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RESEARCH ARTICLE

Investigating the antioxidant, anti-inflammatory and anti-cancer potential of *Ononis natrix* L. with phytochemical analysis

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Abstract

Ononis natrix L. (*O. natrix*) belongs to Fabaceae family and grows on the coast of Mediterranean Sea. The current study aims to investigate the antioxidant, anti-inflammatory and anti-cancer activities of the methanolic extract of *O. natrix* and identify the phytochemical constituents responsible for these effects. Gas chromatography-mass spectrometry (GC-MS) was employed to characterize the phytochemicals found in the extract. The human red blood cells (RBCs) membrane stabilization method, ferric reducing antioxidant power (FRAP), total antioxidant capacity (TAC), 1,1-diphenyl -2-picryl hydrazyl (DPPH) radical scavenging assay and MTT viability assay were used to investigate the anti-inflammatory, antioxidant and anti-cancer activities. The results showed that methyl stearate (39.48%) and hexadecanoic acid, methyl ester (35.61%) were the most relatively abundant phytochemicals identified by GC-MS. The percentage of hemolysis inhibition for RBC membrane achieved by *O. natrix* extract was 99.6 ± 1% versus that by indomethacin was 99.9 ± 0.6%. Additionally, the DPPH radical scavenging activity of *O. natrix* extract versus ascorbic acid was 97 ± 0.3% and 98 ± 0.2%, respectively. The *O. natrix* extract showed a moderate anti-cancer activity against DLD-1 cells (IC_{50} = 57.13±4.9 µg/mL), MCF-7 cells (IC_{50} = 96.69±6.2 µg/mL) and A549 cells (IC_{50} = 126.63±10.4 µg/mL), when compared with the anti-cancer drug; Cisplatin. Therefore, *O. natrix* extract showed significant anti-inflammatory, antioxidant and anti-cancer properties, which could be attributed to methyl stearate and hexadecanoic acid, methyl ester constituents.

Keywords

anti-inflammatory; antioxidant; cancer; GC-MS; *O. natrix*

Introduction

Several anti-inflammatory medications such as ibuprofen, ketoprofen and ketorolac show serious adverse effects, including stomach and duodenum ulcers, bleeding hypertension, nephrotoxicity and ototoxicity (1-4). However, they are currently being used widely due to their over-the-counter availability (5). Additionally, anti-inflammatory medications are being prescribed by different medical specialists for several diseases as painkillers (6). Different plants have been investigated to find phytochemicals with anti-inflammatory potencies and lower adverse effects. For example, coffee leaf

extract was shown to reduce the levels of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-8; which were induced by lipopolysaccharide treatment of co-cultured colorectal adenocarcinoma (Caco-2) and myeloid leukemia (U937) cell lines (7).

Oxidative stress has been linked with cardiovascular and neurodegenerative diseases, such as hypertension (8), myocardial infarction (9), atherosclerosis (10), Alzheimer's disease (11) and Parkinson's disease (12). Oxidative stress is defined as a disproportion between elevated reactive oxygen species (ROS) and inadequate cellular antioxidant activity. Elevated oxidative stress can harm cellular structure and even cause tissue destruction (13). Remarkably, the antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase are responsible for scavenging the oxidative free radicals from the cell (14). Several plant species have been explored for their antioxidant properties with the isolation of antioxidant phytochemicals from different plant extracts. For example, *Tephrosia purpurea* (L.) subsp. *apollinea* extract showed a potential antioxidant activity (15).

Cancer is the second primary cause of death worldwide, with 10 million deaths reported annually (16). Cancer is characterized by abnormal and uncontrolled rapid cell growth affecting any part of the human body. It is viewed as a serious disease that requires consistent efforts for drug discovery (17). Interestingly, more than half of the anti-cancer drugs are isolated from plants. These medicinal plants mostly contain several polyphenolic compounds which inhibit cancer cell growth (18).

There are 20000 species in the Fabaceae family. The genus *Ononis*, a member of the Fabaceae family, has 17 species. The majority of those species are found in Middle Eastern and Mediterranean regions (19). One of the species, *O. natrix*, is primarily found in Tunisia, Libia, Egypt, Palestine, Lebanon and Jordan (20). Remarkably, various chemical constituents of *O. natrix* have shown different therapeutic properties. For instance, 3,4-dihydroisocoumarin derivatives and 5-alkylresorcinol extracted from *O. natrix* acetonitrile sub-fraction of acetone showed moderate anti-microbial and anti-protozoal activities against several protozoa and microorganism strains (21). In another study, 21 components, including 6-(2'R-acetoxypentadecyl)-2-hydroxy-4-methoxybenzoicacid isolated from *O. natrix* were analyzed for anti-bacterial, anti-leishmanial and anti-trypanosomal properties (22). Quercetin, kaempferol and flavones were among the other chemical components discovered in the *O. natrix* leaf extract which exhibited broad spectrum anti-microbial activities (23). *O. natrix* has been renowned for its potential anti-microbial (23), anti-Alzheimer, anti-diabetic (20), antioxidant (24) and anti-hypertensive properties (24). Other studies demonstrated significant biological activities of *Ononis* species, including wound healing, cytotoxicity, anti-spasmodic and analgesic properties (25). Therefore, several *Ononis* species have been traditionally used for the treatment of gout, diabetes, renal and skin disorders (25). *Ononis* species, either alone or in combination with other diuretic plants, have been marketed as herbal teas (26). Very recently, *O. natrix* was found to have anti-proliferative effects against hepatocellular carcinoma and prostate cancer (20).

This study aims to investigate the antioxidant, anti-inflammatory and anti-cancer activities of the methanolic extract of *O. natrix* and identify the phytochemical constituents responsible for these effects.

Materials and Methods

Plant materials

During spring, the *O. natrix* aerial parts were assembled from the City of Mutah, Al-Karak governorate, Jordan. The plant was cleaned using tap water and was dehydrated by incubation at 25ºC, for 10 days in a dark, ventilated room. A grinder (Moulinex, France) was employed to triturate the dried plant (15). A descriptive list was used to identify the plant (27). The Biology Department, Faculty of Agriculture, Mutah University, authenticated the plant and voucher specimens (711/5/1998) deposited in the department.

Preparation of crude extract

The cold percolation technique extracted 250 g of powder from the air-dried plant. In three separate containers, the powder underwent agitation for 3 days at 25°C with 70% methanol (500 mL). The methanol extract was then filtered using a Buchner funnel. Subsequently, a rotary evaporator was utilized to eliminate methanol under reduced pressure at 40°C. Traces of solvent were evaporated using a desiccator, resulting in the acquisition of 20 g per 100 g of dry weight of crude extract of *O. natrix*, which was subsequently subjected to chemical compound characterization via Gas chromatography/Mass spectrometry (GC/MS) (15).

Quantitative analysis of chemical compounds by GC/MS

The previously prepared crude extract underwent analysis utilizing a GC/MS (Thermo Scientific TRACE 1310) instrument (J & W Scientific). The grade of the flow of helium was 1 mL/min. The oven temperature was gradually elevated from 40°C to 280°C at a grade of 5°C per min. The quantities of injection were conserved at 1 µL, with a hold time of 7.5 min. The temperature of the ion source was set to 280°C and the ionization voltage of 70 eV was applied. Samples were ionized in the mode of electron impact using a range of mass of m/z 50–650. Data interpretation was based on database sources from the NIST and Wiley libraries.

Evaluation of anti-inflammatory activity

Human red blood cells (RBCs) membrane stabilization technique

Owing to the similarities between membranes of RBCs and lysosomes, phenylhydrazine ($PhNHNH_2$), heat or hypotonic medium may cause membrane lysis and content release. Indomethacin prevents lysosomal membrane breakdown and enzyme release. The anti-inflammatory properties of *O. natrix* were assessed by comparing its ability to prevent RBC membrane lysis caused by hypotonic solution and indomethacin as the positive control (28).

