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Integrated Chemical and Biological Characterization of *Hypericum perforatum* Extract Using LC-MS/MS and in Vitro Functional Assays

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Abstract

Hypericum perforatum L. (St. John's wort) is a medicinal plant known for its diverse secondary metabolites and pharmacological potential. This study provides a comprehensive evaluation of the phytochemical composition and bioactivities of methanolic extracts from wild *H. perforatum*. LC-MS/MS analysis identified 36 compounds, including phenolic acids (chlorogenic acid, protocatechuic acid) and flavonoids (rutin, isoquercitrin, quercetin), with the first-time detection of genkwanin, vicenin-2, schaftoside, and afzelin, to the best of our knowledge. The extract demonstrated significant antioxidant activity, with a total phenolic content of 203.04 mg GAE/g DW and strong radical scavenging in DPPH (544.78 mg TE/g DW) and ABTS (312.15 mg TE/g DW) assays. In vitro assays showed marked cytotoxicity across multiple cancer cell lines, with IC₅₀ values as low as 3.57 µg/mL in A549 cells, indicating stronger activity compared to cisplatin. Flow cytometric analysis revealed substantial apoptosis induction (up to 53.67% in A549 cells) and G₀/G₁ cell cycle arrest (76.37% in A549). The extract also exhibited moderate antibacterial activity against *E. coli* and *S. aureus*, but no activity against *P. aeruginosa* or *Candida* species. These results position *H. perforatum* as a rich source of bioactive compounds with antioxidant, anticancer, and antimicrobial potential, and suggest further research into its therapeutic applications.

Keywords: Antioxidant activity, Apoptosis, Cell cycle arrest, Cytotoxicity, *Hypericum perforatum*, LC-MS/MS, Phytochemical composition

Introduction

In recent decades, there has been a growing scientific interest in plant-derived bioactive compounds due to their diverse therapeutic potential and generally favorable safety profiles. Among these, *H. perforatum* L. (commonly known as St. John's Wort) has emerged as a prominent medicinal herb with a well-documented ethnopharmacological background and a broad spectrum of biological activities [1]. This perennial herb, belonging to the Hypericaceae

family, comprises over 450 species globally and is widely distributed across Europe, Asia, and parts of North Africa and the Americas [2, 3]. In Türkiye alone, 119 taxa of *Hypericum* have been reported, 49 of which are endemic [4, 5].

H. perforatum has been traditionally used for centuries to treat wounds, burns, gastrointestinal ailments, and mental health conditions such as mild to moderate depression [6-10]. The pharmacological potential of this plant has been attributed to its rich phytochemical profile, which includes naphthodianthrone (e.g., hypericin, pseudohypericin), phloroglucinols (e.g., hyperforin), flavonoids (e.g., quercetin, hyperoside), and other phenolic compounds [2, 5]. These constituents confer antioxidant, anti-inflammatory, antidepressant, antiviral, antimicrobial, and anticancer effects, as documented in numerous in vitro and in vivo studies [11, 12].

Of particular interest is the potential anticancer activity of *H. perforatum*. Recent studies have shown that its extracts can exert cytotoxic and antiproliferative effects on various cancer cell lines, including glioblastoma, leukemia, breast, lung, and colon cancers, often through induction of apoptosis and oxidative stress mechanisms [13-15]. Moreover, bioactive components such as hyperforin and hypericin have been investigated as radiosensitizers, potentially enhancing the efficacy of radiotherapy while minimizing the need for high-dose radiation [13].

While numerous studies have investigated the phytochemical content or biological effects of *H. perforatum* separately, few have combined detailed LC-MS/MS-based chemical profiling with broad-spectrum in vitro bioactivity screening. This integrated approach offers a more holistic view to the current literature on the plant's therapeutic potential.

The present study aimed to perform an integrated chemical and biological characterization of *H. perforatum* using a methanolic extract prepared with 0.05% HCl to enhance phytochemical solubility. The extract was evaluated for its total phenolic content (TPC) and antioxidant activity via DPPH and ABTS assays. In vitro assays were conducted to assess cytotoxicity (MTT), apoptosis, and cell cycle effects across a panel of cancer (A549, SH-SY5Y, HCT116, HeLa, MCF-7, MDA-MB-231) and normal (BEAS-2B) cell lines. Additionally, LC-MS/MS analysis was performed to identify major phytochemicals, and antimicrobial activity was evaluated against clinically relevant microorganisms (*Candida albicans*, *Candida glabrata*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*).

Results

TPC and Antioxidant Activity

The TPC of the *H. perforatum* extract was quantified to assess its polyphenol richness, a key indicator of antioxidant potential. The TPC value was determined to be 203.04 ± 4.36 mg GAE/g extract, based on the calibration curve obtained using gallic acid as the standard. In addition, the antioxidant capacity of the extract was evaluated using two widely accepted radical scavenging assays: DPPH and ABTS. According to the DPPH assay, the extract demonstrated a radical scavenging capacity of 544.78 ± 5.17 mg TE/g extract, while the ABTS assay yielded a value of 312.15 ± 11.32 mg TE/g extract. These data are summarized in Table 1.

Table 1. TPC and antioxidant activities of *H. perforatum* extract

	TPC (mg GAE/g)	DPPH (mg TE/g)	ABTS (mg TE/g)
Extract	203.04 ± 4.36	544.78 ± 5.17	312.15 ± 11.32

TPC: Total phenolic content.

LC-MS/MS Analysis of Phytochemical Compounds

The phytochemical composition of the *H. perforatum* extract was comprehensively characterized using LC-MS/MS, which allowed for the identification and quantification of a total of 36 distinct compounds (Table 2). Standard concentrations of the phytochemical compounds used in the analysis ranged from 10 to 100 ng/mL. External calibration curves were generated by analyzing three replicates of each concentration level. The total ion chromatogram of the phytochemical standard mixture at 100 ng/mL is presented in Figure S1A, while the total ion chromatogram of the *H. perforatum* extract is shown in Figure S1B (provided as supplementary material).

The major classes of compounds detected included phenolic acids, flavonoids, and other bioactive constituents. Among the phenolic acids, chlorogenic acid was the most abundant (17673.90 $\mu\text{g/g}$ dry extract), followed by quinic acid (10795.86 $\mu\text{g/g}$), protocatechuic acid (635.47 $\mu\text{g/g}$), and vanillic acid (398.73 $\mu\text{g/g}$). Other phenolic acids such as gallic acid (94.83 $\mu\text{g/g}$), caffeic acid (178.95 $\mu\text{g/g}$), ferulic acid (302.67 $\mu\text{g/g}$), and coumaric acid (210.73 $\mu\text{g/g}$) were also quantified.

Among the flavonoid compounds, rutin hydrate was present in the highest concentration (14844.99 $\mu\text{g/g}$), followed by isoquercitrin (11789.00 $\mu\text{g/g}$), quercetin (2844.80 $\mu\text{g/g}$), and catechin (1873.21 $\mu\text{g/g}$). Notably, genkwanin was also present at a substantial level (2016.40 $\mu\text{g/g}$). Other flavonoids identified included luteolin (708.60 $\mu\text{g/g}$), narcissin (40.68 $\mu\text{g/g}$), kaempferol (53.06 $\mu\text{g/g}$), and apigenin (35.70 $\mu\text{g/g}$).

Additional minor compounds identified included vanillin, emodin, oleuropein, acacetin, and beta-resorcylic acid, with concentrations generally below 150 $\mu\text{g/g}$ dry extract. Several glycosylated flavonoids, such as luteolin 7-rutinoside, vicianin-2, schaftoside, and afzelin,

were also detected. Overall, the LC-MS/MS data indicate a chemically diverse profile, with a predominance of flavonoids and phenolic acids in the *H. perforatum* extract (Table 2).

