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Evaluation of Phytochemicals, Antioxidant Capacity and Apoptosis-Inducing Potentials of *Hydrocotyle vulgaris* in A431 Human Skin Carcinoma Cells

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This study evaluated the phytochemical profile, antioxidant activity and anticancer potential of aqueous and methanolic extracts of *Hydrocotyle vulgaris* leaf. Qualitative analysis revealed the presence of carbohydrates, tannins, saponins, flavonoids, quinones, phenols, terpenoids, anthraquinones and proteins in both extracts, while glycosides were detected only in the aqueous extract. Quantitative estimation showed higher levels of total phenolics, flavonoids, tannins and total antioxidant capacity in the aqueous extract compared to the methanolic extract. Antioxidant assays demonstrated concentration-dependent DPPH and ABTS radical scavenging activities, with the aqueous extract exhibiting lower IC_{50} values (303.5 and 324.8 µg/mL, respectively). GC-MS analysis of the aqueous extract identified 28 bioactive compounds, indicating chemical diversity. The aqueous extract exhibited significant cytotoxicity against A431 human skin carcinoma cells with an IC_{50} of 107.92 µg/mL. Morphological assessment, fluorescence staining, flow cytometry and DNA fragmentation analysis confirmed apoptosis-mediated cell death. Collectively, these findings highlight the antioxidant efficiency and apoptosis-inducing anticancer activity of *H. vulgaris* leaf extract, supporting its potential as a promising source of natural bioactive compounds for therapeutic applications.

Keywords: *Hydrocotyle vulgaris*, Phytochemical analysis, Antioxidant activity, Anticancer activity, A431 skin carcinoma cells.

INTRODUCTION

Traditional medicines have been utilised the beneficial properties associated with these compounds for centuries, highlighting their potential to become novel drug candidates [1]. Plant-derived secondary metabolites such as flavonoids, phenolic acids, terpenoids and carotenoids exhibit diverse biological activities including antioxidant, anti-inflammatory, antimicrobial, immunomodulatory and anticancer effects, which are highly relevant to the multifactorial pathogenesis of skin disorders [2-4]. These compounds serve crucial roles in plant defense and have long been recognised for their therapeutic properties in traditional medicine systems such as Ayurveda, traditional Chinese medicine (TCM) and Indigenous medicinal practices worldwide [5]. Consequently, the phytochemical studies have become an important area of investigation for identifying novel bioactive molecules and understanding the relationship between chemical composition and biological activity.

Phytochemical analysis and characterisation are fundamental for the identification, isolation and quantification of bioactive constituents present in plant extracts that contribute to their pharmacological activities. These investigations are essential for scientifically validating the therapeutic potential of medicinal plants used in traditional healthcare systems. Furthermore, phytochemical studies support the standardisation, quality assurance, safety evaluation and efficacy of herbal formulations, thereby strengthening the credibility of plant-based medicines [6]. Modern analytical techniques such as gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and spectroscopic methods enable the comprehensive identification and profiling of plant metabolites. Among these approaches, GC-MS is widely applied for the detection and characterisation of bioactive constituents in plant extracts and essential oils, facilitating the discovery of compounds with potential therapeutic significance. Advances in GC-MS technology over the past

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two decades have further improved its capability to analyze a broad spectrum of volatile and non-volatile metabolites including non-polar, semi-polar and polar compounds after suitable derivatisation [7].

Medicinal plants and their bioactive phytochemicals are gaining increasing interest for preventing and managing skin disorders, including skin cancer. Approximately one-third of traditional remedies worldwide are used for the dermatological and wound-healing purposes [8]. In addition to general therapeutic effects, certain plant-derived compounds exhibit anticancer activity by promoting apoptosis, suppressing abnormal cell proliferation and angiogenesis and regulating tumor-related signalling pathways. Compounds such as epigallocatechin gallate (EGCG), genistein and curcumin target key oncogenic regulators including STAT3, Bcl-2 and matrix metalloproteinases, which are crucial in skin cancer development and metastasis [2,9,10]. These observations support further investigation of phytochemicals as potential alternatives or complementary options to conventional cancer treatments [8].

Current therapeutic approaches for skin disorders include corticosteroids, immunomodulators, retinoids, antimicrobial agents and phototherapeutic interventions such as psoralen-UVA (PUVA) and photodynamic therapy [11]. Although these treatments are clinically effective, their long-term use is often limited by adverse effects, drug resistance, high treatment costs and inconsistent outcomes in chronic and recurrent conditions [12]. Consequently, there is growing interest in the alternative and complementary therapeutic strategies that are safer, cost-effective and capable of targeting multiple pathogenic pathways simultaneously [13].

Hydrocotyle vulgaris (marsh pennywort) is a creeping, mat-forming wetland plant with a long history of traditional medicinal use. The species is rich in secondary metabolites including flavonoids, tannins, saponins, terpenoids, steroids and reducing sugars and has traditionally been used to treat skin ailments, burns, respiratory conditions and gastrointestinal disorders. Moreover, several species within the genus Hydrocotyle are reported to possess diuretic, anti-rheumatic, dermatological, vasodilatory and wound-healing properties, highlighting their ethnopharmacological relevance [14,15]. Despite the therapeutic promise of plant-based dermatological agents, their clinical translation remains limited by challenges such as variability in phytochemical composition, lack of standardisation, limited bioavailability and insufficient large-scale clinical validation [13].

In this context, the present study aimed to systematically evaluate the phytochemical composition, antioxidant capacity and anticancer potential of aqueous and methanolic leaf extracts of *H. vulgaris*. Specifically, the study sought to quantify key phytochemical constituents, assess free radical scavenging activity using established antioxidant assays and characterize the chemical composition of the most bioactive extract through GC-MS analysis. Furthermore, the anticancer efficacy of the extract was investigated against A431 human skin carcinoma cells, with emphasis on elucidating the underlying mechanism of cell death through morphological assessment, fluorescence staining, flow cytometry and DNA fragmentation analysis.

EXPERIMENTAL

Sample collection and preparation: Fresh leaves of *Hydrocotyle vulgaris* were collected from Coimbatore, India and authenticated by the qualified Botanist. The plant material was thoroughly washed with sterile distilled water to remove surface contaminants, shade-dried at room temperature in a well-ventilated environment and ground into a fine powder using a mechanical grinder. A 10 g of powdered leaves were subjected to sequential Soxhlet extraction using methanol and distilled water. The extracts were filtered through Whatman No. 1 filter paper, concentrated to dryness at room temperature and stored in airtight containers at 4 ºC until further analysis.

Phytochemical screening: Qualitative phytochemical screening of aqueous and methanolic extracts of *H. vulgaris* leaf was performed using standard protocols as described by Oloyede *et al.* [16]. The extracts were analysed for the presence of carbohydrates, tannins, saponins, alkaloids, flavonoids, glycosides, quinones, phenols, steroids, anthraquinones and terpenoids.

Quantitative estimation of phytochemicals: The total phenolic content was determined using the Folin-Ciocalteu method following Lee *et al.* [17] and expressed as mg gallic acid equivalents (GAE)/g extract. Total flavonoid content was estimated by the $AlCl_3$ colorimetric method described by Muthia *et al.* [18] and expressed as mg quercetin equivalents (QE)/g extract. Total alkaloid content was quantified using the bromocresol green (BCG) method as described by Shamsa *et al.* [19]. Absorbance was measured at 470 nm, atropine was used as the standard and results were expressed as mg atropine equivalents (AE)/g extract. Total tannin content was determined using the vanillin-HCl method according to Jones & Broadhurst [20]. Absorbance was recorded at 500 nm using catechin as the reference compound and results were expressed as mg catechin equivalents (CE)/g extract.

DPPH radical scavenging assay: The antioxidant activity of aqueous and methanolic extracts of *H. vulgaris* was evaluated using the DPPH radical scavenging assay with slight modifications following Wakhidah *et al.* [21]. A 0.1 mM DPPH solution was prepared in methanol. Extract concentrations (100-500 µg/mL) were mixed with 2 mL of DPPH solution and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a UV-visible spectrophotometer. Ascorbic acid was used as the standard.

