations. Indeed, this study highlighted the overall composition of the

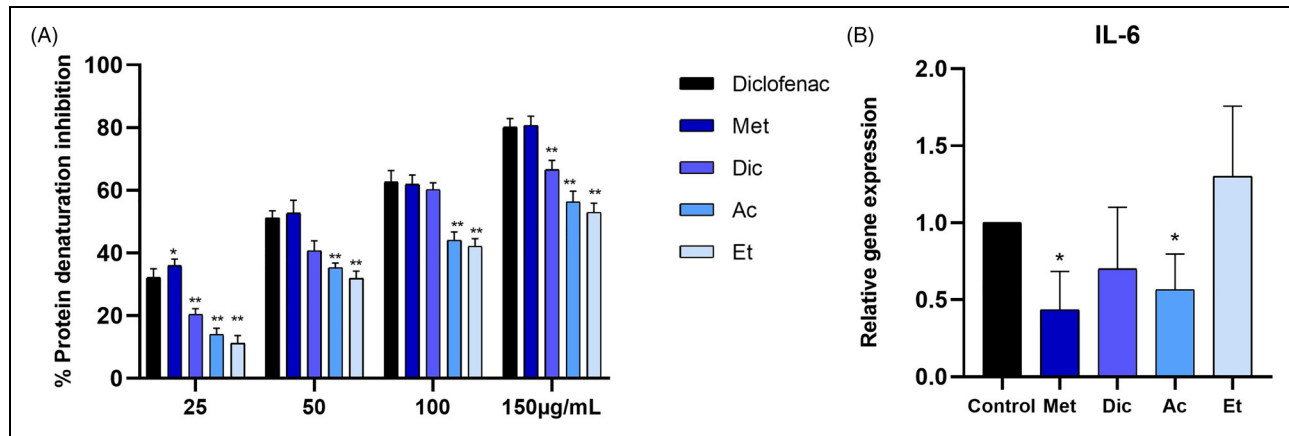


Figure 3. (A) BSA protein denaturation inhibition by Met, Dic, Ac, and Et fruit extracts from *Ammodaucus leucotrichus*, compared with the positive control diclofenac. (B) The relative expression of pro-inflammatory gene *IL-6* in MCF-7 after treatment with 100 µg/mL of extracts. Data are presented as mean ± SEM, n = 3-6, * $P \leq 0.05$, ** $P \leq 0.001$. BSA: Bovine Serum Albumin.

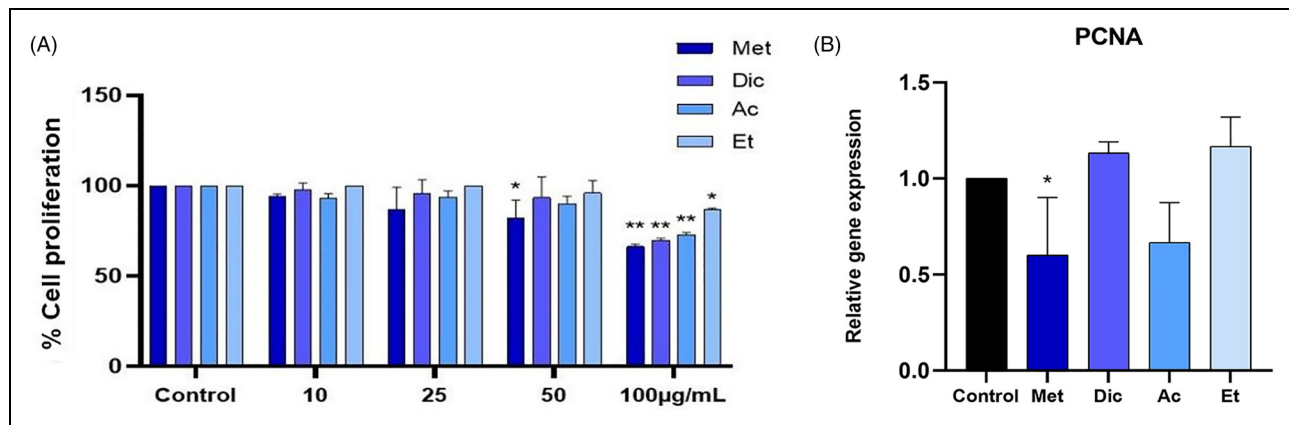


Figure 4. (A) Proliferation of MCF-7 breast cancer cells treated with 10, 25, 50, and 100 µg/mL of Met, Ac, Dic, and Et extracts of *Ammodaucus leucotrichus*. (B) The relative expression of proliferative gene *PCNA* in MCF-7 after treatment with 100 µg/mL of extracts. Data are presented as mean ± SEM, n = 3, * $P \leq 0.05$; ** $P \leq 0.001$ compared to the control value. PCNA: Proliferating Cell Nuclear Antigen.

