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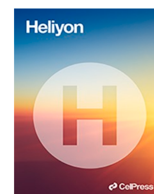

Research article

Metabolomic insights and bioactive efficacies of *Tragopogon dubius* root fractions: Antioxidant and antiproliferative assessments

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ABSTRACT

Tragopogon dubius is commonly consumed as a vegetable and used in traditional medicine for treating inflammatory skin conditions and cutaneous swelling. Despite known pharmacological properties of its leaves and roots, many of its biological characteristics and active phytochemicals remain unexplored. The present study investigates the phytochemical composition, antioxidant, and anticancer properties of methanolic root extracts and isolated fractions (TdRM-1 and TdRM-2) of *T. dubius*. Utilizing preparative thin-layer chromatography, the crude extract was successfully separated into TdRM-1 and TdRM-2, characterized by GC-MS and FTIR analysis, revealing a diverse range of bioactive compounds including terpenes, flavonoids, and phenolic acids. Qualitative phytochemical screening indicated the presence of carbohydrates, tannins, alkaloids, and other phytoconstituents. Advanced UPLC-ESI-QTOF-MS analysis identified 54 metabolites, significantly contributing to the chemical profiling of the extract. The antioxidant activities of the fractions were quantitatively assessed using ABTS, DPPH, and superoxide radical scavenging assays, where TdRM-2 exhibited superior activity with IC_{50} values ranging from 51.29 to 60.03 μg/mL. Anticancer potential was evaluated against A549, LN-18, and MCF-7 cancer cell lines, demonstrating that TdRM-2 significantly inhibited cell proliferation with GI_{50} values as low as 31.62 μg/mL for A549 cells. Additionally, fluorescence microscopy revealed that TdRM-2 induces apoptosis, indicated by changes in nuclear morphology and loss of mitochondrial membrane potential. Annexin V-FITC/PI double staining indicate that the TdRM-2 fractions from *T. dubius* can significantly inhibit the growth of A-549, LN-18, and MCF-7 cancer cell lines by inducing apoptosis These findings suggest that *T. dubius* root extracts, particularly the TdRM-2 fraction, hold promising therapeutic potential due to their significant antioxidant and anticancer activities, underpinned by their rich phytochemical composition. This study underscores the importance of *T. dubius* as a source of natural bioactive compounds with potential health benefits.

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1. Introduction

In recent years, there has been a noticeable surge in interest surrounding natural substances as potential reservoirs of bioactive compounds with diverse pharmacological properties [1]. *Tragopogon dubius*, commonly known as yellow salsify or western Goat's beard and belonging to the Asteraceae family, has garnered attention for its purported medicinal benefits [2–4]. In certain regions, it serves dual purposes as both a culinary ingredient and a remedy for various health ailments [3,4]. The aerial parts of the plant are valued for their cleansing, sweat-inducing, diuretic, antidiarrheal, anti-inflammatory, antirheumatic, and antidiabetic properties and are frequently employed in traditional medicinal practices [5–8] Additionally, it is applied topically to address inflammatory skin issues, skin swellings, and to aid in wound healing [9,10]. Indigenous communities in Jammu and Kashmir, India, utilize the plant parts for wound healing [5,7]. Extracts from various *T. dubius* plant parts, such as water, methanol, and ethyl acetate, have demonstrated antioxidant potential, antibacterial and antifungal activities, and enzyme inhibitory effects, yet studies on its anticancer potential remain scarce [6,11,12]. Previous research has identified the presence of flavonoids, phenyl methane derivatives, and esters of phenyl propanoic acid in *T. dubius* plants [13]. However, there is limited scientific evidence supporting the pharmacological properties of *T. dubius*, and the medicinal phytochemicals present in this plant have yet to be fully elucidated. The search for new anticancer phytochemicals with enhanced efficacy and fewer side effects continues as an effort to overcome the limitations of chemical-based therapies. Our study indicates significant antioxidant and antiproliferative properties in root extracts, though the specific mechanisms and active compounds responsible remain largely unexplored. Thus, there is an urgent need to identify these pharmacologically active compounds from *T. dubius* to harness its potential as a medicinal resource. Cancer remains a significant global health challenge, ranking among the top ten causes of disease-related deaths worldwide, with breast, lung, and colon cancers contributing to the highest incidence of new cases in 2020 [14–16]. The uncontrolled proliferation of cells underlies the pathology of cancer, emphasizing the importance of inhibiting this process in potential treatments [17,18]. However, conventional medications such as cisplatin and doxorubicin are associated with adverse effects such as kidney damage and heart toxicity [19,20]. Natural products offer a promising avenue for the discovery of novel anticancer compounds, highlighting the need to explore potent antiproliferative agents derived from nature [16,21].