Radical scavenging activity (%) was calculated as:

$$\text{Radical scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c is the control absorbance and A_s is the sample absorbance.

ABTS radical cation scavenging assay: ABTS radical scavenging activity was assessed according to the method of Re *et al.* [22]. The $ABTS^{\bullet+}$ stock solution was prepared by mixing 7 mM ABTS with 2.4 mM potassium persulphate and incubating the mixture in the dark for 16 h. The working solution was diluted with 80 % ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. A 300 µL of extract were incubated with 2.7 mL of ABTS solution at 30 ºC for 30 min and absorbance was recorded at 734 nm. Ascorbic acid served as the reference antioxidant. The percentage inhibition was calculated using the same equation as described for the DPPH assay.

Total antioxidant capacity: Total antioxidant capacity was determined by the phosphomolybdenum method described

by Prieto *et al.* [23]. A 3 mL of aqueous or methanolic extract were mixed with 3 mL of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The reaction mixture was incubated at 95 ºC for 90 min. After cooling to room temperature, absorbance was measured at 695 nm against a blank. Antioxidant capacity was expressed as ascorbic acid equivalents.

GC-MS analysis: The aqueous extract of *H. vulgaris* leaf was analysed by GC–MS for the identification of volatile bioactive compounds using an Agilent GC-MS system (Model: CH-GCMSMS02) equipped with MassHunter software. Helium was used as the carrier gas. The oven temperature program was set at 50 ºC (1 min), increased to 120 ºC at 5 ºC/min (1 min), then to 210 ºC at 10 ºC/min (1 min) and finally to 280 ºC at 10 ºC/min with a 5 min hold. Compounds were identified by comparing retention times and mass spectra with those in the NIST mass spectral library and published literature.

In vitro anticancer activity

Cell culture: Human epidermoid carcinoma (A431) cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in Dulbecco's modified eagle medium supplemented with 10 % fetal bovine serum and antibiotics and incubated at 37 ºC in a humidified atmosphere containing 5 % CO_2.

Cell viability assay (MTT): Cell viability was evaluated using the MTT assay following Mosmann's method with minor modifications. A431 cells were seeded in 96-well plates and treated with aqueous *H. vulgaris* extract (18.75-300 µg/mL) for 24 h. After treatment, MTT solution (0.5 mg/mL) was added and incubated for 4 h. Formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm. Cell viability was calculated relative to untreated controls and IC_{50} values were determined by regression analysis.

Morphological assessment: Morphological alterations in treated A431 cells were examined under an inverted phase-contrast microscope. Cytotoxicity was assessed based on cell rounding, shrinkage, detachment and loss of normal cellular morphology compared with untreated controls.

Fluorescence microscopy for apoptosis: Apoptotic cell death was analysed using acridine orange/ethidium bromide (AO/EB) dual staining. Cells treated with IC_{50} concentrations for 24 and 48 h were stained and observed under a fluorescence microscope to differentiate viable, apoptotic and necrotic cells. Nuclear morphology was further assessed by DAPI staining to detect chromatin condensation and nuclear fragmentation.

Cell cycle and apoptosis analysis: For flow cytometric analysis, A431 cells treated with IC_{50} concentrations for 24 h were fixed in 70 % ethanol, treated with RNase and stained with propidium iodide. Cell cycle distribution and apoptosis were analysed using a FACS Canto II flow cytometer (BD Biosciences).

DNA fragmentation analysis: DNA fragmentation was analysed by phenol-chloroform-isoamyl alcohol extraction. Isolated DNA was resolved on a 0.8 % agarose gel using a 100 bp ladder and visualised with a gel documentation system (Bio-Rad, USA).

Statistical analysis: All experiments were performed in triplicate and repeated independently at least three times. Results were expressed as mean ± standard deviation. Statistical analysis was carried out using SPSS version 17.0 and differences were considered statistically significant at $p < 0.01$.

RESULTS AND DISCUSSION

Phytochemical results: Qualitative phytochemical analysis of *H. vulgaris* leaf extracts prepared with aqueous and methanolic solvents revealed the presence of carbohydrates, tannins, saponins, flavonoids, quinones, phenols, terpenoids, anthraquinones and proteins (Table-1), indicating a rich profile of polar and moderately polar bioactive compounds. Alkaloids, steroids, phytosterols and carotenoids were absent in both extracts, while glycosides were detected only in the aqueous extract, reflecting solvent-dependent extractability. The predominance of phenols, flavonoids, tannins and terpenoids suggests strong antioxidant and pharmacological potential. These findings align with previous reports showing that polar and semi-polar solvents efficiently extract bioactive compounds from plant materials [24-26]. Thus, both aqueous and methanol extracts are effective for obtaining therapeutically relevant phytoconstituents from *H. vulgaris* leaf supporting their potential for further bioactivity studies.

TABLE-1
PHYTOCHEMICAL SCREENING OF *H. vulgaris* LEAF

Phytochemical	Aqueous extract	Methanolic extract
Carbohydrates	Presence	Presence
Tannins	Presence	Presence
Saponins	Presence	Presence
Alkaloids	Absence	Absence
Flavonoids	Presence	Presence
Glycosides	Presence	Absence
Quinones	Presence	Presence
Phenols	Presence	Presence
Steroids	Absence	Absence
Terpenoids	Presence	Presence
Anthraquinones	Presence	Presence
Proteins	Presence	Presence
Phytosterols	Absence	Absence
carotenoids	Absence	Absence

Quantitative phytochemical analysis: The quantitative estimation of major phytochemical groups in *H. vulgaris* leaf extracts is shown in Fig. 1. The aqueous extract exhibited higher total phenolic content (TPC: 37.59 mg GAE/g), total flavonoid content (TFC: 14.07 mg QE/g), total antioxidant capacity (TAC: 2.38 mg AE/g) and total tannin content (TTC: 30.34 mg CE/g) compared to the methanolic extract, which showed TPC of 25.87 mg GAE/g, TFC of 10.82 mg QE/g, TAC of 1.20 mg AE/g and TTC of 17.78 mg CE/g. These results indicate that aqueous extraction is more effective in recovering phenolics, flavonoids, tannins and antioxidant compounds, while methanol also extracts bioactive constituents at moderate levels.

The variation in the phytochemicals yield between solvents underscores the influence of solvent polarity on extraction efficiency and supports the complementary use of aqueous and methanolic systems for comprehensive recovery of bioactive compounds from *H. vulgaris* leaves. The high levels of flavo-

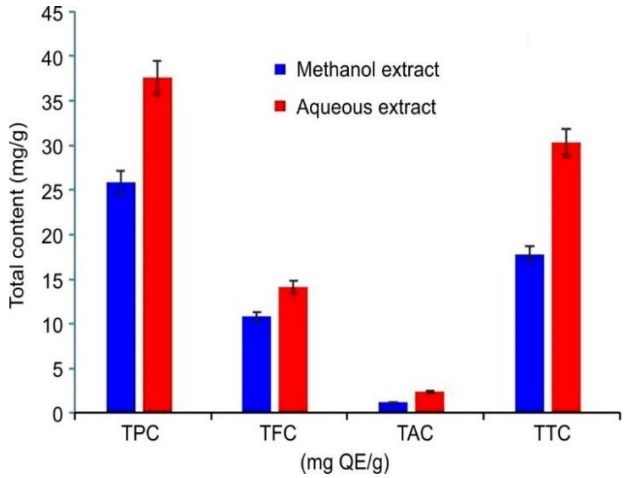

Fig. 1. Quantitative phytochemicals analysis of extracts of *H. vulgaris* leaf

noids and alkaloids are particularly significant, as previous studies have associated these compounds with strong antioxidant and radical-scavenging activities [14,27]. Moreover, tannins, though present in lower amounts, may contribute to antioxidant potential despite their reported dietary limitations [28]. These quantitative data confirm that *H. vulgaris* is a rich source of bioactive phytochemicals with significant medicinal potential.

