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In vitro Assessment of Biological and Cytotoxic Activity of Methanol Seed Extract of Jordanian *Mirabilis jalapa* L

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ABSTRACT

Mirabilis jalapa L. has been used traditionally in herbal medicine for various purposes, including as a laxative, diuretic, and treatment of inflammations and fever, as it contains various phytochemical compounds. The present study aims to analyze the phytochemical and lipidic profiles of *M. jalapa* and characterize its methanol seed extract. It also investigates the antioxidant activity, total protein and phenol contents, and anti-inflammatory, antihemolytic, cytotoxic, and antioxidant activities using established methods. The phytochemical profile of the plant was determined with LC-MS and FTIR. Results of the study revealed that the methanol seed extract showed high anti-inflammatory (92.7%) and antioxidant (59.97%) potentials, with moderate antihemolytic (29.3%) activity. Further analysis of the extract showed antiproliferative activity against various cancer cell lines, namely, Prostate DU-145 (49.3%), Lung cancer HCC95 (45.4%), Breast Cancers MDA-MB-231 (38.2%) and 600MPE (24.1%). The methanol seed extract of *M. jalapa* contains significant quantities of various phytochemicals that may have been responsible for its antiproliferative, antioxidant, anti-hemolytic, and anti-inflammatory properties.

Keywords: Antioxidant, Phytochemicals, Lipidic acid profile, Cytotoxicity, Antiproliferative activity

Introduction

Plants are a rich source of diverse secondary metabolites, including well-known compounds like polyphenols, tannins, flavonoids, anthocyanins, hydroquinone, organic acids, acylated sugars, and other aromatic compounds.[1,2,3] Most of the plant components have been employed as extraction and could have antioxidant and anti-inflammatory qualities connected to conditions like diabetes, atherosclerosis, cancers, or neurological illnesses. Additionally, plant extractions can control the makeup of the gut microbiota by acting as anti-inflammatory medications.[4,5,6] Plants may prevent several diseases whose genesis includes immunological dysfunctions or systemic inflammation. Additionally, the bioactive components of plants can control the oxidative stress brought on by imbalanced reactive oxygen species (ROS) generation and cell enzymes' capacity to act as antioxidants.[7,8] Polyphenols, because of their distinctive design, have an increased level of antioxidant activities that can indirectly or directly stop the generation of free radicals.[9] Polyphenols are naturally occurring compounds known for their potent antioxidant properties that protect the body against harmful free radicals. They are bioactive components that can combat and prevent stress-related illnesses.[10]
Phytosterols are highly homologous to cholesterol and are members of the triterpenoid family.

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They are known for their capacity to decrease cholesterols as well as for their antioxidant, anti-inflammatory, and immune-boosting qualities.[11]
Miralbilis jalapa L., the umbrella worts or four-o'clock, belongs to the Nyctaginaceae plant family. *M. jalapa* is the most well-known species of the *Mirabilis* genus, boasting numerous varieties primarily found in the Americas.[12],[13] This flowering wonder holds a hidden treasure within its root: an antiviral protein with immense potential. It is a triple threat against various ailments, with antibacterial, antiviral, and anti-tumor properties. Its extracts find their way to cosmetics and dermo-pharmaceuticals, aiding in the fight against inflammation and dry skin.[14] *M. jalapa* even contributes to environmental clean-up, effectively co-remediating soil contaminated with heavy metals and organic pollutants.[15] In traditional medicine, *M. jalapa* boasts a long wound healing,[16] inflammation relief, and acute arthritis,[16,17] digestive aid and purgative.[18]
Previous studies have identified a treasure trove of compounds within *M. jalapa* extracts, including D-glucan, terpenoids, fatty acids, *β*-sitosterol, steroids, daucosterols, 2-O- methylabronisoflavone, Oxymethyl anthraquinone, and *β*-sitosterol acetates.[18,19]
This new study focuses on *M. jalapa* grown in Jordan, aiming to estimate the phytochemical composition, quantify the levels of various bioactive compounds like phenols, analyze the lipid profile, and assess the extract's potential for antioxidant, anti-inflammatory, antiproliferative (anticancer), and anti-hemolytic (red blood cell protection) activities.