Erythrocyte suspension preparation

Fresh whole blood (3 mL) was collected into heparinized tubes from participants who had not taken analgesics for two weeks before the study. A complete blood count (CBC) was conducted to rule out any hematological disorders. The tubes were then centrifuged for 10 min at 3000 rpm. The RBC pellets were solubilized in normal saline and centrifuged again at 3000 rpm for 10 min to wash off the supernatant. This step was repeated thrice and the final RBC pellet was reconstituted using isotonic solution (phosphate buffered saline containing 1.15 g of Na_2HPO_4, 0.2 g of 10 mM NaH_2PO_4, 9 g of NaCl and pH 7.4 in 1 L of distilled water) to give 40% v/v suspension. This cellular suspension was used for the anti-inflammatory assay (29).

Hemolysis induced by hypotonicity

5 mL of the hypotonic solution was used to dissolve 1 g of *O. natrix* methanolic extract. The centrifuge tubes were loaded with dilutions ranging from 100–1000 µg/mL. One g of the extract was solubilized in 5 mL of the isotonic solution and the extract concentrations (100–1000 µg/mL) were also added to the centrifuge tubes. The control tubes received 5 mL of hypotonic solution and 5 mL of indomethacin (200 µg/mL). The previously prepared suspension of erythrocyte (100 µL) was added to each tube and mixed. The mixtures were incubated for 1 hr at 37ºC and then centrifuged at 1300 rpm, for 3 min. The supernatant (hemoglobin) was then collected and its absorbance at 540 nm was recorded using Biosystm 310 plus spectrophotometer. Equation 1 was used to calculate the percentage of hemolysis inhibition induced by *O. natrix* (29).

$$\%\text{Hemolysis inhibition} = 1-\left(\frac{\text{Sample Abs. in hypotonic solution -Sample Abs. in isotonic solution}}{\text{Control Abs. in hypotonic solution -Sample Abs. in isotonic solution}}\right) \text{X } 100 \quad \text{.....(Eqn 1)}$$

Antioxidant activity evaluation

Total antioxidant capacity assay (TAC)

3 mL of reagent solution (28 mM Sodium phosphate (NaH_2PO_4), 0.6 M H_2SO_4 and 4 mM Ammonium molybdate) were mixed with 1 mL of 0.5 mg/mL of each sample of *O. natrix* extract. The mixture was incubated for 2.5 hr at 95°C and post incubation, the samples were cooled to 25ºC, followed by recording of absorbance at 630 nm using a microtiter plate reader (Biotek ELX800; Biotek, Winooski, VT, USA). Ascorbic acid (1 mg/mL) was used as the reference drug. Values were represented as ascorbic acid equivalent (AAE) µg/mg of extract (30).

Ferric reducing antioxidant power (FRAP) assay

40 mL of extract samples were mixed with 50 mL 10% trichloroacetic acid, 50 mL (0.2 mol/L) Na_2HPO_4. $2H_2O$ buffer and 50 mL 1% potassium ferricyanide ($K_3Fe(CN)_6$) in Eppendorf tubes. At 3000 rpm, the mixture was centrifuged for 10 min. The 96-well plates were loaded with the supernatant (166.66 µL) of each sample and three wells were kept for the negative control, DMSO, followed by the addition of 33.3 µL of ferric chloride ($FeCl_3$ 1%) to each well. The antioxidant compounds in the extract can donate electrons or hydrogen; therefore, the reduction of Fe^{3+} to Fe^{2+} by these compounds was detected colormetrically using a microtiter plate reader (Biotek ELX800; Biotek, Winooski, VT, USA) at 630 nm. Ascorbic acid (1 mg/mL) was used as the reference drug. The values were expressed as ascorbic acid equivalent (AAE) µg/mg of extract (31).

1,1-diphenyl-2-picryl hydrazyl (DPPH) assay

The antioxidant potential of *O. natrix* was determined using the DPPH assay. A solution of 0.1 mM DPPH in ethanol was prepared; 1 mL of this solution was combined with 3 mL of *O. natrix* extract at various concentrations (ranging from 3.9–1000 µg/mL). The resulting mixture was vigorously shaken and incubated at 25ºC for 30 min before measuring the optical densities at 517 nm employing a UV-VIS spectrophotometer (Milton Roy). Ascorbic acid was used as the reference drug. The experiment was carried out in triplicates. The percentage of DPPH radical scavenging action was computed as follows:

$$\text{DPPH radicals scavenging (\%)} = \frac{\text{Absorbance of control - Absorbance of extract samples}}{\text{Absorbance of control}} \text{X100} \quad \text{.....(Eqn 2)}$$

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability assay

The cell lines of human dermal fibroblasts (HDF), human ER-positive breast adenocarcinoma (MCF-7), colorectal adenocarcinoma cell line (DLD-1) and human lung adenocarcinoma (A549) were cultured and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine. The cells were maintained under standard culture conditions of 95% humidity and 5% CO_2 at 37°C.

For the viability assay, the cells were seeded at a density of 1×10^4 cells in each well of a 96-well plate for 24 hr. *O. natrix* extract was diluted with RPMI 1640 medium to produce serial dilutions from 1–1000 µg/mL, 100 µL of each dilution was added to the corresponding well in triplicates and incubated for four days. After 4 days, 5 mg/mL of MTT solution was added to the treated cells to form formazan products only by the living cells and finally, dimethyl sulfoxide was used to dissolve the purple formazan crystals. Absorbance of the formed color was measured spectrophotometrically by a plate reader at 500–600 nm according to an earlier study (32).

The half-maximal inhibitory concentration (IC_{50}) and the selectivity index (SI) of the *O. natrix* extract were calculated according to the following equations:

$$IC_{50}\% = (100 - \text{OD of test})/\text{OD of control} \times 100 \quad \text{.....(Eqn 3)}$$

$$SI = IC_{50} \text{ for } HDF \text{ cells}/IC_{50} \text{ for cancer cells} \quad \text{.....(Eqn 4)}$$

Microscopic examination

The cells were examined after treatment with *O. natrix* extract under a light microscope (Nikon, 118810) at 400X magnification power) to reveal any cellular abnormalities.

Results

GC/MS analysis

GC-MS was used to detect the chemical composition of *O. natrix* extract. GC-MS analyses allowed identification of 23 compounds belonging to the chemical classes of fatty acid esters, fatty acids, phthalate esters, sterols, aromatic compounds, aliphatic ketones and flavonoids (Table 1 and Fig. 1). The major compounds of *O. natrix* extract were fatty acid esters methyl stearate (Relative abundance (R.A)=39.48%) and hexadecanoic acid, methyl ester (R.A=35.61%). Other fatty acid esters such as octadecanoic acid, ethyl ester (ethyl stearate) (R.A=0.67%), hexadecanoic acid, 14-methyl -, methyl ester (R.A=0.61%), octadecanoic acid,4-hydroxy-, methyl ester (R.A=0.56%), hexadecanoic acid,1-(hydroxyl-methyl)-1,2-ethane diyl ester (R.A=0.37%), glyceryl 1,3-distearate (R.A= 0.31%), pentadecanoic acid, 14-methyl-, methyl ester (R.A=0.27%) and hexadecanoic acid,2,3-dihydroxypropyl ester (R.A=0.27%) were identified in *O. natrix* extract but in small quantities. The identified fatty acids were octadecanoic acid (Stearic acid) (R.A=1.93%), hexadecanoic acid (R.A=0.71%), oleic acid (R.A=0.40%) and n-hexadecanoic acid (R.A=0.15%). Furthermore, the phthalate esters diisooctyl phthalate (1,2-benzene dicarboxylic acid, dioctyl ester) (R.A=0.34%) and glyceryl 1,3-distearate (R.A=0.31 %) were identified in the extract. The detected sterols were cholest-5-en-3-yl myristate (R.A=0.14%), cholesta-4,6-dien-3-ol, (3α)- (R.A=0.26%), cholest-3,5-diene (Oxysterol) (R.A=0.93%), stigmasta-5,22-dien-3-ol, acetate, (3α)- (R.A=0.23%) and stigmast-5-en-3-ol, oleate (R.A=0.69%). The plant extract also contained the aromatic amine compound: 1H-purin-6-amine, [(2 fluoro-phenyl) methyl]- (R.A=0.25%), the flavonoid: 6,8-Di-C-á-glucosylluteolin (R.A=0.21%) and the aliphatic ketone: 18-pentatriacontanone (R.A=0.39%).

