

Chemical compounds investigation and profiling of antimicrobial and antiviral constituents of *Tephrosia purpurea* subsp. *apollinea*

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Abstract

Tephrosia purpurea subsp. *apollinea* belongs to the family Fabaceae, and it is distributed in the delta region of Egypt. It has been used in traditional medicine for the management of various diseases. This study aimed to identify the phytochemicals utilizing

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Contributions: AMMY performed the antiviral assay, interpreted the data, and wrote and designed the manuscript. DAM extracted the plant, performed the GC/MS analysis, interpreted the results, and revised the manuscript. YG conducted the microbiology assay, interpreted the data, and revised the manuscript.

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LC/MS, and biological properties of a methanolic extract of *Tephrosia purpurea* subsp. *apollinea*. The analysis identified 31 compounds representing various phytochemical classes, including fatty acids, sterols, phenolic acids, polyphenols, terpenoids, and flavonoids. Among the major compounds detected, linoleic acid (13.2%) and campesterol (10.1%) were the most abundant. The extract displayed antibacterial, antifungal, and antiviral activities. The methanolic extract demonstrated varying degrees of antimicrobial activity against the tested microorganisms, with *Bacillus subtilis* showing the highest susceptibility. Significant antiviral activity was observed against herpes simplex and hepatitis C viruses. These findings highlight the potential of *T. purpurea* subsp. *apollinea* as a valuable source of natural bioactive compounds including antiviral agents. Additional investigation is required to explore the active principles responsible for the observed biological properties and their therapeutic applications in combating bacterial, fungal, and viral infections.

Introduction

Plants are a source of active metabolites with different biological properties, including antibacterial, antifungal, and antiviral.¹ Plant-derived natural products have been used throughout history to treat a variety of infectious diseases, and modern science has revealed the active compounds responsible for many of their medicinal properties. These compounds, such as flavonoids, terpenoids, phenolic acids, and alkaloids, have been studied for their antibacterial and antiviral activities against a variety of organisms.² Plant essential oils and extracts of individual compounds have been reported to combat bacterial and viral infections, including those caused by antibiotic-resistant organisms.³ Plant-derived antimicrobials are effective against microorganisms including bacteria, fungi, and viruses. In addition, they have been used to prevent various diseases, including malaria, typhoid fever, tuberculosis, and HIV.⁴

Herbal plants have been used for centuries to treat a variety of medical conditions, and their active constituents may have the potential for use as antibacterial and antiviral agents.⁵ The family Fabaceae is a great source of phenolic compounds reported to have antimicrobial and antiviral properties.^{6,7} *Tephrosia purpurea* subsp. *apollinea* (Delile) El-Karemy and Hosni is a herbaceous plant belonging to the Fabaceae family.⁸ It is distributed in different areas in Egypt such as Nile Delta. The genus *Tephrosia* contains phenolic compounds with anticancer and antioxidant properties, which have several pharmacological activities.⁵ Alkaloids, terpenoids, fatty acids, phenolics, and essential oils were the identified compounds in different *Tephrosia* species.⁵ These compounds showed antibacterial and antiviral properties.⁹ In the current report, we aimed to char-

acterize the active ingredients found in *T. purpurea* subsp. *apollinea*, an herbal plant growing in the delta region of Egypt, and to investigate their potential antimicrobial and antiviral activities compared to the positive controls.

Materials and Methods

Plant material

The plant of the current study, *T. purpurea* subsp. *apollinea*, was collected from canals and ponds located in Minyat El-Nasr City, Dakahlia Governorate, Egypt, in March 2022. The plant was recognized by the taxonomist Dr. Iman Al-Gohary, Herbarium Department, Desert Research Center (DRC), Cairo, Egypt. A representative sample number (CAIH-21/23-5) was kept at the Herbarium, Desert Research Center, Cairo, Egypt. The plant was kept at room temperature to dry and then pulverized to a powder to prepare the extract.¹⁰

Methanol extract preparation

The extraction was performed using the percolation technique, where 200 g of the dried plant powder was added to 600 mL of methanol (70%) three times and shaken for three days using a shaker. The methanolic extract was filtered using a Buchner funnel. A rotary evaporator with a reduced pressure and a temperature of 40°C was applied to attain the residue of *T. purpurea* subsp. *apollinea*.^{10,11}

Liquid chromatography-mass spectrometry analysis of methanolic extract

The LC-MS-8030 Liquid Chromatography/Mass spectrometer (LC-MS) (Shimadzu CBM-20A, Kyoto, Japan) was used to identify the different chemical classes found in the extract. The MS conditions were programmed as follows: the drying and nebulizing gases were 20 L/min and 3 L/min, respectively. The temperatures of the heat block and desolvation line (DL) were 450°C and 300°C, respectively. Liquid chromatography LC-30AD Solvent Delivery Unit (Shimadzu, Kyoto, Japan) was used for chemical compound separation. High-Performance Liquid Chromatography (HPLC)-grade water and acetic acid (0.1%) were the components of the mobile phase (A). Methanol and acetic acid (0.1%) were the components of the mobile phase (B). Acetonitrile and acetic acid (0.1%) were the components of the mobile phase (C). An ACE 5 C18 (250×4.6 mm; 5µm) column (Altmann Analytik, München, Germany) at 30°C was used, and the temperature of the column was maintained by column oven CTO-30A (Shimadzu, Kyoto, Japan). The rate of flow was adjusted to be 0.8 mL/min, the volume of injection was 10 µL, and the analysis was performed using the gradient elution method. The elution method was programmed, as shown in Table 1. A Millipore filtration instrument (Millipore Corp., Billerica, MA, USA) was used to filter the HPLC grade solvents and the methanolic extract through a 0.22 µm filter paper before injection into the Liquid