Materials and Methods

Plant collection and identification
M. jalapa was obtained from local gardens in Amman, Jordan (Latitude: 31° 57' 33.372" N, Longitude: 35° 51' 27.936" E) in April 2022. The plant was authenticated by Prof. Saleh Al-Quran, a Taxonomist at the Department of Biological Sciences, Mutah University, Jordan. A voucher specimen with a voucher No. AM2022 was kept at the Biochemistry laboratory at Mutah University, Jordan.

The plant seeds were washed, dried, crushed, and ground into fine powder.

Plant extraction
About 35 g of the powdered material was macerated with 200 mL of 80% methanol over a hotplate and stirred for two cycles of 6 hours each. The extracted plant material was filtered with filter paper and gauze and centrifuged for 20 minutes at 1500 rpm. The filtrate was evaporated to obtain a dried crude extract (9.6 g). The extract was stored in a refrigerator at 2-8°C until further use.[20]

The phytochemical analysis
Phytochemical Analysis was done by triple-quadrupole Liquid Chromatography Mass Spectrometry (LC-MS) model LC-MS-8030, Shimadzu, Japan [BM-20A Control Bus Module, CTO-30A Column oven, LC-30AD Liquid ChromatographySIL-30 AC Auto Sampler, interface ESI, Column C-18 (4.6x25 cm, 5µm]. The solvents used in the experiment were a two-component eluent system consisting of 90% acetic acid and water as component A and 10% methanol as component B. The elution process was done for 5 minutes using 10% B following an isocratic gradient that was further followed by a gradient increase from 10% to 100% B over the next 20 minutes, 100% B for 6 minutes, and finally, re-equilibration of the column. The entire process was conducted at a 200 µL/min flow rate. The scan Form was 50 to 1200 m/z, and the injection volume was 10 µL.[21]

Fourier Transform Infrared Spectroscopy (FTIR)
The FTIR analysis was performed using an FTIR 4100 instrument (JASCO Corporation, Japan). The analysis was carried out with a resolution of 4 cm^{-1} and using KBr pellets in the diffuse reflectance mode. The seed extract from the *M. jalapa* was purified, dried, and pelleted with potassium bromide before analysis.[22] The FTIR spectrophotometer was set in transmittance mode with a spectral range of 350-4500 cm^{-1} to obtain the FTIR spectra.

Determination of total protein content
The total protein content of the extract was determined using the Biuret method.[23] The concentrations ranging between 0.1 to 1.2 mg/mL were used to create a calibration standard curve of bovine serum albumin. The absorbance was measured at 540 nm using a UV-visible Spectrophotometer (Biotech Engineering Management Co. Ltd. in the UK).

Determination of total phenol content
The sample's total phenol content was determined using the modified Folin-Ciocalteu test. Firstly, a mixture of 0.1 mL of the extract's supernatant and 0.5 mL of Folin Ciocalteu's reagent was prepared and incubated at room temperature for 5 minutes. Then, a 2.5 mL aqueous solution of sodium carbonate was added to the extract, and absorbance was measured with a UV-visible spectrophotometer at 765 nm. Various concentrations of gallic acid resulted in the creation of a calibration curve. The overall phenol concentration was determined using the gallic acid calibration curve.[24]

Antioxidant activity using the DPPH radicals-scavenging assay
The method previously described by Sohemat *et al.* was used to determine the antioxidant activity of the extract. Briefly, a reaction mixture was prepared with the combination of 50 µL the plant extract with 1.0 mL of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) solution. This mixture was incubated at room temperature for 30 minutes, and the absorbance was measured against a blank sample of methanol solution at 517 nm. Gallic acid was used as a standard, and the inhibition percentage was determined based on the following equation.[25]

Inhibition activity (%) = (Ac-As) x 100/Ac, where Ac is the blank absorbance, and As is the sample's absorbance.
Half-maximal inhibitory concentration (IC_{50}) is defined as the concentration of the extract that scavenges 50% of DPPH free radicals calculated from the concentration–response curve. The AAA Bioquest IC_{50} calculator was utilized to determine the IC_{50} value.