Table 2. Results of phytochemical compound analysis of *H. perforatum* extract

Compounds	µg/g dry extract	Compounds	µg/g dry extract
4-Hydroxybenzoic acid	53.28	Acacetin	5.25
Salicylic acid	25.70	Vicenin 2	40.30
3-hydroxybenzoic acid	110.93	Genkwanin	2016.40
Gallic acid	94.83	Vitexin	10.86
Protocatechuic acid	635.47	Schaftoside	76.02
Protocatechuic aldehyde	0.63	Rutin hydrate	14844.99
beta-Resorcylic acid	14.71	Luteolin	708.60
Vanillic acid	398.73	Diosmetin	49.25
Vanillin	145.79	Luteolin 7-rutinoside	32.65
trans-Cinnamic acid	10.38	Quercetin	2844.80
Coumaric acid	210.73	Isoquercitrin	11789.00
Caffeic acid	178.95	Narcissin	40.68
Ferulic acid	302.67	Kaempferol	53.06
Sinapic acid	25.01	Afzelin	117.22
Chlorogenic acid	17673.90	Nicotiflorin	535.62
Quinic acid	10795.86	Naringenin	15.91
Catechin	1873.21	Oleuropein	27.53
Apigenin	35.70	Emodin	6.88

Cytotoxic Activity

The cytotoxic effects of the *H. perforatum* extract were evaluated using the MTT assay across six human cancer cell lines (A549, HCT116, SH-SY5Y, HeLa, MCF-7, MDA-MB-231) and one non-cancerous epithelial cell line (BEAS-2B). Cells were exposed to a concentration range of 7.8 to 2000 µg/mL of the extract, and cell viability was determined after 24 hours. As shown in Table S1, the extract of *H. perforatum* exhibited cytotoxic activity across all tested cell lines. The lowest IC₅₀ value was observed in the A549 lung cancer cell line (3.57 ± 0.11 µg/mL), followed by MDA-MB-231 (4.03 ± 0.19 µg/mL), MCF-7 (4.16 ± 0.10 µg/mL), HeLa (4.15 ± 0.12 µg/mL), HCT116 (5.45 ± 0.21 µg/mL), and SH-SY5Y (14.18 ± 1.44 µg/mL). In the non-cancerous BEAS-2B epithelial cell line, the IC₅₀ value was 5.44 ± 0.19 µg/mL. For comparison, the IC₅₀ values of cisplatin ranged from 27.30 ± 0.51 µg/mL in BEAS-2B to 76.33 ± 0.59 µg/mL in HCT116 cells.

These results provide a comparative overview of the extract's cytotoxic potential relative to a standard chemotherapeutic agent. All data are summarized in Supplementary Table S1 (provided as supplementary material) and visualized in Figure 1.

Flow Cytometric Evaluation of Apoptosis

The apoptotic effects of the *H. perforatum* extract were evaluated in six human cancer cell lines (A549, SH-SY5Y, HCT116, HeLa, MCF7, MDA-MB-231) and one non-cancerous

epithelial cell line (BEAS-2B) using Annexin V-FITC/PI double staining followed by flow cytometric analysis. Cells were treated with the extract at a concentration of 2.5 $\mu\text{g/mL}$ for 24 hours, and total apoptosis rates (early + late apoptosis) were quantified (Table 3, Figure 2). Compared to the control groups, extract treatment significantly increased the percentage of apoptotic cells in all tested cell lines ($p < 0.001$). Among cancer cell lines, the highest

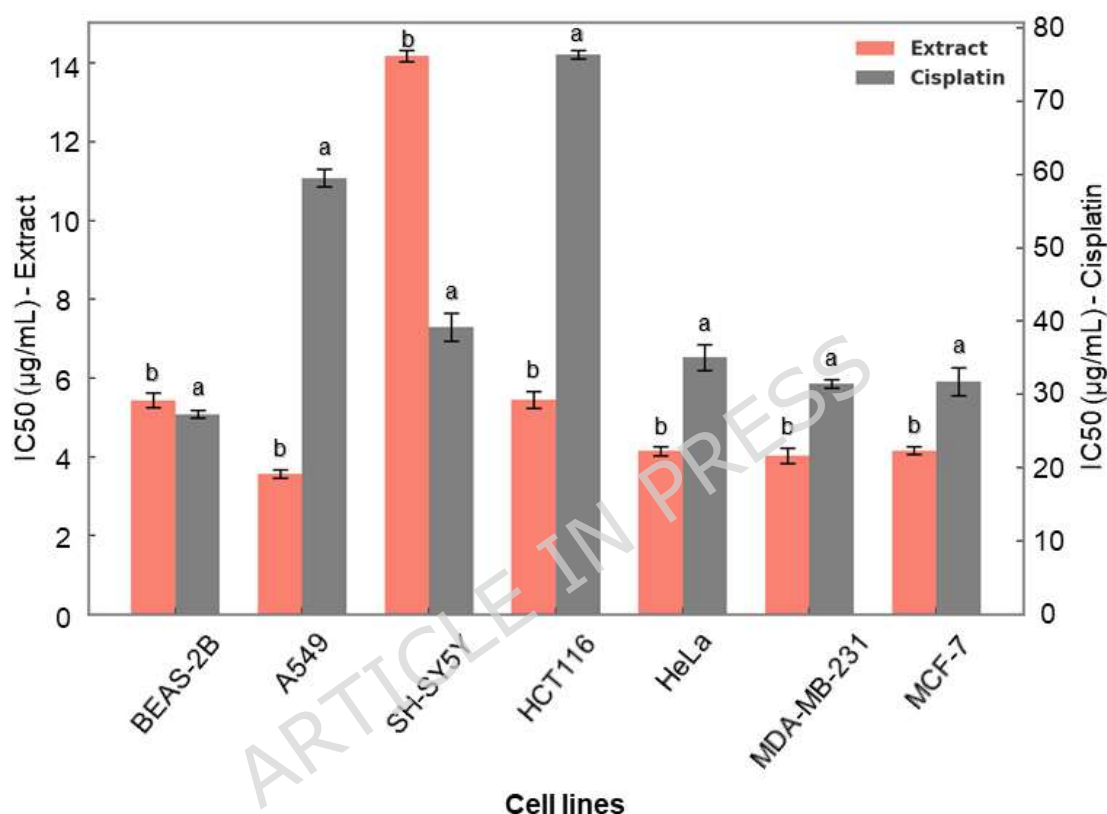


Figure 1. The cytotoxic effects of *H. perforatum* extract and cisplatin on healthy and cancerous cells (BEAS-2B, A549, SH-SY5Y, HCT116, HeLa, MDA-MB-231, MCF-7): The bar plots represent the mean IC₅₀ values ($\mu\text{g/mL}$) $\pm$ standard deviation (SD) from three independent experiments. Different letters indicate statistically significant differences ($p < 0.001$).

apoptotic response was observed in A549 cells ($53.67 \pm 1.00\%$), followed by MDA-MB-231 ($42.00 \pm 1.71\%$), MCF7 ($37.47 \pm 1.10\%$), HeLa ($31.10 \pm 1.60\%$), HCT116 ($29.30 \pm 1.23\%$), and SH-SY5Y ($12.53 \pm 0.60\%$). In the non-cancerous BEAS-2B cells, the extract induced a total apoptosis rate of $27.20 \pm 0.75\%$.

In contrast, the control groups exhibited low baseline apoptosis levels ranging from $3.70 \pm 0.26\%$ in BEAS-2B to $6.23 \pm 0.42\%$ in MCF7. Cisplatin treatment (2.5 $\mu\text{g/mL}$), used as a reference compound, also increased apoptosis compared to control, but the levels were

lower than those observed for the extract in several cell lines, including A549 and MDA-MB-231.

Table 3. Percentage of total apoptotic cells (early + late apoptosis) on healthy and cancerous cells (BEAS-2B, A549, SH-SY5Y, HCT116, HeLa, MDA-MB-231, MCF-7) following 24-hour treatment with *H. perforatum* extract and cisplatin

	BEAS-2B	A549	SH-SY5Y	HCT116	HeLa	MDA-MB-231	MCF-7
Control	3.70±0.26 ^c	4.80±0.50 ^b	4.13±0.25 ^c	5.07±0.15 ^b	5.37±0.50 ^b	4.47±0.51 ^c	6.23±0.42 ^c
Extract	27.20±0.75 ^a	53.67±1.00 ^a	12.53±0.60 ^a	29.30±1.23 ^a	31.10±1.60 ^a	42.00±1.71 ^a	37.47±1.10 ^a
Cisplatin	11.67±0.71 ^b	5.23±0.42 ^b	5.77±0.42 ^b	6.23±0.40 ^b	7.30±0.46 ^b	8.60±0.62 ^b	10.03±0.83 ^b
<i>p</i>	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data are presented as mean ± standard deviation (SD) from three independent experiments. All treatment groups showed significantly higher apoptosis rates compared to the untreated control group ($p < 0.001$).