Metabolomics, a powerful analytical technique for profiling and quantifying small molecules in biological samples, provides a comprehensive view of the metabolic landscape, offering valuable insights into the bioactive constituents of medicinal plants [22]. By integrating chemical profiles with biological activity through multivariate analysis, it becomes possible to identify metabolites associated with specific biological effects, such as antioxidant and antiproliferative properties [23,24]. Therefore, our research aims to unveil the metabolomic profile of *T. dubius* root fractions and evaluate their antioxidant and antiproliferative potential. Given the pivotal role of antioxidants in combating oxidative stress, a major contributor to various chronic diseases including cancer, cardiovascular disorders, and neurodegenerative conditions, understanding the antioxidant properties of natural products is crucial. Similarly, compounds exhibiting antiproliferative activity hold significant therapeutic promise, particularly in the context of cancer treatment [25,26]. Our findings offer insights into the metabolomic composition and bioactive properties of *T. dubius* root fractions, highlighting its potential as a valuable source of pharmacologically active compounds and laying the groundwork for the development of new antioxidant and anticancer agents from natural reservoirs.

2. Material and methods

2.1. Chemicals and reagents

The chemicals and reagents utilized in this study were of analytical grade and procured from Sigma-Aldrich Chemicals Pvt Ltd, USA. Enzymes and dyes were supplied by Hi-media Laboratories Mumbai, India. Deionized water was used for solution preparation.

2.2. Plant collection and identification

Plant material of *T. dubius* was collected in the morning from mountainous plateaus of Pulwama district, Jammu & Kashmir, India (33° 52' 21.1296'' N, 74° 53' 34.2708'' E,1669.0 m above sea level). The plant material was washed thrice with fast moving tap water to remove dust, followed by drying at temperature below 30 °C to avoid the decomposition of thermolabile compounds. This drying process was carried in an open room with air circulation around the plant material to prevent heat and moisture accumulation. The plant species was identified by taxonomist Mr. Akhtar Malik at the University of Kashmir. A plant voucher specimen [No.: (2940-(KASH)] was deposited at the Centre of Taxonomy and Biodiversity, University of Kashmir, Hazratbal, Srinagar, India.

2.3. Preparation of plant extracts

The roots of *T. dubius* were ground into fine powder for extraction. and 250 g of powder were macerated [27] in solvents with increasing polarity: hexane, chloroform, ethyl acetate, and methanol. The mixture was left undisturbed for 48 h at room temperature, yielding the following fractions: hexane fraction TdRH (3.6 %), chloroform fraction TdRC (6.4 %), ethyl acetate fraction TrRE (2.4 %) and methanol fraction TdRM (18.4 %), respectively. The polarities of the solvents facilitated the partitioning and separation of the plant secondary metabolites (PSMs) of the fractions according to their solubility. To prevent the thermal breakdown of volatile substances, the mixture was passed through Whatman No. 1 filter paper, and a rotary evaporator (Buchi Rotavapor R-210, Flawil,

Switzerland) was used to concentrate the filtrate at 40 °C. After evaporation, 46 g of methanolic root extract of black greenish color was collected and kept at 4 °C for further analysis.