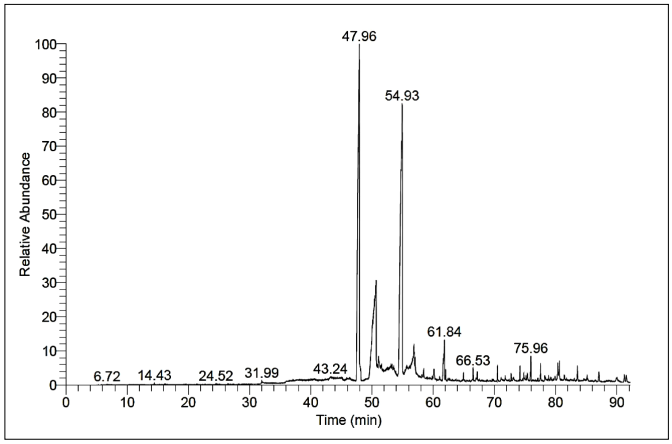

Fig. 1. The methanolic extract GC-MS chromatogram of *O. natrix.*

Anti-inflammatory potential

The human RBCs membrane stabilization technique was employed to determine the anti-inflammatory effect of *O. natrix* methanolic extract compared to a reference drug, indomethacin. As a result, at the concentrations from

Table 1. Phytochemical characterization by GC/MS analysis

S. No.	Characterized phytochemicals	Weight (g/mol)	Formula	Category	Retention time (min)	Relative abundance (%)
1	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$	Fatty acid ester	47.96	35.61
2	Pentadecanoic acid, 14-methyl-, methyl Ester	270	$C_{17}H_{34}O_2$	Fatty acid ester	48.17	0.27
3	Hexadecanoic acid	256	$C_{16}H_{32}O_2$	Fatty acid	50.07	0.71
4	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	Fatty acid	50.20	0.15
5	Hexadecanoic acid, 14-methyl-, methyl ester	284	$C_{18}H_{36}O_2$	Fatty acid ester	51.10	0.61
6	Hexadecanoic acid, 2,3-dihydroxypropyl ester	330	$C_{19}H_{38}O_4$	Fatty acid ester	51.59	0.27
7	Methyl stearate	298	$C_{19}H_{38}O_2$	Fatty acid ester	54.93	39.48
8	Oleic acid	282	$C_{18}H_{34}O_2$	Fatty acid	55.63	0.40
9	Octadecanoic acid (Stearic acid)	284	$C_{18}H_{36}O_2$	Fatty acid	56.86	1.93
10	Octadecanoic acid, ethyl ester (ethyl stearate)	312	$C_{20}H_{40}O_2$	fatty acid ester	57.04	0.67
11	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethane diyl ester	568	$C_{35}H_{68}O_5$	Fatty acid ester	58.46	0.37
12	Octadecanoic acid, 4-hydroxy-, methyl ester	314	$C_{19}H_{38}O_3$	Fatty acid ester	60.13	0.56
13	Glyceryl 1,3-distearate	625	$C_{39}H_{76}O_5$	Fatty acid ester	64.95	0.31
16	Diisooctyl phthalate (1,2-benzene dicarboxylic acid, dioctyl ester)	390	$C_{24}H_{38}O_4$	Phthalate ester	67.21	0.34
17	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	390	$C_{24}H_{38}O_4$	Phthalate ester	72.76	0.23
18	Cholest-5-en-3-yl myristate	596	$C_{41}H_{72}O_2$	Sterol	75.20	0.14
19	Cholesta-4,6-dien-3-ol, (3α)-	384	$C_{27}H_{44}O$	Sterol	75.42	0.26
20	Cholest-3,5-diene	368	$C_{27}H_{44}$	Oxysterol	75.96	0.93
21	Stigmasta-5,22-dien-3-ol, acetate, (3α)-	454	$C_{31}H_{50}O_2$	Sterol	79.90	0.23
22	Stigmast-5-en-3-ol, oleate	678	$C_{47}H_{82}O_2$	Sterol	80.37	0.69
23	1H-purin-6-amine, [(2 fluorophenyl)methyl]-	243	$C_{12}H_{10}FN_5$	Aromatic amine compound	81.43	0.25
24	6,8-Di-C-á-Glucosylluteolin	610	$C_{27}H_{30}O_{16}$	Flavonoids	85.16	0.21
25	18-Pentatriacontanone	506	$C_{35}H_{70}O$	Aliphatic ketone	91.23	0.39

100–1000 µg/mL, the % hemolysis inhibition of *O. natrix* and indomethacin ranged from 73.8 ± 0.6 to 99.6 ± 1% and 92.5±0.3 to 99.9±0.6%, respectively. Remarkably, there was no statistically significant difference between the % hemolysis inhibition of *O. natrix* and indomethacin at a concentration of 1000 µg/mL (Fig. 2). This suggests that *O. natrix* extract may have an anti-inflammatory effect.

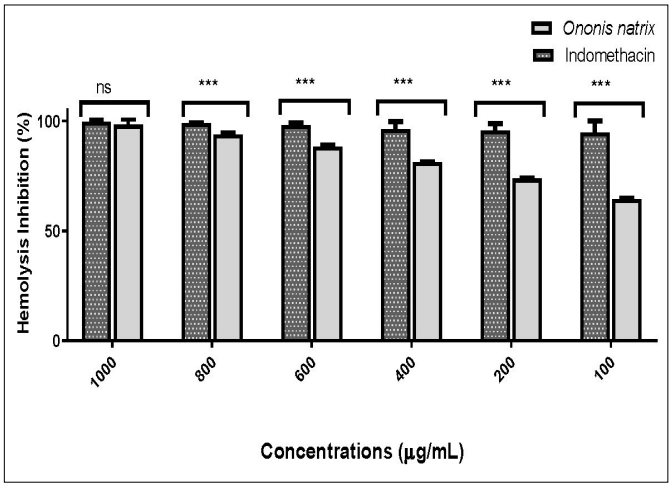

Fig. 2. The hemolysis inhibition percentage of *O. natrix* and indomethacin. The t-test was used to compare the *O. natrix* and indomethacin. *** p = 0.0001 revealed significant differences related to indomethacin, ns: non-significant.

Antioxidant potential

The total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP) assays were applied to estimate the antioxidant activity of *O. natrix* compared to a reference drug, ascorbic acid. The ascorbic acid concentrations for TAC and FRAP assays were plotted on the X-axis while the measured absorbances were plotted on the Y-axis (Fig. 3). As a result, TAC and FRAP assays demonstrated 512.59 ± 0.51 and 411.56 ± 0.48 µg/mg ascorbic acid equivalent (AAE) of the extract, respectively. This may indicate the potential antioxidant activity of *O. natrix*.