Determination of Anti-inflammatory activity
The anti-inflammatory activity of the extract was determined by a colorimetric method described by Naz et al.[26] In this assay, 0.05 mL of each extract and 0.45 mL of bovine serum albumin (BSA) were mixed. The negative control solution was a mixture of 0.05 mL of the sample in 0.45 mL of distilled water. Diclofenac standard stock solution (1.0 mg/mL) incubated at 37 °C for 20 minutes in the first step and at 70 °C for 10 minutes in the second step was used as the positive control. Phosphate buffer solution (2.5 mL) was added to the mixtures before the absorbance was measured at 660nm.
Anti-inflammatory activity was measured according to the following equation:

Anti-inflammatory activity (%) = 100-(AT- (AP/AC)) x100, where AT is the test solution; AP is the negative control; AC is the positive control. The IC_{50} value was computed using the AAA Bioquest IC_{50} calculator.

Determination of the Antihemolytic activity by H_2O_2 method
In this determination, different concentrations of each extract (0.25 mL) were combined with 1.0 mL of RBCs and 1.25 mL saline and incubated for 10 minutes at 37°C before adding 0.23 mL H_2O_2. The incubation was extended for a further 2 hours at room temperature, then centrifuged for 10 minutes at 3000 rpm. After centrifugation, its absorbance was measured at 540 nm. The antihemolytic activity was calculated using the formula of Naz *et al.* [26], as shown below.

Antihemolytic activity (%) = (Ap – (As-Ac/Ap)) x 100, where Ap is the positive control absorbance, Ac/ is the negative control absorbance, and As is the sample's absorbance. The IC_{50} value was computed using the AAA Bioquest IC_{50} calculator.

Cytotoxic activity
The cytotoxic activity was done in Smart Lab (Ibn Khaldoon Street, Amman). The cell lines (Breast cancer 600MPE (Invasive ductal carcinoma cancer cell line), Lung cancer HCC95 (Squamous carcinoma), Breast Cancer MDA-MB-231 (Adenocarcinoma cell line), and Prostate DU-145 (standard prostate cancer cell line)) used in this study were obtained from the American Type Culture Collection (ATCC), Rockville, USA. These cancer cell lines were chosen because the percentage of Breast, prostate, and lung cancer is high in Jordan, and patients often resort to treatment with medicinal plants. Different media were used to culture cancer cell lines, and all media were supplemented with penicillin and streptomycin. The solvent medium used for the plant extract was a mixture of RPMI 1640 and OptiMEM in a 1:1 ratio, and it was then incubated with 5% CO_2 at 37°C in a humid atmosphere. The antiproliferative assay was performed using Cisplatin as the control, with concentrations ranging between 0.01 and 1000 µg/mL. The plant extract was weighed, dissolved in ethanol or distilled water to make it 100 mg/mL, and then diluted to obtain concentrations of 1-10 µg/mL for injection.[27]

Antiproliferative assay (MTT assay)
The antiproliferative tests were performed on the four cancer cell lines: Breast cancer 600MPE, Breast Cancer MDA-MB-231, Lung cancer HCC95, and Prostate DU-145. At $1.0x10^5$/mL density, cells were seeded in 96 well cell culture plates and then treated with different concentrations of *M. jalapa* extract. After 72-96-hour incubation, the tested extract's *in vitro* cytotoxicity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The cytotoxic activity was compared to the activity of Cisplatin. The solution's absorbance was checked at 540nm wavelength. The concentration at which 50% of cell growth is inhibited was determined by analyzing the concentration-response curves.[28]
Cytotoxic activity was measured using the following formula:

Cell survival (%) = [As-Ab/Ac-Ab] x 100, where As is the sample's absorbance, Ab is the blank's absorbance, and Ac is the positive control absorbance.
Cell death (%) = 100- Cell survival (%).
All the determinations were done in triplicate.

