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Research Article

Qualitative chemical compounds analysis and *in vitro* estimation of antiproliferative, antidiabetic and anti-Alzheimer's disease effects of *Ononis natrix* (L.) family Fabaceae

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Abstract

The *O. natrix* belongs to the family Fabaceae and is distributed in Jordan. Different species of the family Fabaceae contain chemical compounds that may have potential antiproliferative, antidiabetic, and anti-Alzheimer's disease. High-performance liquid chromatography (HPLC) was applied to analyse the phenolic compounds found in *O. natrix* methanolic extract. Using the MTT assay, the antiproliferative action was studied. The enzymes α-glucosidase and butyrylcholinesterase inhibition assays were used to study the antidiabetic and anti-Alzheimer's disease actions, respectively, of methanolic extract of *O. natrix*. Eleven phenolics and seven flavonoids were identified in the methanolic extract of *O. natrix* by HPLC. The highest phenolics and flavonoids were gallic acid (1.25 mg/100 g dry weight) and rutin (1.44 mg/100 g dry weight), respectively. The most cancer cell lines influenced by the extract of *O. natrix* were PC-3 (IC_{50} = 55 ± 2 µg/mL) and HepG-2 (IC_{50} = 68 ± 2 µg/mL) compared to positive control cisplatin. However, the cancer cell lines CaCo-2, MCF-7, and HeLa showed IC_{50} values of 109 ± 2 µg/mL, 123 ± 2 µg/mL, and 79 ± 1 µg/mL, respectively, related to cisplatin. The *O. natrix* extract inhibited the α-glucosidase enzyme and butyrylcholinesterase enzyme by 84% and 86%, respectively compared to positive controls acarbose and rivastigmine. The *O. natrix* may possess antiproliferative effects against prostate cancer and hepatocellular carcinoma. It also may have antidiabetic and anti-Alzheimer's disease effects.

Keywords

Antiproliferative, *O. natrix*, Antidiabetics, Anti-Alzheimer's, HPLC

Introduction

Cancer chemotherapeutic drugs are currently administered for the treatment of different tumor types (Al-Saraireh et al. 2021a). However, their severe adverse effects such as immunosuppression (Harris et al. 1976), nephrotoxicity (Gupta et al. 2021), and secondary malignancies (Vega-Stromberg 2003) have been associated

with serious patient complications. Additionally, the tumor-induced resistance to chemotherapeutic agents may decrease their efficacies and increase their toxicities. For example, the enzymes cytochrome P450, and glypican 1 and 3 overexpressed in the tumor microenvironment may inactivate and rapidly metabolize the chemotherapeutics, leading to their ineffectiveness (Al-saraireh et al. 2021b; Alshammari et al. 2021). Therefore, the adverse effects of, and the resistance to, chemotherapeutics have directed the researchers to investigate chemical compounds from natural sources (Bernardini et al. 2018).

Diabetes is a group of different heterogeneous disorders because of relative or absolute deficiency of insulin, and it can be classified according to the aetiologies into type 1 diabetes, and type 2 diabetes (Kumar et al. 2020). Several oral hypoglycemic drugs have been currently used for type 2 diabetes. However, these synthesised drugs have been reported with serious adverse effects. For example, the sulfonylureas glipizide, the biguanide metformin, and the thiazolidinedione rosiglitazone have been associated with hypoglycemia (Ansari et al. 2019), lactic acidosis (Blumenberg et al. 2020), and myocardial infarction (Wallach et al. 2020), respectively. Therefore, discovering natural antidiabetic compounds has received a lot of attention recently. For example, the flavonoids found in several plant species have been investigated for their antidiabetic actions (Ghorbani 2017).

Alzheimer's disease is a type of dementia. The incidence of Alzheimer's disease has been increasing in recent years in elderly patients (Lopez and Kuller 2019). The acetylcholinesterase inhibitors donepezil and rivastigmine have been used for mild to moderate cases of Alzheimer's disease (Islam et al. 2019). Additionally, the glutamate receptor antagonist memantine has been also used for severe cases of Alzheimer's disease (Li et al. 2019). However, these medications' adverse effects such as rhabdomyolysis and insomnia may reduce the patient's adherence to their use (Fleet et al. 2019). Therefore, the researchers extensively investigated several compounds raised from natural products to find a potential compound for Alzheimer's disease treatment (Chen et al. 2021).

The Fabaceae family comprises 20,000 species (Arya et al. 2018). The taxonomist Carl Linnaeus identified 17 species for the genus of *Ononis* which belongs to the family Fabaceae. Most of those species are distributed in Mediterranean areas and Middle East countries (Arya et al. 2018). The *O. natrix* is one of the genus *Ononis* and is mostly present in Jordan (Kherissat and Al-Esawi 2019). *O. natrix* has been known for its diuretic, antioxidant, and antimicrobial actions (Sayari et al. 2016). Additionally, some of the chemical components in *O. natrix* were isolated and their biological activities were investigated. For example, 5-alkylresorcinol and three 3,4-dihydroisocoumarins derivatives were isolated from *O. natrix* acetonitrile subfraction of acetone and investigated against different strains of microorganism and protozoal (Yousaf et al. 2015). The 6-(2'R-acetoxypentadecyl)-2-hydroxy-4-methoxybenzoic acid and 21 constituents were also isolated from *O. natrix* and tested for their antimicrobial, antileishmanial, antioxidant, antitrypanosomal and antiproliferative actions (Al-Rehaily et al. 2014). Other chemical compounds were also found in the *O. natrix* leaf extract such as quercetin, amentoflavone, flavones and kaempferol, and the whole extract was studied for antioxidant and antimicrobial actions (Mhamdi et al. 2015). Besides, the (2E,6E)-farnesol, dodecanal, and 2-phenyl ethyl tiglate were the identified essential oils of *O. natrix* by GC-MS (Al-Qudah et al. 2014). Therefore, the objectives of this research are to identify chemical compounds in *O. natrix* and to investigate its actions as potential antiproliferative, antidiabetic, and anti-Alzheimer's disease.