2.4. Preparative thin layer chromatographic (TLC) separation of plant extract

Preparative thin layer chromatography (TLC) was employed to isolate bioactive components from crude methanolic root extract (TdRM) and assess their antioxidant and apoptotic inducing potential. TLC plates (20 × 20 cm) were prepared using silica gel GF254 (E-Merck) with particle size 5–40 μm. 30 g of finely powdered silica gel GF254 were thoroughly mixed with 60 mL of dH_2O. The resulting slurry was applied to the TLC applicator, which was then gradually moved onto a clean glass plate to form a uniform layer approximately 0.5 mm thick. The glass plate was allowed to dry for 1 h. Subsequently, the silica gel coated plate was dried for 2 h at 110 °C. Following activation of the TLC plate, 100 μL of the plant extract was spotted using a capillary tube, ensuring the integrity of silica gel layer, and left undisturbed to facilitate the separation of major bands from the methanolic root extract of *T. dubius*. Various solvent systems were employed as the mobile phase to separate different components or achieve optimal banding of the crude extract mixture. The spotted TLC plate was placed in the TLC tank, with the mobile phase added, ensuring immersion of approximately 0.5 mm of the plate in the solvent at the bottom. The tank was sealed with a glass lid to ensure complete filling of the chamber with solvent vaoprs. The plates were submerged in the TLC tank until the solvent front reached the top three-quarters of the plate. Subsequently, the plate was removed from the tank and allowed to dry outside at 37 °C to facilitate solvent evaporation. The TLC run plates were examined under bright light, and the separated spots were noted. Relative front values (RF) were calculated using the following formula:

$$\text{RF value} = \frac{\text{Solute Front}}{\text{Solvent Front}}$$

2.5. Preliminary screening of extracts for phytoconstituents

The phytochemical screening for phytoconstituents of TdRM involved a qualitative analysis to detect various plant metabolites including tannins, carbohydrates, anthraquinones, saponins, flavonoids, resins, steroids, phenols, glycosides, alkaloids, and triterpenes. Standard procedures as described in previous studies [28–30].

2.6. UPLC-ESI-QTOF-MS analysis

The samples for metabolic analysis were analyzed using the MS Q-TOF Component Model G6550A with Dual AJS ESI Ion Source, maintaining a flow rate of 0.3 mL/min at 35 °C having flow rate 5 μl maintained at 35°. Two mobile phases, 0.1 % (v/v) aqueous formic acid (mobile phase A) and acetonitrile (mobile phase B), were used to achieve a gradient elution. Q-Exactive Plus Biopharma-High Resolution Orbitrap with a Direct Infusion Mass and ESI & APCI Positive & Negative mode ionization column G1316C were utilized for mass spectrometric analyses. This enables in detailed characterization of metabolites for understanding antioxidant and antiproliferative properties, thereby facilitating insights into its bioactive efficacies for potential therapeutic applications. A capillary voltage of 2.5 kV was used to spray the liquid and charge the droplets, with a cone voltage of 25 V, cone gas flow rate of 50 L/h, desolation gas flow rate of 13 L/min, desolation gas temperature of 250 °C, and source temperature of 150 °C. Collision energies of 30–35 eV were optimized for fragmentation and high resolution, accurate mass, and high sensitivity. The mass range of 120–1200 *m/z* was set. Compound Discoverer software 2.1 SP1 (Thermo Fisher Scientific, Chicago, IL, USA) was employed for data processing on all UPLC-ESI-Q-TOF-MS data. For metabolite identification, spectral libraries as well as compound databases such as ChemSpider and mzCloud were utilized.

2.7. Gas chromatography-mass spectrometry (GC-MS) analysis

Samples were subjected to GC-MS analysis using an Agilent 7890A gas chromatograph coupled with an Agilent 5975C inert XL MSD mass spectrometer equipped with a triple-axis detector and Mass Hunter Workstation software (USA). The DB-5 column (30 m × 0.25 mm i. d. × 0.25 μm film thickness) was employed for the analysis. A helium carrier gas flow rate of 0.5 mL/min was utilized. The temperature was programmed to initially hold at 50 °C for 1 min, then raised up to 50–250 °C at a rate of 50 °C/min, followed by 5 min hold at 250 °C. Mass spectra were recorded in electron impact (EI) mode with an ionization energy of 70 eV and a scan rate of 0.5 s/scan, covering a scan range of 50–600 AMU. Inlet and transfer line temperatures were set to 250 °C. A 50:1 split ratio was employed. Identification of components was accomplished using the Wiley and NIST libraries. Additionally, compounds were identified through peak enrichment via co-injection with readily available genuine standards. Peak area percentages (%) were obtained electronically via access to the extracted ion chromatogram (EIC) response, eliminating the need for correction factors.