Data Analysis

The data obtained from the experiment was recorded in triplicate. Microsoft Excel 2016 software was utilized to determine the mean value and standard deviation (SD) and establish a linear correlation between total phenol content (TPC) and biological activities.

Results and Discussion

M. jalapa has excellent pharmacological properties and has some biologically active molecules. The study investigated the phytochemical composition and potential cytotoxic effects of *M. jalapa* extract, as well as evaluating its pharmacological properties, such as anti-hemolytic, antioxidant, and anti-inflammatory activities.

The extraction yield of *M. Jalapa's* seed sample was 27.43 % (w/w) (Table 1). The Biuret method, which uses bovine serum albumin as a standard, was used to determine the protein content in the extracts of *M. jalapa*. The *M. jalapa* extract contained 0.7118±0.035 mg/mL of proteins (Table 1). The total phenolic contents (TPC) of the extract was 353.22± 8.61 mg GAE/g (Table 1). The TPC result of this study is in agreement that reported by Hajji *et al*.[21,] which varied between 21.45 – 364 mg/g dry extract weight.

The phytochemical and lipid profiles of the methanol seed extract of *M. jalapa* were realized by the LC-MS technique. The compounds were identified according to the chromatographic and spectral data using retention times, molecular weight, and molecular formula in comparison with reference standards and literature data. Results indicated the presence of a diverse assortment of plant secondary metabolites (Table 2) and various quantities of fatty acids (Table 3). These biologically and pharmacologically active compounds are widely recognized for their role in various activities, including antioxidant, anti-hemolytic, anticancer, antimicrobial, and anti-inflammatory activities. Results of this current study revealed the presence of different biological bioactive compounds, namely, Ursolic acid, Brassicasterol, *β*-sitosterol, Stigmasterol, Cadinol, Cyclodecanone, Thymol, limonene, and cymene. These bioactive molecules belong to tannins, alkaloids, phenolic compounds, terpenes, steroids, glycosides, and Saponins.[29] The fatty acid constituents of the seed extract indicated the presence of oleic acid, linoleic acid, linoleic acid, palmitic acid, palmitoleic acid, behenic acid, cis-11-eicosenoic acid, and stearic acid. The composition of these fatty acids illustrated the biological importance of the seed extract on some pharmaceutical potentials such as anti-inflammatory, antioxidant, antihemolytic, and anti-cancer activities. Behenic acid (docosanoic acid) and 11-eicosanoic acid (gondoic acid) are used in skincare, cosmetic applications, and drug formulation to treat various diseases like cancer.[30]

The result of the FTIR analysis is shown in Figure 1. The IR spectrum shows the presence of C=O peaks at 1631.78 cm^{-1} for a carbonyl functional group, C-H stretch at 2927.94 $cm^{-1,}$ and carboxylic acid OH stretch at 3360.00 $cm^{-1.}$ Furthermore, peaks at 1620.21 cm^{-1}, 3425.58 cm^{-1}, and 3600-2500 cm^{-1} are related to the C=C aromatic ring, N-H stretch, and OH stretch, respectively. These analyses revealed the presence of different functional groups that belonged to ketones, sterols, acids, alcohols, terpenes, and sitosterols.[29]

In the DPPH free radical scavenging assay, *M. Jalapa's* crude extract exhibited good antioxidant activity of 59.97% ± 1.85 with an IC_{50} value of 0.6034 µg/mL (Table 4). This value was higher as compared to the results of Rumzhum *et al*.[31] (598. 02 µg/mL) and Zachariah *et al*.[32] (500.0 µg/mL). The higher antioxidant potential may be due to bioactive polyphenols in the extract. As presented in Table 1, the high content of phenolic compounds in the extract correlated well with its antioxidant potential. It has been reported that antioxidant activity depends on the number of hydroxyl groups on the aromatic ring of phenolic compounds in the extract.[33] The ability of *M. jalapa* extracts to prevent oxidative damage to erythrocyte membranes by neutralizing red blood cells using H_2O_2 was also examined. The experiment showed that *M. jalapa* extracts inhibited hemolysis of RBCs by 29.30 ± 2.12% with an IC_{50} value of 0.0605 µg/mL (Table 4). Additionally, the crude extract of *M. jalapa* exhibited a strong anti-inflammatory effect, with a percentage of 92.17 ± 5.62%, while the IC_{50} value was 1.054 µg/mL (Table 4). The findings of this study demonstrated a potent inhibition of protein denaturation, as inflammation is typically linked to protein denaturation. This high anti-inflammatory potential of the extract may be attributed to its phytoconstituents (steroids, alkaloids, proteins, and terpenes). This effect may be due to individual or combined action of the constituents depending on the type and concentration of bioactive molecules present in the extract.[34,35] In addition, the seed extract exerts its effects on the stabilization of RBC membranes by preventing their hemolysis.