Materials and methods

Plant material

The aerial parts of *O. natrix* were gathered in the flowering phase in April 2023 from Mutah City, Al-Karak, Jordan. The plant was dried for ten days after it was cleaned with tap water. The drying conditions were as follows: the room temperature was 25 °C, and the room was well-ventilated and dark. The dried plant was then milled (Youssef et al. 2023a).

Methanol extract preparation

The cold percolation method was used to extract 200 g of air-dried plant powder. The powder was shaken for 72 hours at 25 °C using 70% methanol (500 mL) three times. The extract of methanol was filtrated by a Buchner funnel. A rotary evaporator (Buchi rotavapor r-215, Marshal Scientific, Switzerland) was used to completely remove the 70% methanol under decreased pressure at 40 °C. A desiccator was used to evaporate the traces of solvent, and 20 g/100 g dry weight of *O. natrix* crude was obtained and stored in a refrigerator. Then, the methanolic crude extract was used for the characterisation of the chemical compounds by HPLC (Al-Saraireh et al. 2021c).

Determination of the total phenolics and flavonoids

The typical Folin-Ciocalteu approach was applied to quantify phenolics and flavonoids. After an hour, the optical densities of the blue solution were recorded at 725 nm utilizing a Unicam UV-visible spectrometer (ATi Unicam, UV4-200, United Kingdom). Distilled water was used as a blank. The gallic acid calibration curve was plotted. For each gram of extract, gallic acid equivalents (GAE) in mg were determined (Youssef et al. 2023b).

The total amount of flavonoids was calculated using a colorimetric technique with aluminum chloride. Distilled water was added to the extract to reach a dilution of 1:6 (v:v); thereafter, 150 µL of 10% $AlCl_3.6H_2O$ and 75 µL of 5% $NaNO_2$ were added to the mixture. The mixture was

allowed to reside for 6 min. A 1 M NaOH solution (500 µL) and distilled water (2.5 mL) were added to the mixture. The optical densities were recorded relative to distilled water (a blank) by a spectrometer Unicam UV-visible (ATi Unicam, UV4-200, United Kingdom) at 510 nm. Using (+)-catechin, a standard calibration curve was created. For each gram of extract, catechin equivalents (CE) in mg were determined (Youssef et al. 2023b).

Quantitative analysis of phenolic compounds by HPLC

Standards

The phenolic compounds and trifluoroacetic acid were provided by Merck (Darmstadt, Germany). The HPLC grade of acetonitrile and MeOH were obtained from Sigma-Aldrish. Authentic phenolic compounds: caffeic acid, kaempferol, daidzein, methyl gallate, chlorogenic acid, catechin, pyrocatechol, syringic acid, ferulic acid, ellagic acid, quercetin, naringenin, coumaric acid, apigenin, rutin, cinnamic acid, gallic acid, rosamarinic acid vanillin, and hesperetin were acquired from Sigma-Aldrish. The purity level of all phenolic standards was 98%.

HPLC

In 2 mL of acetonitrile, 0.25 g of the *O. natrix* meOH extract was dissolved. An Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA) was applied for HPLC analysis. An Eclipse C18 column (5 µm, 4.6 mm × 250 mm ID) was used for the separation. The component of the mobile phase (A) was water and the components of the mobile phase (B) were 0.05% trifluoroacetic acid and acetonitrile, and the rate of flow was 0.9 ml/min. The linear gradient was used to program the mobile phases as shown in Table 1. The multi-wavelength detector was used at 280 nm. For each sample, 5 µL was inserted into the HPLC. The column was kept at a stable temperature of 40 °C. Methanol was utilized to prepare the stock solutions of the authentics to give a working concentration of 10 mg/50 mL. After being diluted, the authentics were subjected to HPLC (Youssef et al. 2023b).

The following equation (1) was applied to characterise and measure the phenolics and flavonoids in the *O. natrix* methanolic extract, and the findings were represented by mg/100 g Dry weight (DryW.) (Youssef et al. 2023b).

$$\text{The identified compound's concentration}\left(\frac{\mu g}{mL}\right) = \frac{\text{Area of the sample} \times \text{Conc of the standard}}{\text{Area of standards}} \quad (1)$$

Table 1. The linear gradient method used for mobile phases of HPLC analysis.