2.8. FTIR analysis

FTIR spectra of *T. dubius* roots TdRM, as along with the separated fractions TdRM-1 and TdRM-2, were acquired using a Shimadzu Fourier Transform Infrared (FTIR) spectrometer (Model IR Tracer-100). The IR spectra were recorded in absorbance mode across the 400–4000 cm^{-1} frequency range. Prior to sample measurement, the ATR crystal was meticulously cleaned with ethanol and allowed to

dry. Subsequently, the IR spectra were processed in Lab Solutions IR V2 (Shimadzu Corporation) for normalization. The Happ-Genzel apodization function was employed to strike a balance between ripples and resolution.

2.9. Total phenolic content

To determine the phenolic content of the *T. dubius* extract/fractions, the Folin-Ciocalteu method was employed with minor modifications [31]. In this experiment, 0.1 mL of the test sample (200 μg/mL) was added in 900 μL of dH_2O, followed by the addition of 500 μL of Folin-Ciocalteu reagent. Then, 1.5 mL of 20 % sodium carbonate was added to this mixture and thoroughly shaken manually. The reaction mixture was incubated for 2 h. After incubation period, dH_2O was added to bring the volume up to 10 mL. Finally, the absorbance was measured at 765 nm. Gallic acid (25–400 μg/mL), a standard phenolic compound, was used to create the calibration curve.

2.10. Total flavonoid content

The total flavonoid content of the extract (TdRM) and isolated fractions TdRM-1 and TdRM-2 was determined using the aluminium chloride method [32]. A working reaction mixture was prepared by combining 1 mL of the test extracts with 4 mL of dH_2O. To this mixture, 300 μL of 5 % $NaNO_2$ and 300 μL of 10 % $AlCl_3$ were added, followed by 5 min incubation period. Subsequently, 2 mL of 1 M NaOH were added to bring the total volume to 10 mL. The absorbance of the resulting mixture was measured at 510 nm. To establish a calibration curve, rutin (25–400 μg/mL), a standard flavonoid compound, was used. Using the regression equation derived from this calibration curve, the total flavonoid content of the extract or fraction was calculated and expressed as rutin equivalents (RE) in mg/g of the dry weight of extracts.

2.11. Antioxidants activity

2.11.1. DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the methanolic root extract and fractions was determined using the method described by Blois [32] with slight modifications. This method relies on the ability of antioxidants to scavenge the DPPH cation radical. Briefly, 1 mL of the sample extract (ranging from 25 to 400 μg/mL of lyophilized extract) or standards was added to 3 mL of 0.5 mM DPPH solution and vigorously vortexed. After a 30 min dark incubation period of mixture at room temperature, the decolorization of DPPH was measured at 517 nm relative to a blank. The sample's capacity to scavenge the DPPH radical was determined using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

Here, Abs Control represents the absorbance of DPPH radical in methanol, and Abs Sample represents the absorbance of DPPH radical mixed with the sample extract/standard.

2.11.2. Superoxide anion radical scavenging assay

The superoxide anion radical scavenging activity of the extract/fractions was assessed using the method Nishikimi et al. [33] with minor modifications. Superoxide anions were generated by adding 1 mL of NBT solution (156 μM) and 1 mL of NADH (nicotinamide adenine dinucleotide) solution (468 μM). The NADH will serve as the electron donor, while NBT will act as an electron acceptor. Following the addition of 100 μL of a solution of 60 μM phenazine methosulphate (PMS) prepared in 100 mM phosphate buffer (pH 7.4), the reaction was allowed to proceed for 5 min at 25 °C. The non-enzymatic phenazine methosulfate/nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, and NBT serves as a reference to determine production of superoxide anions by its reduction of NBT into a purple-colored formazan. The absorbance was measured at 560 nm using a multimode microplate reader. The inhibition percentage was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

Where, Abs Control represents the absorbance of superoxide radicals in methanol, and Abs sample represents the absorbance of superoxide radicals mixed with the sample extract or standard.