Met extract of *Ammodaucus leucotrichus* but did not isolate the main molecules involved in the antioxidant, anti-inflammatory and antiproliferative effects of our extracts. Furthermore, these results demonstrated anti-inflammatory and antiproliferative effects on MCF-7 breast cancer cells but further studies are needed to determine the molecular mechanisms and signaling pathways responsible for the regulation of inflammation and triggering of apoptosis. Finally, this study was carried out on MCF-7 2D model, and it would be interesting to test the effects of these extracts in co-culture 3D breast cancer model.

Conclusion

In summary, we demonstrated that rhodojaponin VI, flavonoids such as luteolin and cynaroside, iridoids derived from loganin and phenolic acids constituted the major compounds in *Ammodaucus leucotrichus* fruit extracts. These substances showed significant antioxidant, anti-inflammatory, and

antiproliferative properties, resulting in up-regulation of proapoptotic gene expression and cell death in MCF-7 breast cancer cells. Going forward, an evaluative study will be necessary to improve our understanding of the potential anticancer effect of *Ammodaucus leucotrichus* fruit compounds on human breast cancer cell lines.

Experimental

Plant Materials

The fruit samples of *Ammodaucus leucotrichus* Coss. & Dur. were collected from the Province of Bechar (southern Algeria at an altitude of 475 m; 30°05'00''N, 2°06'00''W; arid, hot climate) in October 2020. The plant was identified in the laboratory of Pharmacognosy, University of Tlemcen, by Dr Dali-Yahia Kamel. After drying at room temperature, the fruit compounds were extracted.

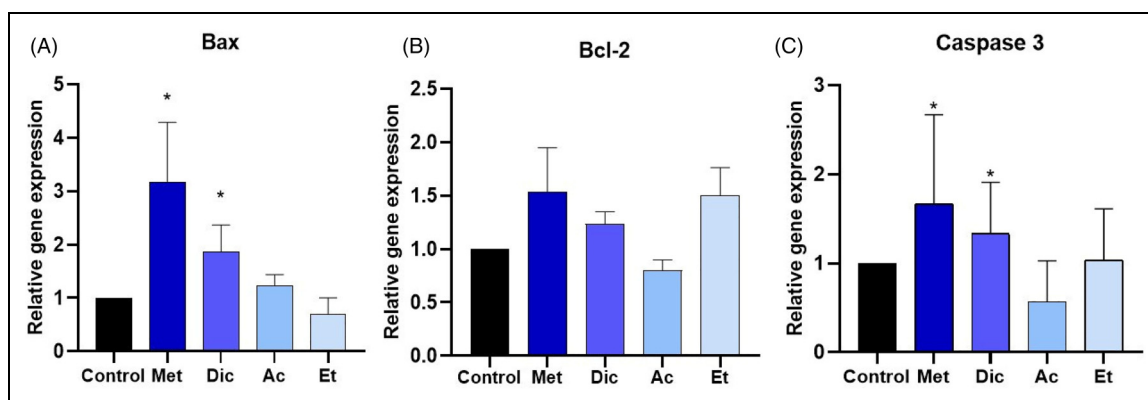


Figure 5. The relative expression of proapoptotic gene *Bax* (A), anti-apoptotic gene *Bcl-2* (B), and *Caspase 3* (C) and after transcription by qRT-PCR analysis. MCF-7 cells were treated with 100 $\mu\text{g}/\text{mL}$ of Met, Dic, Ac, and Et extracts of *Ammodaucus leucotrichus* fruits, * $P \leq 0.05$, compared to the control group. Experiments were performed in triplicate. qRT-PCR: quantitative real-time polymerase chain reaction.

Table 3. Primer Sequences.