Table 1. LC-30AD pump gradient program. (A), (B), (C): mobile phases.

Time (min)	(A)	(B)	(C)
0-8	80	12	8
8-40	80	12	8
40-45	25	60	15
45-50	80	12	8

Chromatography. The libraries used to investigate the chemical compounds in the methanolic extract were the WILEY and NIST Mass Spectral Databases.¹⁰ The experiment was conducted thrice.

Antimicrobial assay

Microorganisms

The following microbial strains and fungus were used in the antibacterial and antifungal assays: the bacterial strains *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 13883), and the yeast *Candida albicans* (ATCC 10221). All strains were attained from the American Type Culture Collection (ATCC; Rockville, MD, USA).

Preservation and inoculum preparation

The microorganisms were preserved on -80°C glycerol stocks and agar slants at 4°C. To prepare the inoculum, a loop full of bacterial culture was suspended in 10 mL of nutrient agar broth and incubated at 37°C for 24h. Pure isolated colonies were selected from an 18- to 24-hour agar plate, preferably a non-selective medium such as blood agar. Direct broth or saline suspensions were prepared from these colonies. The suspension was adjusted using sterile saline solution to achieve a turbidity equivalent to a McFarland standard of 0.5, ensuring a consistent inoculum size of approximately 1-2×10⁸ Colony Forming Units (CFU)/mL. To ensure accuracy, a photometric device or visual comparison against a card with a white background and contrasting dark lines was used.

To prepare the *Candida albicans* inoculum, similar steps were followed with some modifications. Cultures were stored on Sabouraud Dextrose agar slants at 4°C and in -80°C glycerol stocks. For inoculum preparation, a loop of yeast culture was suspended in Sabouraud Dextrose broth and incubated at 30°C until visible growth occurred (24-48 hours). The resultant yeast suspension was adjusted to a 0.5 McFarland standard, corresponding to 1-5×10⁶ CFU/mL, utilizing a spectrophotometer (Thermo Fisher Scientific, Hampton, USA) for accuracy. The prepared inoculum was used immediately or stored refrigerated for up to 24 hours.

Minimum inhibitory concentration determination

The Minimum Inhibitory Concentration (MIC) of the plant methanolic extract against different microorganisms was determined using a standard broth microdilution protocol. Dilutions of the antimicrobial agent were prepared in Mueller-Hinton broth (bacterial strains) or Sabouraud (*C. albicans*) broth to create a series of two-fold dilutions. For the positive control, gentamicin or fluconazole, a starting working solution was prepared at 100-fold of the expected MIC value. The diluted methanolic extract solutions were dispensed (100 µL) into sterile, optically clear 96-well plates using a pipette, and 100 µL of the inoculum was added. The plates were incubated for 18-24 hours at 37°C and 30°C for 48 h for the bacterial strains and *Candida albicans*, respectively. The MIC values were recorded visually and were defined as the lowest concentration needed to inhibit the growth of each tested microorganism.

After noting the MIC values, the Minimum Bactericidal Concentration (MBC) values were evaluated. The dilutions representing the MIC and at least three of the more concentrated test product dilutions were plated on suitable media, incubated, and enumerated to determine the viable CFU/mL. The MBC is the lowest concentration of the test substance that demonstrates 99.9% viable count reduction.

Antiviral assay

Plant extract

The previously prepared crude plant extract was added to methanol (70%) to reach a working concentration of 50 mg/mL. The solution was kept in the dark at 4°C. Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, Cairo, Egypt) was utilized to attenuate the extract to a working concentration of 1 mg/mL. DMEM was supplemented with 0.1% fetal bovine serum. The diluted extract (1 mg/mL) was filtered *via* a cellulose acetate filter (0.2 µm pore size).

Test viruses in the cell cultures

Vero cells (kidney epithelial cells obtained from the monkey *Cercopithecus aethiops*) were purchased from VACCERA, Giza, Egypt and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Tunisia). A 10% v/v fetal bovine serum, L-glutamine (2 mM), 100 µg/mL streptomycin, and 100 U/mL penicillin were added to the RPMI 1640 medium. The cells were incubated at 37°C with CO₂ (5%). Herpes simplex type 1 virus (HSV1), coxsackie (COXB4) adenovirus, and Hepatitis A Virus (HAV) were obtained as kind gifts from the College of Medicine, University of Al-Azhar, Egypt.