DPPH radical scavenging activity: The antioxidant potential of extracts of *H. vulgaris* leaf was evaluated using the DPPH radical scavenging assay. Both methanolic and aqueous extracts exhibited a clear concentration dependent increase in free radical scavenging activity over the range of 100-500 μg/mL. At the lowest tested concentration (100 μg/mL), the aqueous extract showed higher scavenging activity (26.79 ± 1.10%) than the methanolic extract (23.28 ± 1.24%), while ascorbic acid, used as a standard, exhibited the highest activity (33.69 ± 2.30%). With increasing concentrations, both extracts demonstrated enhanced radical scavenging capacity. At 500 μg/mL, the aqueous extract exhibited significantly higher activity (72.18 ± 2.50%) compared to the methanolic extract (67.37 ± 1.90%), though both remained lower than the standard ascorbic acid (83.19 ± 1.00%). The IC_{50} values further supported these findings, with the aqueous extract had a lower IC_{50} (303.5 μg/mL) than the methanolic extract (425.5 μg/mL), indicating superior antioxidant potency (Table-2). These results suggest that the aqueous extract of *H. vulgaris* is more effective in neutralizing DPPH radicals, likely due to its higher content of phenolic and flavonoid compounds, as supported by quantitative phytochemical analysis.

TABLE-2
DPPH FREE RADICAL SCAVENGING ACTIVITY OF THE DIFFERENT SOLVENT EXTRACTS FROM *H. vulgaris* LEAF

Concentration (μg/mL)	Methanolic extract	Aqueous extract	Ascorbic acid
100	23.28 ± 1.24	26.79 ± 1.1	33.69 ± 2.3
200	31.19 ± 1.08	35.87 ± 1.7	41.89 ± 1.6
300	37.43 ± 1.08	50.38 ± 1.9	52.84 ± 1.09
400	44.98 ± 2.7	59.76 ± 1.5	65.68 ± 1.1
500	67.37 ± 1.9	72.18 ± 2.5	83.19 ± 1.0
IC_{50} (μg/mL)	425.5	303.5	274.3

ABTS radical cation scavenging activity: Similar to the DPPH assay, both methanolic and aqueous extracts displayed a concentration-dependent increase in radical scavenging activity. At 100 μg/mL, the aqueous extract exhibited higher inhibition (29.47 ± 1.50%) than the methanolic extract (21.37 ± 1.08%), with ascorbic acid showing the highest activity (32.47 ± 1.70%). At the highest concentration (500 μg/mL), the aqueous extract maintained superior activity (70.12 ± 1.90%) relative to the methanolic extract (52.26 ± 1.54%), though it remained slightly lower than ascorbic acid (74.16 ± 1.10%). The IC_{50} values indicated stronger antioxidant activity of the aqueous extract (324.8 μg/mL) compared to the methanolic extract (475.6 μg/mL) (Table-3). These findings are consistent with the DPPH assay results, reinforcing the role of solvent polarity in the efficient extraction of antioxidant phytochemicals from *H. vulgaris* leaves.

TABLE-3
ABTS RADICAL CATION SCAVENGING ACTIVITY (%) OF EXTRACTS OF *H. vulgaris* LEAF AND ASCORBIC ACID

Concentration (μg/mL)	Methanolic extract	Aqueous extract	Ascorbic acid
100	21.37 ± 1.08	29.47 ± 1.5	32.47 ± 1.7
200	29.46 ± 1.22	37.68 ± 1.9	39.54 ± 1.1
300	34.29 ± 1.38	46.17 ± 1.02	48.96 ± 1.2
400	41.09 ± 1.5	57.74 ± 1.03	62.14 ± 1.46
500	52.26 ± 1.54	70.12 ± 1.9	74.16 ± 1.1
IC_{50} (μg/mL)	475.6	324.8	298.2

Values are expressed as mean ± SD.

Total antioxidant capacity: Both extracts showed the concentration-dependent increases in antioxidant activity from 100-500 μg/mL. Interestingly, at lower concentrations (100 and 200 μg/mL), the methanolic extract exhibited slightly higher activity than the aqueous extract. However, at higher concentrations, the aqueous extract demonstrated marginally superior antioxidant capacity, with 58.04 ± 2.10% at 500 μg/mL, compared to 54.48 ± 0.90% for the methanolic extract. As expected, ascorbic acid showed the highest antioxidant potential across all concentrations, reaching 84.14 ± 1.30% at 500 μg/mL. The IC_{50} values confirmed slightly better efficacy of the aqueous extract (396.5 μg/mL) compared to the methanolic extract (410.6 μg/mL) (Table-4).

TABLE-4
TOTAL ANTIOXIDANT ACTIVITY OF *H. vulgaris* LEAF EXTRACTS AND ASCORBIC ACID EVALUATED USING THE PHOSPHOMOLYBDENUM ASSAY

Concentration (μg/mL)	Methanolic extract	Aqueous extract	Ascorbic acid
100	30.14 ± 0.71	28.82 ± 1.3	57.76 ± 1.3
200	36.27 ± 1.9	35.09 ± 1.41	62.08 ± 1.7
300	42.09 ± 2.4	43.12 ± 0.7	70.57 ± 1.1
400	47.97 ± 1.3	50.87 ± 1.2	76.06 ± 0.9
500	54.48 ± 0.9	58.04 ± 2.1	84.14 ± 1.3
IC_{50} (μg/mL)	410.6	396.5	22.4

Values are expressed as mean ± SD.

The concentration-dependent antioxidant response observed across DPPH, ABTS and total antioxidant assays aligns

with previous studies reporting that higher extract concentrations typically correlate with increased bioactive compound availability [29,30]. The superior performance of the aqueous extract may be attributed to its enhanced solubility and extraction efficiency for hydrophilic phenolic and flavonoid compounds, which are known to contribute significantly to radical scavenging activity. Moderate thermal treatment has been reported to improve phenolic compound release by disrupting cell wall structures and protein-phenolic interactions [31,32], potentially explaining the robust activity observed.

Previous research on *H. vulgaris* has highlighted its rich content of flavonoids, alkaloids, β-carotene and vitamins C and E, supporting its strong free radical scavenging capacity [14]. Similarly, other plant studies have demonstrated comparable trends, with methanolic and aqueous extracts exhibiting differential antioxidant efficiencies due to solvent polarity and extraction methods [33,34]. Aqueous extraction is particularly effective for isolating highly polar phytochemicals, including phenolic compounds, flavonoids, tannins and glycolsides, which were prominently detected during the phytochemical screening and are well known for their antioxidant and potential anticancer activities [35]. Moreover, the extracts prepared using water are generally regarded as more biocompatible for the biological investigations since they avoid the possible toxicity associated with residual organic solvents. From an applied perspective, aqueous extracts also better reflect the forms commonly used in the traditional herbal remedies and functional food preparations [36]. Based on its comparatively higher bioactivity, the aqueous extract was selected for further GC-MS analysis to characterize its major bioactive constituents and for subsequent evaluation of its potential anticancer activity.

GC-MS analysis of aqueous leaf extract: The GC-MS was performed to characterize the phytochemical composition of the aqueous leaf extract of *H. vulgaris*. A total of 28 compounds were identified based on the retention times and mass spectral matching, revealing a chemically diverse profile (Table-5 and Fig. 2). Glycerin was identified as the dominant compound in the extract showing the highest peak area (3,226,320), which indicates its relatively high abundance. This compound has been reported to possess moisturizing and protective properties that may help maintain cellular integrity and support metabolic functions [37]. In addition to glycerin, other constituents detected in the extract included benzene, (2-butenyloxy)-, bicyclo[2.2.1]heptane, 2-(2-propenyl)-, cyclopentane, 1,1,3-trimethyl-, cyclotetradecane and several fatty acid esters such as dodecanoic acid ethyl ester, ethyl 9-hexadecenoate and 12-methyl tetradecanoic acid methyl ester. Fatty acid esters like dodecanoic acid ethyl ester (ethyl laurate) and ethyl 9-hexadecenoate have previously been reported to exhibit antimicrobial, antioxidant and anti-inflammatory activities and some studies have also suggested their potential cytotoxic effects against certain cancer cell lines [38]. The extract also contained alcohols, aldehydes, ketones, hydrocarbons, esters and

