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Comparative liquid chromatography–electrospray ionization–tandem mass spectrometry profiling and bioactivity evaluation of *Ebenus macrophylla* Jaub. & Spach leaf, stalk and root extracts: an *in vitro* and *in silico* approach

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Abstract

BACKGROUND: *Ebenus macrophylla* Jaub. & Spach (EM), an endemic Turkish plant, is a rich source of bioactive compounds with potential therapeutic applications. This study aimed to analyze the phytochemistry of its leaf, stalk, and root extracts and evaluate their antioxidant, antimicrobial, enzyme inhibitory, and antiproliferative effects.

RESULTS: Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) analysis identified 39 phenolic compounds. Among the major phenolics identified, hesperidin (in the leaf and stalk), isoquercitrin (in the leaf), catechin (in all parts), and vanillic acid (in the root) were particularly abundant. The leaf extract showed the strongest antioxidant activity (2,2-Diphenyl-1-picrylhydrazyl (DPPH) Half maximal inhibitory concentration (IC_{50}): 14.08 μg mL^{-1}) and the highest phenolic content (62.27 mg GAE g^{-1}), which is attributed to its high levels of hesperidin and shikimic acid. Antimicrobial assays showed significant activity by the leaf extract against *Clostridium perfringens* and *Pseudomonas aeruginosa* (Minimum Inhibitory Concentration (MIC): 31.3 μg mL^{-1}). In enzyme inhibition studies, the leaf extract showed stronger effects than standard inhibitors on α-glucosidase (IC_{50}: 1.56 μg mL^{-1-1}) and tyrosinase (IC_{50}: 18.01 μg mL^{-1}). Molecular docking analyses confirmed that hesperidin forms multiple stabilizing interactions with α-glucosidase and blocks substrate access. Antiproliferative studies showed that the root extract was significantly active against A549, A2780, and HeLa cancer cell lines (Growth Inhibition 50% (GI_{50}): 1.00–3.22 μg mL^{-1}) and minimal toxicity toward normal BEAS-2B cells.

CONCLUSION: The distribution of phenolic compounds detected in different plant parts is the main factor determining the strong antioxidant, antimicrobial, antidiabetic, and antiproliferative potential of EM. This study demonstrates that the species is a pharmaceutically promising natural bioactive source and provides the scientific basis for further *in vivo* research.
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Supporting information may be found in the online version of this article.

Keywords: *Ebenus macrophylla*; antioxidant activity; antiproliferative activity; antimicrobial activity; enzyme inhibition; molecular docking

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INTRODUCTION

Plant-derived secondary metabolites are essential for identifying and developing novel medications. Natural products are valuable sources for pharmaceutical research because of their structural diversity and biological activity. Plants with strong therapeutic properties – including antioxidant, anti-inflammatory, and antibacterial activity – form the basis of many traditional medicines.[1,2]

Secondary metabolites, particularly phenolic compounds, are widely regarded as key contributors to these therapeutic effects. Phenolics exhibit antioxidant and antibacterial properties that protect against oxidative stress and pathogenic microorganisms. Antioxidants reduce oxidative stress, which is associated with the etiology of numerous illnesses. Phenolic compounds function as antioxidants through metal ion chelation, single electron transfer, and free radical scavenging.[3] They also possess strong antifungal and antibacterial characteristics, which can be utilized to treat infections and illnesses.[4]

The Fabaceae (Leguminosae) is one of the most populous families, with more than 17 000 members across the world. It is important both medically and commercially. The members of this family have a wide range of secondary metabolites such as flavonoids, organic acids, terpenes, and tannins with a broad spectrum of biological activity.[5,6] The *Ebenus* genus is represented by 13 species in Türkiye, 12 of which are endemic. Some *Ebenus* species are traditionally used to treat skin, stomach, and kidney disorders.[7] Previous studies have reported the presence of methylinositol, rutin, catechin, hyperoside, picein, tannic acid, and *p*-coumaric acid in several Ebenus species, including *Ebenus macrophylla* (EM). These phytochemicals are associated with a wide range of biological activity, such as antioxidant, antifungal, anticancer, estrogenic, and anticonvulsant effects.[3,8-10]

Ebenus macrophylla is one of the species endemic to Türkiye and is known as *ulu geven*[3] in Turkish. It reaches 25–35 cm in height. Its silky-pubescent leaves feature three pairs of lanceolate leaflets, each measuring 25–50 × 6–7 mm. The flowers appear in elongated pedunculate spikes, with the peduncle ranging from 15 to 30 cm. The spikes are oblong-cylindrical, reaching dimensions of up to 6 × 3 cm. The outer bracts are oblong and cuspidate. The calyx is slightly longer than the corolla and the petals exhibit a striking purple color while remaining glabrous.[11] As EM is a local endemic species with a limited geographic distribution, studies on its phytochemistry and biological activities remain scarce. To date, the only published investigation by Bektaş *et al*. reported the antioxidant and antimicrobial properties of a methanolic extract and profiled its phenolic content using a limited number of standards with high-performance liquid chromatography with diode-array dedector (HPLC-DAD). However, the Bektaş *et al*. study provided only a preliminary overview of the antioxidant and antimicrobial potential of EM based on a crude methanol extract of aerial parts and a narrow set of phenolic standards.[3]

This study provided a comprehensive and organ-specific investigation, examining the leaf, stalk, and root individually with an advanced validated liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) approach, which measured 39 phenolic compounds. It built on prior research by evaluating EM's enzyme inhibitory characteristics (α-glucosidase and tyrosinase), antiproliferative efficacy against cancer cell lines, and molecular docking to establish enzyme inhibition mechanisms. These are the first reports of enzyme inhibition, docking investigations, and anticancer assessments for EM. Importantly, as the ethnopharmacological use of EM has not been reported in any source, this research expands the phytochemical and bioactivity profile of the plant and establishes a foundation for its potential therapeutic exploration and medicinal application.

MATERIALS AND METHODS

Plant materials

The plant samples (0.7 kg, fresh weight) were collected from Erzincan province in June 2023, during the inflorescence stages, and authenticated by Professor Ali Kandemir, Erzincan Binali Yıldırım University, Faculty of Art and Sciences, Department of Biology. Authentic samples (ID number of EM: 0000006) were stored in the Herbarium of Erzincan Binali Yıldırım University (EBYU). The leaf, stalk, and root parts of EM were separated carefully and dried at room temperature and ground into a fine powder using a mechanical grinder.

Preparation of extracts

One hundred grams of finely powdered, dried plant material was macerated overnight in 500 mL of a methanol–water (7:3, v:v) mixture. The extraction was performed in triplicate. Plant residues were filtered off, and the combined solvents were concentrated to remove methanol. The resulting extracts were lyophilized overnight to yield amorphous solids (7.1 g, 6.8 g, and 9.7 g for the leaf, stalk, and root of EM, respectively). The extracts were stored in dark flasks at 4 °C until use.