Genes	Primer sequences (5'-3')
Nrf2	F: CACATCCAGTCAGAAACCAGTGG R: GGAATGTCTGCGCCAAAAGCTG
IL-6	F: GCTGCAGGCACAGAACCA
PCNA	R: ACTCCTTAAAGCTGCGCAGAA
Bax	F: AGGCACTCAAGGACCTCATCA
Bcl-2	R: GAGTCCATGCTCTGCAGGTTT
Caspase 3	F: TGCTTCAGGGTTTCATCCAG
Actin	R: GGCGGCAATCATCCTCTG F: GGCTGGGATGCCTTTGTG R: CAGCCAGGAGAAATCAAACAGA F: ACATGGCGTGTCAAAAATACC R: CACAAAGCGACTGGATGAAC F: CCTGGCACCCAGCACAAAT R: GCCGATCCACACGGAGTACT

F, Forward; R, reverse; PCNA: Proliferating Cell Nuclear Antigen.

Chemicals and Reagents

DPPH, β -Carotene, BHT, and BSA were obtained from Sigma Aldrich. Human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (Molsheim, France). MCF-7 cells were cultured in RPMI-1640 (Gibco, ThermoFisher, Illkirsh, France), supplemented with 10% heat-inactivated fetal bovine serum (Eurobio, Courtaboeuf, France), 1% L-glutamine (2 mM) (Gibco), 0.5% gentamycin (50 mg/mL) (Fisher Scientific), and 0.05% insulin (100 IU/ml) (Sigma-Aldrich). Cells were kept at 37°C in an environment of 5% CO₂. Stock solution of four extracts was prepared in dimethylsulfoxide (DMSO) and stored at 4°C.

Cold Maceration

The fruits of *Ammodaucus leucotrichus* were chopped into very small pieces; 50 g of dried and crushed fruits were macerated for 48 h, while being stirred, in 500 mL of solvent: methanol,

ethyl acetate, dichloromethane, and diethyl ether. The mixtures were filtered, and solvents were removed by evaporation under reduced pressure. The concentrated extracts were stored at 4°C until use and the extraction yield was calculated according to the following formula:

$$\text{Extraction yield (w/w)(\%)} = \frac{\text{dry extract mass (g)}}{\text{dry initial mass (g)}} \times 100$$

LC-MS Analysis

LC-MS analyses were carried out on an UHPLC Ultimate 3000 RSLC chain and an Orbitrap Q-Exactive (Thermo Scientific, Waltham, MA, USA) using an Uptisphere C₁₈₋₃ (250 mm \times 4.6 mm, 5 μm) column from Interchim (Montluçon, France). Source operating conditions were 3 kV spray voltage; 320°C heated capillary temperature; 400°C auxiliary gas temperature; sheath, sweep and auxiliary gas (nitrogen) flow rate 50, 10, and 2 arbitrary units, respectively; collision cell was used in SIM mode with AGC Target at 1^{e5} and maximum IT at 100 ms. Data were obtained at a resolution of 35,000. Data were processed using Freestyle software (Thermo Fisher Scientific Inc., Waltham, MA, USA). The analysis was carried out using negative ionization mode. For analyses, the mobile phase was a mixture of 0.1% (v/v) formic acid in water (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). The gradient of phase A was 100% (0 min), 80% (10 min), 73% (35 min), 0% (40 min), 0% (50 min), 100% (51 min), and 100% (60 min). The flow rate was 0.8 mL/min, oven temperature was 30°C, the injection volume was 5 μL , and sample concentration was 1 mg/mL prepared in methanol HPLC grade.

Antioxidant Activity Assays

Evaluation of antioxidant activity by DPPH assay. Firstly, the antioxidant activity of Met, Dic, Ac, and Et extracts was tested using the stable radical DPPH. To achieve this, 1 mL of

extract at 0.093, 0.1875, 0.375, 0.75, and 1.5 mg/mL was mixed with 1 mL of ethanolic solution of DPPH at 0.004%. After 30 min of incubation at room temperature, the absorbance was recorded at 517 nm using a spectrophotometer (Thermo Spectronic, England). BHT was used as the positive control and all assays were performed six times. IC₅₀ symbolized the concentration of the sample that reduced 50% of the DPPH.

Evaluation of antioxidant activity by β -Carotene assay. A stock solution of β -Carotene was prepared with 0.5 mg of β -Carotene and 1 mL of chloroform. This latter was mixed with 25 μ L of linoleic acid in 200 mg of Tween 40. The chloroform was completely evaporated under reduced pressure at 45 °C, then 100 mL of oxygen-saturated distilled water was added and the resulting mixture was vigorously shaken to form an emulsion. The reaction medium containing 2.5 mL of β -Carotene/linoleic acid emulsion and 0.5 mL of extract solutions at different concentrations ranging from 0.093 to 1.5 mg/mL was placed in a water bath and incubated at 50 °C for 2 h. The absorbance of samples was measured at 470 nm using a spectrophotometer (Thermo Spectronic, England). A standard BHT was used as a positive control. Experiments were repeated six times.