Effects on Cell Cycle Distribution

The impact of *H. perforatum* extract on cell cycle progression was assessed by flow cytometry following 24-hour treatment. As presented in Table 4 and Figure 3, treatment with the extract resulted in significant alterations in cell cycle distribution across all tested cell lines.

Table 4. Distribution (%) of cells in G0/G1, S, and G2/M phases following 24-hour treatment with *H. perforatum* extract and cisplatin on BEAS-2B, A549, SH-SY5Y, HCT116, HeLa, MDA-MB-231, and MCF-7 cell lines

	BEAS-2B	A549	SH-SY5Y	HCT116	HeLa	MDA-MB-231	MCF-7
G0/G1							
Control	52.27±0.86 ^b	54.83±0.55 ^b	58.80±0.85 ^b	57.67±0.55 ^b	60.70±0.26 ^b	54.77±0.42 ^b	54.37±1.53 ^b
Extract	68.27±1.39 ^a	76.37±0.74 ^a	65.40±0.70 ^a	68.27±0.83 ^a	70.27±0.86 ^a	71.47±0.74 ^a	68.90±1.41 ^a
Cisplatin	45.27±1.37 ^c	10.10±1.25 ^c	60.47±1.20 ^b	47.47±1.02 ^c	51.30±0.62 ^c	26.33±0.71 ^c	38.50±1.44 ^c
<i>p</i> Value	<0.001	<0.001	<0.003	<0.001	<0.001	<0.001	<0.001
S							
Control	21.07±0.23 ^a	19.33±0.31 ^a	21.20±0.89 ^a	20.33±0.70 ^a	18.77±0.31 ^a	25.87±0.65 ^b	20.13±1.33 ^a
Extract	18.37±0.74 ^b	14.20±0.70 ^b	17.70±0.70 ^b	18.50±0.90 ^a	7.30±0.82 ^b	7.37±1.01 ^c	19.50±0.44 ^a
Cisplatin	20.43±0.76 ^a	19.50±0.60 ^a	7.90±0.82 ^c	9.53±1.40 ^b	8.30±0.40 ^b	30.23±0.85 ^a	10.83±0.51 ^b
<i>p</i> Value	<0.0044	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
G2/M							
Control	26.43±1.08 ^b	24.93±0.57 ^b	19.53±0.55 ^b	20.70±0.75 ^b	19.27±0.15 ^b	18.50±0.87 ^b	25.40±0.44 ^b
Extract	10.10±0.20 ^c	6.43±0.47 ^c	15.67±0.49 ^c	11.17±0.42 ^c	17.53±0.67 ^b	16.30±0.92 ^b	11.13±1.59 ^c
Cisplatin	34.43±0.35 ^a	67.43±0.61 ^a	28.70±2.42 ^a	40.40±0.66 ^a	39.10±1.21 ^a	39.80±1.28 ^a	48.73±0.67 ^a
<i>p</i> Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data are presented as mean ± standard deviation (SD) from three independent experiments. Different letters indicate statistically significant differences ($p < 0.001$).

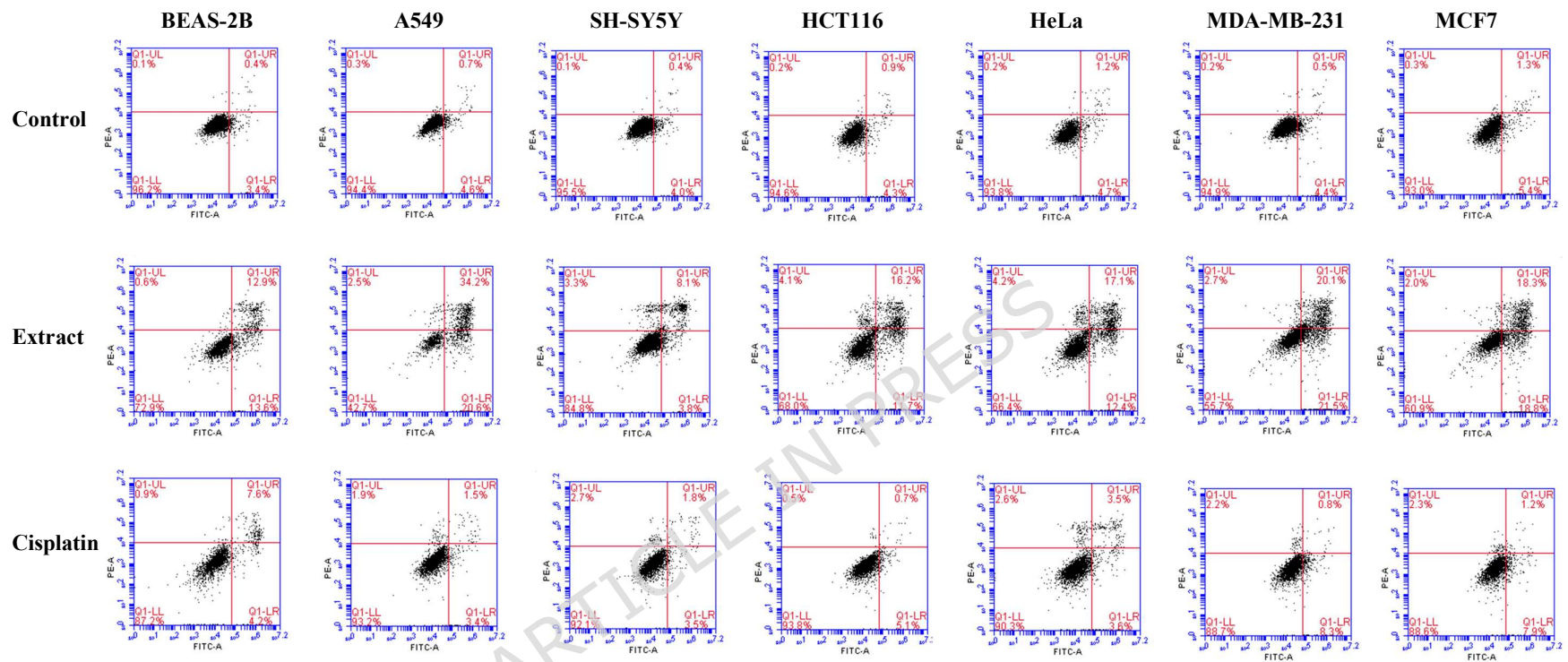


Figure 2. Flow cytometry dot plots showing apoptosis profiles on BEAS-2B, A549, SH-SY5Y, HCT116, HeLa, MDA-MB-231, and MCF-7 cells after 24-hour treatment with *H. perforatum* extract and cisplatin, using Annexin V-FITC/PI staining

In all cell types, the proportion of cells in the G0/G1 phase significantly increased upon extract exposure. The most prominent G0/G1 arrest was observed in A549 ($76.37 \pm 0.74\%$) and MDA-MB-231 ($71.47 \pm 0.74\%$) cells, compared to their respective controls ($54.83 \pm 0.55\%$ and $54.77 \pm 0.42\%$). Similarly, G0/G1 accumulation was detected in non-cancerous BEAS-2B cells ($68.27 \pm 1.39\%$) and other cancer lines including SH-SY5Y ($65.40 \pm 0.70\%$), HCT116 ($68.27 \pm 0.83\%$), HeLa ($70.27 \pm 0.86\%$), and MCF-7 ($68.90 \pm 1.41\%$).

Corresponding to G0/G1 accumulation, a decrease in S phase population was observed in most cell lines. Notably, the S phase percentage dropped from $19.33 \pm 0.31\%$ to $14.20 \pm 0.70\%$ in A549, and from $25.87 \pm 0.65\%$ to $7.37 \pm 1.01\%$ in MDA-MB-231 cells following extract treatment.