Time (min)	Percentage of mobile phase A	Percentage of mobile phase B
0–1	82	18
1–11	75	25
11–18	60	40
18–22	82	18
22–24	82	18
16–20	82	18

Antiproliferative effect

Cell lines

Cell lines of colorectal adenocarcinoma (CaCo-2), breast cancer (MCF-7), prostate cancer (PC-3), hepatocellular carcinoma (HepG-2), cervical cancer (HeLa), and human fetal lung fibroblast (WI-38) were obtained by cell culture laboratory, Faculty of Medicine, Mutah University. The dimethyl sulfoxide was added to the extract of *O. natrix* for the solubilization and serially diluted using a Roswell Park Memorial Institute medium (RPMI 1640) to 1000, 500, 250, 125, 62.5, 31.25, and 1 µg/mL. Penicillin, streptomycin, L-glutamine, amphotericin B, and fetal bovine serum (10%) were added to the medium (Youssef et al. 2023b).

MTT assay

A 96-well microplate was used for seeding the cells at a concentration of 1×10^4 per well. Thereafter, the 96-well microplate was incubated for one day at 37 °C, 95% humidity, and 5% CO_2, to produce a fully formed monolayer sheet. The methanol extract of *O. natrix* was diluted with RPMI medium to obtain the above concentrations. After that, 0.1 mL of each dilution of the extract or cisplatin (a reference drug) or control (medium) was injected into each well once the cells had adhered. Then, the 96-well plates were incubated for four days at 95% humidity, 5% CO_2, and 37 °C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method was applied to assess antiproliferative action of *O. natrix* extract against cell lines of cancer and normal cells. Living cells can only convert MTT to purple formazan due to active metabolism. After the completion of the period of incubation (4 hours), 200 µL of MTT solution (5 mg/mL) was added to the treated cell lines (cancer and normal) and kept until the formation of formazan crystals. The formed formazan crystals were solubilized by adding 150 µL of DMSO, and a multi-well spectrophotometer (Mindray-96 A, Shenzhen, China) was used to measure the optical densities at 500–600 nm (Youssef et al. 2023b).

Calculation of IC_{50}

GraphPad Prism version 8 (San Diego, USA) was applied to estimate the IC_{50} profiles (the half-maximal inhibitory concentration) of *O. natrix* and cisplatin for both the cancerous and normal cell lines. According to the equation 2, the IC_{50} values were calculated (Youssef et al. 2023b).

$$\text{Antiproliferative percentage}\,(\%) = \frac{(100 - \text{mean OD test})}{\text{mean OD control}} \times 100 \quad (2)$$

Microscopy

The morphologies of the studied cell lines after the treatment with *O. natrix* at different concentrations were examined by light microscopy (Nikon, 118811) with an objective lens of 40× and total magnification = 400×.

Antidiabetic effect

α-Glucosidase inhibitory assay

The previously prepared methanolic extract from *O. natrix* was serially diluted to the doses of 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 500, and 1000 µg/mL. An aliquot of 50 µL from each concentration was added to 10 µL of α-glucosidase enzyme (from *Saccharomyces cerevisiae*) solution (Sigma-Aldrich, St. Louis, USA) to reach working concentration of 1 U/mL. The buffer solution of 0.1 M phosphate (125 µL), pH 6.8, was also added. The mixture was then incubated for twenty minutes at a temperature of 37 °C. The substrate *p*-nitrophenyl-*α*-D-glucopyranoside (pNPG) (20 µL) was added to the mixture and incubated for an additional 30 minutes. The α-glucosidase enzyme catalyzes the substrate pNPG to produce a yellow-coloured product p-Nitrophenol. Fifty microliters of 0.1 N of Na_2CO_3 were then added to the mixture to terminate the reaction. At 405 nm, the optical densities were recorded using Biosystm 310 plus spectrophotometer (Bimedis, East Flat Rock, North Carolina, United States). The antidiabetic acarbose was used as a reference drug. All values were obtained in thrice (Bhatia et al. 2019).

Calculation of IC_{50}

GraphPad Prism version 8 (San Diego, USA) was applied to evaluate the IC_{50} profiles (A dose needed to inhibit 50% of the α-glucosidase enzyme activity) of *O. natrix* and acarbose. According to the equation 3, the IC_{50} values were calculated (Bhatia et al. 2019).

$$\alpha-\text{Glucosidase inhibition }\% = \frac{(\text{OD control} - \text{mean OD test})}{\text{mean OD control}} \times 100 \quad (3)$$

Anti-Alzheimer's effect

Butyrylcholinesterase inhibition assay

The *O. natrix* extract was serially diluted to the concentrations of 0.195, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 µg/mL in dimethyl sulfoxide (0.2%) (Li et al. 2021). An aliquot of 10 μL from each concentration was added to 79 μL of 20 mM PBS (pH 7.6) and 1 μL of butyrylcholinesterase enzyme (Biodiagnostic, Giza, Egypt) to give a final enzyme concentration of 0.2 U/mL. The mixture was incubated for 15 minutes at a temperature of 37 °C. A ten microliter of the substrate butyrylthiocholine iodide (Biodiagnostic, Giza, Egypt) was then added to the mixture to give a final substrate concentration of 4 mM. The mixture was then incubated for 30 minutes. The butyrylcholinesterase enzyme catalyzes the substrate butyrylthiocholine iodide to produce a yellow color. A 900 μL of 5,5'-dithiobis-bis-nitrobenzoic acid-phosphate-ethanol reagent (Biodiagnostic, Giza, Egypt) was added to the mixture to terminate the reaction. The yellow colour intensities were measured at 405 nm using Biosystm 310 plus spectrophotometer (Bimedis, East Flat Rock, North Carolina, United States). The anti-Alzheimer's rivastigmine was used as a reference drug. All values were obtained in thrice (Li et al. 2021).