The free radical scavenging was estimated by the DDPH scavenging method. The DDPH scavenging percentage of *O. natrix* was compared to a reference drug, ascorbic acid. The values of IC_{50} of *O. natrix* and ascorbic acid were computed by GraphPad Prism version 8 (Fig. 4). It was observed that the DDPH radical scavenging percentage of *O. natrix* and ascorbic acid at concentrations from 2–1000 µg/mL were from 38 ± 0.2% to 97 ± 0.3% and 47 ± 0.2% to 98 ± 0.2%, respectively. There was no statistically significant difference in the DPPH radical scavenging percentage for *O. natrix* and ascorbic acid at the highest concentration of 1000 µg/mL (p value = 0.5). On the contrary, there was a significant difference between *O. natrix* and ascorbic acid at other concentrations (2–500 µg/mL, p-value = 0.0001) as shown in Fig. 5. Additionally, the IC_{50} values for *O. natrix* extract and ascorbic acid were 6 ± 0.2 µg/mL and 3 ± 0.2 µg/mL, respectively. This may suggest that *O. natrix* may possess an antioxidant effect.

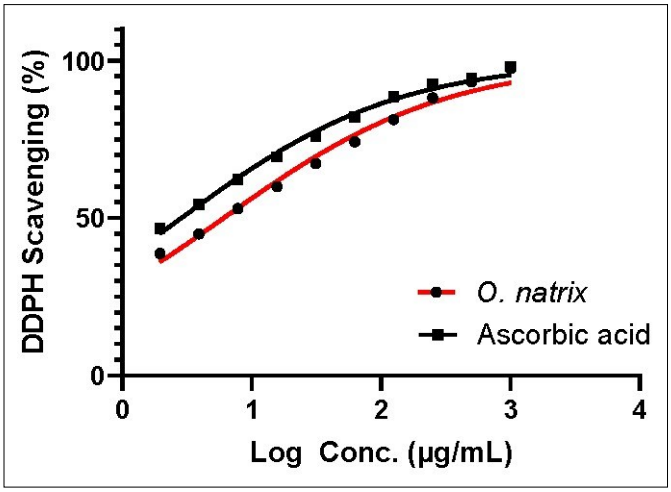

Fig. 4. IC_{50} values estimation of *O. natrix* and ascorbic acid for DDPH scavenging percentage.

Anti-cancer potential

To determine the anti-cancer activity of *O. natrix* extract, the MTT assay was employed to determine the IC_{50} values. The concentrations of the methanolic extract of *O. natrix* were plotted against the % survival of various cell lines (normal and cancerous) such as HDF, MCF-7, DLD-1 and A549. The standard anti-cancer drug, Cisplatin, was used as a positive control and for comparison purposes (Fig. 6 A and B).

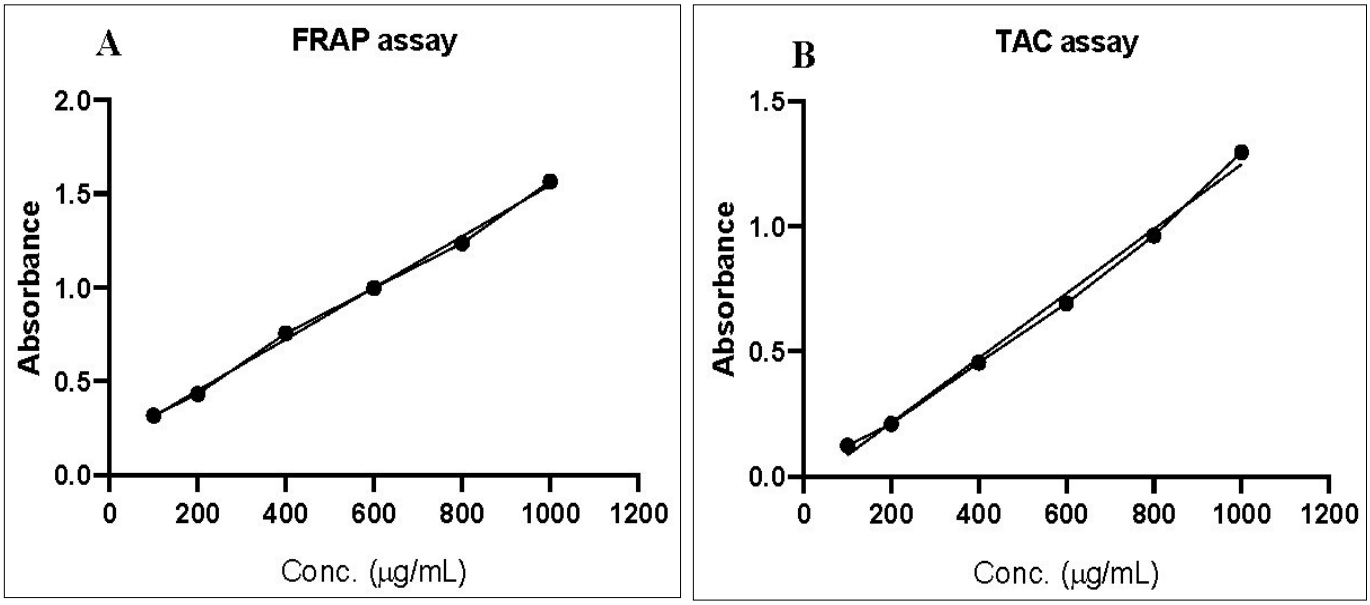

Fig. 3. Determination of free radical scavenging effect of *O. natrix*. (**A**) Ferric reducing antioxidant power (FRAP) and (**B**) Total antioxidant capacity (TAC).

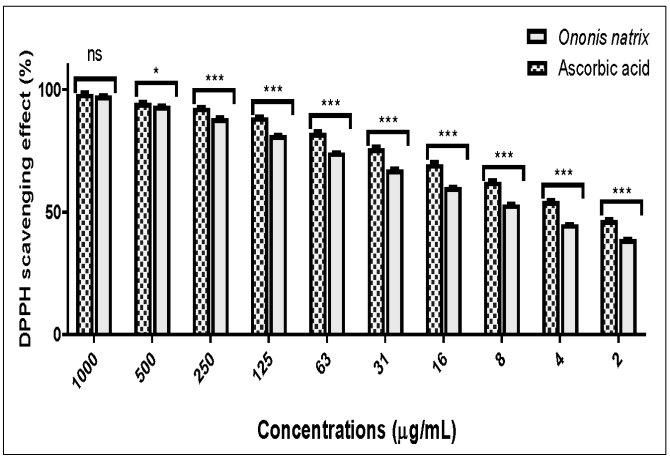

Fig. 5. The DPPH scavenging effect of *O. natrix* and ascorbic acid. The t-test was employed to compare the *O. natrix* and ascorbic acid. *** p = 0.0001 illustrates significant differences compared to ascorbic acid, ns: non-significant.

colorectal adenocarcinoma cell line of 3.54, while negligible cytotoxic selectivity was observed against MCF-7 or A549 cell lines (SI = 2.09 and 1.59, respectively). Remarkably, the cisplatin drug showed a prominent cytotoxic selectivity against all three DLD-1, MCF-7 and A549 cell lines (SI> 3) (Table 3).

Furthermore, the different cell lines were treated with *O. natrix* extract and examined under a light microscope to reveal the cellular changes following treatment. As shown in Fig. 7, the control cells from HDF, MCF-7, DLD-1 and A549 cell lines demonstrated normal intact cellular structures and features. However, the *O. natrix* extract-treated cells exhibited shrunken, necrotic and abnormal cellular structures. The cisplatin-treated cells showed severely destroyed cells. These results confirm the anti-cancer activity of *O. natrix* extracts against a panel of cancer cell lines.