TABLE-5
IDENTIFIED PHYTOCONSTITUENTS OF *H. vulgaris* AQUEOUS LEAF EXTRACT USING GC-MS

Peak#	Retention time	Area	Height	Name
1	3.747	68913	10652	(S)-(+)-2-Amino-3-methyl-1-butanol
2	3.908	15440	3537	Propargylalcohol
3	5.200	202514	26807	Benzene,(2-butenyloxy)-
4	5.804	3226320	141311	Glycerin
5	6.442	30428	4997	2-Pyrrolidinone,1-methyl-
6	7.272	20381	5362	Hexane,2,2,5,5-tetramethyl-
7	9.931	7996	2612	2-Pentenal,(E)-
8	10.535	15843	4588	(Benzyloxy)(methyl)amine,N-trifluoroacetyl-
9	11.118	12376	3645	1-Pentanone,1-(*p*-anisyl)-3-methyl-
10	11.275	13881	2266	1,1,1,3,5,7,9,11,11,11-Decamethyl-5-(trimethy
11	13.165	70223	14902	Bicyclo[2.2.1]heptane,2-(2-propenyl)-
12	14.253	13506	3977	4-Fluoro-2-trifluoromethylbenzoicacid,neope
13	15.367	9074	1956	4-Trifluoroacetoxyoctane
14	15.496	25039	6659	Phthalicacid,cyclobutyltridecylester
15	16.598	46891	12310	Cyclopentane,1,1,3-trimethyl-
16	17.127	25277	8058	Z-2-Dodecenol
17	18.224	14514	4920	Butane,2,2-dimethyl-
18	19.848	13042	4240	Tetradecanoicacid,12-methyl-,methylester,(
19	20.260	30707	8951	Isoamylnitrite
20	20.673	73199	18926	Dodecanoicacid,ethylester
21	22.379	44765	13760	Cyclotetradecane
22	22.589	12137	3875	Isoamylnitrite
23	22.740	80706	22711	Ethyl9-hexadecenoate
24	22.958	52281	14221	Dodecanoicacid,ethylester
25	25.078	9957	2936	3-Hexanone,2,2-dimethyl-
26	26.058	13887	4401	Sulfurousacid,2-ethylhexylhexylester
27	26.398	21827	6528	Pentanenitrile,4,4-dimethyl-5-oxo-
28	27.010	15811	4654	Aceticacid,trifluoro-,2,2-dimethylpropyleste
		4186935	363762	

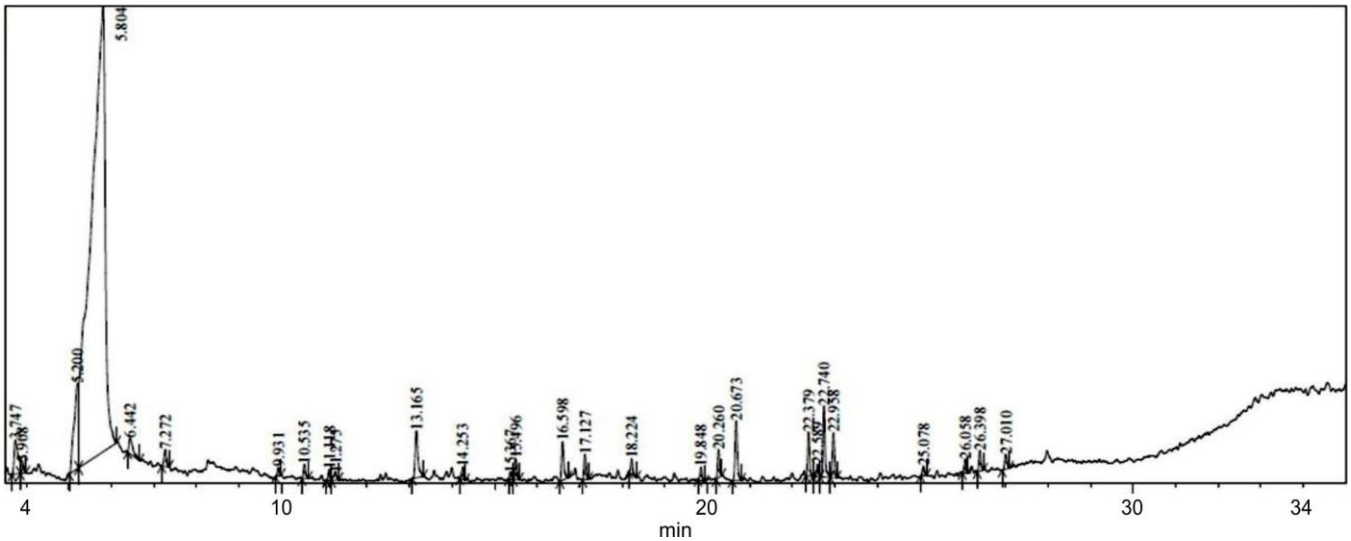

Fig. 2. Gas chromatography–mass spectrometry (GC-MS) chromatogram of *H. vulgaris aqueous* leaf extract

nitrogen-containing compounds, reflecting a complex chemical composition. Low-molecular-weight compounds including (S)-(+)-2-amino-3-methyl-1-butanol, propargyl alcohol and 2-pentenal (E), were detected at earlier retention times, whereas long-chain hydrocarbons and fatty acid derivatives eluted at higher retention times. The diversity of these phytochemicals likely contributes to the observed antioxidant activity of *H. vulgaris*, as demonstrated in the DPPH, ABTS and phosphomolybdenum assays. Many of the identified compounds have been reported to possess antioxidant, anti-inflammatory and antimicrobial properties, supporting the traditional medicinal uses of the plant [24]. Thus, the GC-MS profile confirms that the aqueous leaf extract of *H. vulgaris* is rich in bioactive constituents, highlighting its potential as a source of natural therapeutic agents and reinforcing the results obtained from the *in vitro* antioxidant assays.

Cytotoxic studies: The cytotoxic potential of *H. vulgaris* aqueous leaf extract was assessed in A431 human skin carcinoma cells using the MTT assay. Treatment with the extract resulted in a clear, dose-dependent reduction in cell viability across the concentration range of 18.75-300 μg/mL. Increasing extract concentrations progressively decreased metabolic activity indicating effective inhibition of cell proliferation and suggesting that the extract disrupts cellular metabolic processes essential for survival (Fig. 3) [39]. The half-maximal inhibitory concentration (IC_{50}) of the extract was determined to be 107.92 μg/mL, reflecting significant cytotoxic efficacy against A431 cells. These findings suggest that the aqueous extract of *H. vulgaris* can reduce cancer cell viability *in vitro* and may contain bioactive constituents with antiproliferative properties. Nevertheless, considering the moderate IC_{50} value, the extract should be regarded as a potential preliminary lead rather than a strongly potent anticancer agent. Further studies, including isolation of active compounds and detailed mechanistic investigations, are required to better understand and validate its therapeutic potential [40]. Previous studies have reported comparable cytotoxic effects of plant extracts in human cancer models. For example, Thiagarajan *et al.* [41] observed an IC_{50} of 48.89 μg/mL against oral cancer cells, while

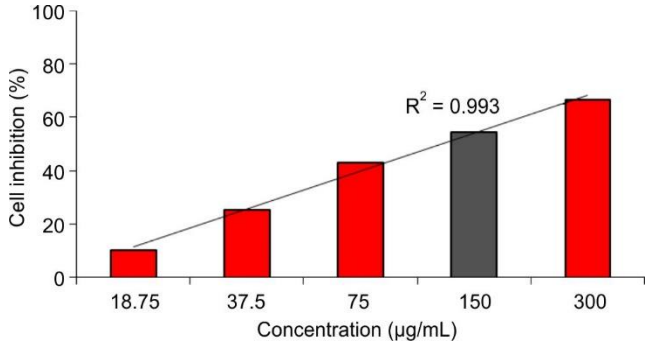

Fig. 3. IC_{50} determination of *H. vulgaris* aqueous extract against skin carcinoma (A431) cell lines

doxorubicin, used as a positive control, exhibited higher potency (IC_{50} = 9.62 μg/mL). Similarly, vanilla leaf extract was shown to suppress proliferation in A431 cells [42], highlighting the promising role of phytochemicals as potential alternative or adjunct anticancer agents.