In the G2/M phase, extract-treated cells generally showed reduced percentages compared to control groups. For instance, G2/M phase distribution in BEAS-2B decreased from $26.43 \pm 1.08\%$ to $10.10 \pm 0.20\%$, and in A549 from $24.93 \pm 0.57\%$ to $6.43 \pm 0.47\%$. In contrast, cisplatin treatment led to marked G2/M phase accumulation across all cell lines, consistent with its known mechanism of action.

These results indicate that treatment with *H. perforatum* extract altered the distribution of cells across the G0/G1, S, and G2/M phases in all tested cell lines. The most notable shift was an increase in the G0/G1 phase population accompanied by a decrease in S and G2/M phase percentages, as shown in Table 4 and Figure 3.

Antimicrobial Activity

The antimicrobial potential of *H. perforatum* extract was assessed using the disc diffusion method against selected fungal, Gram-negative, and Gram-positive strains. As shown in Table 5, the extract exhibited antibacterial activity against *E. coli* (11.67 ± 0.46 mm) and *S. aureus* (10.23 ± 0.90 mm), while no inhibition was observed against *P. aeruginosa*, *C. albicans*, or *C. glabrata*. In contrast, the positive controls showed larger inhibition zones: ampicillin showed activity against *E. coli* (14.27 ± 0.61 mm), *P. aeruginosa* (12.50 ± 0.40 mm), and *S. aureus* (15.33 ± 0.21 mm), while caspofungin was effective against *C. albicans* (14.30 ± 0.15 mm) and *C. glabrata* (20.57 ± 0.61 mm).

The antimicrobial screening revealed selective inhibitory effects of the *H. perforatum* extract, with measurable activity against specific Gram-positive and Gram-negative bacterial strains. No antifungal activity was observed under the experimental conditions.

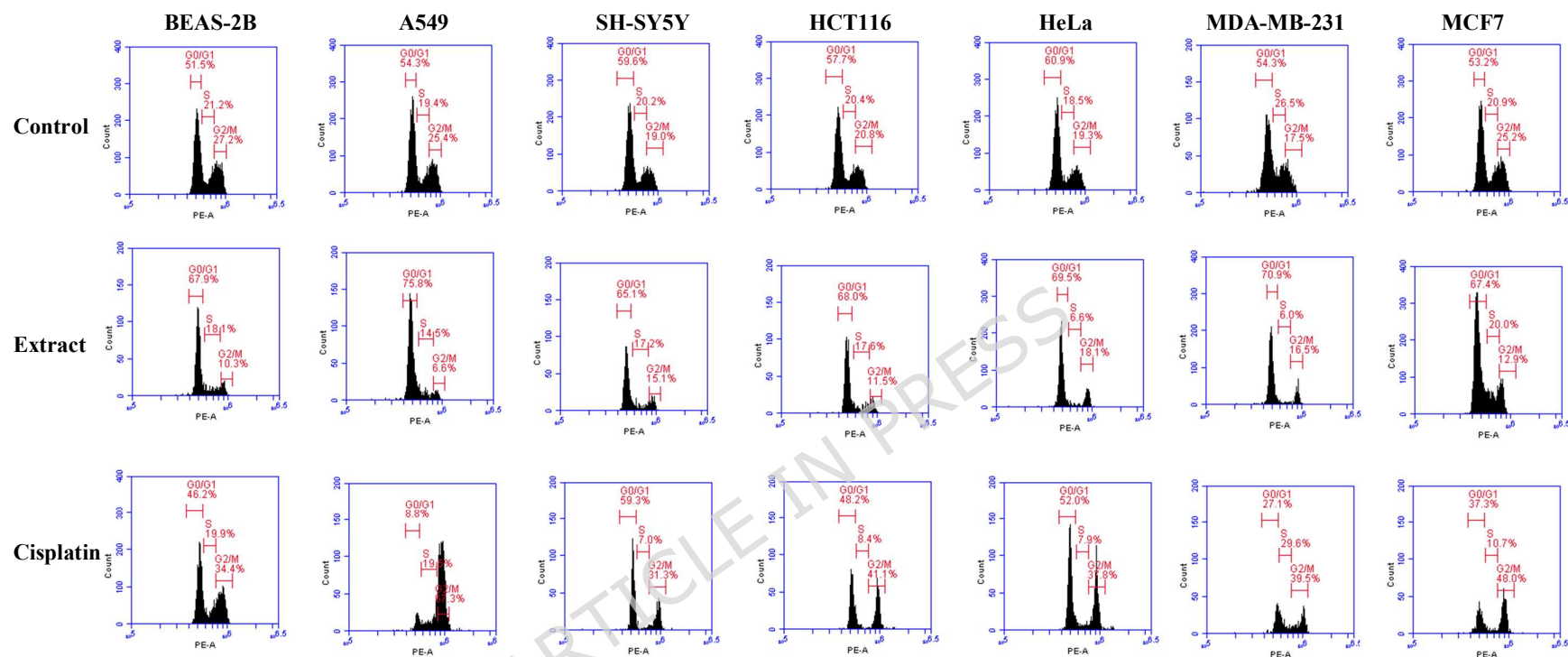


Figure 3. Representative histograms showing the distribution of cell cycle phases (G0/G1, S, and G2/M) in BEAS-2B, A549, SH-SY5Y, HCT116, HeLa, MDA-MB-231, and MCF-7 cells following 24-hour treatment with *H. perforatum* extract, and cisplatin

Table 5. Inhibition zone diameters (mm) representing the antimicrobial activity of *H. perforatum* extract and standard antibiotics (casprofungin and ampicillin) against selected microbial strains

	Fungi		Gram-negative		Gram-positive
	<i>C. albicans</i>	<i>C. glabrata</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
Extract	-	-	11.67±0.46 ^b	-	10.23±0.90 ^b
Casprofungin	14.30±0.15	20.57±0.61	-	-	-
Ampicillin	-	-	14.27±0.61 ^a	12.50±0.40	15.33±0.21 ^a

Results are presented as mean ± standard deviation (SD) from three independent experiments. Different letters indicate statistically significant differences ($p < 0.001$).

Discussion

The present study investigated the phytochemical composition and biological activities of *H. perforatum* extract, focusing on its antioxidant, antimicrobial, and anticancer properties. LC-MS/MS phytochemical profiling revealed a broad spectrum of secondary metabolites, including phenolic acids, flavonoids, and anthraquinones, such as gallic acid, chlorogenic acid, rutin, isoquercitrin, and emodin—compounds that have been previously associated with diverse pharmacological effects. These findings were supported by in vitro analyses that demonstrated notable antioxidant capacity, selective antimicrobial effects particularly against Gram-positive bacteria, and measurable cytotoxicity against various human cancer cell lines. The results obtained from TPC and antioxidant activity measurements offer valuable information on the phenolic profile and antioxidant properties of the *H. perforatum* extract. The quantified TPC value (203.04 ± 4.36 mg GAE/g) reflects a notable richness in phenolic substances, which are widely associated with biological functions including antioxidative, antimicrobial, anti-inflammatory, and anticancer effects [16]. Previous studies have reported a broad range of TPC outcomes depending on the type of solvent and environmental factors affecting the plant. For example, Tahirović et al. [17] observed 274.5 mg GAE/100 mL in infusion tea made from wild flora collected in Serbia, while water extracts from cultivated plants in Iran yielded only 0.051 mg/g GAE [18]. In comparison, our methanolic extract shows substantially higher phenolic content, underlining the efficiency of acidified methanol as an extraction solvent. Kilibarda et al. [19] also confirmed solvent-based variation, noting 26.48 mg/g GAE (infusion tea) and 31.38 mg/g GAE (methanolic extract), both markedly lower than our findings. Likewise, Błońska-Sikora et al. [20] reported values depending on solvent choice, with 599.74 mg GAE/100 mL (glycerol–water extract) and 265.64 mg GAE/100 mL (propylene glycol–water extract), further emphasizing the influence of extraction medium. Regarding antioxidant performance, our extract exhibited strong radical scavenging activity, with 544.78 ± 5.17 mg TE/g (DPPH) and 312.15 ± 11.32 mg TE/g (ABTS). Interestingly, the higher DPPH activity compared to ABTS suggests that the methanol-based extract is richer

in lipophilic compounds, which typically react more readily with DPPH radicals [21, 22]. This contrasts with aqueous extracts, which often display superior ABTS activity due to their higher hydrophilic phenolic content. Kilibarda et al. [19] reported much lower activities for methanolic extracts—149.99 $\mu\text{mol Trolox/g DW}$ (DPPH) and 130.49 $\mu\text{mol Trolox/g DW}$ (ABTS)—supporting the notion that solvent polarity and extraction technique critically determine antioxidant capacity. Their infusion tea extracts also showed 132.96 $\mu\text{mol Trolox/g DW}$ (DPPH) and 176.48 $\mu\text{mol Trolox/g DW}$ (ABTS), illustrating a reverse pattern compared to our methanolic findings, consistent with solvent-dependent selectivity.