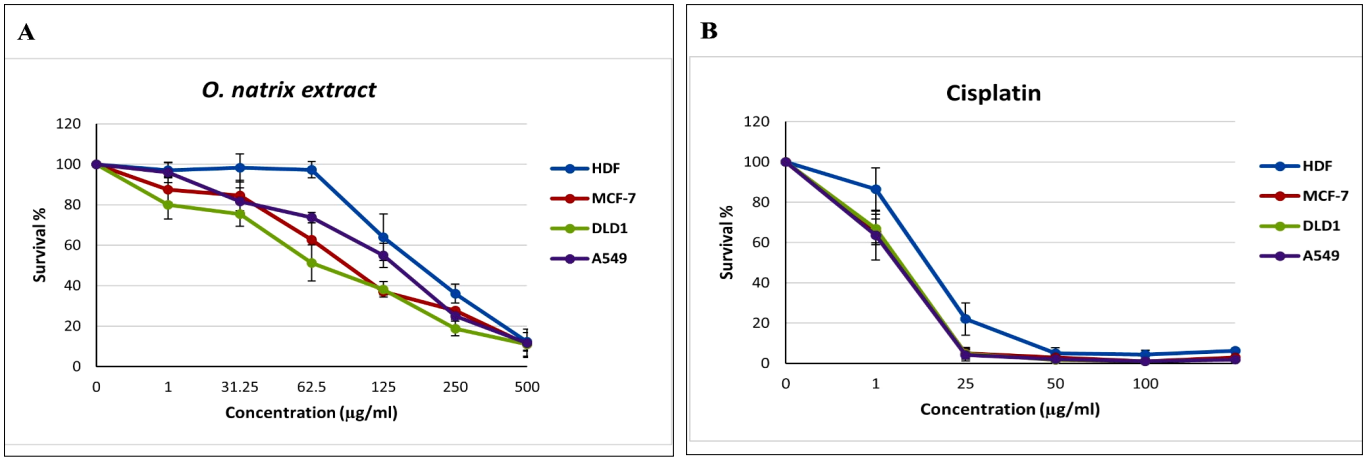

Fig. 6. Determination of the half-maximum inhibitory concentration (IC_{50}) of *O. natrix* extract. (**A**) The concentrations of the methanolic extract of *O. natrix* were plotted against the survival % of HDF, MCF-7, DLD-1 and A549 cell lines. (**B**) The concentrations of anti-cancer drug; cisplatin were plotted against the survival % of HDF, MCF-7, DLD-1 and A549 cell lines. HDF: human dermal fibroblasts; MCF-7: human ER positive breast adeno-carcinoma cell line; DLD-1: colorectal adeno-carcinoma cell line; A549: human lung adenocarcinoma cell line.

Table 2 shows the detailed IC_{50} values observed after *O. natrix* extract and cisplatin treatments of cell lines. The results revealed that the anti-cancer activity of *O. natrix* extract was lower than cisplatin with a moderate anti-cancer activity observed against the DLD-1 colorectal adenocarcinoma cell line (IC_{50} = 57.13±4.9 µg/mL) followed by MCF-7 breast cancer cell line (IC_{50} = 96.69±6.2 µg/mL) and finally the A549 human lung adenocarcinoma cell line (IC_{50} = 126.63±10.4 µg/mL).

Table 2. The IC_{50} of *O. natrix* extract and cisplatin on cancer and non-cancer cell lines

Cell line	IC_{50} (µg/mL)	
	O. natrix	**Cisplatin**
HDF	202.3 ± 7.3	7.87 ± 2.9
MCF-7	96.69 ± 6.2	2.35 ± 1.1
DLD-1	57.13 ± 4.9	1.85 ± 0.7
A549	126.63 ±10.4	1.78 ± 0.4

IC_{50}: the half-maximal inhibitory concentration; **HDF**: human dermal fibroblasts; **MCF-7**: human ER-positive breast adenocarcinoma cell line; **DLD**-1: colorectal adenocarcinoma cell line; **A549**: human lung adenocarcinoma cell line.

The methanolic extract of *O. natrix* showed the highest selectivity index (SI) of cytotoxicity against the DLD-1

Table 3. Selectivity index of *O. natrix* extract against cancer cell lines

Cell line	Selectivity index (SI)	
	O. natrix	**Cisplatin**
MCF-7	2.09	3.35
DLD-1	3.54	4.25
A549	1.60	4.40

SI: selectivity index; **MCF-7**: human ER-positive breast adenocarcinoma cell line; **DLD-1**: colorectal adenocarcinoma cell line; **A549**: human lung adenocarcinoma cell line. The selectivity index (SI) was calculated by dividing the IC_{50} value of human dermal fibroblasts (HDF) by the IC_{50} values for each cancer cell line. The SI values > 3 represent significant anti-cancer agents.

Discussion

In this study, the analysis of GC-MS for *O. natrix* extract showed the existence of several active components, which explains the observed antioxidant and anti-inflammatory properties of *O. natrix*. The fatty acid ester hexadecanoic acid, methyl ester and octadecanoic acid, ethyl ester (ethyl stearate) are known for possessing antioxidant, anti-inflammatory and anticancer activities. These compounds are found in plants at the highest concentrations as compared to other identified compounds. Other fatty acid esters such as pentadecanoic acid, 14-methyl-, methyl ester, methyl stearate and hexadecanoic acid,1-(hydroxymethyl) -1,2-ethane diyl ester have been recognized for possessing

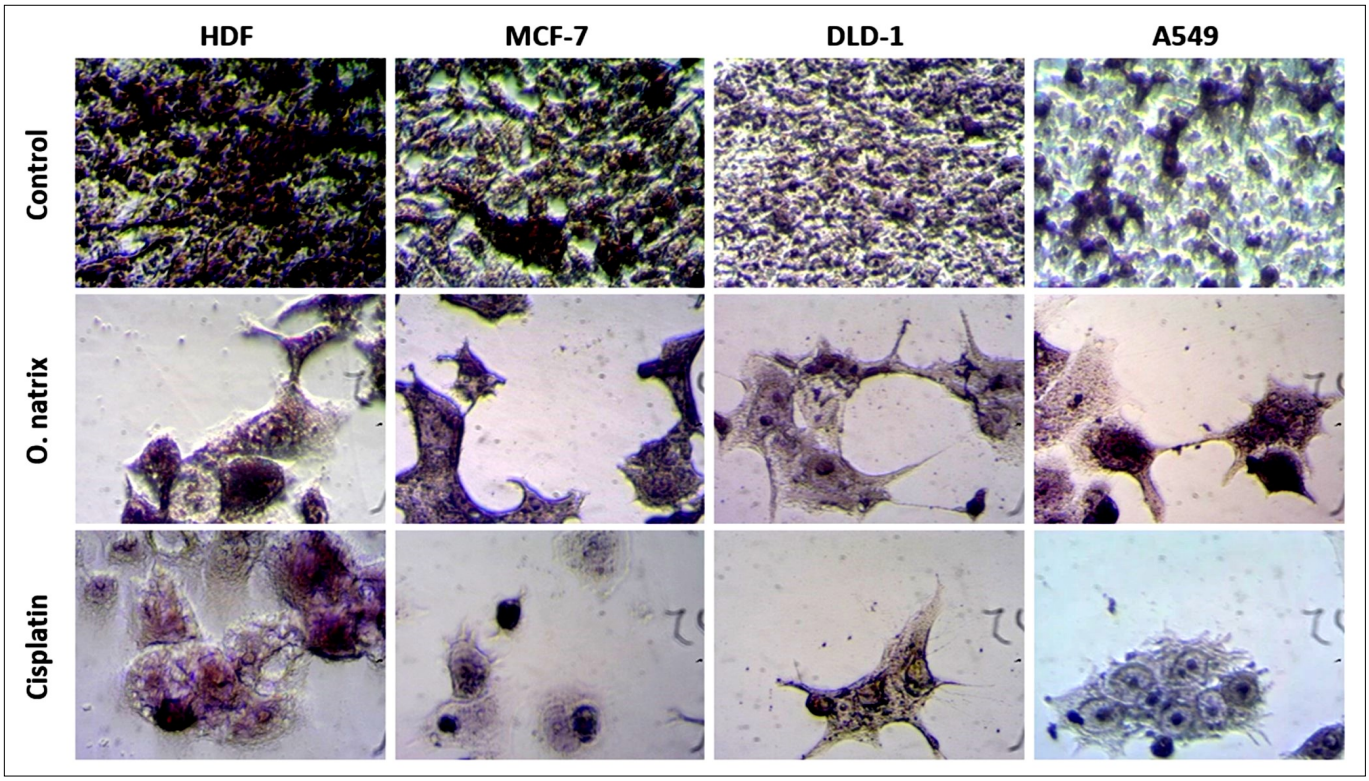

Fig. 7. The anti-cancer effects of *O. natrix* extract against cancer cell lines. Complete monolayer cell sheets from the studied cancer cell lines were examined under the microscope in case of no treatment (control), after *O. natrix* extract treatment and after cisplatin treatment. The control untreated cells from HDF, MCF-7, DLD-1 and A549 cell lines showed normal intact cellular structures and features. However, the *O. natrix*-treated cells exhibited shrunken, necrotic and abnormal cellular structures. The cisplatin-treated cells showed severely destroyed cells.