Anti-Inflammatory Activity Assay

The anti-inflammatory effects of Met, Dic, Ac, and Et fruit extracts was evaluated by BSA assay, in accordance with the method described by Williams et al.³⁷ Briefly, 0.05 mL of plant extract or sodium diclofenac (positive control) solutions were diluted to concentrations of 25, 50, 100, and 150 μ g/mL, then mixed with 0.45 mL of BSA (0.5% w/v). The mixtures were heated to 37 °C for 5 min, and then to 57 °C for 3 min. After cooling, phosphate buffer (2.5 mL) was added to each solution and absorbance was measured at 660 nm using a UV/VIS spectrophotometer. The negative control represented 100% of protein denaturation. The percentage inhibition of protein denaturation was determined as follows:

$$\% \text{ inhibition} = (A_c - A_s) / A_c \times 100$$

A_c represented the absorbance of negative control and A_s the absorbance of the extracts.

Cell Proliferation Assay

MCF-7 cells were seeded in 96-well plates (1 × 10⁴/well) in complete media for 24 h before treatment with different extract solutions ranging from 10 to 100 μ g/mL at 37 °C in a 5% CO₂ atmosphere. The vehicle solvent DMSO at concentration < 0.1% (v/v) was used as control. After 72 h of treatment, a resazurin solution (Sigma-Aldrich) (25 μ g/mL) was added to each well for 2 h of incubation at 37 °C. Fluorescence was measured on an automated 96-well plate reader (Fluoroskan Ascent FL; Thermo Fisher Scientific, Wilmington, NC, USA) at 530 nm and 590 nm, and the OD value was proportional to

the number of living cells in the well. Experiments were repeated in triplicate. Data were normalized to the control group fluorescence.

Total RNA Extraction

Cells were seeded in 6-well plates (4 × 10⁵ cells/well) and treated as previously with 100 μ g/mL of Met, Dic, Ac, Et extracts, or with the vehicle solvent (control) for 72 h. After harvesting cells with trizol (Sigma Aldrich), the mixture was separated by chloroform into three phases, and the aqueous phase, containing RNA, was recovered. The RNA was precipitated with isopropanol, then frozen overnight at -20 °C. Thereafter, two centrifugations at 12,000 × g for 15 min were necessary to obtain concentrated RNA, which was then suspended in DEPC-treated water. The RNA concentration and purity were determined using a spectrophotometer (Nanodrop 2000; Thermo Scientific).

Real-Time Polymerase Chain Reaction

DNase treatment was applied, using DNase I Amplification grade (Invitrogen). Reverse transcription of 1 μ g of RNA in cDNA was then carried out with the MultiScribe reverse transcriptase (HighCap cDNA RT Kit with RNase inhibitor, Invitrogen) following the manufacturer's protocol. Expression of *Nrf2*, *IL-6*, *PCNA*, and of the pro- and anti-apoptotic genes *Bax*, *Bcl-2* and *Caspase 3*, was measured in MCF-7 cells treated with 100 μ g/mL of Met, Dic, Ac and Et extracts by quantitative real-time polymerase chain reaction (qRT-PCR) (Table 3). The *Actin* gene was used for normalization and relative *Nrf2*, *IL-6*, *PCNA*, *Bax*, *Bcl-2* and *Caspase 3* gene expression of samples was evaluated by $\Delta\Delta CT = [\Delta CT (\text{sample1}) - \Delta CT (\text{sample2})]$ and $\Delta CT = [CT (\text{target gene}) - CT (\text{reference gene})]$. The values were obtained from three independent experiments.

Statistical Analysis

Tests were conducted, at least, in triplicate, and results were expressed as mean values \pm SEM. All analyses were performed by Student's t-test using GraphPad Prism software version 8 (GraphPad Software, San Diego, USA). Differences at $P \leq 0.05$ (flagged as *) or $P \leq 0.001$ (flagged as **) were considered statistically significant.

Acknowledgements

The Authors are grateful to the ECREIN team (INRAE, UNH, CRNH of Clermont Auvergne University), ICCF-SIGMA of Clermont-Ferrand and Scientific and Technical Research Center in Physico-Chemical Analysis (CRAPC) of Algeria for the achievement of this work.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical Approval is not applicable for this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article, so informed consent is not applicable.

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