Liquid chromatography-electrospray ionization-tandem mass spectrometry conditions

An Agilent 1260 Infinity II liquid chromatography (LC) system (Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used to conduct a quantitative analysis of the phytochemicals. Chromatographic separation was performed on an Agilent Poroshell120 EC-C18 (Agilent Technologies, Santa Clara, CA, USA) analytical column (100 mm × 3.0 mm, 2.7 µm) at 40 °C. A mobile phase consisting of water with 0.1% formic acid and 5 mM ammonium formate (A) and acetonitrile with 0.1% formic acid (Sigma-Aldrich, St. Louis, MO, USA) and 5 mM ammonium formate (Sigma-Aldrich, St. Louis, MO, USA) (B) was used. The gradient elution was programmed as follows: starting at 3% B (0–1 min), increasing to 25% B (1–3 min), then to 50% B (4–12 min), further to 90% B (13–21 min), and finally decreasing to 3% B (22–25 min). The flow rate was 0.5 mL min^{-1} and the analysis time was 25 min. Mass analysis was performed in both negative and positive modes. The multiple reaction monitoring (MRM) mode was optimized to detect and quantify phytochemical molecules accurately using precursor phytochemical-to-fragment ion transitions identified through screening. The collision energies (CE) were adjusted to provide the best fragmentation of the phytochemicals and maximum transmission of the product ions. The mass spectrometry (MS) operation parameters were as follows: drying gas (N_2) flow, 15 L min^{-1}; nebulizing gas (N_2) flow, 11 L min^{-1}; capillary voltage of 4000 V; gas temperature of 350 °C; heat block temperature, 400 °C, and interface temperature, 350 °C. Agilent Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA) was used to acquire and process the LC-ESI-MS/MS data. The method was validated by evaluating the limit of detection (LOD), limit of quantification (LOQ), and linearity range.[12]

Prior to analysis, 50 mg of the extract was dissolved in 1 mL of methanol and then added to 1 mL of *n*-hexane. The mixture was vortexed at 4 °C for 2 min and centrifuged at 10 500 × *g* for 10 min to remove the fats. The upper phase was discarded. The methanol phase was diluted at a ratio of 1:9 in distilled water. The samples were injected into the system via autosampler after filtration over a 0.45 µm syringe filter.

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Table 1. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) quantitative results for the leaf, stalk, and root extracts of *Ebenus macrophylla* Jaub. & Spach (EM) (μg analyte/g extract)

No.	Compound name	*RT*	Leaf	Stalk	Root	No.	Compound name	*RT*	Leaf	Stalk	Root
1	Shikimic acid	1.29	3212.12	2003.12	1483.95	21	Hesperidin	11.90	59 471.63	8314.66	356.48
2	Gallic acid	3.30	601.33	78.51	65.83	22	Isoquercitrin	11.93	5793.18	1603.30	63.99
3	Protocatechuic acid	5.52	334.06	196.00	572.10	23	Rutin	12.74	58.45	87.16	124.18
4	Epigallocatechin	6.82	123.51	64.32	88.89	24	Quarcetin-*3*-xyloside	12.49	206.85	106.45	52.47
5	Catechin	6.94	2076.59	3582.40	3503.45	25	Kaempferol-*3*-glucoside	13.27	634.77	148.91	148.62
6	Chlorogenic acid	7.44	364.18	63.29	54.30	26	Fisetin	13.27	26.46	59.45	39.12
7	Hydroxybenzaldehyde	7.74	20.17	387.12	452.83	27	Baicalin	13.61	ND	ND	ND
8	Vanillic acid	7.84	124.25	184.56	5897.95	28	Chrysin	14.25	14.45	16.06	27.29
9	Caffeic acid	7.90	89.16	23.32	36.55	29	Daidzein	14.20	ND	ND	ND
10	Syringic acid	8.46	1584.56	1436.25	2492.35	30	*trans*-Cinnamic acid	14.34	1.74	1.45	1.88
11	Caffein	8.44	1.85	1.95	1.23	31	Quercetin	14.87	796.18	103.38	206.45
12	Vanillin	8.69	33.61	16.15	23.54	32	Naringenin	15.05	254.45	326.45	385.12
13	*o*-Coumaric acid	9.50	707.12	137.01	374.74	33	Hesperetin	15.84	ND	ND	ND
14	Salicylic acid	9.82	1085.99	798.20	348.34	34	Morin	15.90	ND	ND	ND
15	*trans*-Ferulic acid	10.20	426.36	123.17	989.81	35	Kaempferol	16.46	ND	ND	ND
16	Sinapic acid	10.45	ND	ND	ND	36	Baicalein	17.03	ND	ND	ND
17	Scutellarin	11.18	1.95	1.88	1.12	37	Luteolin	18.21	384.41	404.52	496.48
18	*p*-Coumaric acid	11.51	ND	ND	ND	38	Biochanin A	17.89	1.21	1.16	10.70
19	Coumarin	11.19	ND	ND	ND	39	Diosgenin	23.37	ND	ND	ND
20	Protocatechuic ethyl ester	11.66	ND	ND	ND						

Abbreviations: EM, *Ebenus macrophylla* Jaub. & Spach; ND, not detected.

Biological activity of EM extracts

Experimental procedures for the measurement of the antioxidant, antiproliferative, antibacterial, and enzyme activity of EM extracts are given in the Supporting Information.

RESULTS AND DISCUSSION

Phytochemical investigation of EM by LC-ESI-MS/MS

The phytochemical profiling of the hydromethanolic extracts of EM was conducted using a previously developed and validated LC-ESI-MS/MS method.[12] This analytical method assessed the extracts for their bioactive phytochemical content, quantifying 39 phenolic compounds. Table 1 presents a quantitative analysis of these extracts. Figure 1 displays the LC-ESI-MS/MS TIC chromatograms of the EM extracts.

Hesperidin, an important phenolic compound found in this study, possesses a number of well documented pharmacological properties, including antioxidant, antimicrobial, anticancer, antihypertensive, and antiulcer effects. Due to its protective effects on liver, kidney, and heart functions, it has significant potential for medical and dietary uses.[22] In this study, high concentrations of hesperidin were observed in the leaf extracts of EM (59 471.63 μg g^{-1} extract), and in the stalk extract (8314.66 μg g^{-1} extract). Considering the known pharmacological properties and broad applications of hesperidin, its verification through LC-ESI-MS/MS could contribute significantly to a better understanding of EM's potential biological effects. Confirming its presence and concentration is therefore essential for assessing the therapeutic value of EM extracts.

Shikimic acid plays an essential role in the biosynthetic pathways leading to aromatic amino acids, including L-phenylalanine, L-tryptophan, and L-tyrosine. Many studies have documented the diverse biological properties exhibited by shikimic acid and its derivatives. These include antioxidant, anti-inflammatory, antiviral, antidiabetic, hypolipidemic, cutaneous protective, neuroprotective, and antibacterial effects.[22] Moderate levels of shikimic acid were found in leaf extracts (3212.12 μg g^{-1} extract), stalks (2003.12 μg g^{-1} extract), and root (1483.95 μg g^{-1} extract) of EM. In a study by Bektaş *et al.*,[3] shikimic acid and hesperidin were not found in the methanolic extract of EM. In this study, higher 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging ability was observed in the leaf extract than in the stalk and root extracts, as evidenced by the fact that shikimic acid, which has antioxidant properties, was mostly found in the leaf.