Determination of maximum non-toxic concentration in cell culture

The 96-well Microtiter™ plates were used to seed the Vero cells (1×10⁴ cells/well, 100 µL/well). The growth medium was removed when the cells were at confluency. The DMEM was used to wash the cell monolayers two times. The methanolic extract was serially diluted (10 mg/mL to 312.5 µg/mL/well) and added to the cell monolayers. The studied sample was diluted twice in DMEM, and the various wells received 0.1 mL of each extract dilution. Three wells served as controls and received maintenance media. For up to two days, the plates were incubated at 37°C in a 5% CO₂ environment while being checked constantly. The granulation, rounding, and shrinkage of the cells were microscopically examined. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test was applied. Five mg of MTT powder was added to 1 mL of phosphate-buffered saline (PBS). An aliquot of twenty microliters of MTT solution was injected into each well. The plate was then vigorously shaken for 5 min at 150 rpm using a shaker. Thereafter, the plates were incubated at 37°C for 4 h with 5% CO₂. The medium was decanted, and the paper towels were used to clean and dry the plate consistently. Two hundred microliters of dimethyl sulfoxide (DMSO) were injected into each well to reconstitute the formazan, a metabolic product of the MTT reagent. The formazan crystals and DMSO were mixed using a shaker at 150 rpm for 10 min. The optical densities were measured at 560 nm by UV-Vis Spectrophotometer (Thermo Fisher Scientific, Hampton, USA). The maximum nontoxic concentration (MNTC) was also determined. All values were measured thrice.¹¹

Antiviral activity

The infective titer of stock solution was 10⁻⁷ tissue culture infective dose (TCID) 50 mL (50% tissue culture infective dose), and the Vero cells were used to propagate the herpes simplex type 1 virus (HSV1), coxsackie (COXB4), and hepatitis A (HAV)

viruses independently. Using a cytopathic effect (CPE) inhibition experiment, the MNTC of the methanolic extract of *T. purpurea* subsp. *apollinea* was examined for antiviral properties. One milliliter of DMSO was added to the MNTC of the studied sample to form a solution. A maintenance medium (10 mL) was added to the solution to produce a 1 mg/mL concentration of the solution of stock. The stock solution was then sterilized by filtration and different dilutions were prepared from the solution of stock. For the cytotoxicity assay 10,000 cells suspended in 0.1 mL of Median (50%) Tissue Culture Infectious Dose (TCID50) were seeded in each well of 96-well Microtiter™ plates (Thermo Fisher Scientific, USA). Three wells were left for the control (DMSO). The plates were incubated for two days at 37°C, with 95% humidity and 5% CO₂. The cell granulation and rounding were microscopically examined constantly, and the cytopathogenic effect (CPE) was calculated. The optical densities of the formazan yielded by the MTT reagent were used to assess the viability of infected and non-infected cells, as previously mentioned. The suppression of cytopathic effects in comparison to the reference, *i.e.*, the level of safety provided to the cells by the test samples, was used to measure anti-HSV1, anti-HAV, and anti-COXB4 properties. After the cells were treated with extract, the yielding of the virus was estimated.¹¹

Evaluation of CC50 value of the extract on Vero cells

The 50% cytotoxic concentration (CC50) values of various doses of *T. purpurea* subsp. *apollinea* on Vero cells were computed utilizing GraphPad Prism version 8 software. Equation [1] was used to compute the percentage of cell viability:¹¹

$$\text{Cell viability (\%)} = \left[\frac{\text{Mean absorbance of extract treated cells}}{\text{Mean absorbance of control cells}} \right] \times 100 \quad [1]$$

Evaluation of the maximum non-toxic concentration (MNTC)

The concentration of *T. purpurea* subsp. *apollinea* extract was plotted on the X-axis, and the percentage of cell toxicity was designed on the Y-axis to estimate the MNTC. The MNTC applied for antiviral studies was 250±8 µg/mL for *T. purpurea* subsp. *apollinea*.

Evaluation of viabilities of Vero cells infected by viruses

Equation [2] was employed to compute the viabilities of virus-infected Vero cells at MNTC (250±8 µg/mL) following the treatment of the cells with *T. purpurea* subsp. *apollinea* extract):

$$\text{Cell viability (\%)} = \left[\frac{\text{absorbance of treatment}}{\text{absorbance of Vero control}} \right] \times 100 \quad [2]$$

Evaluation of the rate of the cell protection (CPR) of *T. purpurea* subsp. *apollinea* extract against virus-infected Vero cells

Equation 3 was applied to calculate the cell protection rate or the level of protection provided to the cells by the extract against Vero cells infected with HSV1, COXB4, and HAV viruses utilizing MNTC (250±8 µg/mL).

$$\text{CPR \%} = \left[\frac{\text{absorbance of treatment} - \text{absorbance of virus control}}{\text{absorbance of Vero control} - \text{absorbance of virus control}} \right] \times 100 \quad [3]$$