Calculation of IC_{50}

GraphPad Prism version 8 (San Diego, USA) was applied to compute the IC_{50} profiles (A dose needed to inhibit 50% of the butyrylcholinesterase enzyme activity) of *O. natrix* and rivastigmine. According to the equation 4, the IC_{50} values were calculated (Li et al. 2021).

$$\text{Butyrylcholinesterase inhibition }\% = \frac{(\text{OD control} - \text{mean OD test})}{\text{mean OD control}} \times 100 \quad (4)$$

Statistical analysis

Unpaired t-test was used to study the statistically significant difference between the *O. natrix* methanol extract and the reference drugs cisplatin, acarbose and rivastigmine.

Results

High-Performance Liquid Chromatography (HPLC) analysis

The colorimetric methods Folin-Ciocalteu and aluminum chloride were used for estimating the total phenolic and flavonoid contents, respectively. As a result, the total phenolics and flavonoids were 45 ± 0.2 mg GAE/g DryW. and 28 ± 0.4 mg CE/g DryW., respectively. Additionally, the phenolic compounds in *O. natrix* extract were investigated quantitatively by HPLC analysis (Table 2, Fig. 1). Therefore, gallic acid (1.25 mg/100 g DryW.), syringic acid (0.3 mg/100 g DryW.), caffeic acid (0.99 mg/100 g DryW.), ellagic acid (0.26 mg/100 g DryW.), coumaric acid (0.03 mg/100 g DryW.), ferulic acid (0.71 mg/100 g DryW.) and rosmarinic acid (0.49 mg/100 g DryW.) were the detected phenolic acids. The polyphenol chlorogenic acid (0.66 mg/100 g DryW.) was also identified in the extract. Furthermore, the phenolic compounds methyl gallate (0.28 mg/100 g DryW.) and vanillin (0.22 mg/100 g DryW.) were also identified in the *O. natrix* extract. The cinnamic acid (0.07 mg/100 g DryW.) was the identified monocarboxylic acid. Additionally, the flavanol catechin (1.03 mg/100 g DryW.), and the flavonols rutin (1.44 mg/100 g DryW.), quercetin (0.28 mg/100 g DryW.) and kaempferol (0.22 mg/100 g DryW.) were the identified flavonoids. The isoflavone daidzein (0.31 mg/100 g DryW.), and flavanones naringenin (0.14 mg/100 g DryW.) and hesperetin (0.45 mg/100 g DryW.) were also the identified flavonoids.

Antiproliferative effect

In Fig. 2, the X-axis contained the logarithmic concentrations of the *O. natrix* extract or cisplatin while the Y-axis contained the antiproliferative percentage. Therefore, IC_{50} values of tested cell lines were obtained using GraphPad Prism version 8. For example, the IC_{50} for *O. natrix* against cell lines of colorectal adenocarcinoma (CaCo-2) was 109 ± 2 µg/mL, while IC_{50} for cisplatin, a reference drug, was 84 ± 2 µg/mL.

Table 2. Quantitative HPLC analysis for phenolic compounds.

No	Chemical Compounds	Molecular Weight (g/mol)	Molecular Formula	Category	Retention Time (min)	Conc. (mg/100 g DryW.)
1	Gallic acid	170	$C_7H_6O_5$	phenolic acids	3.596	1.24
2	Chlorogenic acid	354	$C_{16}H_{18}O_9$	polyphenol	4.252	0.66
3	Catechin	290	$C_{15}H_{14}O_6$	flavanol	4.504	1.03
4	Methyl gallate	184	$C_8H_8O_5$	phenolic compound (galloyl esters)	5.525	0.28
5	Coffeic acid	180	$C_9H_8O_4$	phenolic acids	5.957	0.99
6	Syringic acid	198	$C_9H_{10}O_5$	phenolic acids	6.45	0.30
8	Rutin	610	$C_{27}H_{30}O_{16}$	glycoside flavonol	6.943	1.44
8	Ellagic acid	302	$C_{14}H_6O_8$	phenolic acids	7.27	0.26
9	Coumaric acid	164	$C_9H_8O_3$	phenolic acid	8.739	0.03
10	Vanillin	152	$C_8H_8O_3$	phenolic (aldehyde)	9.153	0.22
11	Ferulic acid	194	$C_{10}H_{10}O_4$	phenolic acids	9.787	0.71
12	Naringenin	580	$C_{27}H_{32}O_{14}$	flavanones	10.456	0.14
13	Rosmarinic acid	360	$C_{18}H_{16}O_8$	phenolic acids	11.879	0.49
14	Daidzein	254	$C_{15}H_{10}O_4$	isoflavone	16.083	0.31
15	Quercetin	302	$C_{15}H_{10}O_7$	flavonol	17.38	0.97
16	Cinnamic acid	148	$C_9H_8O_2$	monocarboxylic acid	19.314	0.07
17	Kaempferol	286	$C_{15}H_{10}O_6$	flavonol	20.658	0.22
18	Hesperetin	302	$C_{16}H_{14}O_6$	flavanon-glycoside	21.252	0.45