2.11.3. ABTS assay

The method described by Suseela et al. [34] was employed with slight modifications to assess the radical scavenging activity of extract and fractions based on their ability to scavenge ABTS radicals. Two stock solutions were prepared: a 2.4 mM potassium persulfate solution and a 7 mM ABTS solution. These solutions were mixed in equal proportions to create the working solution, which was then allowed to react for 14 h at room temperature in the dark. Following this, the solution was diluted with 1 mL of ABTS solution and 60 mL of methanol. The absorbance was measured, yielding a value of 0.706 ± 0.01 units at 734 nm. Fresh ABTS solution was prepared for each assay solution. The absorbance at 734 nm was measured after 7 min of reaction between 1 mL of plant extracts and 1 mL of the ABTS solution. The percentage of inhibition was calculated as the ABTS radical scavenging activity, and the extract's ability to

scavenge ABTS was compared to that of rutin using the following formula:

$$\text{Inhibition } (\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

where Abs Control represents the absorbance of ABTS radical in methanol, and Abs Sample represents the absorbance of ABTS radical solution mixed with sample extract or standard.

2.12. MTT assay

The antiproliferative potential of methanolic extract or fractions from *T. dubius* root against lung (A549), glioblastoma (LN-18) and human breast (MCF-7) cancer cell lines was assessed using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [35]. Cell lines were obtained from NCCS Pune, India, and cultured in RPMI1640 and DMEM (Dulbeccos Modified Eagle Medium) supplemented with 20 % FBS (Fetal Bovine Serum), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C under carefully monitored conditions. The selection of A-549, LN-18, and MCF-7 cell lines was based on their relevance to the study's objectives, reflecting distinct tissue origins and characteristics pertinent to the research question. For each cell line, cells were seeded at a density of 8×10^3/0.1 mL in 96-well plates, and allowed to adhere for 24 h. Subsequently, cells were exposed to varying concentrations (31.25–1000 μg/mL) of each fraction and incubated for an additional 24 h. Following this incubation period, cells were treated with 20 μl MTT solution (5 mg/mL) for 3 h. The formazan crystals formed were dissolved by adding 0.1 mL of DMSO after removing the supernatant, and the absorbance was measured at 570 nm. The GI_{50}, which represents the concentration of extract and fractions that inhibits 50 % of cell growth during the 24 h of treatment, was calculated using the following equation:

$$GI_{50} = (T_{24} - C+) \,/\, (C^- - C^+) \times 100 = 50$$

whereas T_{24}, C^+, and C^- correspond to the number of treated cells, the number of cells in the positive control, and the number of cells in the negative control after 24 h of treatment, respectively. Percentage growth inhibition indicates the extent to which the extract or fractions suppress the growth of cells in a given experiment. Higher percentages suggest greater efficacy in inhibiting growth, indicating the potential effectiveness of the extract or fractions in combating the proliferation of the cells under study. DMSO and culture medium were utilized as positive and negative controls, respectively.

2.13. Morphological assessment of cancerous cells

2.13.1. Nuclear staining with DAPI

The nuclear morphology and apoptosis inducing potential of the TdRM-2 fraction were assessed in A549, LN-18 and MCF-7 cell lines using DAPI (40′,6-diamidino-2-phenylindole) staining [36]. Briefly, cancer cells were grown in 24 well plate at a density of 4×10^5 cells/well. After 24 h, they were treated with the GI_{50} value of TdRM-2. Following a 24 h treatment period, cells were washed with 1X PBS (Phosphate buffered saline) and fixed with 4 % paraformaldehyde for 15–20 min in the dark. Subsequently, cells were washed again with 1X PBS. DAPI (4 μg/mL) was used for nuclear staining, and cells were incubated for 30 min in the dark. Stained cells were washed twice with 1X PBS to remove excess dye, and coverslips were mounted on glass slides using fluoromount. The cells were examined under Nikon A1R Fluorescence Microscope system (Nikon Eclipse T2 Japan).