Evaluation of the rate of virus inhibition (I) of the *T. purpurea* subsp. *apollinea* extracts against virus-infected Vero cells

The rates of virus inhibition (I%) of *T. purpurea* subsp. *apollinea* extract at MNTC (250±8 µg/mL) against the Vero cells infected with the HSV1, COXB4, and HAV viruses were computed utilizing Equation 4:

$$I \% = \left[\frac{\text{absorbance of treatment} - \text{absorbance of virus control}}{\text{absorbance of virus control}} \right] \times 100 \quad [4]$$

Evaluation of the cell growth inhibition rate (IR)

The cell growth inhibition rate (IR%) was computed utilizing Equation [5]:

$$\text{IR \%} = \left[\frac{\text{absorbance of control} - \text{absorbance of treatment}}{\text{absorbance of control}} \right] \times 100 \quad [5]$$

Statistical analysis

GraphPad Prism version 8 (San Diego, USA) was used for statistical analysis. The t-test analysis was utilized. Statistically significant differences are represented as * ($p < 0.05$), ** ($p < 0.001$), and *** ($p < 0.0001$). The values are represented as mean±standard deviation.

Microscopy

An inverted microscope, 8× objective (Nikon, 118811), was used to investigate the morphological changes in non-treated and treated Vero cells with *T. purpurea* subsp. *apollinea* extract at various concentrations.

Results

Chemical compound identification in the methanolic extract by LC/MS

In the current study, many components were identified in the methanolic extract of *T. purpurea* subsp. *apollinea* utilizing LC/MS technique. Table 2 shows the presence of 31 compounds from differ-

Table 2. Biological compound identification in the methanolic extract from *Tephrosia purpurea* subsp. *apollinea* using LC/MS.

No.	Compounds	Molecular weight	Molecular formula	Category	Retention time (min)	Relative abundance %
1	Succinic acid	118	C ₄ H ₆ O ₄	dicarboxylic acid	1.5	5.8
2	Tyrosol	138	C ₈ H ₁₀ O ₂	phenol	2.4	3.4
3	Caffeic acid	180	C ₉ H ₈ O ₄	polyphenol	4	2.1
4	Loliolide	196	C ₁₁ H ₁₆ O ₃	monoterpenoid	6.9	2.3
5	Daidzein	254	C ₁₅ H ₁₀ O ₄	isoflavone	9	0.4
6	Purpurin	256	C ₁₄ H ₈ O ₅	anthraquinones	9.2	0.2
7	Coumestrol	268	C ₁₅ H ₈ O ₅	polyphenol (phytoestrogen)	11	0.5
8	Apigenin	270	C ₁₅ H ₁₀ O ₅	flavones	12.3	8.2
9	Naringenin	272	C ₁₅ H ₁₂ O ₅	flavanone	13	2.4
10	Linoleic acid	280	C ₁₈ H ₃₂ O ₂	fatty acid	13.2	13.2
11	Aureusidin	286	C ₁₅ H ₁₀ O ₆	flavonoid	15	1.1
12	Catechin	290	C ₁₅ H ₁₄ O ₆	flavanol	15.3	0.1
13	Ellagic acid	302	C ₁₄ H ₆ O ₈	tannins	16.3	1.5
14	Quercetin	302	C ₁₅ H ₁₀ O ₇	flavonol	17	6.7
15	Isorhamnetin	316	C ₁₆ H ₁₂ O ₇	flavonol	18.9	0.1
16	Myricetin	318	C ₁₅ H ₁₀ O ₈	flavone	19	8.2
17	Purpurenone	336	C ₂₁ H ₂₀ O ₄	flavonoid	19.8	1.3
18	Tephrosone	352	C ₂₁ H ₂₀ O ₅	chalcones	21.1	0.4
19	Nitenin	356	C ₂₂ H ₂₈ O ₄	flavan	21.9	0.5
20	Pongamol	362	C ₂₃ H ₂₂ O ₄	flavone	23	2.2
21	Quercetol B	368	C ₂₃ H ₂₈ O ₄	flavan	24.4	0.6
22	Rotenone	394	C ₂₃ H ₂₂ O ₆	isoflavone	26.1	5.1
23	Deguelin	394	C ₂₃ H ₂₂ O ₆	flavonoid	26.5	2.5
24	Isotephrosin	398	C ₂₂ H ₂₂ O ₇	isoflavone	27	1.2
25	Campesterol	400	C ₂₈ H ₄₈ O	sterols	29	10.1
26	Tephrosin	410	C ₂₃ H ₂₂ O ₇	isoflavone	29.2	2.5
27	α-tocopherol	430	C ₂₉ H ₅₀ O ₂	vitamin E	30	5.2
28	Rubixanthin	552	C ₄₀ H ₅₆ O	carotenoid	31.2	0.8
29	Rutin	610	C ₂₇ H ₃₀ O ₁₆	flavonol glycoside	32	8.1
30	Hesperidin	610	C ₂₈ H ₃₄ O ₁₅	flavanone	35	2.2
31	4-coumaroyl	909	C ₃₀ H ₃₈ N ₇ O ₁₈ P ₃ S ₄	coenzyme A	35.3	1.1

ent phytochemical classes such as fatty acids, sterols, dicarboxylic acid, vitamin E, phenol, polyphenol, tannins, anthraquinone, monoterpenoid, coenzyme A, carotenoid, flavonoids (flavones, flavonol glycoside, flavonols, isoflavones, flavanones, flavans, chalcone and flavanol). The main identified bioactive compounds in the methanolic *T. purpurea* subsp. *apollinea* extract were linoleic acid (13.2%) and sterol campesterol (10.1%). However, the relative abundances of other identified compounds in the extract were ranging from 0.1% to 8% (Figure 1).