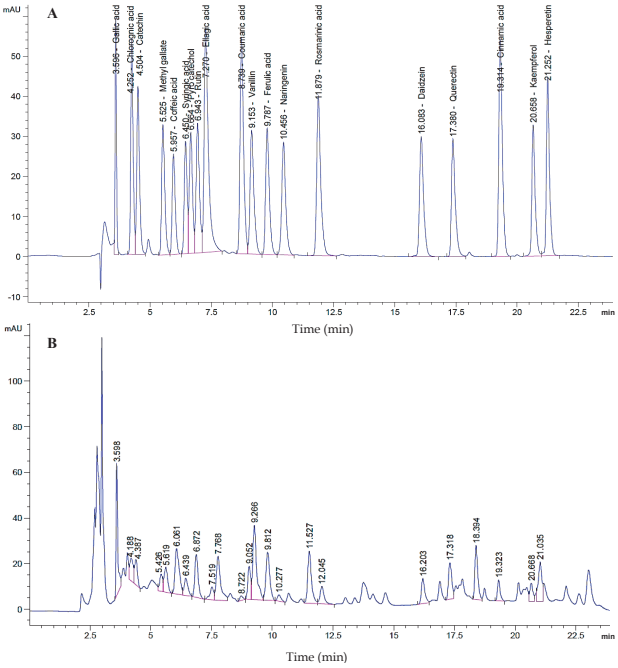

Figure 1. The HPLC chromatograms. **A.** Standard chromatogram; **B.** *O. natrix* chromatogram.

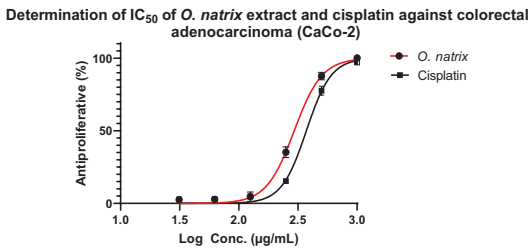

Figure 2. Determination of IC_{50} of *O. natrix* extract and cisplatin against colorectal adenocarcinoma cell lines (CaCo-2).

The MTT method was carried out to study the antiproliferative effects of *O. natrix* against cancerous cell lines of colorectal adenocarcinoma (CaCo-2), breast cancer (MCF-7), prostate cancer (PC-3), hepatocellular carcinoma (HepG-2), and cervical cancer (HeLa). The values of the IC_{50} for *O. natrix* extract were compared to those IC_{50} values for cisplatin by applying a t-test analysis. As a result, there is a statistically significant difference ($p < 0.0001$) between the IC_{50} values for *O. natrix* and cisplatin against CaCo-2 (109 ± 2 µg/mL and 84 ± 2 µg/mL, respectively), MCF-7 (123 ± 2 µg/mL and 64 ± 1 µg/mL, respectively), and HeLa (79 ± 1 µg/mL and 58 ± 2 µg/mL, respectively). In contrast, there was no statistically significant difference ($p > 0.5$) between the IC_{50} values for *O. natrix* and cisplatin against PC-3 (55 ± 2 µg/mL and 57 ± 2 µg/mL, respectively) and HepG-2 (68 ± 2 µg/mL and 64 ± 2 µg/mL, respectively) as shown in Fig. 3. This indicates that *O. natrix* may have a potential antiproliferative effect against prostate cancer and hepatocellular carcinoma but not against colon, breast, or cervical cancers. Additionally, there is a statistically significant difference between the IC_{50} values for *O. natrix* and cisplatin, 122 ± 4 µg/mL and 104 ± 2 µg/mL, respectively, toward the normal human fetal lung fibroblast (WI-38). This indicates that *O. natrix* could possess lower cytotoxicity to normal cells than cisplatin. The antiproliferative effects of *O. natrix* and cisplatin against CaCo-2, MCF-7, PC-3, HepG-2, HeLa, and WI-38 cell lines and after four days of treatment were microscopically examined as shown in Fig. 4.

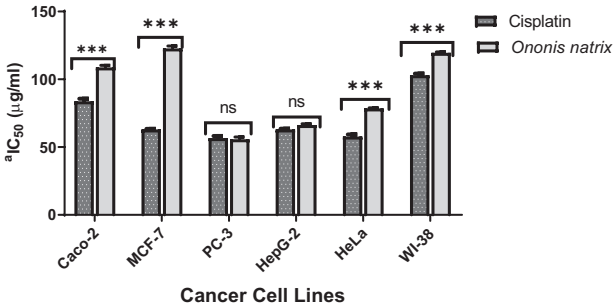

Figure 3. The antiproliferative actions of *O. natrix* and cisplatin against cancerous cell lines colorectal adenocarcinoma (CaCo-2), breast cancer (MCF-7), prostate cancer (PC-3), hepatocellular carcinoma (HepG-2), and cervical cancer (HeLa), and normal human fetal lung fibroblast (WI-38). $^{ns}P = 0.5$ and $^{***}p = 0.0001$ demonstrate significant differences related to cisplatin. The *t*-test was applied to compare the *O. natrix* and cisplatin.