Wholly, the comparatively high TPC, DPPH, and ABTS values obtained in this study highlight the superior antioxidant potential of *H. perforatum* methanolic extract from spontaneous flora. The variability observed across different reports can be explained by a combination of geographic origin, plant stress factors, and solvent systems [23]. Our results reinforce the central role of phenolic compounds in antioxidant defense and further support the traditional and pharmacological significance of *H. perforatum* as a valuable natural source of bioactive molecules [16].

The LC-MS/MS analysis demonstrated a chemically rich extract, with 36 identified compounds predominantly consisting of phenolic acids (e.g., chlorogenic acid, quinic acid, protocatechuic acid) and flavonoids (e.g., rutin hydrate, isoquercitrin, quercetin), all well-documented for their potent antioxidant and cytotoxic activities. These findings are consistent with previous reports highlighting the dominance of similar phytochemicals in *H. perforatum* extracts. In particular, Budantsev et al. [1] reported the prevalence and biological relevance of chlorogenic acid and rutin in *H. perforatum* extracts, while K1sa et al. [5] demonstrated that chlorogenic acid, rutin, and isoquercitrin were the major compounds detected when using a range of solvents, including methanol, hexane, butanol, and ethyl acetate.

Comparable findings have also been reported in recent literature. Kilibarda et al. [19], employing a UHPLC-QToF-MS platform, identified a wide range of phenolic compounds in *H. perforatum* methanolic extract and infusion, including hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid, caffeic acid, chlorogenic acid, catechin, kaempferol, quercetin, rutin, naringenin, and biapigenin/apigenin (apigenin also exists as a dimer, biapigenin, mainly isolated from the buds and flowers of *H. perforatum*). In contrast to our study, additional compounds such as rosmarinic acid, syringic acid, epicatechin, procyanidin B2, myricetin, quercitrin, and hyperoside were also detected. Similarly, B1ońska-Sikora et al. [20] analyzed *H. perforatum* extracts prepared with glycerol–water and propylene glycol–water as solvents, using HPLC, and reported the presence of neochlorogenic acid, *p*-

coumaric acid, chlorogenic acid, rutin, isoquercitrin, and hyperoside. Distinct from our findings, neochlorogenic acid and hyperoside were identified in their extracts. These discrepancies may be attributed to differences in analytical platforms and compound databases, as certain metabolites may not be included or defined within specific LC-MS/MS libraries.

Among the identified compounds, genkwanin was detected at a notably high concentration (2016.40 $\mu\text{g/g}$ dry extract). Genkwanin is a non-glycosylated flavone [24] previously reported in various medicinal plants such as *Vernonia fasciculata* [25], *Daphne genkwa* [26], *Ocimum basilicum* [27], *Salvia officinalis* [28], *Rosmarinus officinalis* [29], and *Artemisia iwayomogi* [30]. To the best of our knowledge, this is the first report of genkwanin in *H. perforatum*. The pharmacological relevance of this compound has been well-documented, with studies demonstrating its anti-inflammatory potential [31], antibacterial activity [32], chemopreventive properties [33], and significant anti-tumour effects [34, 35]. The presence of genkwanin at such a substantial concentration in the extract may therefore contribute synergistically to the observed bioactivities of *H. perforatum*, particularly in terms of anti-inflammatory and anticancer effects.

In addition to genkwanin, our analysis revealed several other compounds identified in *H. perforatum* for the first time, to the best of our knowledge. These include vicenin-2, previously isolated from *Artemisia capillaris* [36] and reported to exhibit potent anti-diabetic activity; schaftoside, a flavonoid glycoside known from *Clinacanthus nutans* [37], and afzelin, a flavonol glycoside identified in *Nymphaea odorata* [38], associated with both antibacterial and antitumor effects. The discovery of these metabolites in *H. perforatum* significantly broadens its phytochemical spectrum and highlights novel contributors to its biological activity profile. Such findings not only enrich our understanding of the plant's chemical diversity but also provide valuable leads for future pharmacological investigations.

Furthermore, the detection of vitexin, a flavonoid that has been quantified in *Hypericum montbretii* at 0.96 mg/g [39], and acetin, previously identified in *H. perforatum* callus cultures at 0.10 mg/L [40], adds significance to our results. These findings confirm the potential of *H. perforatum* to produce a wide array of secondary metabolites beyond the commonly studied constituents, underlining the plant's diverse pharmacological potential and possible applications in phytotherapy.

In addition to the major constituents, several phenolic acids from the hydroxybenzoic and hydroxycinnamic acid classes were also detected in the extract, with concentrations ranging between 14.71 and 635.47 $\mu\text{g/g}$ dry weight. These included 4-hydroxybenzoic acid

(antihypertensive) [41], β -resorcylic acid, vanillic acid, protocatechuic acid (hepatic and cardiovascular protection) [42], p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid. Although these compounds were present at lower levels compared to chlorogenic acid or rutin, they are widely recognized for their shared therapeutic properties, including anti-inflammatory [43-45], antioxidant [46, 47], antitumour [48, 49], metabolic regulatory [50, 51], neuroprotective [52], and antimicrobial [53, 54]. The coexistence of such a broad spectrum of bioactive phenolic acids suggests that the pharmacological profile of *H. perforatum* is shaped not only by its dominant metabolites but also by the synergistic contributions of these minor compounds.

The extract of *H. perforatum* demonstrated pronounced cytotoxicity across all tested cancer cell lines, with IC₅₀ values ranging from 3.57 μ g/mL (A549) to 14.18 μ g/mL (SH-SY5Y). These values are markedly lower than those of the standard chemotherapeutic agent cisplatin, whose IC₅₀ values in our study ranged from 31.44 μ g/mL (MDA-MB-231) to 76.33 μ g/mL (HCT116), measured after 24 hours of incubation, highlighting the comparatively higher cytotoxicity of the extract based on IC₅₀ values measured under the same experimental conditions. The IC₅₀ values obtained for cisplatin in our study (27.30–76.33 μ g/mL, depending on the cell line) are comparable to those reported in the literature for similar cancer cell models. For instance, cisplatin has been shown to exert IC₅₀ values of 4.00 μ M in HeLa cells and 12.74 μ M in A549 cells [60], while the LD₅₀ for MCF-7 breast cancer cells was reported as 20 μ g/mL [63]. In the present study, cisplatin IC₅₀ values were 27.30 μ g/mL in BEAS-2B, 31.44 μ g/mL in MDA-MB-231, and 76.33 μ g/mL in HCT-116 cells (Uğur et al., 2025). These data demonstrate that our cisplatin results are within the expected range observed in previous studies, confirming the reliability and reproducibility of the cytotoxicity assays used.

Comparable findings have been reported in previous studies: methanolic and lipophilic extracts of *H. perforatum* exhibited strong cytotoxic effects against bladder cancer cells (T24, NBT-II), with LC₅₀ values as low as 4–5 μ g/mL [55]. Similarly, extracts showed dose-dependent growth inhibition and apoptosis induction in leukemia and glioblastoma cells, with GI₅₀ values between 0.43 and 1.77 mg/mL depending on plant material and extract type [56]. The cytotoxicity observed in our study is also consistent with reports in colon carcinoma cells, where *H. perforatum* extracts suppressed NF- κ B signaling and significantly reduced proliferation [57], and in breast and colorectal tumor cells, where methanolic extracts of *Hypericum* species decreased viability in MCF-7 and SW480 cell lines [58]. Furthermore, in prostate cancer xenografts, methanolic extracts of *H. perforatum* significantly reduced tumor

growth and metastatic spread in vivo [59]. Recent studies also confirm selective cytotoxic effects on 2D and 3D models of HeLa, K562, and A549 cells, demonstrating induction of apoptosis and reduced metastatic markers such as MMP2 and VEGFA [60].