Antimicrobial results

The antimicrobial property of the methanolic extract was assessed. The methanolic extract exhibited varying degrees of activity against the tested microorganisms (Table 3). *Bacillus subtilis* (ATCC 6633) showed the highest susceptibility, with a MIC of 7.8 ± 0 $\mu\text{g/mL}$ and a MBC of 15.62 ± 0 $\mu\text{g/mL}$. The MBC/MIC ratio was calculated to be 2.00. The positive control, gentamicin, displayed a MIC value of 7.8 ± 0 $\mu\text{g/mL}$ for this strain. *Staphylococcus aureus* (ATCC 6538) demonstrated moderate susceptibility, with a MIC of 15.62 ± 4.51 $\mu\text{g/mL}$ and a MBC of a similar value. The MBC/MIC ratio was 1, and the positive control gentamicin exhibited a MIC of 10.41 ± 4.51 $\mu\text{g/mL}$. *Escherichia coli* (ATCC 8739) displayed the highest MIC value of 62.5 ± 0 $\mu\text{g/mL}$ and a MBC of 145.83 ± 95.47 $\mu\text{g/mL}$, resulting in a MBC/MIC ratio of 2.33. Gentamicin had a MIC of 62.5 ± 0 $\mu\text{g/mL}$ for this strain. *Klebsiella pneumoniae* (ATCC 13883) showed MIC and MBC of 26.04 ± 9.02 $\mu\text{g/mL}$, with a MBC/MIC ratio of 1.00, which matched the MIC value of the positive control gentamicin. *Candida albicans* (ATCC 10221) demonstrated a MIC of 15.62 ± 0 $\mu\text{g/mL}$ and a MBC of 23.43 ± 13.54 $\mu\text{g/mL}$, resulting in a MBC/MIC ratio of 1.50. The positive control, fluconazole, exhibited a MIC of 15.62 ± 0 $\mu\text{g/mL}$ for this strain. In summary, the methanolic extract displayed promising antibacterial and antifun-

gal activities against the tested microorganisms, with *Bacillus subtilis* being the most susceptible strain.

Antiviral results

The MNTC of *T. purpurea* subsp. *apollinea* extract on Vero cells (a negative control) was 250 ± 8 $\mu\text{g/mL}$, which was applied to evaluate the rate of cell protection (CPR) of the extract (Figure 2). The *T. purpurea* subsp. *apollinea* extract revealed different antiviral activities against HSV1, HAV, and COXB4 viruses. The computed CC50 of the *T. purpurea* subsp. *apollinea* extract on Vero cells was 499.8 ± 1.7 $\mu\text{g/mL}$ as compared to the untreated controls. The calculated cell viability percentages of the treated HSV1- and HAV-infected Vero cells were $79.32\% \pm 6.7$ and $71.33\% \pm 2.5$, respectively, compared to the non-treated HSV1- ($45.85\% \pm 1.0$) and HAV-infected ($49.84\% \pm 1.8$) ($p < 0.001$) Vero cells; however, the percentage of cell viability of the treated COXB4-infected Vero cells was $49.67\% \pm 0.6$ compared to the non-treated COXB4-infected Vero cells ($41.36\% \pm 1.9\%$, $p < 0.05$). Therefore, there is a very high statistically significant antiviral effect of *T. purpurea* subsp. *apollinea* extract towards HSV1 and HAV in infected Vero cells, while a statistically significant difference was observed for the antiviral effect of *T. purpurea* subsp. *apollinea* extract against COXB4. Additionally, the *T. purpurea* subsp. *apollinea* methanolic extract exhibited a greater cell protection rate (CPR) against HSV1 ($61.9 \pm 3.4\%$) and HAV ($42.67 \pm 7.1\%$) than COXB4 ($14.09 \pm 3.8\%$). The extract from *T. purpurea* subsp. *apollinea* also exhibited higher inhibitory activity against HSV1 ($73.06 \pm 2.3\%$) and HAV ($43.36 \pm 9.1\%$) than COXB4 ($20.3 \pm 6.7\%$). This indicates that *T. purpurea* subsp. *apollinea* could have potential antiviral activities against HSV1 and HAV but not COXB4 viruses (Table 4 and Figure 3). The morphological changes of non-infected Vero cells treated with *T. purpurea* subsp. *apollinea* at different concentrations were examined microscopically (Figure 4). As a result, the