Isoquercitrin was the second most abundant phenolic compound in the leaf extract, with a concentration of 5793.18 μg g^{-1} extract. Its antioxidant activity involves inhibition of lipid peroxidation through interference with xanthine oxidase, chelation of redox-active metals, enhanced vitamin C absorption, and direct scavenging of reactive oxygen species (ROS). Isoquercitrin has also been reported to exhibit stronger antiproliferative effects than other flavonoids, particularly against colon, breast, and hepatocellular cancers.[22] Despite the absence of isoquercitrin in the methanol extract of EM in previous studies,[3] this compound was identified in the leaves of EM grown in Erzincan in the present study. Concentrations in the stalk and root extracts were lower than in the leaf extract. These findings indicate that isoquercitrin is one of the phytochemicals contributing to the antiproliferative activity of EM.

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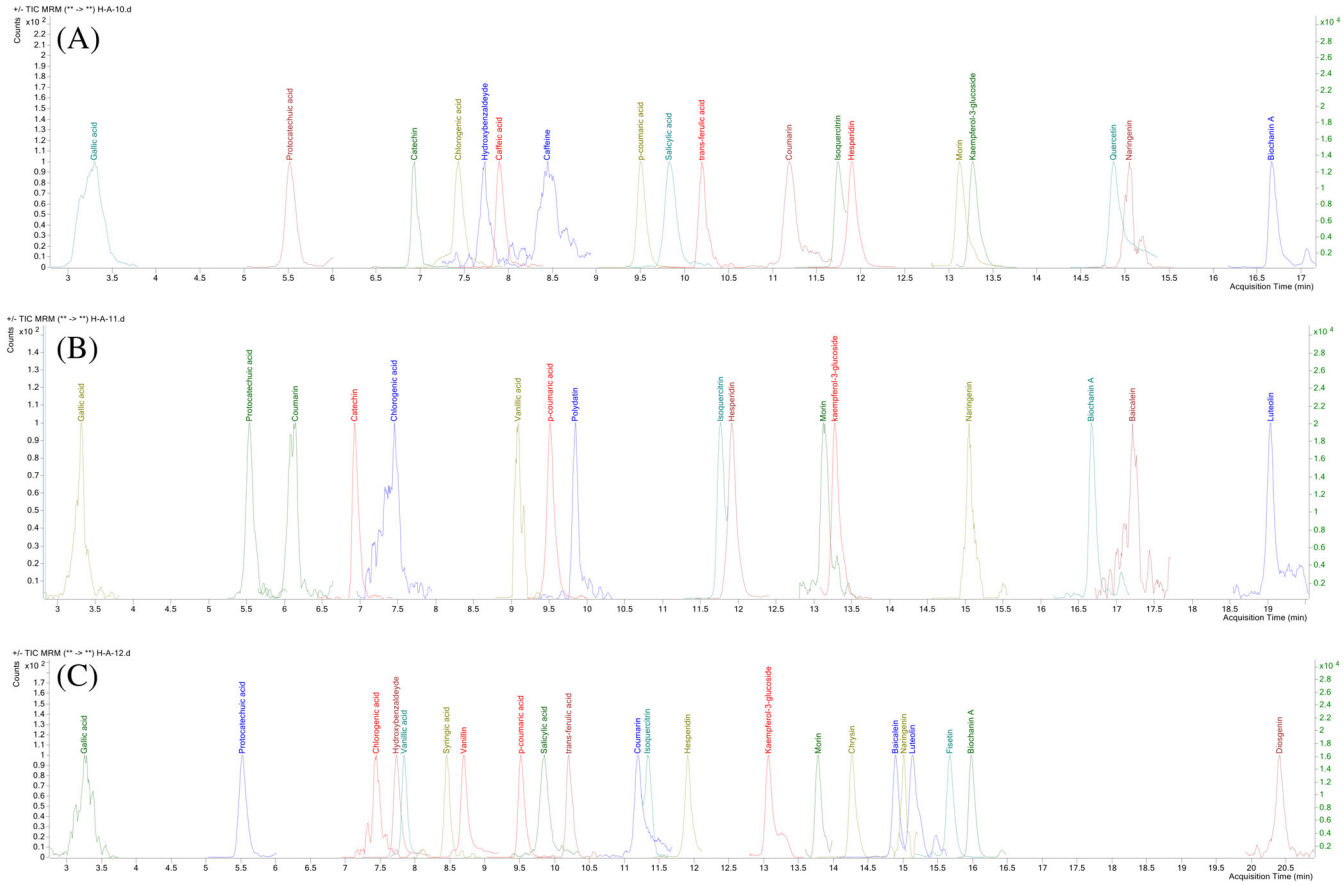


Figure 1. Total ion chromatograms of the (A) leaf, (B) stalk and (C) root extracts of *Ebenus macrophylla* Jaub. & Spach (EM).

Vanillic acid is known to exhibit antidiabetic, cardioprotective, anticancer, hepatoprotective, and antioxidant properties in experimental animal models.[23] Liquid chromatography–electrospray ionization–tandem mass spectrometry analysis detected a high concentration of vanillic acid in the root extract (5897.95 μg g^{-1} extract), whereas only low levels were observed in the leaf (124.25 μg g^{-1} extract) and stalk (184.56 μg g^{-1} extract). In a previous study,[3] vanillic acid was reported at 2965 μg g^{-1} in the methanol extract of the aerial parts of EM. The broader term 'aerial parts' likely included a mixture of leaves, stems, and possibly flowers, which may explain the higher concentration reported compared with the individual leaf and stalk values in the present study. Overall, these results indicate that vanillic acid accumulates predominantly in the root of the plant rather than in the aerial parts, whereas the lower levels found in the leaf and stalk extracts reflect organ-specific distribution.

In addition to antioxidant activity, catechin shows protective effects in oxidative stress-induced diseases such as cancer, cardiovascular diseases, and neurodegenerative diseases.[23] The results of the current study showed the presence of high levels of catechin in the extracts of leaf (2076.6 μg g^{-1} extract), stalk (3582.4 μg g^{-1} extract), and root (3503.5 μg g^{-1} extract), highlighting their potential as valuable sources of catechin for therapeutic applications. The stalk and root extracts have higher levels of catechin than leaves. This suggests that catechin is distributed differently among the different parts of the plant, which may indicate variations in their functional roles or metabolic activity.

Syringic acid is an invaluable compound with a wide range of therapeutic benefits. It has been shown to be effective in the treatment of various diseases, including diabetes, cardiovascular disorders, cancer, cerebral ischemia, neuropathies, and liver damage. It also exhibits antioxidant, antimicrobial, anti-inflammatory, and antiendotoxic properties, making it a promising candidate for various therapeutic applications.[24] Compared with the previous study, a higher concentration of syringic acid was found in the extracts of leaf (1584.6 μg g^{-1} extract), stalk (1436.3 μg g^{-1} extract), and root (2492.4 μg g^{-1} extract). In addition to these phenolic compounds, naringenin, protocatechuic acid, quercetin, *o*-coumaric acid, gallic acid, and luteolin were detected at very low concentrations in the methanolic extract of EM, as reported previously[3] (Table 1).

Antioxidant activity

The antioxidant activity – measured using DPPH and ferric reducing antioxidant power (FRAP) – together with the total phenolic content (TPC), and the total flavonoid content (TFC) of the extracts are reported in Table 2. The DPPH• scavenging ability, expressed as the half maximal inhibitory concentration (IC_{50}) (μg mL^{-1}), was 14.08 ± 1.76, 19.45 ± 2.47, and 62.57 ± 1.38 for the leaf, stalk, and root extracts. Trolox showed an IC_{50} of 11.95 ± 0.15. All plant parts differed significantly in scavenging activity ($P < 0.05$), and the leaf extract exhibited the strongest effect.