antioxidant activities. Furthermore, glyceryl 1,3-distearate is renowned for possessing anti-inflammatory activity (33). Additionally, hexadecanoic acid (34), oleic acid (35, 36) and n-hexadecanoic acid (37, 38) are known to have antioxidant, anti-inflammatory and anti-cancer activities. The *O. natrix* plant contains other compounds that have exhibited antioxidant activities such as 1,4-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester (39) and 1H-purin-6-amine, [(2 fluorophenyl)methyl]- (40). Interestingly, the hexadecanoic acid, 2,3-dihydroxypropyl ester (41), octadecanoic acid (Stearic acid) (42) and di-isooctyl phthalate (1,2-benzene dicarboxylic acid, dioctyl ester) (43) have exhibited antibacterial activities while cholesta-4,6-dien-3-ol, (3α)- has shown antimicrobial activity (44). The plant also contains cholest-3,5-diene which is known to promote wound healing (45).

The *O. natrix* extract showed a potential anti-inflammatory effect in the current study. The findings of the human RBC membrane stabilization method revealed that *O. natrix* and indomethacin had no significant difference in the inhibition of RBC membrane hemolysis at the concentration of 1000 µg/mL. Additionally, the anti-inflammatory effect of *O. natrix* was enhanced by increasing the extract doses. These observations are compatible with the anti-inflammatory effects of other *Ononis* species that are closely affiliated with the *O. natrix*. For example, the *n*-butanol, ethyl acetate and petroleum ether constituents of *O. mitissima* showed anti-inflammatory and anti-hemolytic effects at a concentration of 500 µg/mL (46). However, there are no earlier reports about the anti-inflammatory effects of *O. natrix*, yielding a novel finding for the current study.

The antioxidant assays of FRAP, TAC and DPPH were used to estimate the antioxidant capacity of *O. natrix.* The difference in DPPH radical scavenging activities between the *O. natrix* and ascorbic acid was not statistically significant at the highest concentration of 1000 µg/mL. Therefore, our results show that the potential antioxidant effect of *O. natrix* is similar to that of ascorbic acid. The current observations were directly compared with the previously reported findings on the antioxidant effect of *O. natrix* leaf extract which displayed an IC_{50} value of 29 µg/mL of DPPH radical scavenging ability and a broad spectrum antibacterial effect (23). In the previous study, the methanolic extract of *O. natrix* leaves was found to have kaempferol, amentoflavone, flavones and quercetin as the main chemical components (23). The extracted essential oils from *O. natrix* also showed a moderate antioxidant effect (47). The chloroform, ethyl acetate, hexane and methanol extracts from *O. natrix* leaves showed DPPH radical scavenging ability with IC_{50} values of 13.45 µg/mL, 20.03 µg/mL, 43.92 µg/mL and 49.97 µg/mL, respectively. Therefore, the chloroform extract had a greater antioxidant capacity than ethyl acetate, hexane and methanol (24). Moreover, the ethanol and aqueous extracts from *O. natrix* demonstrated DPPH radical scavenging ability with IC_{50} values of 80.2 ±1.6 µg/mL and 78.1 ± 0.9 µg/mL, respectively (48). However, in the present study, the IC_{50} value of DPPH radical scavenging ability is 6 ± 0.2 µg/mL; indicating antioxidant effect for *O. natrix* extract stronger than the previously available literature. Therefore, our results confirm the potential antioxidant effect of *O. natrix*.

The cytotoxic effects of *O. natrix* extract on the PC-3 prostate cancer cell line and HeLa cervical cancer cells were previously assessed using the MTT cell viability assay in which 1 mg of the methanol extract induced apoptosis

in the PC-3 cell line (25). Remarkably, the anti-cancer effect may be related to the occurrence of hexadecanoic acid, methyl ester, octadecanoic acid, ethyl ester and oleic acid found in the *O. natrix* extract. In a similar study, an ethyl acetate extract of *O. natrix* showed cytotoxicity against the MDA MB-231 breast cancer cells in a dose-dependent mode, achieving IC_{50} = 28.75 ± 2.5 µg/mL. The ethyl acetate extract included terpenes and flavonoids, which may explain the cytotoxic effect (49). Moreover, the cytotoxic effects of *O. natrix* on other cancer cell lines such as HeLa, MCF-7, CaCo-2, PC-3 and HepG-2 were examined in previous studyand were found variable (20). For example, the *O. natrix* extract showed a higher cytotoxic effect against HepG-2 and PC-3 cells, when compared to MCF-7, HeLa and CaCo-2 cells. This study is more relevant to our findings, the anti-cancer effect of *O. natrix* extract against the MCF-7 cells showed an IC_{50} of 123±2 µg/mL, compared with an IC_{50} of 96.69±6.2 µg/mL observed in the current study. Our results confirm the anti-cancer activity of *O. natrix* as a promising agent for the treatment of various cancer types.

In a recent study, the aerial parts of *O. natrix* were extracted using methanol, ethyl acetate and water to analyze the phytochemical composition and the various therapeutic activities they posses like antioxidant, anti-Alzheimer, anti-diabetic and tyrosinase activities. The spectrophotometric analysis revealed that the water extract had the highest concentration of total flavonoids (27.88 mg rutin equivalents/g dry weight) while the methanol extract had the highest concentration of total phenolics (74.60 mg gallic acid equivalents/g dry weight). Quantitative phytochemical tests revealed the presence of significant amounts of quercetin, eriodictiol, hyperoside and taxifolin (50). The methanol extract showed the highest activity in all tests, i.e., Phosphomolybdenum, 2,2′-Azinobis-(3-Ethylbenzo-thiazoline-6-Sulfonic acid (ABTS) radical scavenging assay, Copper-reducing antioxidant capacity (CUPRAC) method, Ferric ion reducing antioxidant power (FRAP) assay and metal chelating assays; except for the DPPH radical scavenging activity. The methanol extract also demonstrated greater inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and α-glucosidase, while the ethyl acetate extract was most potent in inhibiting α-amylase and tyrosinase activities (50).

Conclusion

The phytochemicals identified in the methanolic extract of *O. natrix* such as hexadecanoic acid, methyl ester, octadecanoic acid and ethyl ester may be responsible for the anti-inflammatory, antioxidant and anti-cancer activities. The anti-inflammatory and antioxidant effects of the *O. natrix* were similar to the authentic drugs indomethacin and ascorbic acid. However, the anti-cancer activity of *O. natrix* extract was moderate against DLD-1, MCF-7 and A549 cell lines compared to cisplatin. Therefore, the *O. natrix* extract possesses therapeutic potential against oxidative stress and inflammation, as well as colon, breast and lung cancers with selective cytotoxicity against colon cancer.