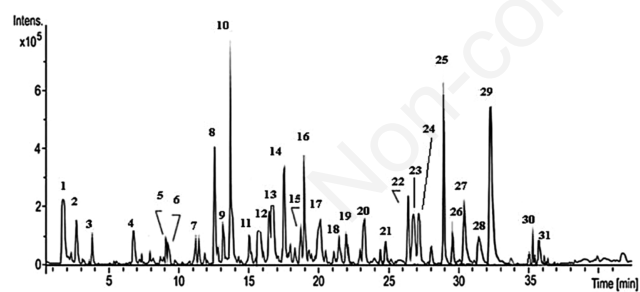


Figure 1. Spectra of methanolic extract from *Tephrosia purpurea* subsp. *apollinea* by liquid chromatography/mass spectrometry.

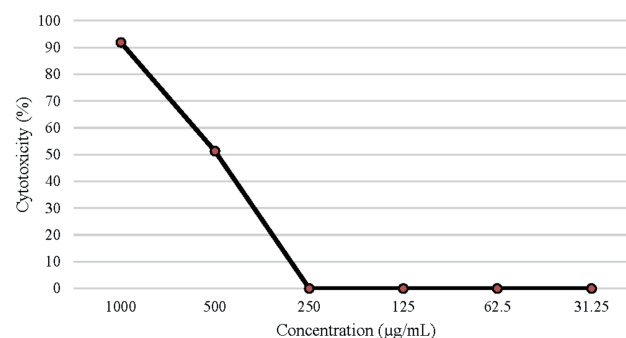


Figure 2. Estimation of the maximum non-toxic concentration (MNTC) of *T. purpurea* subsp. *apollinea* extract.

Table 3. MIC and MBC values recorded for the *Tephrosia purpurea* subsp. *apollinea* extract and comparison of MIC with the positive control values.

Microorganisms (strain number)	MIC ($\mu\text{g/mL}$)	Methanolic extract MBC ($\mu\text{g/mL}$)	MBC/MIC	The positive controls MIC ($\mu\text{g/mL}$)
<i>Bacillus subtilis</i> (ATCC 6633)	7.8 ± 0	15.62 ± 0	2.00	Gentamicin (7.8 ± 0)
<i>Staphylococcus aureus</i> (ATCC 6538)	15.62 ± 4.51	15.62 ± 4.51	1.00	Gentamicin (10.41 ± 4.51)
<i>Escherichia coli</i> (ATCC 8739)	62.5 ± 0	145.83 ± 95.47	2.33	Gentamicin (62.50 ± 0)
<i>Klebsiella pneumoniae</i> (ATCC 13883)	26.04 ± 9.02	26.04 ± 9.02	1.00	Gentamicin (26.04 ± 9.02)
<i>Candida albicans</i> (ATCC 10221)	15.62 ± 0	23.43 ± 13.54	1.50	Fluconazole (15.62 ± 0)

non-infected Vero cells were shrunk and rounded at a concentration of 500 $\mu\text{g/mL}$ of the extract compared to its control.

Discussion

This study was conducted to find out the active compounds that are present in the methanolic extract of *Tephrosia purpurea* by using LC/MS. The results revealed the existence of different compounds that exert biological properties including antibacterial, antifungal, and antiviral. For instance, campesterol has been reported to possess

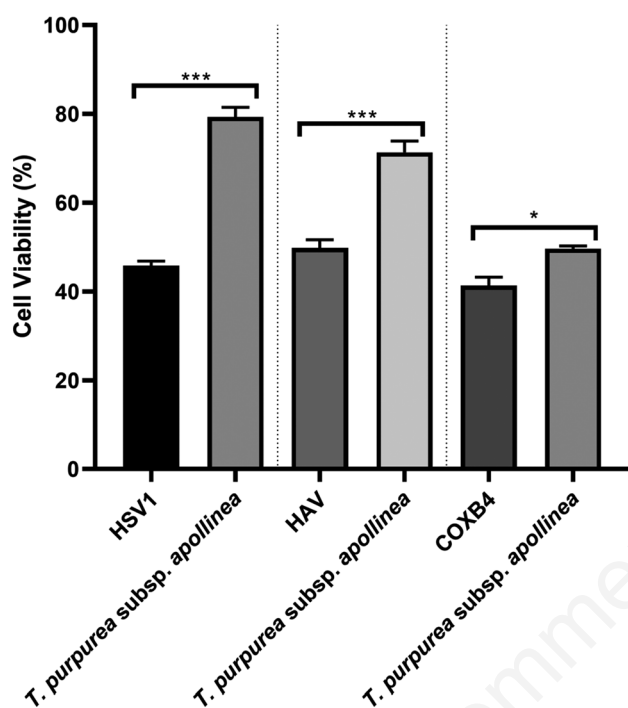


Figure 3. Antiviral effects of *T. purpurea* subsp. *apollinea* on Vero cells infected with HSV1, HAV and COXB4. The values are expressed as the mean \pm SD. * $p < 0.05$ and *** $p < 0.0001$ indicate the statistically significant differences compared to untreated cells. The percent of cell viability of the *T. purpurea* subsp. *apollinea* extract on HSV1-, HAV- and COXB4-infected Vero cells were compared with untreated cells *via* the t-test analysis.