Overall, these findings suggest that the cytotoxicity of *H. perforatum* extract is not confined to a single cancer type but rather reflects a broad anticancer potential. The relatively low IC₅₀ values observed in our study, particularly against A549 and MDA-MB-231 cells, support the notion that polyphenolic-rich extracts of *H. perforatum* could represent a promising multi-targeted therapeutic option.

Annexin V/PI staining demonstrated that the *H. perforatum* extract induced apoptosis in a dose- and cell line-dependent manner. The most pronounced effect was observed in A549 lung carcinoma cells, where the proportion of apoptotic cells reached 53.67%, compared to 27.20% in non-malignant BEAS-2B epithelial cells. A moderate apoptotic response was also detected in MDA-MB-231 and MCF-7 breast carcinoma cells, whereas SH-SY5Y neuroblastoma cells exhibited the lowest sensitivity (12.53% apoptosis). These findings indicate a degree of selectivity toward cancer cells, consistent with the relatively low cytotoxicity against normal cells observed in MTT assays.

Comparable apoptotic effects of *H. perforatum* extracts have been documented in several malignancies. Hostanska et al. [56] reported that aqueous-ethanolic extracts induced apoptosis in leukemia (K562, U937) and glioblastoma (LN229) cells, confirmed by Annexin V/PI flow cytometry, even in the absence of light exposure. Similarly, Roscetti et al. [61] observed significant apoptotic death in K562 leukemia cells treated with *H. perforatum* methanolic flower extract, whereas purified hypericin alone failed to induce comparable apoptosis, suggesting a synergistic contribution of multiple metabolites. Valletta et al. [62] further confirmed that *H. perforatum* total extracts reduce growth and trigger apoptosis in K562 cells, with variability depending on extraction method and plant origin.

Evidence from other cancer models supports these pro-apoptotic effects. In breast carcinoma, hypericin treatment induced up to 52% apoptosis in MCF-7 cells, a level comparable to cisplatin treatment (60%), through modulation of p53 and Bcl-2 expression [63]. In basal cell carcinoma, *H. perforatum* extract increased the expression of pro-apoptotic proteins (caspase-3, Bax, AIF) and decreased Bcl-2, while simultaneously suppressing inflammatory mediators such as NF- κ B and COX-2 [64]. More recently, Pazarcı & Kaplan [65] demonstrated that extracts upregulated GRP78, GADD153, Bax, and cleaved caspase-3 in osteosarcoma cells, further corroborating activation of intrinsic apoptotic pathways.

Collectively, these data, together with previous reports, suggest that *H. perforatum* extracts can promote apoptosis. Earlier studies demonstrated the involvement of caspases, p53, and Bcl-2 family proteins in this process, which may also underlie the apoptotic effects observed in our experiments. The relatively high apoptotic indices observed in A549 and MDA-MB-231 cells in our study support the hypothesis that the anticancer potential of *H. perforatum* arises not only from growth inhibition but also from the active promotion of programmed cell death.

Flow cytometry revealed that *H. perforatum* extract induced marked alterations in cell cycle progression. The most prominent effect was observed in A549 lung carcinoma cells, where G0/G1 arrest increased to 76.37%, accompanied by a reduction in S and G2/M phase populations. Similar but less pronounced effects were also evident in MDA-MB-231 and MCF-7 breast carcinoma cells. In contrast, cisplatin treatment in our experiments predominantly resulted in G2/M phase accumulation, indicating that the extract and the chemotherapeutic agent may act through distinct mechanisms.

Our results align with previous reports showing that *H. perforatum* extracts and related species can interfere with cell cycle progression. For instance, Shiverick et al. [66] demonstrated that methanolic fractions of *H. perforatum* increased the proportion of cells in the G0/G1 phase in bladder carcinoma, which was associated with changes in p21 and cyclin expression. Similarly, Oezmen et al. [67] showed that *Hypericum adenotrichum* extract suppressed cyclin D1 and induced p21, leading to G0/G1 arrest in tumor cells. In another study, Haake et al. [13] reported that *H. perforatum* extract in combination with radiotherapy promoted G2/M arrest in breast and colon carcinoma cells, suggesting that the precise checkpoint targeted may vary depending on extract composition and treatment context.

Taken together, our findings indicate that *H. perforatum* extract preferentially halts proliferation at the G0/G1 checkpoint, while literature evidence points to multiple possible mechanisms—including modulation of cyclins and CDK inhibitors—that warrant further investigation. As we did not assess gene or protein expression in this study, mechanistic confirmation will require additional molecular analyses in future work.

Although our experiments did not explore molecular pathways, previous research provides insights into how specific phytochemicals identified in our extract may contribute to the observed effects. For instance, 4-hydroxybenzoic acid has been reported to modulate the PI3K/Akt, MAPK3, and STAT3 pathways to exert antitumour activity [68], β -resorcylic acid has been shown to inhibit CDK1 and arrest cell cycle progression [69], and genkwanin has been highlighted as a potent anticancer agent by down-regulating p38, JNK, and AP-1

signaling, as well as suppressing pro-inflammatory cytokines [24]. These mechanisms, described in the literature, may help explain the observed cytotoxic, pro-apoptotic, and cell cycle regulatory effects.

In summary, our findings demonstrate that *H. perforatum* exerts anticancer effects by simultaneously reducing cell viability, promoting apoptosis, and altering cell cycle dynamics. Importantly, the apoptotic activity we observed aligns with previous findings in breast cancer models, where *H. perforatum* extracts were shown to activate the mitochondrial apoptotic pathway while suppressing proliferative signaling through AMPK/mTOR inhibition [15]. These complementary insights highlight that the anticancer activity of *H. perforatum* likely results from the combined action of multiple mechanisms, reinforcing its potential value as a source of bioactive compounds for therapeutic development.

H. perforatum extract exhibited moderate antibacterial activity in our study, with inhibition zones against *E. coli* (11.67 mm) and *S. aureus* (10.23 mm), while no activity was detected against *P. aeruginosa* or *Candida* species. These results are in agreement with previous findings reporting selective antimicrobial effects of *H. perforatum*. For instance, Orhan et al. [70] observed only weak antibacterial effects from aerial parts macerated in olive oil, with limited activity against *S. aureus* and *Trypanosoma brucei rhodesiense*. In contrast, ethanolic and aqueous fractions demonstrated stronger activity against oral pathogens such as *Streptococcus sobrinus* and *Lactobacillus plantarum* [71], while alcoholic extracts and hypericin were reported to inhibit *Lactobacillus acidophilus*, suggesting its potential use as oral disinfectants [72]. Collectively, these findings emphasize that the antimicrobial spectrum of *H. perforatum* is both strain- and solvent-dependent, and its moderate antibacterial effects observed in our study may still hold therapeutic relevance, particularly against Gram-positive pathogens.

Conclusion

This study provides an integrated chemical and biological characterization of *H. perforatum* extract, highlighting its richness in phenolic acids and flavonoids, including high levels of chlorogenic acid, rutin, isoquercitrin, quercetin, and the newly reported presence of genkwanin, vicianin-2, schaftoside, and afzelin. The extract exhibited remarkable antioxidant potential, with high total phenolic content and strong radical scavenging activity, confirming the central role of polyphenolic compounds in its bioactivity. Importantly, the extract demonstrated potent cytotoxic effects across multiple cancer cell lines, outperforming cisplatin in terms of IC₅₀ values, while promoting apoptosis and inducing G0/G1 cell cycle arrest, suggesting potential anticancer effects in vitro that warrant further in vivo validation.

Although mechanistic studies were not performed here, literature evidence supports the involvement of multiple molecular pathways through which key constituents may exert anticancer activity. Furthermore, moderate antibacterial effects against *E. coli* and *S. aureus* underline the selective antimicrobial potential of the extract.