The TPC values of the leaf, stalk, and root extracts were 62.27 ± 1.74, 33.82 ± 0.22, and 32.56 ± 3.75 mg GAE g^{-1} extract. The leaf extract contained significantly more phenolics than the other parts ($p < 0.05$). The leaf extract also showed the highest TFC

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Table 2. Total phenolic, flavonoid contents and antioxidant activity of *Ebenus macrophylla* Jaub. & Spach (EM) (leaf, stalk, and root)

Plants and standards	Part used	DPPH[1]	TPC[2]	TFC[3]	FRAP[4]
Ebenus macrophylla	Leaf	14.08 ± 1.76^{c}	62.27 ± 1.74^{a}	32.10 ± 2.16^{a}	88.69 ± 2.11^{d}
	Stalk	19.45 ± 2.47^{d}	33.82 ± 0.22^{b}	8.10 ± 0.87^{c}	60.90 ± 2.36^{e}
	Root	62.57 ± 1.38^{e}	32.56 ± 3.75^{b}	27.05 ± 0.44^{b}	53.21 ± 0.97^{e}
Trolox		11.95 ± 0.15^{b}	-	-	-
BHA		8.04 ± 0.69^{a}	-	-	338.57 ± 0.31^{b}
BHT		10.70 ± 0.73^{b}	-	-	257.80 ± 1.24^{c}
Ascorbic acid		9.91 ± 0.87^{ab}	-	-	383.66 ± 0.98^{a}

Note: [1]IC_{50} in μg mL^{-1}, [2]mg GAE g^{-1} extract, [3]mg QE g^{-1} extract, [4] mg TE g^{-1} extract. [a–e]Different letters in the same column indicate statistically significant differences ($P < 0.05$), DPPH: 2,2-diphenyl-1-picrylhydrazyl; TPC: Total phenolic content; TFC: Total flavonoid content; FRAP: Ferric reducing antioxidant power; BHT: Butylated hydroxytoluene; BHA: Butylated hydroxyanisole.

Table 3. Antimicrobial activity of *Ebenus macrophylla* EM (leaf, stalk, and root)

Samples and standard	*Clostridium perfringens*	*Pseudomonas aeruginosa*	*Listeria monocytogenes*	*Bacillus cereus*	*Staphylococcus aureus*	*Salmonella enteritidis*
	Inhibition zone (in mm)					
Leaf	19.1 ± 0.4^{b}**	18.5 ± 0.3^{b}	14.2 ± 0.2^{b}	15.4 ± 0.4^{b}	n.i.	9.4 ± 0.1^{b}
Stalk	12.5 ± 0.2^{c}	13.2 ± 0.1^{c}	11.4 ± 0.1^{c}	11.5 ± 0.3^{c}	n.i.	8.9 ± 0.1^{b}
Root	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Imipenem	32.4 ± 0.8^{a}	28.1 ± 0.7^{a}	35.8 ± 1.2^{a}	33.8 ± 1.8^{a}	26.4 ± 0.5	31.2 ± 1.6^{a}
	MIC (in μg mL^{-1})					
Leaf	31.3	31.3	250	62.5	>500	>500
Stalk	125	125	250	125	>500	>500
Root	>500	>500	>500	>500	>500	>500
Imipenem	<3.91	3.91	<3.91	<3.91	3.91	<3.91

Note: *Different letters within the same column indicate statistically significant differences at $P < 0.05$. *$P < 0.05$; **$P < 0.01$; n.i., no inhibition.

(32.10 ± 2.16 mg QE g^{-1} extract), followed by the root and stalk, with significant differences among all groups.

The FRAP of the leaf extract (88.69 ± 2.11 mg TE g^{-1} extract) exceeded that of the stalk (60.90 ± 2.36 mg TE g^{-1} extract) and root (53.21 ± 0.97 mg TE g^{-1} extract) ($P < 0.05$), although all values remained below that of ascorbic acid (383.66 ± 0.98 mg TE g^{-1} extract). These differences reflect the distinct phytochemical profiles of the plant parts.[13,15,16]

Bektaş *et al.*[3] reported on the antioxidant activity of the methanolic extract of aerial parts of EM.[3] However, there are no studies examining the activity in different parts of the plant. It has been proven in previous studies that hesperidin displays high antioxidant activity.[25,26] The identification, using LC-ESI-MS/MS analysis, of hesperidin as the major compound in the stalks and leaves of EM indicates that this is the primary compound responsible for the high antioxidant activity of these two extracts.

Antimicrobial activity

Plant extracts are emerging as effective antibacterial agents against microbes, offering potential alternatives for managing infections and wounds caused by bacteria that have become difficult to treat with conventional therapies.[27] This development is particularly important given the rapid rise of antibiotic resistance and the spread of resistant strains in healthcare settings and the wider community.[28] The antibacterial activity of the leaf, stalk, and root extracts of EM is presented in Table 3.

Leaf extract demonstrated good antibacterial activity against *perfringens, Pseudomonas aeruginosa*, and *Bacillus cereus*, and showed moderate inhibition against *Listeria monocytogenes*, with significantly greater inhibition zone diameters than stalk and root extracts ($P < 0.05$). The stalk extract also exhibited moderate activity against the same bacteria. None of the extracts showed antibacterial activity against *Staphylococcus aureus*, and the root extract showed no inhibitory effects on any microorganisms. As Table 2 shows, the leaf extract had significantly higher TPC, TFC, and FRAP values. This rich and complex phytochemical profile of the leaf extract in terms of phenolic and flavonoid compounds is considered to be the main reason for its stronger antioxidant capacity and more pronounced antibacterial activity in comparison with root and stalk extracts.

The antibacterial activity of the extracts was classified as good if the MIC was less than 100 μg mL^{-1}, moderate at 100–500 μg mL^{-1}, weak at 500–1000 μg mL^{-1}, and inactive above 1000 μg mL^{-1}.[29] According to Table 3, the leaf extract showed good antibacterial activity against *C. perfringens*, *P. aeruginosa*, and *B. cereus*. The stalk extract showed moderate activity against *C. perfringens*, *P. aeruginosa*, *L. monocytogenes*, and *B. cereus*. Previous studies have reported antimicrobial activity in various *Ebenus* species, which supports these findings.[3,30]

Enzyme activity

Plant-derived natural products are a valuable resource in the fight against many diseases as they are less toxic and have fewer side

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Table 4. Inhibitory effect of *Ebenus macrophylla* Jaub. & Spach (EM) (leaf, stalk, and root) on α-glucosidase and tyrosinase activity

Sample	α-glucosidase IC_{50} (μg mL^{-1})	Tyrosinase IC_{50} (μg mL^{-1})
Leaf	$1.56 \pm 0.13^{a*}$	18.01 ± 1.40^{a}
Stalk	n.i.	n.i.
Root	509.67 ± 8.67^{c}	67.29 ± 2.43^{c}
Acarbose (positive control)	49.21 ± 1.87^{b}	-
Kojic acid (positive control)	-	26.47 ± 0.82^{b}

Note: *Different letters within the same column indicate statistically significant differences at $P < 0.05$. n.i., no inhibition.

effects. α-Glucosidase is a hydrolase enzyme that plays a role in the breakdown of carbohydrates in the digestive system and hydrolyzes polysaccharides into simpler sugars such as glucose. Thus, inhibition of this enzyme helps to lower the blood glucose level.[31]

Tyrosinase is an enzyme responsible for melanin production and is widely found in microorganisms, animals, and plants. It plays an important role in various processes, including antibiotic resistance, skin pigment formation, the sclerotization of the cuticle, and neurodegeneration. Due to its metabolic function, it is considered an important therapeutic target enzyme in the development of anti-browning, skin-whitening, and other therapeutic agents.[32] The α-glucosidase and tyrosinase enzyme inhibitory activities of the extracts were evaluated *in vitro*. Table 4 presents the results, and Fig. 2 shows the IC_{50} graphs.