antifungal, antibacterial, and antiviral activities¹². Linoleic acid also possesses antimicrobial activity¹³. Apigenin is also known to exhibit antibacterial, antifungal, and antiviral activities,¹⁴ as well as myricetin,¹⁵ hesperidin,¹⁶ tyrosol, and ellagic acid.¹⁷ Rutin was found to have antifungal and antiviral activities,¹⁸ as well as rotenone.¹⁹ Additionally, α -tocopherol has been reported to possess antimicrobial activity,²⁰ as well as tephrosin, pongamol, purpurenone, and tephrosone.²¹ Deguelin has been also reported to possess antimicrobial activities²² and inhibit human cytomegalovirus and hepatitis C virus²³ but naringenin was found to have antifungal,²⁴ antibacterial properties²⁵ and antiviral activity against white spot syndrome virus.²⁶ The plant also contained loliolide, which is known to have antibacterial,²⁷ antifungal properties and antiviral activity against hepatitis C virus,²⁸ daidzein which inhibits the infection by influenza virus²⁹ and prevents the infection by hepatitis C virus³⁰ and coumestrol that inhibits herpes simplex virus types 1 and 2.³¹ Moreover, succinic acid was shown to possess antibacterial activity³² and aureusidin to possess antibacterial activity against *Staphylococcus aureus*.³³ The plant also contained quercetin which exhibits antimicrobial and antiviral activities,² as well as caffeic acid,³⁴ catechin,³⁵ and isorhamnetin.³⁶ Purpurin is also known to have antifungal and antibacterial activities.³⁷ Furthermore, some identified compounds, such as flavan, nitenin, quercetol B, isotephrosin, rubixanthin, and 4-coumaroyl, have not been assessed for their biological properties. This study revealed that *T. purpurea* subsp. *apollinea* has antibacterial, antifungal, and antiviral activities which may be due to the existence of many compounds that have antibacterial, antifungal, and antiviral activities. The total phenolics and flavonoids of *T. purpurea* subsp. *apollinea* was 39.12 mg gallic acid equivalent (GAE)/g dry weight (DW) and 17.83 mg catechin equivalent (CE)/g DW, respectively, as previously determined by Youssef *et al.*¹

The antimicrobial activity of the methanolic extract against tested Gram-positive and Gram-negative strains showed promising results. The MBC/MIC ratios highlighted the extract's ability to eradicate bacteria at concentrations equal to or surpass the MIC. Lower MBC/MIC ratios indicated potent bactericidal effects, while higher ones suggested weaker activity. Particularly noteworthy activity was observed against the *Bacillus subtilis* strain. The observations align with findings from the *T. purpurea* flowers, which also revealed antibacterial properties against Gram-negative and Gram-positive bacteria.⁹ Studies conducted by Khan showed *T. purpurea* efficacy against *Propionibacterium acnes* and *Staphylococcus epidermidis* using the disc diffusion method.³⁸ A tri-terpenoid glycoside from the butanolic seed extract even completely inhibited the growth of *Streptococcus pneumoniae* and a fungal species,

Table 4. Cell viability of Vero cells and antiviral activity of *T. purpurea* subsp. *apollinea* extract.

	MNTC $\mu\text{g/mL}$	Virus	Percent of cell viability	Percent of IR	CC50 ($\mu\text{g/mL}$)	Percent of CPR	Percent of I	Percent of Viral activity
Negative Control (Vero cells)	250	HSV1	45.85 \pm 1.0	54.16 \pm 1.0		0	-	100
<i>T. purpurea</i> subsp. <i>apollinea</i> extract			79.34 \pm 2.1***	20.67 \pm 2.1	499.8 \pm 1.7	61.9 \pm 3.4	73.06 \pm 2.3	26.94 \pm 2.3
Negative Control (Vero cells)		HAV	49.84 \pm 1.8	50.17 \pm 1.8		0	-	100
<i>T. purpurea</i> subsp. <i>apollinea</i> extract			71.33 \pm 2.5***	28.68 \pm 2.5	499.8 \pm 1.7	42.67 \pm 7.1	43.36 \pm 9.1	65.64 \pm 9.1
Negative Control (Vero cells)		COXB4	41.36 \pm 1.9	58.65 \pm 1.8		0	-	100
<i>T. purpurea</i> subsp. <i>apollinea</i> extract			49.67 \pm 0.6*	50.34 \pm 0.6	499.8 \pm 1.7	14.09 \pm 3.8	20.3 \pm 6.7	79.7 \pm 6.7

MNTC, maximum non-toxic concentration; IR, cell growth inhibition rate; CC50, 50% cytotoxic concentration; CPR, cell protection rate; I, virus inhibition rate; the values are expressed as mean \pm SD. * $p < 0.05$, and *** $p < 0.0001$ indicates significant differences compared with the control cells. A t-test analysis was used for the comparison between the percentage of cell viability of *T. purpurea* subsp. *apollinea* on vero-infected HSV1, COXB4 and HAV viruses and the untreated cells.