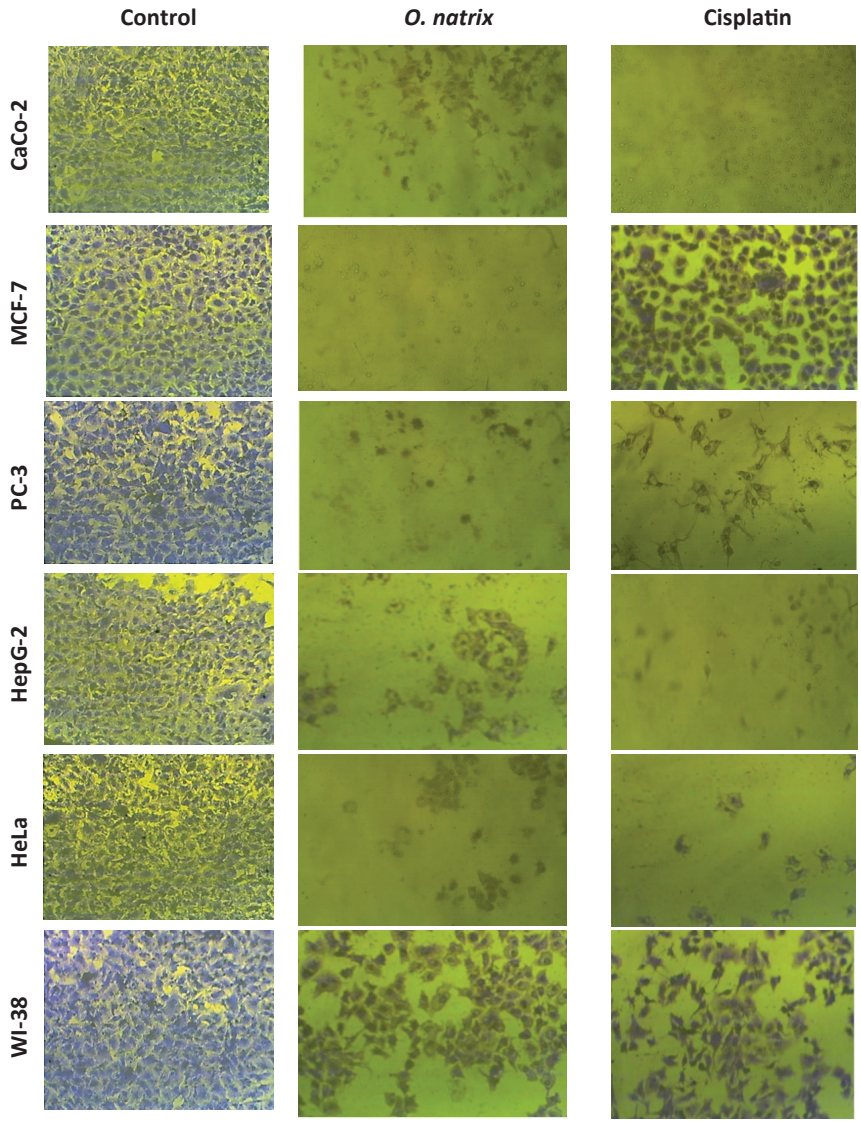

Figure 4. Antiproliferative effects of *O. natrix* and cisplatin against normal cell line (WI-38) and cancer cell lines (CaCo-2, MCF-7, PC-3, HepG-2, and HeLA) after 4 days of treatment at a concentration of 125 µg/mL. Treatment was performed at the respective IC_{50} of the *O. natrix* extract and cisplatin for each cell line (40× objective lens, total magnification = 400×).

Antidiabetic effect

The IC_{50} values for *O. natrix* and acarbose (a reference drug) were obtained using GraphPad Prism 8, where, the X-axis contained the logarithmic doses of *O. natrix* or acarbose and the Y-axis contained the inhibition percentages of the α-glucosidase enzyme, as shown in Fig. 5. As a result, the IC_{50} values for *O. natrix* and acarbose were 12 ± 3 µg/mL and 5 ± 2 µg/mL, respectively. The percentages of α-glucosidase enzyme inhibition for *O. natrix* and acarbose at doses from 1.9 µg/mL to 1,000 ranged from 35% to 84% and 42% to 90%, respectively (Fig. 6). Therefore, there is a directly proportional effect between the enzyme inhibition percentages and dose increases for *O. natrix*. This indicates that *O. natrix* could have a potential antidiabetic effect.

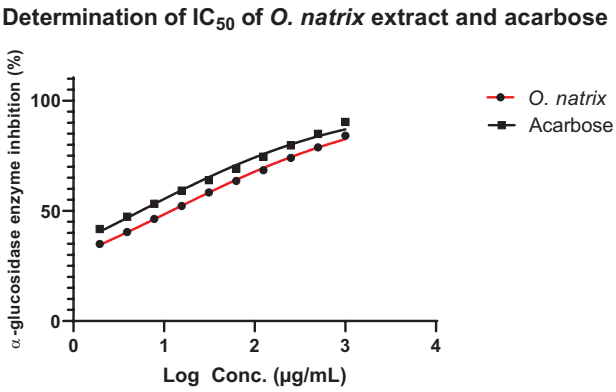

Figure 5. Determination of IC_{50} values of *O. natrix* and acarbose for inhibition of α-glucosidase enzyme.