The leaf extract demonstrated a strong inhibitory effect on both α-glucosidase and tyrosinase enzyme activity, with IC_{50} values of 1.56 ± 0.13 μg mL^{-1} and 18.01 ± 1.40 μg mL^{-1}. Its α-glucosidase inhibition was significantly stronger than that of acarbose ($IC_{50} = 49.21 \pm 1.87$ μg mL^{-1}), indicating substantial antidiabetic potential. Its tyrosinase inhibition also exceeded that of kojic acid ($IC_{50} = 26.47 \pm 0.82$ μg mL^{-1}), although the difference was smaller. These results show that the leaf extract, especially through α-glucosidase inhibition, exhibited superior enzyme inhibitory activity compared with the standards ($P < 0.05$).

The root extract showed moderate tyrosinase inhibition ($IC_{50} = 67.29$ μg mL^{-1}) but weak α-glucosidase inhibition. The stalk extract showed no inhibitory activity against either enzyme (Table 4).

The LC-ESI-MS/MS analysis found hesperidin to be the most abundant phenolic in leaf extract. It has been reported in multiple studies that hesperidin has a strong inhibitory effect on the α-glucosidase enzyme, with IC_{50} values ranging from 10 to 16 μg mL^{-1}.[33,34] Accordingly, the leaf extract can be suggested as an antidiabetic agent candidate.

Previous studies reported that hesperidin and isoquercitrin, major compounds of the leaf extract, demonstrated a weak inhibitory effect on the tyrosinase enzyme at concentrations ranging from 0.5 to 2.41 mM.[35,36] The strong tyrosinase inhibitory effect of the leaf extract in this study can be attributed to other minor phenolics or unidentified compounds. Among the other 37 phenolic compounds identified in the LC-ESI-MS/MS analysis, there is evidence in the literature that chrysin, naringenin, and quercetin have significant inhibitory effects on tyrosinase.[37] These compounds are known to exhibit synergistic effects even at low concentrations. The strong tyrosinase inhibitory effect of EM leaf extract is therefore thought to be due not only to the major compounds hesperidin and isoquercitrin but also to the contribution of these minor compounds and their possible synergistic interactions. The root extract showed weak activity on the α-glucosidase enzyme but exhibited stronger inhibitory activity against tyrosinase. This suggests that different plant organs have unique chemical compositions, and the compounds in the extract demonstrate different binding models to the active sites of various enzymes.[38] These findings suggest that extracts derived from plants are complex and necessitate further investigation into both major and minor compounds to better understand their mechanisms of enzyme inhibition and potential applications in food and pharmaceuticals.

Molecular modeling

A docking study was carried out to reveal the interactions of inhibition at the molecular level for the two main components found in the leaf extract with strong inhibitory effects. The results are given in Table 5 and the 2D interaction diagrams are given in Fig. 3.

The findings of the current study indicated that hesperidin interacted more with amino acids than with isoquercitrin. It formed conventional hydrogen bonds with GLN279, ARG315x3, ASN415, ARG442, PRO312, SER311, GLU411, TYR158, and ASP307, and carbon-hydrogen bonds with ARG315x2, HIS280, and PHE314. It demonstrated electrostatic interaction with ASP352 and hydrophobic interactions with PHE303, ARG315, TYR72, PHE314, TYR316, and VAL216.

Isoquercitrin formed conventional hydrogen bonds with GLN279, ASP307x2, ASP352, ASP215, GLU277, ASP69, and TYR158. It also formed carbon-hydrogen bridges over the following residues: HIS280 and ARG315. Finally, it formed a π-donor hydrogen bond with TYR72. ASP352x2 formed electrostatic interactions through GLU411, while hydrophobic interactions were mediated by TYR72, VAL216, and ARG315.

TYR158, HIS280, and loop 310–315 are the entrance to the α-glucosidase active site.[17] Table 5 and Fig. 3 show the docking results and indicate that the glucose units of hesperidin hinder substrate access by forming both hydrogen bonds and hydrophobic interactions with these residues. Previous studies reported that isoquercitrin has lower α-glucosidase inhibitory activity than hesperidin.[36,39] The docking results are consistent with this: although isoquercitrin binds in the same region as hesperidin and interacts with TYR158 and HIS280, which are important for active-site access, it does not interact with loop 310–315 on the opposite side of the active region.

Antiproliferative activity

The antiproliferative effects of the extract were evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and the NCI-60 procedures. The total growth inhibition (TGI) values of plant extracts are classified as effective at 1.00–100.0 μg mL^{-1}, moderately effective at 100.1–499.9 μg mL^{-1}, and ineffective above 500 μg mL^{-1}. High lethal concentration 50% (LC_{50}) values indicate low cytotoxicity, and low growth inhibition 50% (GI_{50}) and TGI values indicate strong cytostatic activity. The root extract showed potent antiproliferative effects against all tested cancer cell lines except MCF7 and HT29, with GI_{50} values of 1.00–20.1 μg mL^{-1}, TGI values of 4.79–208.6 μg mL^{-1}, and LC_{50} values above 500 μg mL^{-1}.

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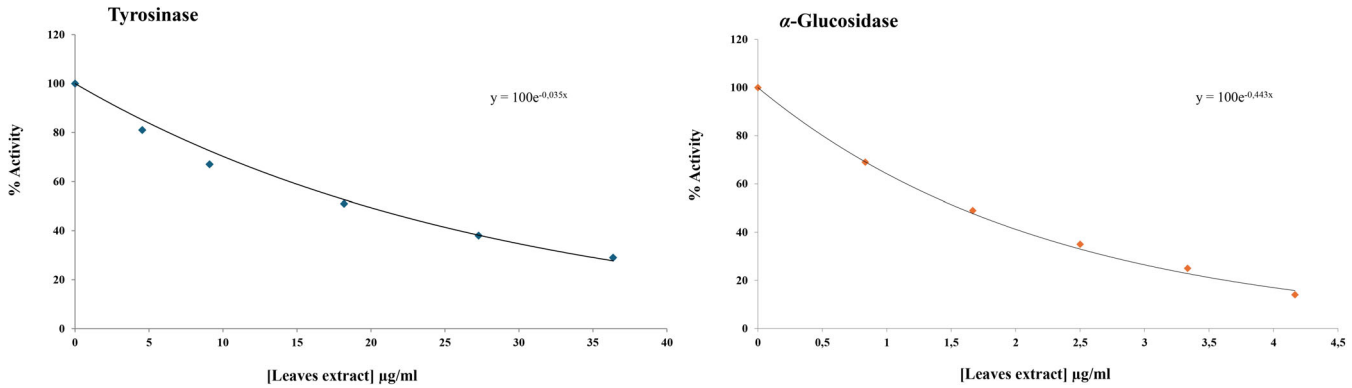


Figure 2. The inhibitory activities of *Ebenus macrophylla* Jaub. & Spach (EM) (leaf, stalk, and root) against (A) α-glucosidase and (B) tyrosinase.