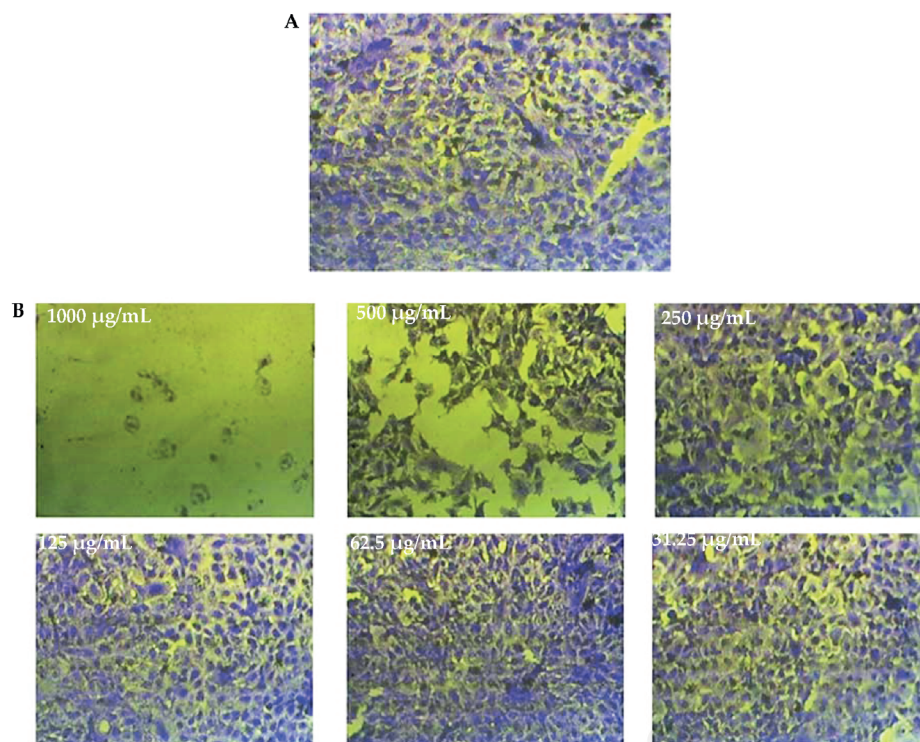


Figure 4. Effect of *T. purpurea* subsp. *apollinea* extract on non-infected Vero cells at different concentrations (A) complete monolayer sheets of Vero cells that have not been treated; (B) non-infected Vero cells (a negative control) that were treated with *T. purpurea* subsp. *apollinea* extract at different concentrations.

Alternaria alternata.³⁸ Moreover, the organic extracts of *T. purpurea* seeds and roots inhibited several bacterial strains, including *B. subtilis*, *E. coli*, and *P. aeruginosa*.^{39,40} Additionally, the ethanolic root extract was able to inhibit two strains of *E. coli* and three isolates of *P. aeruginosa*.⁴¹ Further studies have reported alcoholic extracts of *T. purpurea* exhibiting antimicrobial activity against numerous Gram-positive, Gram-negative, and fungal species.⁴² For example, the methanolic extract exhibited remarkable activity at a concentration of 100 mg/mL.⁴² The alcoholic extract from *T. purpurea* aerial parts affected the growth of *E. coli*, *Serratia marcescens*, and *S. epidermidis*.⁴³ Flavonoids in *T. purpurea* are believed to be a major contributor to its antimicrobial properties.⁴⁴ Previous research has also shown the potential of *T. purpurea* to inhibit *Helicobacter pylori*.⁴⁵ As such, our observations are in line with previous reports supporting the antimicrobial potential of *T. purpurea* and its possible therapeutic applications.

On the other hand, our antiviral results revealed that *T. purpurea* subsp. *apollinea* had a strong antiviral effect against HSV1. Similar results are also achieved by the flowers of *T. purpurea* that showed great antiviral activities against various virus cultures in HeLa, HEL, and Vero cells.⁴⁶ The *T. purpurea* flowers were also investigated against the herpes simplex-1 and 2, and vaccinia viruses. As a result, they had antiviral activities against those tested viruses.⁴⁷

Conclusions

This study identified active principles in the methanolic extract of *T. purpurea* subsp. *apollinea* revealing a wide range of antibac-

terial, antifungal, and antiviral activities. Compounds such as campesterol, linoleic acid, apigenin, rutin, and α -tocopherol were found to possess these activities. The extract demonstrated antimicrobial effectiveness, eradicating bacteria at concentrations equal to or higher than the MIC. These results align with previous research on *Tephrosia purpurea* and related species, supporting their antimicrobial and antiviral properties. Overall, these findings highlight the potential of *T. purpurea* subsp. *apollinea* as a natural source of therapeutic agents. Further research is needed to explore their mechanisms and clinical applications.

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