Morphological changes in A431 cells: Phase-contrast microscopy was used to evaluate the morphological changes in A431 human skin carcinoma cells following 24 h exposure to *H. vulgaris* aqueous leaf extract (Fig. 4a-f). Untreated control cells displayed typical epithelial morphology, characterised by well-spread, adherent cells forming a confluent monolayer with intact plasma membranes. Treatment with lower concentrations of the extract (100 and 200 μg/mL) induced early cytopathic effects, including mild reductions in cell density, slight cell rounding and initial membrane irregularities. At 300 μg/mL, cells exhibited more pronounced morphological changes such as shrinkage, loss of adherence and disruption of the monolayer architecture. Higher concentrations (400 and 500 μg/mL) caused severe cytotoxic effects including extensive rounding, membrane blebbing, detachment from the substratum and formation of apoptotic bodies.

These dose-dependent morphological alterations correlate well with the reduction in cell viability observed in the MTT assay, suggesting that *H. vulgaris* extract induces apoptosis in A431 cells. The observed features-cell shrinkage, detach-

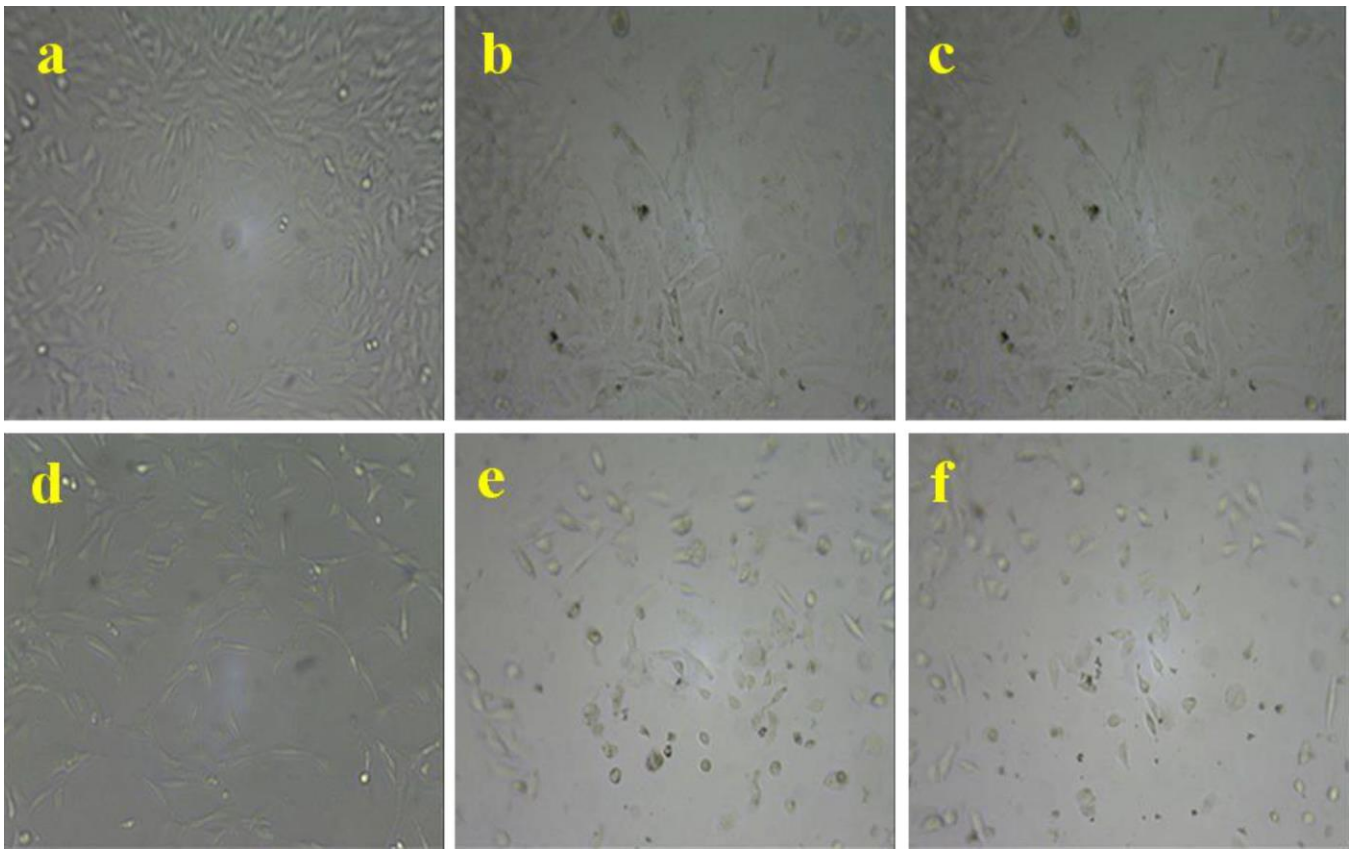

Fig. 4. Morphological analysis of aqueous leaf extract of *H. vulgaris* treated A431 cells for 24 h (a) control (untreated), (b)100 µg/mL, (c) 200 µg/mL, (d) 300 µg/mL, (e) 400 µg/mL and (f) 500 µg/mL

ment, membrane blebbing and formation of apoptotic bodies are consistent with the apoptotic cell death and align with previous reports on A431 cells treated with plant-derived extracts [43-46].

Apoptotic cell death induced by *H. vulgaris* in A431 cells: The pro-apoptotic effect of *H. vulgaris* aqueous leaf extract on A431 human skin carcinoma cells was evaluated using acridine orange/ethidium bromide (AO/EtBr) dual staining and fluorescence microscopy (Fig. 5a-b). AO/EtBr staining allows discrimination between viable, early apoptotic, late apoptotic and necrotic cells based on membrane integrity and nuclear morphology. Untreated control cells predominantly

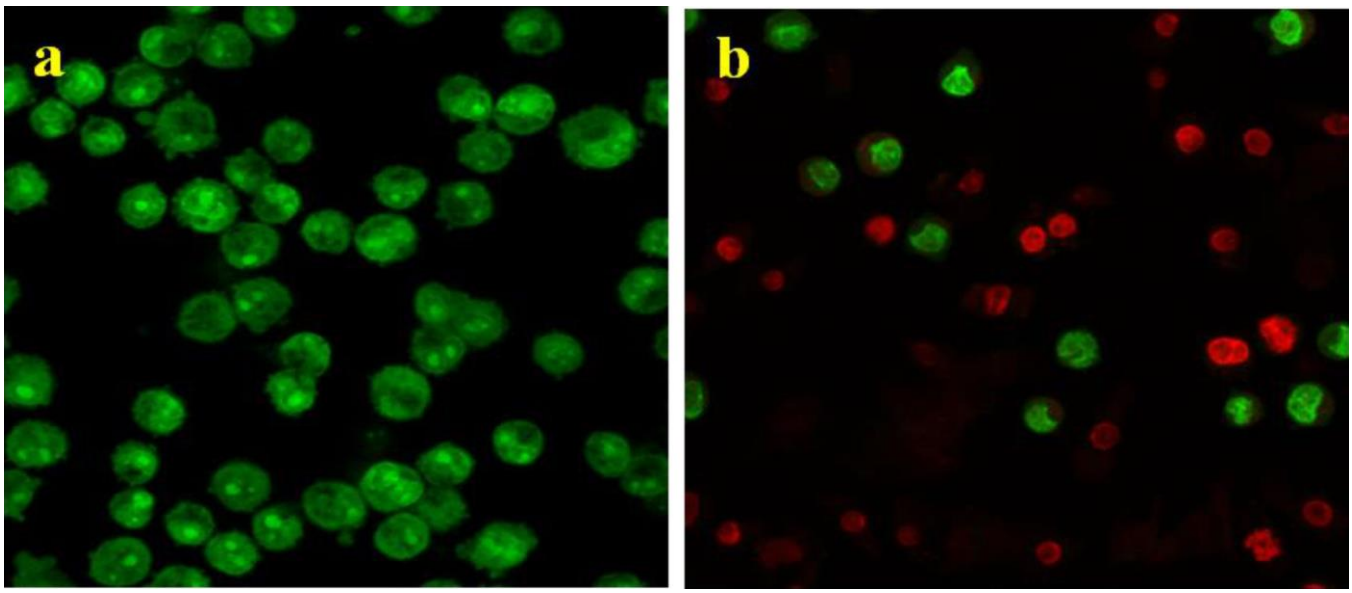

Fig. 5. AO/EtBr staining assay depicting apoptotic changes in A431 cells treated with aqueous extracts of *H. vulgaris* for 24 h: (a) untreated control and (b) treated

exhibited uniform bright green fluorescence with round, evenly stained nuclei demonstrating intact membranes and normal nuclear architecture. There was no evidence of chromatin condensation or fragmentation, confirming the absence of apoptosis or necrosis.