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Authors' contributions

AY and NE were involved in conceiving and planning the research. AY and MA performed phytochemical analysis by GC/MS and the data acquisition/collection. KA and RH performed the antioxidant assays and calculated the experimental data. MA performed the MTT assay and NE performed anti-inflammatory assay. AY, NE, MA, KA and RH drafted the manuscript and designed the figures. AY, NE, MA, KA and RH aided in interpreting the results. All authors took part in giving critical revision of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

References

1. Laine L, Kivitz AJ, Bello AE, Grahn AY, Schiff MH, Taha AS. Double-blind randomized trials of single-tablet ibuprofen/high-dose famotidine vs. ibuprofen alone for reduction of gastric and duodenal ulcers. The American Journal of Gastroenterology. 2012;107(3):379.https://doi.org/10.1038/ajg.2011.443
2. Aljadhey H, Tu W, Hansen RA, Blalock SJ, Brater DC, Murray MD. Comparative effects of non-steroidal anti-inflammatory drugs (NSAIDs) on blood pressure in patients with hypertension. BMC Cardiovasc Disord. 2012;12(1):1-10.https://doi.org/10.1186/1471-2261-12-93
3. Musu M, Finco G, Antonucci R, Polati E, Sanna D, Evangelista M, et al. Acute nephrotoxicity of NSAID from the foetus to the adult. Eur Rev Med Pharmacol Sci. 2011;15(12):1461-72.
4. Curhan SG, Shargorodsky J, Eavey R, Curhan GC. Analgesic use and the risk of hearing loss in women. Am J Epidemiol. 2012;176(6):544-54.https://doi.org/10.1093/aje/kws146
5. Mullan J, Weston KM, Bonney A, Burns P, Mullan J, Rudd R. Consumer knowledge about over-the-counter NSAIDs: they don't know what they don't know. Aust N Z J Public Health. 2017;41(2):210-14.https://doi.org/10.1111/1753-6405.12589
6. Cavazos J, Naik A, Woofter A, Abraham N. Barriers to physician adherence to nonsteroidal anti-inflammatory drug guidelines: a qualitative study. Aliment Pharmacol Ther. 2008;28(6):789-98.https://doi.org/10.1111/j.1365-2036.2008.03791.x
7. Mei S, Chen X. Investigation into the anti-inflammatory mechanism of coffee leaf extract in LPS-induced Caco-2/U937 co-culture model through cytokines and NMR-based untargeted metabolomics analyses. Food Chem. 2023;404:134592.https://doi.org/10.1016/j.foodchem.2022.134592
8. Amponsah-Offeh M, Diaba-Nuhoho P, Speier S, Morawietz H. Oxidative stress, antioxidants and hypertension. Antioxidants. 2023;12(2):281.https://doi.org/10.3390/antiox12020281
9. Aladağ N, Asoğlu R, Ozdemir M, Asoğlu E, Derin AR, Demir C, et al. Oxidants and antioxidants in myocardial infarction (MI): Investigation of ischemia modified albumin, malondialde-

hyde, superoxide dismutase and catalase in individuals diagnosed with ST elevated myocardial infarction (STEMI) and non-STEMI (NSTEMI). Journal of Medical Biochemistry. 2021;40(3):286.https://doi.org/10.5937/jomb0-28879

10. Negre-Salvayre A, Guerby P, Gayral S, Laffargue M, Salvayre R. Role of reactive oxygen species in atherosclerosis: Lessons from murine genetic models. Free Radical Biol Med. 2020;149:8-22.https://doi.org/10.1016/j.freeradbiomed.2019.10.011

11. Goshtasbi H, Pakchin PS, Movafeghi A, Barar J, Castejon AM, Omidian H, et al. Impacts of oxidants and antioxidants on the emergence and progression of Alzheimer's disease. Neurochem Int. 2022;153:105268.https://doi.org/10.1016/j.neuint.2021.105268

12. Dorszewska J, Kowalska M, Prendecki M, Piekut T, Kozłowska J, Kozubski W. Oxidative stress factors in Parkinson's disease. Neural Regeneration Research. 2021;16(7):1383.https://doi.org/10.4103/1673-5374.300980

13. Preiser JC. Oxidative stress. Journal of Parenteral and Enteral Nutrition.2012;36(2):147-54. https://doi.org/10.1177/0148607111434963

14. Bratovcic A. Antioxidant enzymes and their role in preventing cell damage. Acta Sci Nutr Health. 2020;4:01-7.https://doi.org/10.31080/ASNH.2020.04.0659

15. Youssef AMM, Maaty DAM, Al-Saraireh YM. Phytochemical analysis and profiling of antioxidants and anticancer compounds from *Tephrosia purpurea* (L.) subsp. *apollinea* family Fabaceae. Molecules. 2023;28(9).https://doi.org/10.3390/molecules28093939

16. Chhikara BS, Parang K. Global cancer statistics 2022: The trends projection analysis. Chemical Biology Letters. 2023;10(1):451-51.

17. Zainab L, Hiba T, Hanan A. An updated assessment on anticancer activity of screened medicinal plants in Jordan: Mini review. Journal of Pharmacognosy and Phytochemistry. 2020;9(5):55-58. https://doi.org/10.22271/phyto.2020.v9.i5a.12423

18. Majolo F, Delwing LKdOB, Marmitt DJ, Bustamante-Filho IC, Goettert MI. Medicinal plants and bioactive natural compounds for cancer treatment: Important advances for drug discovery. Phytochemistry Letters. 2019;31:196-207.https://doi.org/10.1016/j.phytol.2019.04.003

19. Arya S, Kumar R, Anju V. A study on the different pigments in *Clitoria ternatea* L. varieties (Fabaceae). Journal of Pharmacognosy and Phytochemistry. 2018;7(1):1107-09.

20. Youssef AMM, Maaty DAM, Al-Saraireh YM. Qualitative chemical compounds analysis and *in vitro* estimation of antiproliferative, antidiabetic and anti-alzheimer's disease effects of *Ononis natrix* (L.) family Fabaceae. Pharmacia. 2024;71:1-11.https://doi.org/10.3897/pharmacia.71.e119755

21. Yousaf M, Al-Rehaily AJ, Ahmad MS, Mustafa J, Al-Yahya MA, Al-Said MS, et al. A 5-alkylresorcinol and three 3, 4-dihydroisocoumarins derived from *Ononis natrix*. Phytochemistry Letters. 2015;13:1-5.https://doi.org/10.1016/j.phytol.2015.05.002

22. Al-Rehaily AJ, Shamim Ahmad M, Yousaf M, Iqrar Khan S, Mustafa J, Tekwani BL, et al. Bioactive chemical constituents of *Ononis natrix*. Journal of the Chemical Society of Pakistan. 2014;36(6).

23. Mhamdi B, Abbassi F, Abdelly C. Chemical composition, antioxidant and antimicrobial activities of the edible medicinal *Ononis natrix* growing wild in Tunisia. Nat Prod Res. 2015;29(12):1157-60.https://doi.org/10.1080/14786419.2014.981188

24. Sayari N, Saidi MN, Sila A, Ellouz-Chaabouni S, Bougatef A. Chemical composition, angiotensin I-converting enzyme (ACE) inhibitory, antioxydant and antimicrobial activities of *Ononis natrix* leaves extracts. Free Radicals and Antioxidants. 2016;6(1):23-33.https://doi.org/10.5530/fra.2016.1.3

25. Yerlikaya S, Zengin G, Mollica A, Baloglu MC, Celik Altunoglu Y, Aktumsek A. A multidirectional perspective for novel functional products: *in vitro* pharmacological activities and *in silico* studies on *Ononis natrix* subsp. *hispanica*. Front Pharmacol. 2017;8:600.https://doi.org/10.3389/fphar.2017.00600

26. Gampe N, Darcsi A, Lohner S, Béni S, Kursinszki L. Characterization and identification of isoflavonoid glycosides in the root of Spiny restharrow (*Ononis spinosa* L.) by HPLC-QTOF-MS, HPLC–MS/MS and NMR. J Pharm Biomed Anal. 2016;123:74-81.https://doi.org/10.1016/j.jpba.2016.01.058