Taken together, these findings reinforce *H. perforatum* as a valuable natural source of bioactive compounds with antioxidant, anticancer, and antimicrobial properties. The discovery of novel phytochemicals expands the known chemical spectrum of this species and provides fresh avenues for pharmacological research. Future studies should focus on mechanistic validations, in vivo efficacy, and safety assessments to further explore its potential as a complementary or adjuvant therapeutic agent.

Materials and Methods

Plant Material

In this study, the plant material (*Hypericum perforatum* L. subsp. *perforatum*) was collected on July 10, 2024, from the vicinity of Çat Dam in the Çelikhan district of Adıyaman province, Türkiye. The geographical coordinates of the collection site are 38°05'16" N latitude and 38°10'30" E longitude, at an elevation of 1928 meters above sea level. Permission for plant collection was granted by the landowner. Plant authentication was performed by Dr. Turgay Kolaç (Pharmacognosist, Department of Pharmacy Services, Inonu University), and a voucher specimen was deposited in the Herbarium of Faculty of Pharmacy, Inonu University, under the voucher number TK1503.

The plant material was thoroughly washed with distilled water to remove debris and potential contaminants, and subsequently dried under shade at ambient room temperature to preserve its phytochemical integrity. Once fully dried, the aerial parts of the plant were ground into a fine powder using a laboratory mill. The powdered samples were stored in airtight containers under cool and dry conditions, protected from light, until further analysis.

Extraction Procedure

The powdered aerial parts of *H. perforatum* were extracted using methanol acidified with 0.05% HCl, which was selected to enhance the solubility and recovery of phenolic acids and flavonoid glycosides. Mild acidification promotes the partial hydrolysis of glycosidic bonds and facilitates the release of bound phenolics from plant tissues, resulting in higher extraction efficiency, increased total phenolic content, and potentially improved antioxidant and cytotoxic properties of the extract. For extraction, 10 g of plant powder was macerated with 100 mL of the acidified methanol (0.05% HCl) at room temperature in the dark for 24 hours [73]. The extraction was repeated three times, and the resulting filtrates were combined. The

combined extracts were filtered through Whatman No. 1 filter paper, and the solvents were evaporated under reduced pressure using a rotary evaporator at 40 °C to obtain the crude dry extracts.

Extraction yields were calculated based on the dry weight of *H. perforatum* aerial parts. The acidified methanolic extraction yielded 1.7041 g of dry extract from 12.63 g of powdered plant material, corresponding to an extraction efficiency of 13.52%.

The dried extracts were stored at 4 °C in amber glass containers protected from light until further analysis. Prior to biological and chemical assays, stock solutions were freshly prepared by dissolving the extract in a 50:50 (v/v) ethanol:water mixture.

Determination of Total Phenolic Content (TPC)

The TPC of the extracts was determined using the Folin–Ciocalteu reagent method as previously described, with minor modifications [74]. Briefly, 0.5 mL of extract solution was mixed with 2.5 mL of 10% Folin–Ciocalteu reagent. After 5 minutes of incubation at room temperature, 2 mL of 7.5% sodium carbonate (Na_2CO_3) solution was added to the mixture. The final solution was incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 765 nm using a UV–Vis spectrophotometer (Shimadzu 2000S Model, Japan). A calibration curve was constructed using gallic acid, and the results were expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g).

Determination of Antioxidant Capacity

DPPH Radical Scavenging Activity

The antioxidant activity of the extracts was evaluated based on their ability to scavenge the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). In the assay, 100 μL of extract was added to 3.9 mL of freshly prepared DPPH solution. The mixture was vortexed and incubated in the dark at room temperature for 30 minutes. The absorbance was then measured at 517 nm using a UV–Vis spectrophotometer (Shimadzu 2000S Model, Japan). Antioxidant capacity was quantified by comparison with a Trolox standard curve and expressed as milligrams of Trolox equivalent per gram of dry extract (mg TE/g) [75].

ABTS Radical Scavenging Assay

The antioxidant capacity of the extract was evaluated using the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging method, as described by Zengin et al. [76]. Briefly, 50 μL of the extract was mixed with 150 μL of the extraction solvent and 3800 μL of the freshly prepared ABTS^{•+} radical solution. The mixture was vortexed and incubated in the dark at room temperature for 15 minutes. Following incubation, the absorbance was measured at 734 nm using a UV-Vis spectrophotometer (Shimadzu 2000S

Model, Japan). The antioxidant activity was expressed as Trolox equivalents (mg TE/g dry weight).

LC-MS/MS Analysis of Phytochemical Compounds

The chemical composition of the extract of *H. perforatum* was analyzed using a Thermo Scientific UHPLC system coupled to a TSQ Quantum Access MAX triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. Chromatographic separation was performed on a Thermo Accucore C18 column (100 mm × 2.1 mm, 2.6 µm) maintained at 30 °C. The mobile phases consisted of solvent A: ultrapure water with 0.1% (v/v) formic acid and solvent B: methanol with 0.1% (v/v) formic acid. A linear gradient elution was applied as follows: 0–2 min, 5% B; 2–10 min, 5–95% B; 10–12 min, 95% B; 12–13 min, return to 5% B; and followed by re-equilibration until 15 min.. The flow rate was 0.3 mL/min and the injection volume was 10 µL. The MS/MS analyses were performed in negative ion mode with the following settings: spray voltage 3500 V, capillary temperature 320 °C, sheath gas 35 (arb), auxiliary gas 10 (arb), and collision gas pressure set to 1.5 mTorr. Multiple Reaction Monitoring (MRM) mode was used for the identification and quantification of target compounds, based on comparison with authentic standards and literature-reported transitions. Sample preparation was carried out by dissolving the dried extract in LC-MS grade methanol at a final concentration of 1 mg/mL. Before injection, the solutions were filtered through a 0.22 µm PTFE syringe filter (Marz Shuller). Compound identification was confirmed by matching retention times, precursor/product ion pairs, and fragmentation patterns [77].

Analytical parameters of the LC-MS/MS method were evaluated using calibration standards prepared for each identified compound. Calibration curves were constructed within the linear range of 10–100 ng/mL, and all analytes showed excellent linearity with correlation coefficients (R^2) ranging from 0.9903 to 0.9976. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on signal-to-noise ratios of 3 and 10, respectively. Recovery studies were performed by spiking 100 ng/mL of standards into pre-analyzed samples and processing them under identical conditions. The analytical parameters, including retention time (RT), parent and fragment ions, R^2 , LOD, LOQ, and recovery percentage, are summarized in Table 6.

MTT Test

The anticancer properties of the extracts were evaluated against a panel of human cancer cell lines: MDA-MB-231 (breast adenocarcinoma), MCF-7 (breast adenocarcinoma), A549 (lung carcinoma), SH-SY5Y (neuroblastoma), HCT116 (colorectal carcinoma), HeLa (cervical

carcinoma), and the non-cancerous BEAS-2B (human bronchial epithelial) cell line, as described by Sharma et al. [78]. All human cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) with the following catalog numbers: MDA-MB-231 (ATCC HTB-26), MCF-7 (ATCC HTB-22), A549 (ATCC CCL-185), SH-SY5Y (ATCC CRL-2266), HeLa (ATCC CCL-2), BEAS-2B (ATCC CRL-3588), and HCT-116 (ATCC CCL-247).

All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h to allow cell attachment. The medium was then replaced with fresh medium containing the test extracts at concentrations ranging from 0.78 to 200 µg/mL, followed by a 24 h incubation under the same conditions. After treatment, the medium was removed and replaced with 0.5 mg/mL MTT solution prepared in culture medium, and the plates were incubated for 4 h at 37 °C. The resulting formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm (reference 650 nm) using a microplate reader (Epoch, Biotek, USA). Cell viability (%) was calculated relative to untreated control cells, and IC₅₀ values were determined from the dose–response curves plotting cell viability against extract concentrations.