It has been reported that most anticancer drugs are cytotoxic to healthy cells. The traditional uses of extracts of *M. jalapa* in treating different diseases and its applications as a nutraceutical in Jordan informed its cytotoxicity screening against different cancer cell lines in this study. The cytotoxic results, according to the MTT assay, revealed that the methanol extract of *M. jalapa* showed different percentage inhibition of cell proliferation against Breast cancer 600MPE, Breast Cancer MDA-MB-231, Lung cancer HCC95, and Prostate DU-145 at 24, 38, 45, and 49%, respectively, (Table 5). The IC_{50} values of the extract indicated the effectiveness of the extract on the selected cancer cell lines at low concentrations of 4.9, 4.8, 5.1, and 5.2 µg/mL, respectively (Table 5). The antiproliferative activity of seed extract in the present work was also reported.

Table 1: Approximate contents of extraction yield, total proteins, and total phenolic contents

Extract	Yield (%)	Total protein(mg/mL)	Total Phenol (mg GAE/g)
M. jalapa	27.43±1.62	0.7118 ± 0.035	353.22 ± 8.61

GAE: Gallic Acid Equivalent. Mean ±SD, n=3.

Table 2: Phytochemicals profiling of crude methanol seed extract of *M. jalapa* using LC-MS technique

Compounds	Molecular formula	Molecular weight	%
Limonene	$C_{10}H_{16}$	136.23	8.3
Thymol	$C_{10}H_{14}O$	150.22	8.5
Cyclodecanone	$C_{10}H_{18}O$	154.25	9
beta-sitosterol	$C_{29}H_{50}O$	414.7	13
stigmasterol	$C_{29}H_{48}O$	412.7	11
Brassicasterol	$C_{28}H_{46}O$	398.7	15
Cadinol	$C_{15}H_{26}O$	222.37	9.6
Ursolic acid	$C_{30}H_{48}O_3$	456.7	18
cymene	$C_{10}H_{14}$	134.22	7.5

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Table 3: Lipidic profiling of crude methanol seed extract of *M. jalapa* using LC-MS technique

Fatty acid	Molecular formula	Molecular weight	%
linoleic acid	$C_{18}H_{32}O_2$	280.4	18.9
oleic acid	$C_{18}H_{34}O_2$	282.5	12.1
palmitic acid	$C_{16}H_{32}O_2$	256.42	9.9
stearic acid	$C_{18}H_{36}O_2$	284.5	10
Linolenic acid	$C_{18}H_{30}O_2$	278.4	14.3
palmitoleic acid	$C_{16}H_{30}O_2$	254.41	10.8
cis-11-eicosenoic acid	$C_{20}H_{38}O_2$	310.5	8.8
behenic acid	$C_{22}H_{44}O_2$	340.6	15.2