Table 5. The details of molecular interaction between amino acids in the active site of α-glucosidase and main compounds

		Hesperidin		Isoquercitrin	
		−155.966 MolDock		−128.569 MolDock	
Types	Category	Residues	Distance	Residues	Distance
Hydrogen bond		ARG442	1.76	GLN279	2.19
		ASN415	2.14	ASP69	2.60
		GLN279	2.19	**TYR158**	2.60
		TYR158	2.33	ASP307	2.62
	Conventional hydrogen bond	GLU411	2.60	GLU277	2.62
		ASP307	2.61	ASP215	3.10
		ARG315	**2.76**	ASP307	3.10
		ARG315	**2.83**	ASP352	3.10
		ARG315	**2.95**		
		PRO312	**3.10**		
		SER311	**3.16**		
		ARG315	**2.44**	**HIS280**	2.89
		ARG315	**2.60**	ARG315	2.91
	Carbon Hydrogen Bond	PHE314	2.62		
		HIS280	2.97		
	Pi-Donor Hydrogen Bond			TYR72	4.10
Electrostatic		ASP352	4.23	GLU411	2.86
	Pi-Anion			ASP352	4.32
				ASP352	4.71
Hydrophobic	Pi-Pi T-shaped	PHE303	4.80	TYR72	5.88
	Alkyl	**ARG315**	**3.80**		
		TYR72	3.82	ARG315	5.22
		PHE314	4.12	VAL216	5.39
	Pi-alkyl	TYR316	4.53		
		VAL216	5.17		

Note: Bold values indicate statistical significance at $P < 0.05$.

This effect is thought to be closely related to the high concentrations of vanillic acid (5897.95 µg g^{-1}), catechin (3503.45 µg g^{-1}), and syringic acid (2492.35 µg g^{-1}) accumulated in the root. Earlier studies reported that vanillic acid activates apoptotic pathways, leading to DNA damage; catechin suppresses cancer cell proliferation, and syringic acid contributes to both antioxidant and anticancer mechanisms.[23,40,41] The combination of these compounds and their possible synergistic effects may explain the selective antiproliferative profile of the root extract. The leaf and stalk extracts were effective only against A549 cells (GI_{50}: 1.05–1.39 µg mL^{-1}, TGI: 3.76–29.4 µg mL^{-1}, LC_{50} > 500 µg mL^{-1}). The extracts were not found to be active against MCF7 and HT29 cell lines. Statistically significant differences were observed between the root extract and the other parts (leaf and

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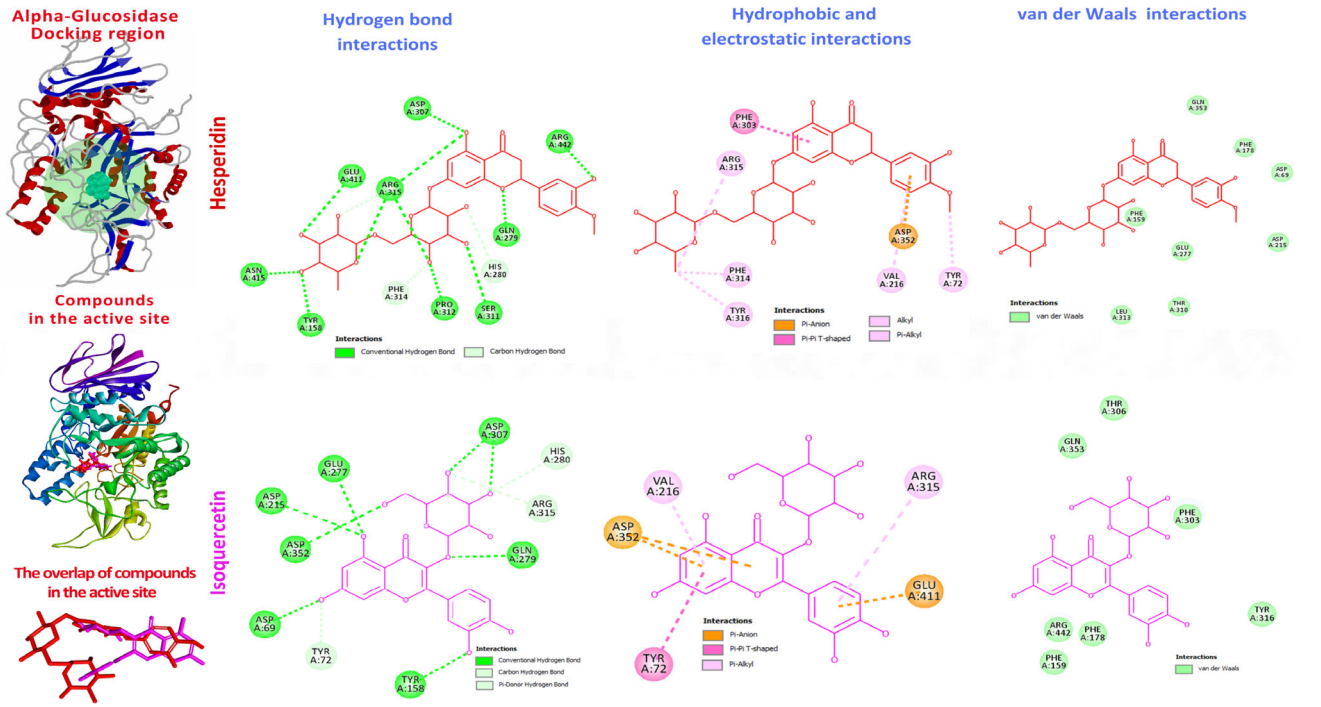


Figure 3. The molecular interaction between amino acids in the active site of α-glucosidase and main compounds.

Table 6. GI_{50}, TGI, and LC_{50} values of EM (leaf, stalk, and root) against MCF7, HeLa, A2780ADR, A2780, A549, BEAS-2B, and HT29

Cells/extract (μg mL^{-1})		Leaf	Stalk	Root	5-FU
MCF7	**GI_{50}**	2.72^{B}	4.14^{C}	20.1^{D}	1.70^{A}
	TGI	>500	>500	>500	61.2 ± 3.4
	LC_{50}	>500	>500	>500	430.1 ± 15.7
HeLa	**GI_{50}**	26.1 ± 1.3^{B}	24.8 ± 1.2^{B}	2.03^{A}	1.72^{A}
	TGI	>500	>500	408.6 ± 14.9^{B}	36.7 ± 1.2^{A}
	LC_{50}	>500	>500	>500	429.1 ± 15.9
A2780ADR	**GI_{50}**	15.3 ± 0.7^{C}	9.05^{B}	1.28^{A}	1.56^{A}
	TGI	>500	>500	11.8 ± 0.5^{A}	52.9 ± 3.5^{B}
	LC_{50}	>500	>500	>500	431.6 ± 13.2
A2780	**GI_{50}**	2.61^{B}	23.5 ± 1.1^{D}	3.22^{C}	1.40^{A}
	TGI	>500	>500	176.8 ± 5.3^{B}	51.2 ± 2.7^{A}
	LC_{50}	>500	>500	>500	413.5 ± 14.2
A549	**GI_{50}**	1.39^{A}	1.05^{B}	1.00^{B}	1.43^{A}
	TGI	29.4 ± 1.1^{C}	3.76^{A}	4.79^{B}	48.1 ± 1.9^{D}
	LC_{50}	>500	>500	>500	462.0 ± 14.6
BEAS-2B	**GI_{50}**	22.7 ± 1.1^{C}	4.59^{B}	1.91^{A}	1.42^{A}
	TGI	>500	>500	58.9 ± 2.9^{B}	41.8 ± 2.3^{A}
	LC_{50}	>500	>500	>500	417.5 ± 12.8
HT29	**GI_{50}**	27.0 ± 1.1^{D}	11.4 ± 0.6^{B}	20.0 ± 1.0^{C}	1.50^{A}
	TGI	>500	>500	>500	52.0 ± 2.7
	LC_{50}	>500	>500	>500	421.9 ± 15.1