In contrast, cells treated with the aqueous extract showed a significant increase in apoptotic populations. Early apoptotic cells displayed bright green fluorescence with condensed or fragmented nuclei, while late apoptotic cells exhibited orange to red fluorescence due to compromised membrane integrity and enhanced ethidium bromide uptake. Morphological features characteristic of apoptosis, such as nuclear shrinkage, chromatin condensation, membrane blebbing and formation of apoptotic bodies, were clearly observed. The shift from green to orange-red fluorescence in treated cells indicates the activation of programmed cell death rather than nonspecific necrosis, supporting the cytotoxic and antiproliferative effects detected in MTT and morphological assays [44-47].

Nuclear morphology changes observed by DAPI staining: DAPI staining further confirmed the apoptotic nuclear changes in A431 cells (Fig. 6a-b). Untreated cells displayed uniform, intact nuclei with regular shape and homogeneous fluorescence, indicative of healthy, viable cells. In contrast, the extract-treated cells exhibited nuclear condensation, chromatin fragmentation, reduced nuclear size and the presence of intensely fluorescent, fragmented nuclei. Some cells also displayed punctate or dispersed nuclear staining patterns, reflecting late stage apoptosis. These nuclear alterations validate the findings from AO/EtBr staining, MTT assays and phase-contrast morphological observations, signifying that *H. vulgaris* aqueous extract induces apoptosis in A431 skin carcinoma cells. The observed apoptotic effects are consistent with the reports showing that plant-derived compounds as well as rosmarinic acid and other phytochemicals, can promote programmed cell death in cancer cells through nuclear condensation, chromatin fragmentation and membrane alterations [44,47].

Cell cycle arrest and apoptosis induction by *H. vulgaris*: Flow cytometric analysis was performed to assess the impact of *H. vulgaris* aqueous leaf extract on cell cycle progression and apoptosis in A431 human skin carcinoma cells (Fig. 7a-b). In untreated control cells, most of the population resided in the G_0/G_1 phase, with minimal representation in the sub-G_0/G_1 region, reflecting high cell viability and negligible apoptotic or necrotic events. Treatment with the extract at its IC_{50} concentration (107.92 µg/mL) induced significant changes in cell cycle distribution. There was a marked increase in the sub-G_0/G_1 population, which is indicative of DNA fragmentation and apoptotic cell death. This was accompanied by a reduction in the G_0/G_1 phase population and alterations in S and G_2/M phases, representing the disruption of normal cell cycle progression [48]. The accumulation of cells in sub-G_0/G_1 and the concomitant decrease in proliferative phases suggest that *H. vulgaris* extract triggers cell cycle arrest, thereby limiting cellular replication and promoting apoptosis. Annexin V–FITC/PI staining confirmed the pro-apoptotic effect of the extract, showing a significant increase in both early and late apoptotic cell populations compared to controls. These findings align with previous reports on the anti-melanoma and anticancer potential of *H. vulgaris* with the inhibition of metastasis and angiogenesis, as well as cytotoxic effects in various tumor models [49]. Similar apoptotic mechanisms have been observed with other plant-derived bioactive molecules; for example, peptides from *Acanthus ebracteatus* induced apoptosis in cancer cells *via* Annexin V-based pathways [50].

DNA fragmentation confirms apoptosis in A431 cells: The apoptotic effect of *H. vulgaris* aqueous leaf extract on A431 human skin carcinoma cells was further evaluated through DNA fragmentation analysis. In untreated control cells, geno-

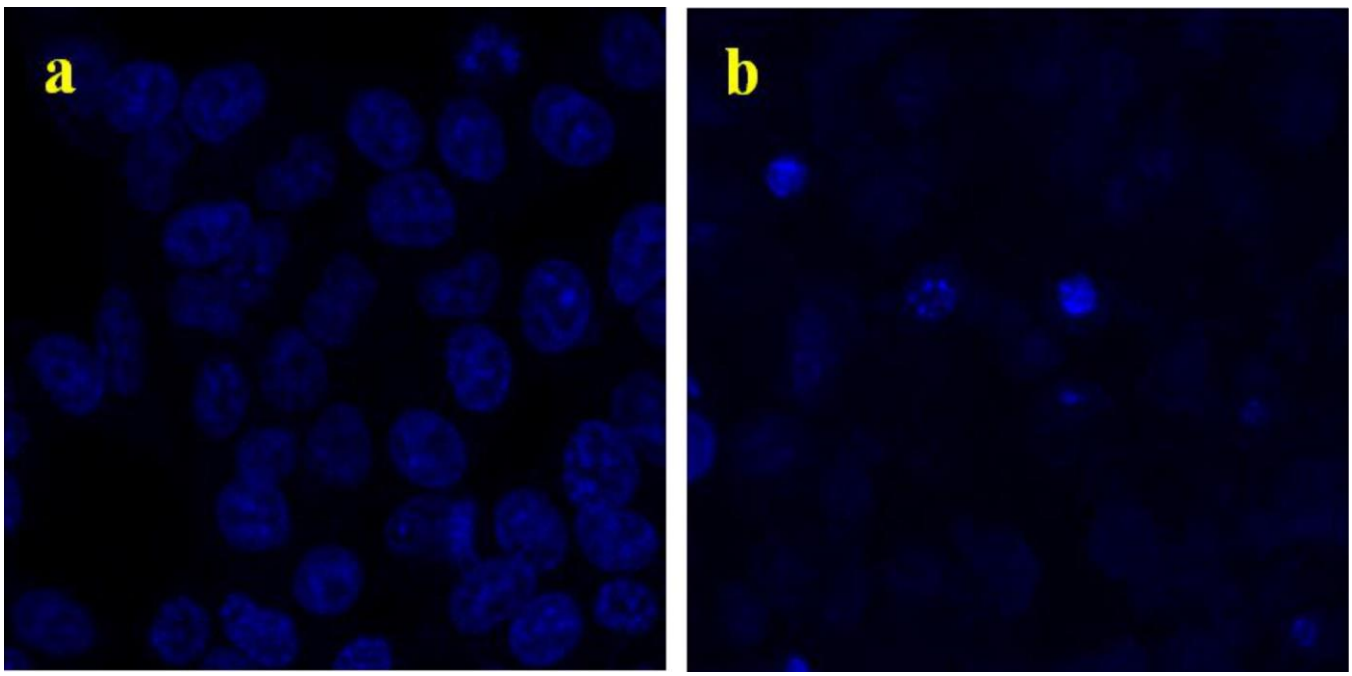

Fig. 6. DAPI staining assay depicting apoptotic changes in A431 cells treated with aqueous extracts of *H. vulgaris* for 24 h: (a) untreated control and (b) treated