Flow Cytometric Apoptosis Analysis

Apoptotic changes in the plasma membrane were evaluated after 24-hour treatment with 2.5 µg/mL of each extract using the Annexin V-FITC/PI Apoptosis Detection Kit (E-CK-A211, Elabscience), following the manufacturer's protocol. Briefly, treated cells were harvested and transferred into 12x75 mm polystyrene tubes, followed by centrifugation at 1100 rpm for 5 minutes at room temperature (RT). The resulting cell pellets were resuspended in 1–2 mL of Annexin V Binding Buffer (AVBB) and centrifuged again under the same conditions. After aspirating the supernatant, each pellet was resuspended in 100 µL of propidium iodide (PI) working solution prepared in AVBB, and 5 µL of Annexin V-FITC was added. The samples were incubated for 15 minutes at RT in the dark. Following incubation, 400 µL of AVBB was added, and the samples were kept on ice until analysis. Stained cells were analyzed using a BD Accuri C6 Plus flow cytometer equipped with the appropriate laser and filter settings. Based on dual staining, cell populations were classified as: viable (Annexin V⁻/PI⁻), early apoptotic (Annexin V⁺/PI⁻), late apoptotic or secondary necrotic (Annexin V⁺/PI⁺), and necrotic (Annexin V⁻/PI⁺). Data were expressed as percentage of total cells in each quadrant [79].

Cell Cycle Analysis by Propidium Iodide Staining

The cell cycle assay was performed as previously described [80]. Briefly, 12x75 mm centrifuge tubes were pre-filled with 4.5 mL of 70% ethanol and kept on ice. Approximately 10^6 to 10^7 cells were harvested and suspended in 5 mL of phosphate-buffered saline (PBS), then centrifuged at $\sim 200 \times g$ (1000 rpm) for 6 minutes. The resulting pellet was gently resuspended in 0.5 mL of PBS using a Pasteur pipette to ensure a single-cell suspension, which is essential to prevent irreversible cell aggregation during fixation. The suspension was then transferred to the pre-chilled ethanol tubes and incubated at $+4^\circ\text{C}$ for a minimum of 2 hours for fixation.

Following fixation, the cells were centrifuged for 5 minutes at $200 \times g$ and the ethanol was carefully decanted. The pellet was resuspended in 5 mL of PBS, incubated for 60 seconds, and centrifuged again under the same conditions. Finally, the cells were resuspended in 1 mL of PI staining solution containing RNase A, and incubated either for 15 minutes at 37°C or for 30 minutes at room temperature in the dark. Cell cycle distribution was analyzed using a BD Accuri C6 Plus flow cytometer equipped with appropriate laser and filter settings.

Antimicrobial Activity Assay

The antibacterial and antifungal activities of extracts were evaluated using the disc diffusion method. The tested microorganisms included four bacterial strains—*Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), and *Pseudomonas aeruginosa* (ATCC 27853)—and two fungal strains—*Candida albicans* (SC5314/ATCC MYA-2876) and *Candida glabrata* (ATCC 2001).

For the assay, 100 mg of extract was dissolved in 100% DMSO to prepare a stock solution at a concentration of $10 \mu\text{g}/\mu\text{L}$. From this solution, $100 \mu\text{L}$ was loaded onto sterile paper discs (6 mm diameter), which were then placed onto agar plates previously inoculated with test microorganisms. Bacterial suspensions ($\sim 1 \times 10^8$ CFU/mL) were prepared in LB broth, while fungal suspensions ($\sim 1 \times 10^7$ CFU/mL) were prepared in YPD broth. The inoculated media were evenly spread onto Petri dishes (90 mm in diameter) under aseptic conditions. The plates were incubated at 37°C for 24 hours. Ampicillin ($100 \mu\text{g}/\text{disc}$) was used as the positive control for bacteria, and caspofungin ($100 \mu\text{g}/\text{disc}$) for fungi. Discs loaded with DMSO alone served as negative controls. Following incubation, antimicrobial activity was assessed by measuring the diameter of the inhibition zones (in millimeters) surrounding each disc.

Statistical Analysis

All experimental data were obtained from three independent replicates and are presented as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine statistically significant differences between control and treatment groups. When

significant differences were found, Tukey's multiple comparison test was applied for post hoc analysis. A p -value of less than 0.05 was considered statistically significant.

Table 6. Validation parameters for the LC-MS/MS quantification of phytochemical standards in *H. perforatum* extract, including retention time (RT), parent and fragment ions, linearity (R^2), limit of detection (LOD), limit of quantification (LOQ), and recovery percentage (%)

Compounds	RT	Parent Ion (m/z)	Fragment Ions (m/z)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%)
4-Hydroxybenzoic acid	7.82	137.02442	93.03471	0.9921	1.03	3.44	104.89
Salicylic acid	10.93	137.02442	93.03468	0.9946	1.16	3.87	105.31
3-hydroxybenzoic acid	8.66	137.02442	93.03471	0.9926	1.71	5.70	103.14
Gallic acid	3.70	169.01425	125.02461	0.9903	2.94	9.78	105.96
Protocatechuic acid	6.24	153.01933	109.02949	0.9908	0.73	2.44	103.64
Protocatechuic aldehyde	7.29	137.02442	136.01671	0.9909	0.46	1.55	101.52
β -Resorcylic acid	8.42	153.01933	67.01888	0.9935	0.84	2.79	99.39
Vanillic acid	8.57	167.03498	108.02173	0.9938	1.87	6.24	101.12
Vanillin	9.23	151.04007	108.02178	0.9913	1.61	5.37	104.71
<i>trans</i> -Cinnamic acid	12.31	147.04515	47.04515	0.9952	2.25	7.52	102.50
Coumaric acid	9.80	163.04007	119.05027	0.9928	1.01	3.37	100.41
Caffeic acid	8.62	179.03498	135.04509	0.9917	0.63	2.09	103.81
Ferulic acid	10.05	193.05063	134.03751	0.9933	1.39	4.63	101.37
Sinapic acid	10.02	223.06120	193.01436	0.9934	1.03	3.44	101.24
Chlorogenic acid	8.07	353.08781	191.05624	0.9976	0.80	2.65	101.66
Quinic acid	0.93	191.05611	85.02962	0.9939	0.80	2.68	101.67
Catechin	7.62	289.07176	109.02975	0.9943	0.66	2.20	108.18
Apigenin	13.24	269.04555	117.03464	0.9932	1.05	3.51	100.61
Acacetin	14.93	283.06120	268.03717	0.9911	1.92	6.41	101.74
Vicenin 2	9.05	593.15119	473.10941	0.9945	1.09	3.63	108.34
Genkwanin	14.93	283.06120	268.03745	0.9911	1.31	4.37	102.44
Vitexin	10.09	431.09837	311.05603	0.9939	0.92	3.06	109.65
Schaftoside	9.66	563.14063	443.09949	0.9908	2.01	6.69	107.79
Rutin hydrate	10.67	609.14611	300.02777	0.9912	1.28	4.28	103.79
Luteolin	12.53	285.04046	175.04039	0.9932	1.54	5.12	98.29
Luteolin 7-rutinoside	10.40	593.15119	285.04065	0.9923	0.70	2.33	108.50
Isoquercitrin	10.74	463.08820	300.02768	0.9939	0.70	2.32	105.86
Narcissin	11.41	623.16176	315.05139	0.9918	1.26	4.21	107.64
Kaempferol	13.08	285.04046	136.01686	0.9924	0.57	1.91	99.98
Afzelin	11.98	431.09837	285.04028	0.9951	1.96	6.53	103.95
Nicotiflorin	11.31	593.15119	285.04041	0.9915	0.75	2.49	105.07
Naringenin	12.34	271.06120	119.05035	0.9932	1.46	4.85	99.44
Oleuropein	11.78	542.16678	395.07828	0.9929	1.96	6.53	100.84
Emodin	16.35	269.04555	225.05573	0.9909	1.34	4.46	101.65

RT: Retention time, LOD: Limit of detection, LOQ: Limit of quantification, R^2 : Correlation coefficient.

Declarations

Ethics approval and consent to participate

All plant collection and research activities were conducted in accordance with relevant institutional, national, and international guidelines and legislation. The collection of *H. perforatum* was carried out with permission from the landowner, and no protected or endangered species were involved in this study.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare no competing interests.

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Authors' contributions

YU and TK: Project administration, Methodology, Formal analysis and investigation, Writing-original draft; MAG, İNM, and MD: Methodology, Formal analysis and investigation, Writing-review and editing; RZ and AG: Formal analysis and investigation, Writing-original draft.

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