Note: Percentage inhibition is the mean value ± SD of three independent measures. If percentage inhibition is smaller than 10, the SD value is <0.5. Values followed by the same letter in the row are not significantly different ($P < 0.05$).

stalk) in A549, HeLa, A2780, and A2780ADR cell lines in terms of GI_{50} and TGI values ($P < 0.05$). The leaf and stalk extracts showed no toxic effects on normal BEAS-2B cells (TGI > 500 μg mL^{-1}, LC_{50} > 500 μg mL^{-1}) and the root extract exhibited lower toxicity than the positive control, 5-fluorouracil (5-FU) (TGI: 58.9 μg mL^{-1}, LC_{50} > 500 μg mL^{-1}). Comparison of the GI_{50} and TGI values of the extracts with 5-FU suggests that they may be used safely across all cell lines as antiproliferative agents (Table 6). The extracts demonstrated remarkable NCI60 survival parameters for cancer cell lines, with their GI_{50}, TGI, and LC_{50} values all falling within the required ranges. The results indicate that these extracts have a cancer-specific effect. In the treatment of A549, A2780, A2780ADR,

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Table 7. Percentage cytotoxicity of *Ebenus macrophylla* Jaub. & Spach (EM) (leaf, stalk, and root) at TGI concentrations against the cells

Extract	A549	MCF7	HT29	BEAS-2B	A2780	A2780ADR	HeLa
Leaf	11.5 ± 0.6^{C}	3.0^{B}	1.5^{AB}	3.7^{B}	3.1^{B}	2.0^{A}	2.1^{A}
Stalk	26.8 ± 1.3^{C}	3.2^{B}	2.2^{A}	3.4^{B}	3.7^{B}	3.1^{B}	1.9^{A}
Root	26.9 ± 1.4^{D}	2.8^{A}	2.7^{A}	15.6 ± 0.7^{C}	7.3^{B}	26.7 ± 1.5^{D}	7.4^{B}
5-FU	14.5 ± 0.8^{A}	16.6 ± 0.9^{C}	15.4 ± 0.7^{AB}	15.8 ± 0.7^{B}	13.9 ± 0.5^{A}	14.8 ± 0.6^{AB}	15.9 ± 0.7^{B}

Note: Percentage cytotoxicity is the mean value ± SD of three independent measures. Standard deviation of percentage inhibition is <3.0. Values followed by the same letter in the row are not significantly different ($P < 0.05$).

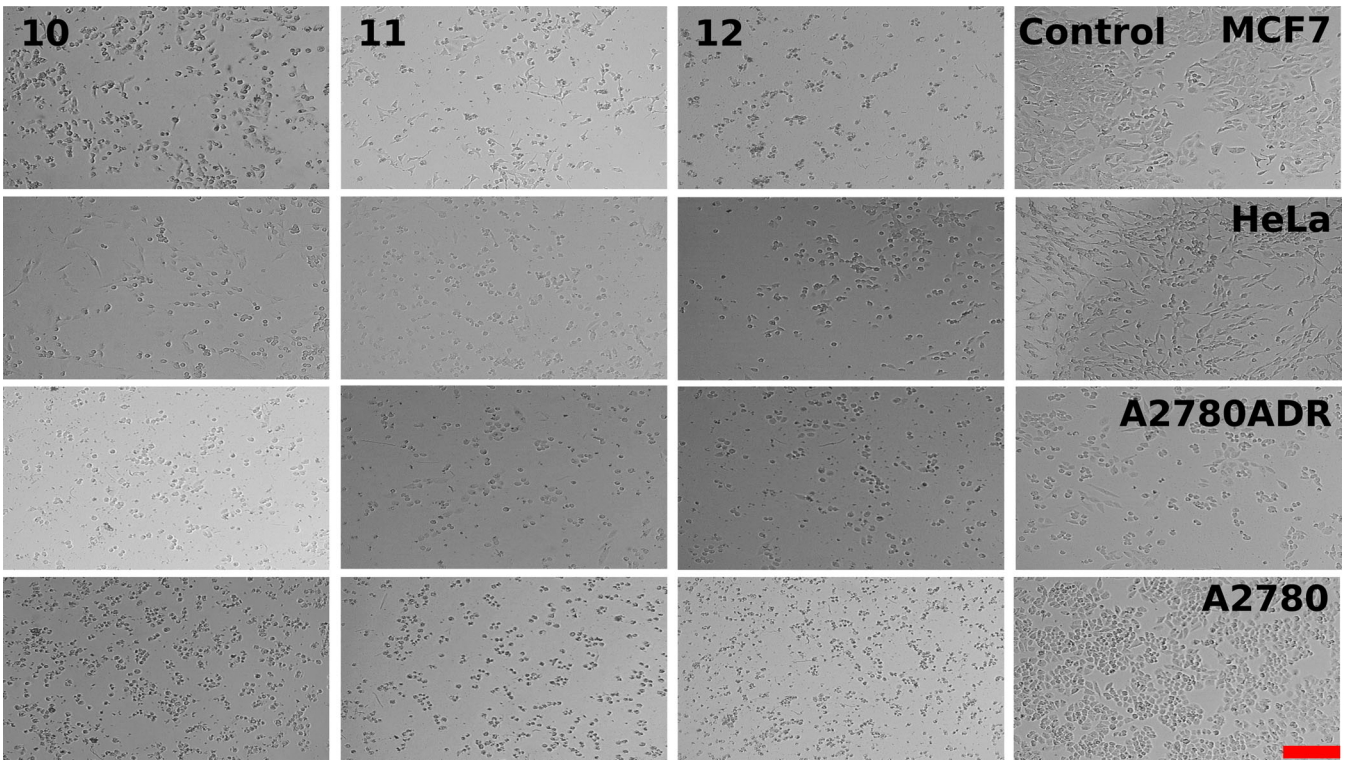


Figure 4. Effects of *Ebenus macrophylla* Jaub. & Spach (EM) leaf, stalk, and root extracts on the morphology of MCF7, HeLa, A2780ADR, and A2780 cell lines. Cells treated with dimethyl sulfoxide (DMSO) served as controls. Scale bars represent 100 μm.

and HeLa cell lines, the root extract showed the most promising antiproliferative characteristics for further preclinical studies (GI_{50}: 1.00–3.22 μg mL^{-1}, TGI: 4.79–208.6 μg mL^{-1}, LC_{50} > 500 μg mL^{-1}).