Anti-Alzheimer's disease effect

The IC_{50} values for *O. natrix* and rivastigmine (a reference drug) were obtained using GraphPad Prism 8, where, the X-axis contained the logarithmic concentrations of *O. natrix* or a reference drug and the Y-axis contained the inhibition percentages of the butyrylcholinesterase (BuCHE) enzyme, as shown in Fig. 7. As a result, the IC_{50} values for *O. natrix* and rivastigmine were 9 ± 1 µg/mL and

2 ± 2 µg/mL, respectively. The percentages of BuCHE enzyme inhibition for *O. natrix* and rivastigmine at concentrations from 0.195 µg/mL to 100 ranged from 1% to 86% and 13% to 94%, respectively (Fig. 8). Therefore, there is a directly proportional effect between the enzyme inhibition percentages and dose increases for *O. natrix*. This indicates that *O. natrix* could have a potential anti-Alzheimer's effect.

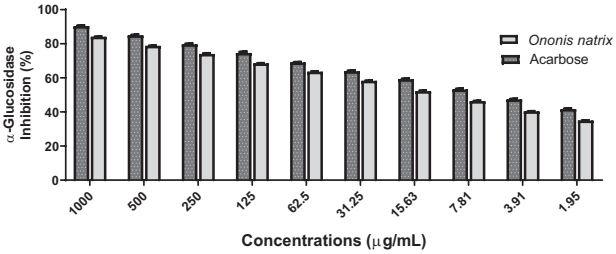

Figure 6. The inhibition effect of *O. natrix* and acarbose for α-glucosidase enzyme. Anti-Alzheimer's disease effect.

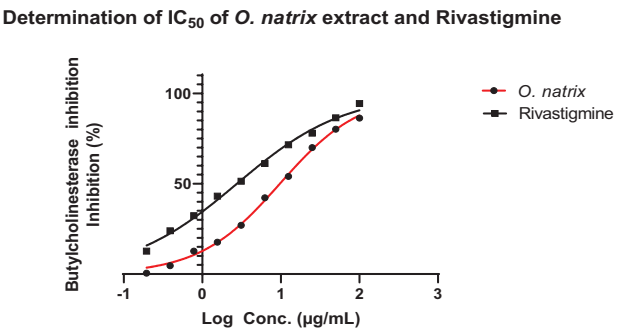

Figure 7. Determination of IC_{50} values of *O. natrix* and acarbose for inhibition of Butyrylcholinesterase enzyme.

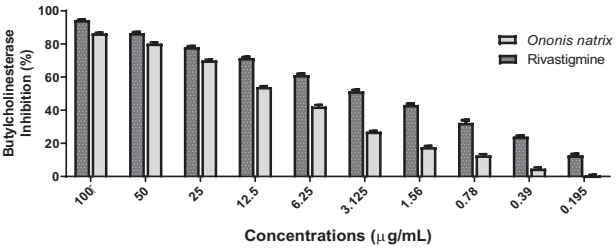

Figure 8. The inhibition effect of *O. natrix* and rivastigmine for Butylcholinesterase enzyme.

Discussion

The quantitative HPLC analysis for *O. natrix* methanolic extract revealed that the greatest amount of phenolic and flavonoid were gallic acid (1.25 mg/100 g DryW.) and rutin (1.44 mg/100 g DryW.), respectively. The gallic acid is the identified phenolic acid and is recognized for its antiproliferative (Jiang et al. 2022), antidiabetic (Variya et al. 2020) and anti-Alzheimer's disease (Obafemi et al. 2021) actions. The caffeic acid is also the identified phenolic acid and it is recognized to have antiproliferative (Kanimozhi and Prasad 2015), antidiabetic (Xu et al. 2020) and anti-Alzheimer's disease (Khan et al. 2013) actions. However, it has not been evaluated for anti-Alzheimer's disease until now. Additionally, the identified phenolic acids with antiproliferative and antidiabetic actions were syringic acid (Srinivasan et al. 2014; Mihanfar et al. 2021), ellagic acid (Fatima et al. 2017; Ceci et al. 2018), coumaric acid (Hu et al. 2020; Abdel-Moneim et al. 2022), ferulic acid (Eroğlu et al. 2015; Narasimhan et al. 2015), and rosmarinic acid. (Ngo and Chua 2018; Anwar et al. 2020) However, the phenolic acids associated with anti-Alzheimer's disease action were ellagic acid (Kiasalari et al. 2017), ferulic acid (Tsai et al. 2015) and rosmarinic acid (Mirza and Zahid 2022). The syringic acid and coumaric acid have not been evaluated for their anti-Alzheimer's disease action yet. The other identified phenolics recognized to have antiproliferative, antidiabetic and anti-Alzheimer's disease actions were chlorogenic acid (Ong et al. 2013; Anggreani and Lee 2017; Santana-Gálvez et al. 2020), methyl gallate (Chaudhuri et al. 2015; Oluwarotimi et al. 2019; Prakashkumar et al. 2021) and vanillin (Naz et al. 2018; Blaikie et al. 2020; Salau et al. 2021). The identified flavanol recognized to possess antiproliferative, antidiabetic and anti-Alzheimer's disease actions was catechin. (Mrabti et al. 2018; Chen et al. 2020; Sun et al. 2020). The identified flavonols rutin (Xu et al. 2014; Ghorbani 2017; Imani et al. 2021), quercetin (Rauf et al. 2018; Bule et al. 2019) and kaempferol (Alkhalidy et al. 2018; Imran et al. 2019) recognized for their antiproliferative, antidiabetic and anti-Alzheimer's disease actions. The other identified flavonoids with antiproliferative, antidiabetic and Anti-Alzheimer's actions were the isoflavone daidzein (Choi et al. 2013; Park and Ju 2013; Hua et al. 2018), and the flavanones naringenin (Zaki et al. 2014; Den Hartogh and Tsiani 2019; Stabrauskiene et al. 2022) and hesperetin (Li et al. 2017; Jayaraman et al. 2018; Sohel et al. 2022). Finally, the detected monocarboxylic acid associated with antiproliferative, antidiabetic and anti-Alzheimer's disease was cinnamic acid (Adisakwattana 2017; Feng et al. 2022; Drakontaeidi and Pontiki 2024). The identified phenolic compounds in this study by HPLC analysis were also reported by LC-MS and GC-MS analysis for *O. natrix* methanolic extract (Al-Mterin et al. 2021). This confirms the presence of these identified chemical compounds in the plant.