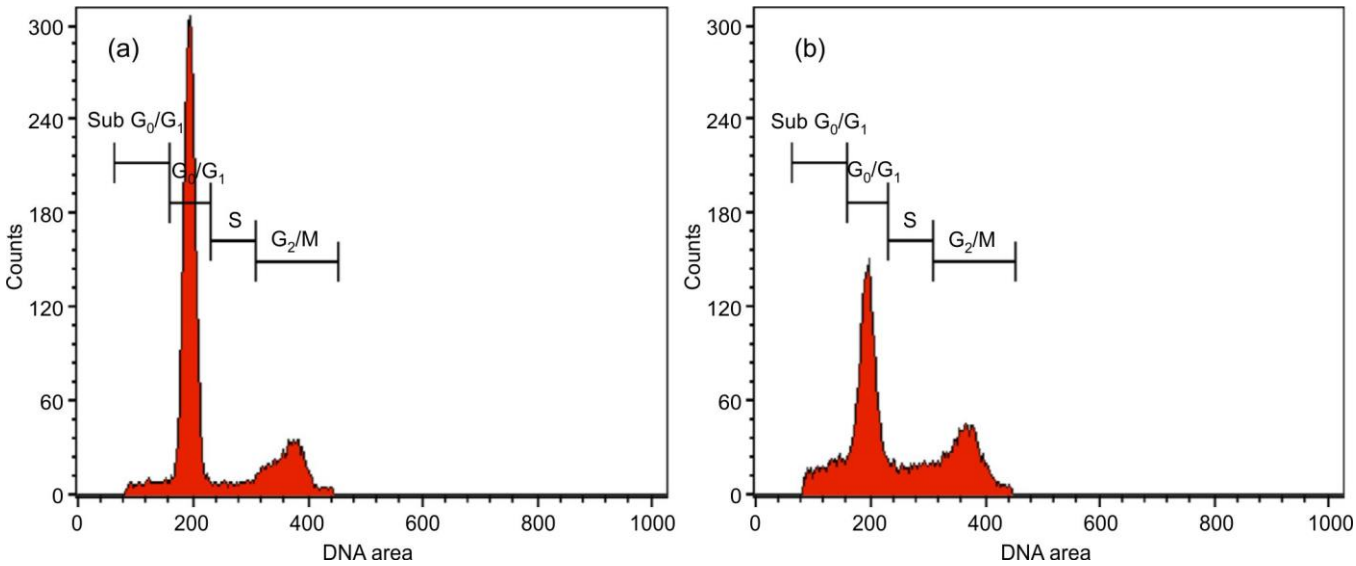

Fig. 7. Apoptosis assay by flow cytometry using PI and Annexin V-FITC double staining method *aqueous* leaf extract of *H. vulgaris* treated A431 cells (a) control, (b)107.92 µg/mL - IC_{50} values

mic DNA appeared as a single, high-molecular-weight band, indicating intact nuclear DNA and the absence of apoptotic cleavage [51]. In contrast, DNA from cells treated with the extract displayed a characteristic laddering pattern accompanied by smeared fragments, indicative of internucleosomal cleavage caused by activation of endogenous endonucleases, a biochemical hallmark of apoptosis (Fig. 8). The 1kb DNA ladder served as a molecular size reference, confirming the fragmented nature of DNA in treated cells. DNA laddering is widely recognised as a definitive marker of apoptosis, reflecting programmed chromosomal DNA degradation rather than random necrotic cleavage [48]. Similar patterns of DNA fragmentation have been reported in A549, U343 and HeLa cells treated with plant-derived compounds or chemotherapeutic agents [52], supporting the capacity of natural products to induce programmed cell death. These findings reinforce the therapeutic potential of plant-derived bioactive compounds in selectively triggering apoptosis while exhibiting relative safety and accessibility.

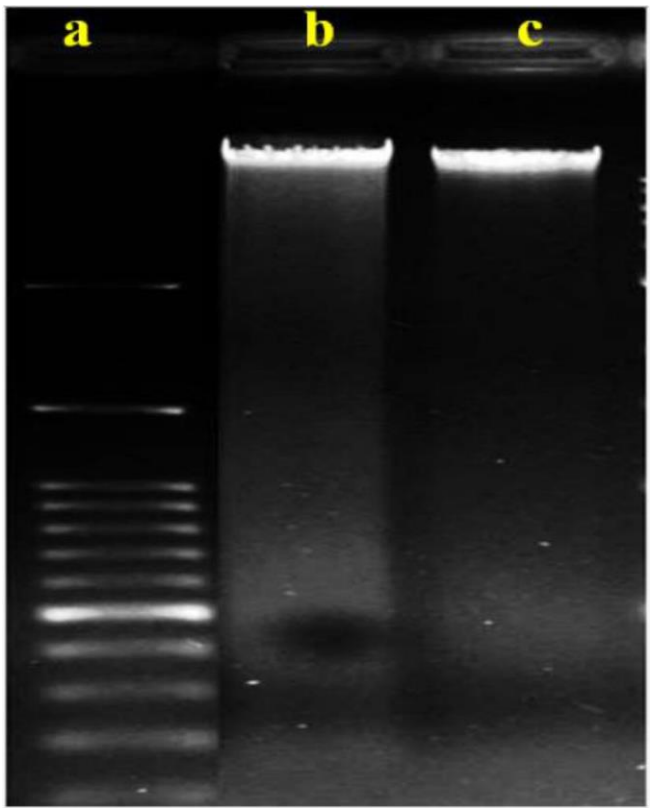

Fig. 8. (a) 1 kb DNA marker; (b) Fragmented DNA of A431 cells treated with aqueous extract of the *H. vulgaris*; (c) DNA profile of untreated A431 cells (undigested)

Conclusion

This study confirms that *Hydrocotyle vulgaris* leaf extracts are rich in bioactive phytochemicals with significant antioxidant and anticancer properties. Aqueous extraction yielded higher concentrations of phenolics, flavonoids and tannins, correlating with superior DPPH, ABTS and total antioxidant capacity values. GC-MS analysis revealed a chemically diverse profile of volatile constituents, supporting the redox potential of the extract. The aqueous extract exhibited pronounced cytotoxicity against A431 human skin carcinoma cells, with evidence of apoptosis-mediated cell death, including nuclear condensation, sub-G_0/G_1 arrest and DNA fragmentation. Based on the results, the *H. vulgaris* can consider as a promising source of chemically active natural compounds with potential relevance in antioxidant and anticancer research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