27. Al-Eisawi DM. Field guide to wild flowers of Jordan and neighbouring countries. Amman: Jordan Press; 1998.

28. Anosike CA, Obidoa O, Ezeanyika LU. Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (*Solanum aethiopicum*). DARU J Pharm Sci. 2012;20:1-7.https://doi.org/10.1186/2008-2231-20-76

29. Yesmin S, Paul A, Naz T, Rahman AA, Akhter SF, Wahed MII, et al. Membrane stabilization as a mechanism of the anti-inflammatory activity of ethanolic root extract of Choi (*Piper chaba*). Clinical Phytoscience. 2020;6:1-10. https://doi.org/10.1186/s40816-020-00207-7

30. Lahmass I, Ouahhoud S, Elmansuri M, Sabouni A, Elyoubi M, Benabbas R, et al. Determination of antioxidant properties of six by-products of *Crocus sativus* L. (saffron) plant products. Waste and Biomass Valorization. 2018;9:1349-57.https://doi.org/10.1007/s12649-017-9851-y

31. Spiegel M, Kapusta K, Kołodziejczyk W, Saloni J, Żbikowska B, Hill GA, et al. Antioxidant activity of selected phenolic acids–ferric reducing antioxidant power assay and QSAR analysis of the structural features. Molecules. 2020;25(13):3088.https://doi.org/10.3390/molecules25133088

32. Youssef AM, Althneibat TH, Maaty DA, Al Hujran TA, Al-Saraireh YM. Anticancer and antioxidant activities of *Euphorbia paralias*. J Pharm Pharmacogn Res. 2024;12(4):786-99.https://doi.org/10.56499/jppres23.1918_12.4.786

33. Thongyim S, Chiangchin S, Pandith H, Tragoolpua Y, Jangsutthivorawat S, Panya A. Anti-Inflammatory activity of glyceryl 1, 3-distearate identified from *Clinacanthus nutans* extract against bovine mastitis pathogens. Antibiotics. 2023;12(3):549.https://doi.org/10.3390/antibiotics12030549

34. Momodu I, Okungbowa E, Agoreyo B, Maliki M. Gas chromatography–mass spectrometry identification of bioactive compounds in methanol and aqueous seed extracts of *Azanza garckeana* fruits. Nigerian J Biotechnol. 2022;38(1):25-38. https://doi.org/10.4314/njb.v38i1.3S

35. Santamarina AB, Pisani LP, Baker EJ, Marat AD, Valenzuela CA, Miles EA, et al. Anti-inflammatory effects of oleic acid and the anthocyanin keracyanin alone and in combination: Effects on monocyte and macrophage responses and the NF-κB pathway. Food Funct. 2021;12(17):7909-22.https://doi.org/10.1039/D1FO01304A

36. Carrillo Pérez C, Cavia Camarero MdM, Alonso de la Torre S. Antitumor effect of oleic acid; mechanisms of action. A review. Nutr Hosp. 2012;27(6):1860-65.

37. Ganesan T, Subban M, Christopher Leslee DB, Kuppannan SB, Seedevi P. Structural characterization of n-hexadecanoic acid from the leaves of *Ipomoea eriocarpa* and its antioxidant and antibacterial activities. Biomass Convers Biorefin. 2022;1-12. https://doi.org/10.1007/s13399-022-03576-w

38. Aparna V, Dileep KV, Mandal PK, Karthe P, Sadasivan C, Haridas M. Anti-inflammatory property of n-hexadecanoic acid: structural evidence and kinetic assessment. Chem Biol

Drug Des. 2012;80(3):434-49.https://doi.org/10.1111/j.1747-0285.2012.01418.x

39. Nurani L, Edityaningrum C, Windarsih A, Riyanto S, Rohman A. Isolation of active compound from *Nephelium lappaceum* L. rind as an antioxidant. Food Res. 2022;6(3):84-91.https://doi.org/10.26656/fr.2017.6(3).331
40. Ramadan KM, El-Beltagi HS, Shanab SM, El-Fayoumy EA, Shalaby EA, Bendary ES. Potential antioxidant and anticancer activities of secondary metabolites of *Nostoc linckia* cultivated under Zn and Cu stress conditions. Processes. 2021;9(11):1972.https://doi.org/10.3390/pr9111972
41. Moni S, Alam M, Sultan M, Makeen H, Alhazmi H, Mohan S, et al. Spectral analysis, *in vitro* cytotoxicity and antibacterial studies of bioactive principles from the leaves of *Conocarpus lancifolius*, a common tree of Jazan, Saudi Arabia. Braz J Biol. 2021;83.https://doi.org/10.1590/1519-6984.244479
42. El-Din SMM, El-Ahwany AM. Bioactivity and phytochemical constituents of marine red seaweeds (*Jania rubens*, *Corallina mediterranea* and *Pterocladia capillacea*). Journal of Taibah University for Science. 2016;10(4):471-84.https://doi.org/10.1016/j.jtusci.2015.06.004
43. Rahman MS, Jaripa Begum JB, Yousuf M, Chowdhury J, Anwar M. Antibacterial activity of 1, 2-benzenedicarboxylic acid, diisooctyl ester isolated from the root of *Plumbago zeylanica* Linn. 2007;23-28.
44. EL-Shial EM, Kabbash A, El-Aasr M, Elekhnawy E, El-Sherbeni SA. GC-MS analysis of bioactive compounds of *Gardenia thunbergia* Thunb. leaves and antibiofilm potential against *Staphylococcus aureus* clinical isolates. Journal of Advanced Medical and Pharmaceutical Research. 2023;4(1):11-18.https://doi.org/10.21608/jampr.2023.170762.1048
45. Al-Hassan JM, Afzal M, Oommen S, Liu YF, Pace-Asciak C. Oxysterols in catfish skin secretions (*Arius bilineatus*, Val.) exhibit anti-cancer properties. Front Pharmacol. 2022;13:1001067. https://doi.org/10.3389/fphar.2022.1001067
46. Besbas S, Mouffouk S, Haba H, Marcourt L, Wolfender J-L, Benkhaled M. Chemical composition, antioxidant, antihemolytic and anti-inflammatory activities of *Ononis mitissima* L. Phytochemistry Letters. 2020;37:63-69. https://doi.org/10.1016/j.phytol.2020.04.002
47. Al-Qudah MA, Al-Ghoul AM, Trawenh IN, Al-Jaber HI, Al Shboul TM, Abu Zarga MH, et al. Antioxidant activity and chemical composition of essential oils from Jordanian *Ononis natrix* L. and *Ononis sicula* Guss. Journal of Biologically Active Products from Nature. 2014;4(1):52-61. https://doi.org/10.1080/22311866.2014.890069
48. Al-Mterin MA, Aboalhaija NH, Abaza IF, Kailani MH, Zihlif MA, Afifi FU. Chromatographic analysis (LC-MS and GC-MS), antioxidant activity, total phenol and total flavonoid determination of *Ononis natrix* L. grown in Jordan. Jordan Journal of Chemistry(JJC).2021;16(1):31-39. https://doi.org/10.47014/16.1.4
49. Al-Zereini WA. *Ononis natrix* and *Salvia verbenaca*: Two Jordanian medicinal plants with cytotoxic and antibacterial activities. J Herbs, Spices Med Plants. 2017;23(1):18-25. https://doi.org/10.1080/10496475.2016.1241200
50. Ertaş E, Aygan A, Sarikurkcu C, İstifli ES, Tepe B. Investigation of the chemical composition, antioxidant and enzyme inhibitory activities of *Ononis natrix* subsp. *natrix*, performance of *in silico* analyses of its major compounds. J Mol Struct. 2025;1319:139428.https://doi.org/10.1016/j.molstruc.2024.139428