Cytotoxic activity of the extracts

Intracellular lactate dehydrogenase (LDH) can be released into the extracellular environment due to extract-induced membrane damage, providing an indicator of cytotoxicity. Cytoplasmic LDH activity was measured using an LDH cytotoxicity kit.

Cytotoxicity induced by the extracts was quantified at TGI concentrations and compared with the positive control, 5-FU. Cytotoxicity levels were classified as toxic (15.0–28.4%), moderately toxic (10.5–14.9%), slightly toxic (5.4–9.6%), or non-toxic (1.1–3.9%). Extracts that were effective in the MTT proliferation assay exhibited cytotoxicity ranging from 1.5 to 26.9% across the tested cell lines at TGI concentrations (Table 7). The LDH assay results indicated that the extracts were non-toxic to MCF7, HT29, A2780, and HeLa cells, with cytotoxicity between 1.5 and 7.4%.

Statistical analysis revealed that the cytotoxicity values of the root extract in A2780 and HeLa cells were significantly higher than those of the leaf and stalk extracts ($P < 0.05$). However, when assessing the cytotoxic effects of the extracts on A549 cells, it was found that the leaf extract was moderately toxic (11.5%) but the extracts of the stalk and root were highly toxic (26.8–26.9%). The root extract also exhibited moderate toxicity (15.6%) for BEAS-2B and high toxicity (26.7%) for A2780ADR. The MTT and LDH assay results, indicating therapeutic and cytotoxic features, respectively, were evaluated together. It was found that the root extract demonstrated the optimal antiproliferative and cytotoxic effect against cancer and normal cells in comparison with the positive control, 5-FU (Tables 6 and 7). Overall, the root extract was found to be sufficiently toxic to cancer cell lines, including A549, A2780, A2780ADR, and HeLa cells, while remaining safe against normal cells. This warrants further preclinical studies.

Effect of the extracts on cell morphology

The morphological effects of the extracts were evaluated on A549, HeLa, A2780, and A2780ADR gynecological cancer cell lines, HT29 colon cancer cells, MCF7 breast cancer cells, and BEAS-2B normal lung cells. Assessments and photographic documentation were

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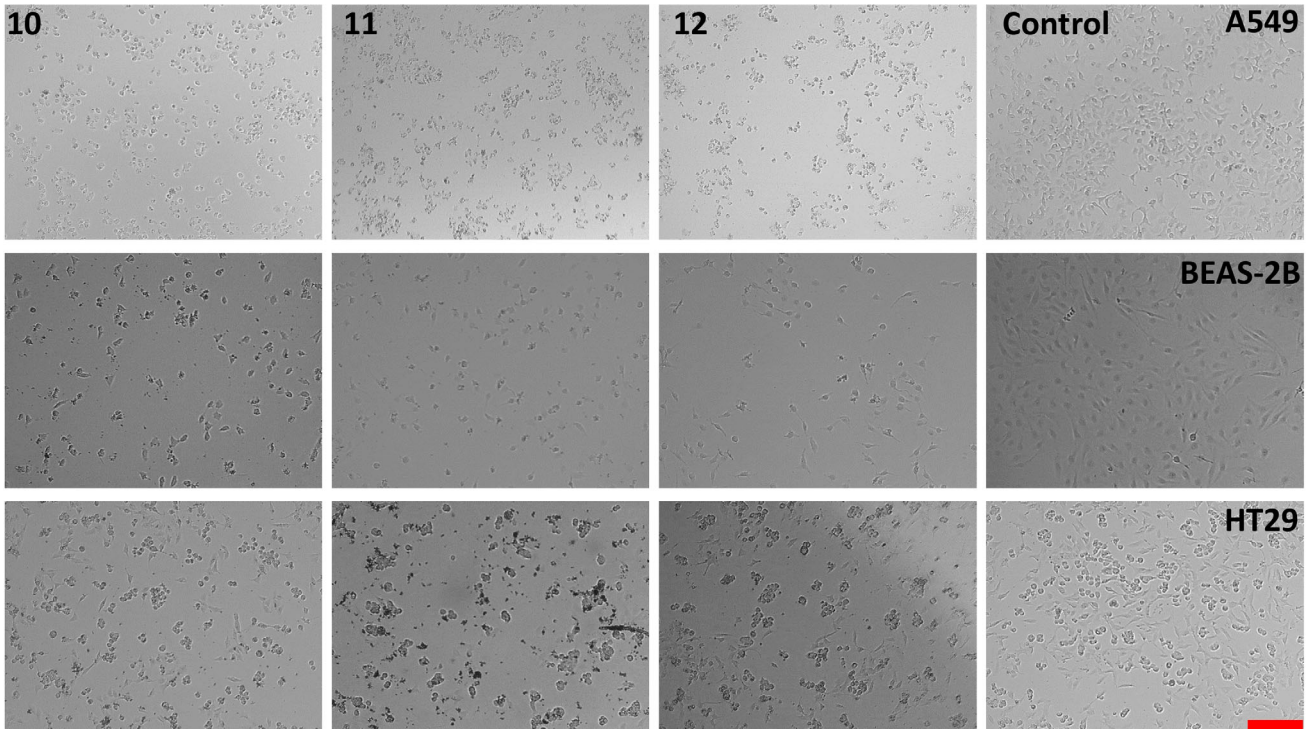


Figure 5. Effects of *Ebenus macrophylla* Jaub. & Spach (EM) leaf, stalk and root extracts on the morphology of A549, BEAS-2B, and HT29 cell lines. Cells treated with dimethyl sulfoxide (DMSO) served as controls. Scale bars represent 100 μm.

performed 24 h after treatment. The images revealed characteristic morphological changes in all treated cell lines, including reduced cell attachment or floating cells, aggregation, rounding, granular formation, blebbing, cellular shrinkage, and disintegration of cell clusters, with most cells exhibiting these alterations (Figs 4 and 5). The induction of these changes at TGI concentrations suggests that the extracts' mechanisms, including apoptosis, are consistent with the results of the MTT and LDH assays.

Beyond the individual activities of phenolic compounds, the synergistic effects of their combined presence should also be considered. The diversity of compounds in extracts from different organs of the EM suggests that biological effects may be the result of multiple compound interactions rather than a single major compound. Future fractionation and biodirected isolation studies could clarify which phenolic combinations contribute most significantly to tyrosinase inhibitory or antiproliferative activity.

CONCLUSION

This study highlighted the significant potential of *E. macrophylla* Jaub. & Spach, an endemic plant species in Türkiye, as a source of bioactive compounds with various biological effects. Phytochemical analysis revealed the presence of high levels of hesperidin, isoquercitrin, catechin, shikimic acid, and vanillic acid, with distinct distributions in different plant organs. The unique chemical profiles of different organ extracts led to various biological activities, such as antioxidant, antiproliferative, antimicrobial, and enzyme-inhibitory effects. The leaf extract showed significant antioxidant activity and strong antibacterial effects. It exhibited potent enzyme inhibition, indicating its potential as an antidiabetic and skin-whitening agent. The root extract showed promising antiproliferative effects against cancer cell lines such as A549, A2780, and HeLa, with low toxicity to normal cells.

In conclusion, *E. macrophylla* Jaub. & Spach can be suggested as a significant natural resource for the development of potent antioxidant, antimicrobial, and anticancer agents. Further studies are needed to confirm *in vivo* efficacy and investigate the detailed mechanisms of biological action.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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