REFERENCES

1. P. Chihomvu, A. Ganesan, S. Gibbons, K. Woollard and M.A. Hayes, *Int. J. Mol. Sci.*, **25**, 8792 (2024); https://doi.org/10.3390/ijms25168792
2. Y.J. Surh, *Nat. Rev. Cancer*, **3**, 768 (2003); https://doi.org/10.1038/nrc1189
3. M. Simsek and K. Whitney, *Foods*, **13**, 1020 (2024); https://doi.org/10.3390/foods13071020
4. G. Annunziata, L. Verde, A. Zink, G. Muscogiuri, C. Albanesi, A. Paganelli, L. Barrea and E. Scala, *Curr. Nutr. Rep.*, **14**, 42 (2025); https://doi.org/10.1007/s13668-025-00632-5
5. J.K. Paul, M. Azmal, A.S.N.B. Haque, O.F. Talukder, M. Meem and A. Ghosh, *Adv. Redox Res.*, **13**, 100113 (2024); https://doi.org/10.1016/j.arres.2024.100113
6. M. Heinrich, *Br. J. Clin. Pharmacol.*, **80**, 62 (2015); https://doi.org/10.1111/bcp.12586
7. N. Frolova, T. Bilova, S. Silinskaia, A. Orlova, A. Gurina and A. Frolov, *Int. J. Mol. Sci.*, **27**, 1343 (2026); https://doi.org/10.3390/ijms27031343
8. D.J. Newman and G.M. Cragg, *J. Nat. Prod.*, **83**, 770 (2020); https://doi.org/10.1021/acs.jnatprod.9b01285
9. Z. Apalla, D. Nashan, R.B. Weller and X. Castellsagué, *Dermatol. Ther. (Heidelb.)*, **7(S1)**, 5 (2017); https://doi.org/10.1007/s13555-016-0165-y
10. S. Kowalski, J. Karska, M. Tota, K. Skinderowicz, J. Kulbacka and M. Drąg-Zalesińska, *Molecules*, **29**, 728 (2024); https://doi.org/10.3390/molecules29030728
11. R. Ganceviciene, A.I. Liakou, A. Theodoridis, E. Makrantonaki and C.C. Zouboulis, *Dermatoendocrinol*, **4**, 308 (2012); https://doi.org/10.4161/derm.22804
12. D.R. Bickers and M. Athar, *J. Invest. Dermatol.*, **126**, 2565 (2006); https://doi.org/10.1038/sj.jid.5700340
13. H.A.R. Suleria, G. Gobe, P. Masci and S.A. Osborne, *Trends Food Sci. Technol.*, **50**, 44 (2016); https://doi.org/10.1016/j.tifs.2016.01.019
14. R. Ureta, S. Mejico and Y. Maranan, *Int. J. Pharmacol. Phytochem. Ethnomed.*, **10**, 1 (2018); https://doi.org/10.18052/www.scipress.com/IJPPE.10.1
15. I. Hazarika, G.K. Mukundan, P.S. Sundari and D. Laloo, *J. Ethnopharmacol.*, **35**, 1847 (2021); https://doi.org/10.1002/ptr.6924
16. G.K. Oloyede, P.A. Onocha and B. Olaniran, *Adv. Environ. Biol.*, **5**, 3700 (2011).
17. Y.H. Lee, C. Choo, M.I. Watawana, N. Jayawardena and V.Y. Waisundara, *J. Sci. Food Agric.*, **95**, 2956 (2015); https://doi.org/10.1002/jsfa.7039
18. R. Muthia, H. Wati, W. B. Jamaludin, K. Kartini, F. Setiawan, M. Fikri, and A. Wahhab, *Pharmacogn. J.*, **13**, 73 (2021); https://doi.org/10.5530/pj.2021.13.11
19. F. Shamsa, H. Monsef, R. Ghamooshi and M. Verdian-Rizi, *Thaiphesatchasan*, **32**, 17 (2008); https://doi.org/10.56808/3027-7922.2196
20. R.B. Broadhurst and W.T. Jones, *J. Sci. Food Agric.*, **29**, 788 (1978); https://doi.org/10.1002/jsfa.2740290908
21. A.Z. Wakhidah, C. Novianti and W.A. Mustaqim, in eds.: F.M. Franco, Ethnobotany of the Mountain Regions of Southeast Asia, Springer, Cham, pp. 1-8 (2021).
22. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radic. Biol. Med.*, **26**, 1231 (1999); https://doi.org/10.1016/S0891-5849(98)00315-3
23. P. Prieto, M. Pineda and M. Aguilar, *Anal. Biochem.*, **269**, 337 (1999); https://doi.org/10.1006/abio.1999.4019
24. R.P. Shirsat, *Eur. J. Med. Plants*, **36**, 129 (2025); https://doi.org/10.9734/ejmp/2025/v36i31269.
25. S. Sasidharan, Y. Chen, D. Saravanan, K.M. Sundram and L.Y. Latha, *Afr. J. Tradit. Complement. Altern. Med.*, **8**, 1 (2011).
26. S. Mondal, M. Das, S. Debnath, B.K. Sarkar and G. Babu, *Nat. Prod. Res.*, **19**, 1 (2024).
27. F. Maiza-Benabdesselam, S. Khentache, K. Bougoffa, M. Chibane, S. Adach, Y. Chapeleur and D. Laurain-Mattar, *Rec. Nat. Prod.*, **1**, 28 (2007).
28. A.E. Hagerman, K.M. Riedl, G.A. Jones, K.N. Sovik, N.T. Ritchard, P.W. Hartzfeld and T.L. Riechel, *J. Agric. Food Chem.*, **46**, 1887 (1998); https://doi.org/10.1021/jf970975b
29. D. Tungmunnithum, A. Thongboonyou, A. Pholboon and A. Yangsabai, *Medicines*, **5**, 93 (2018); https://doi.org/10.3390/medicines5030093
30. İ. Gulcin, *Arch. Toxicol.*, **94**, 651 (2020); https://doi.org/10.1007/s00204-020-02689-3
31. D.A. Ribeiro, S.S. Damasceno, A.A. Boligon, I.R.A. de Menezes, M.M.A. Souza and J.G.M. da Costa, *Asian Pac. J. Trop. Biomed.*, **7**, 739 (2017); https://doi.org/10.1016/j.apjtb.2017.07.009
32. S. Kumar and N. Goel, *Biotechnol. Rep.*, **24**, e00370 (2019); https://doi.org/10.1016/j.btre.2019.e00370
33. S. Baliyan, R. Mukherjee, A. Priyadarshini, A. Vibhuti, A. Gupta, R.P. Pandey and C.M. Chang, *Molecules*, **27**, 1326 (2022); https://doi.org/10.3390/molecules27041326
34. J.G. Shirmila and P.M. Radhamany, *Int. J. Pharma Sci.*, **5**, 161 (2013).
35. H. Harouak, J. Ibijbijen and L. Nassiri, *Chem. Afr.*, **5**, 543 (2022); https://doi.org/10.1007/s42250-022-00333-4
36. A. Kumar, N. P, M. Kumar, A. Jose, V. Tomer, E. Oz, C. Proestos, M. Zeng, T. Elobeid, S. K and F. Oz, *Molecules*, **28**, 887 (2023); https://doi.org/10.3390/molecules28020887
37. H.J. Chen, P.Y. Lee, C.Y. Chen, S.L. Huang, B.W. Huang, F.J. Dai, C.F. Chau, C.S. Chen and Y.S. Lin, *Sci. Rep.*, **12**, 10232 (2022); https://doi.org/10.1038/s41598-022-13452-2
38. G. Mohammadi, M.M. Zangeneh, A. Zangeneh and Z.M.S. Haghighi, *Appl. Organomet. Chem.*, **34**, e5136 (2020); https://doi.org/10.1002/aoc.5136
39. T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983); https://doi.org/10.1016/0022-1759(83)90303-4
40. R.M. Widyanto, R.N. Halimah, Y. Rahmi, B. Utomo, W.D. Proborini and Y. Yunimar, *J. Islam. Med.*, **4**, 123 (2020); https://doi.org/10.18860/jim.v4i2.10109
41. M.K. Thiagarajan, D. Chandrasekharan, R. Mahalingam and A. Ravi, *J. Oral Maxillofac. Pathol.*, **28**, 211 (2024); https://doi.org/10.4103/jomfp.jomfp_32_24
42. K. Vijaybabu and K. Punnagai, *Biomed. Pharmacol. J.*, **12**, 1141 (2019); https://doi.org/10.13005/bpj/1742
43. Y. Nakamura, *J. Med. Assoc. Thai.*, **96**, S60 (2013).
44. A.J. Akindele, Z.A. Wani, S. Sharma, G. Mahajan, N.K. Satti, O.O. Adeyemi, D.M. Mondhe and A.K. Saxena, *Evid. Based Complement. Alternat. Med.*, **2015**, 1 (2015); https://doi.org/10.1155/2015/560404
45. V. Mohansrinivasan, S. Devi C, M. Deori, A. Biswas and J. Naine S, *Braz. Arch. Biol. Technol.*, **58**, 540 (2015); https://doi.org/10.1590/s1516-89132015000076
46. V.N. Yajarla, R.P. Nimmanapalli, S. Parikapandla, G. Gupta and R. Karnati, *J. Herbs Spices Med. Plants*, **20**, 359 (2014); https://doi.org/10.1080/10496475.2013.876698
47. B. Jin, J. Liu, D. Gao, Y. Xu, L. He, Y. Zang, N. Li and D. Lin, *J. BUON*, **25**, 1383 (2020).
48. W.K. Kaufmann and D.G. Kaufman, *FASEB J.*, **7**, 1188 (1993); https://doi.org/10.1096/fasebj.7.12.8375618
49. J. Antony, M. Saikia, V. Vinod, R. Nath, M.R. Katiki, M.S.R. Murty, A. Paul, A. Shabna, H. Chandran, S.M. Joseph, S.K. Nishanth Kumar, E.J. Panakkal, I.V. Sriramya, I.V. Sridivya, S. Ran, S. Sankar, E. Rajan and R.J. Anto, *Sci. Rep.*, **5**, 11107 (2015); https://doi.org/10.1038/srep11107
50. E. Li, T. Zhang, X. Sun, Y. Li, H. Geng, D. Yu and C. Zhong, *Oncol. Lett.*, (2019); https://doi.org/10.3892/ol.2019.10657
51. P. Cos, A.J. Vlietinck, D.V. Berghe and L. Maes, *J. Ethnopharmacol.*, **106**, 290 (2006); https://doi.org/10.1016/j.jep.2006.04.003
52. C. Singh, D. Ahuja and S.C. Mehta, *Int. J. Pharm. Qual. Assur.*, **10**, 361 (2019); https://doi.org/10.25258/ijpqa.10.2.25