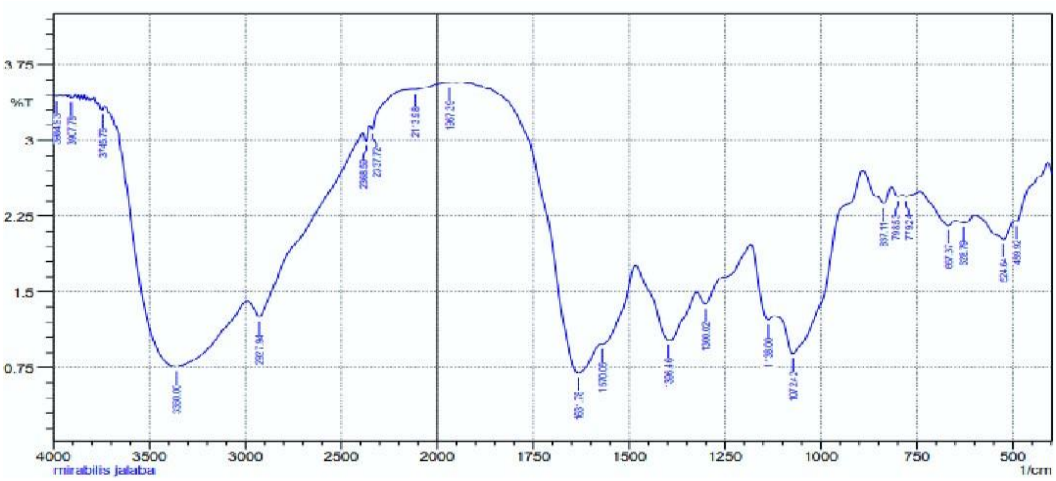

Figure 1. The average spectrum of FT-IR for crude seed extract of *M. Jalapa*.

These results (IC_{50} value = 5.0 µg/mL) agree with those reported by Rumzhum et al., who reported the IC_{50} value of 0.598 µg/mL for *M. jalapa* methanol leaves extract.[31] The methanol extract of *M. jalapa* contained a variety of phytoconstituents in substantial quantities, as indicated by the results. The extract has high phenolic content and showed good varied biological activities. The study emphasizes the biological potential of *M. jalapa* extract. The presence of various phytoconstituents, like polyphenols, sterols, flavonoids, proteins, lipids, and carbohydrates in the methanol extract of *M. jalapa* could be responsible for its diverse biological activities, considering the synergetic interaction of these phytoconstituents.[36,37,38]

The results of this study agree with the findings of previous research in terms of phytochemical contents and biological activities. However, it differs in terms of percentage or values, and this may be due to the difference in the geographical location of the samples collection site (Amman city, Jordan) with moderate weather, over a thousand meters above sea level. Also, there are other differences due to the plant's part used in the extraction. This study utilizes methanol extract from the seeds, whereas other studies used extracts of the leaves or roots. In addition, the differences in values may be attributed to the extraction solvent. This study used methanol, while others used aqueous extract or ethanol and ethyl acetate.

Conclusion

M. jalapa extract is a rich source of diverse compounds (ketones, sterols, acids, alcohols, terpenes, and sitosterols) with potent medicinal properties. The high phenolic content of the extract could be associated with its potent antioxidant, anti-hemolytic, cytotoxic, and anti-inflammatory activities, indicating its potential for various therapeutic applications. The results of this study revealed a need for further investigation of *M. jalapa* seed extract as a source of valuable bioactive compounds with potential applications in pharmacy, ethnomedicine, and cosmetics.

Table 4: Percentage of Antioxidant, Anti-inflammatory, and Antihemolytic activity of methanol extract of *M. jalapa* with IC_{50} values.

Activity Assay	Percentage inhibition	IC_{50} (µg/mL)
Antioxidant	59.97 ± 1.85	0.6034
Anti-inflammation	92.17± 5.62	1.054
Antihemolytic	29.30 ± 2.12	0.0605

Mean ±SD, n=3.

Table 5: Antiproliferative activity (%) and IC_{50} (µg/mL) of *M. jalapa* extract on different cancer cell lines.

Cancer cell line	IC_{50} (µg/mL)	Antiproliferative (%)
Breast cancer 600MPE	4.9±0.32	24± 2.10
Breast Cancer MDA-MB-231	4.8±0.52	38±3.02
Lung cancer HCC95	5.1±0.53	45±3.6
Prostate DU-145	5.2±0.42	49±.84

Mean± SD, n=3.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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