The antiproliferative effects of *O. natrix* against the tested cell lines CaCo-2, MCF-7, PC-3, HepG-2 and HeLa were varied. The antiproliferative effects of *O. natrix* were greater against PC-3 and HepG-2 than CaCo-2, MCF-7 and HeLa compared to a positive control cisplatin. The findings of our study confirm previous observations where the *O. natrix* was investigated against the cell line of breast cancer MDA MB-231. As a result, it showed a potential antiproliferative effect with IC_{50} of 29 ± 3 µg/mL compared to a positive control tamoxifen which had IC_{50} of 11 ± 2 µg/mL (Al-Zereini 2017). However, our results demonstrated that the antiproliferative effect of *O. natrix* against the breast cancer cell line MCF-7 had IC_{50} of 123 ± 2 µg/mL compared to cisplatin which had IC_{50} of 64 ± 1 µg/mL. Despite the differences, these findings provide support that *O. natrix* may have a promising antiproliferative effect according to the National Cancer Institute (NCI) guidelines, which stated that if the IC_{50} value of the plant extract was between 21–200 µg/mL, this indicates that the

plant has a moderate antiproliferative effect (Sriwiriyajan et al. 2014). However, there are no further reports in the literature showing the antiproliferative effects of *O. natrix* against other cancer cell lines. Therefore, this is the first report about *O. natrix* as a potential antiproliferative extract against different cancerous and healthy cell lines.

The antidiabetic action of *O. natrix* was evaluated using an α-glucosidase inhibition assay. As a result, the *O. natrix* extract showed a dose-response inhibition for the α-glucosidase enzyme compared to a positive control acarbose. The α-glucosidase enzyme is responsible for the cleavage of carbohydrates within epithelium cells of the small intestine to glucose which is readily absorbed into the systemic circulation (DiPiro et al. 2017). Therefore, *O. natrix* could possess an antidiabetic effect. This observation was also supported by findings from an *in vivo* study of the antidiabetic effect for *O. natrix*. Diabetes mellitus was induced in Wistar rats using streptozotocin, and the extract was administered by oral gavage to the negative and positive control groups of rats for 2 weeks (Al-Mubideen et al. 2021). The blood glucose concentrations were measured from the blood tails of the rats. The data revealed that *O. natrix* was able to reduce blood glucose concentrations (Al-Mubideen et al. 2021).

The anti-Alzheimer's disease effect for *O. natrix* methanolic extract was also investigated using a butyrylcholinesterase (BuCHE) enzyme inhibition assay. The data demonstrated that the *O. natrix* showed a dose-response inhibition for BuCHE enzyme activity. The inhibition of the BuCHE enzyme may increase the concentration of acetylcholine neurotransmitter, which is responsible for the formation of new memories in the brain (DiPiro et al. 2017). Therefore, *O. natrix* may have a potential anti-Alzheimer's disease. However, there are no reports in the literature on the anti-Alzheimer's disease effect of *O. natrix*. Therefore, this is the first report for *O. natrix* extract as a possible therapy for Alzheimer's disease.

Conclusion

HPLC demonstrated that the *O. natrix* extract included a variety of phenolics and flavonoids. The characterized phenolic compounds are associated with antiproliferative, antidiabetic, and anti-Alzheimer's disease. Therefore, the extract showed a higher antiproliferative action against prostate cancer and hepatocellular carcinoma than colorectal adenocarcinoma, breast cancer, and cervical cancer. Antidiabetic and anti-Alzheimer's disease investigations for the *O. natrix* extract showed that it could have potential antidiabetic and anti-Alzheimer's disease actions. Future studies should focus on chemical compound isolation from *O. natrix*. This work will serve as a platform for future pharmacological investigations on *O. natrix*.

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Conflicts of interest

The authors declare no conflict of interest.

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