2.13.2. Rhodamine 123 (Rh-123) staining

Rhodamine 123 (Rh-123) staining was employed to observe the effects of the TdRM-2 fraction on changes in the mitochondrial membrane in A549, LN-18, and MCF-7 cells, utilizing a fluorescent microscope as described by Puja et al. [36]. Cancer cells were seeded at a density of 4×10^5 cells/well in a 24-well plate and treated with the TdRM-2 fraction (GI_{50}) for a full day. Subsequently, the cells (at a density of 10^5 cells/well) were stained with rhodamine 123 stain (Thermo Fisher, USA) for 1 h at 37 °C in the dark. Observations were made under a fluorescence microscope (Nikon Eclipse Ts2, Japan) at 20X magnification.

2.13.3. Detection of apoptosis by AO/EtBr staining

A549, LN-18 and MCF-7 cells were cultured and seeded at a density of 4×10^5 cells/well in 6-well plate and allowed to adhere. After 24 h, cells were treated with the GI_{50} value. To obtain a pellet, both suspended and adherent cells were combined and centrifuged for 5 min at 1500 rpm. The supernatant was decanted, and the pellet was resuspended in 100 μL of 1X PBS. Next, the cells were incubated with a 5 μL mixture of AO/EtBr (60 μg/mL acridine orange and 100 μg/mL ethidium bromide) for 5 min in the dark [37]. Subsequently, 25 μL of the stained cell mixture was added to a glass slide and covered with a coverslip. The slide was promptly examined under a fluorescence microscope (Nikon Eclipse Ts2, Japan).

2.14. Annexin V-FITC/PI double staining for apoptotic cell death quantification

The apoptotic cell death in TdRM-2 treated cells was quantified using Annexin V-FITC/PI double staining method with a flow cytometer [38]. A-549, MCF-7 and LN-18 cells were grown in 6-well plates and treated with the GI_{50} concentration of the TdRM-2 fraction for 24 h. After the treatment period, both floating and adherent cells were harvested and centrifuged for 5 min. The pellet

cells were washed with PBS and centrifuged again for 5 min. The cell pellets were then resuspended in binding buffer (0.1 M) for 15 min followed by the addition of 5 μL of Annexin V-FITC conjugate and 5 μL propidium iodide (PI). The cells were incubated in the dark for 15 min. Finally, data was acquired using a BD Accuri C6 flow cytometer (BD Biosciences) and analyzed using BD Accuri software.

2.15. Statistical analysis

Each experiment was conducted three times, and the results were expressed as mean ± standard error. One-way analysis of variance (ANOVA) was employed to determine significant differences among all the groups at the $p < 0.05$ level of significance. Tukey's HSD (Honestly Significant Difference) test was utilized for comparing the means. GI_{50} and IC_{50} values were calculated using excel software (2019) by comparing triplicate data variables.

3. Results

3.1. Preparative thin layer chromatography

The crude plant extract was subjected to PTLC to separate it into various fractions potentially present in the methanolic extract. Using a solvent combination of hexane and ethyl acetate in a ratio of 70:30, bands were successfully separated. Approximately 10 mg of two dominant bands, labelled as TdRM-1 and TdRM-2 (Fig. 1), were collected for further analysis of their antioxidant and anticancer activities. Additionally, these two fractions were characterized through GC-MS analysis.

3.2. Qualitative analysis of phytoconstituents

The qualitative phytochemical screening of methanolic root extract of *T. dubius* revealed the presence of various phytoconstituents. Triterpenes, resins, alkaloids, glycosides, phenols, steroids, flavonoids, tannins, saponins, and carbohydrates were identified in the extract. Among these phytoconstituents, triterpenes, resins, and carbohydrates were present in abundant in the extract as compared to others. However, anthraquinones were notably absent in the extract.

3.3. UPLC-ESI-QTOF-MS analysis

Ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry plays a unique role in characterizing, quantifying, and confirming metabolite identification from plant extracts with unmatched confidence. The methanolic root extract of *T. dubius* was analyzed using this method, resulting in identification of 54 plant metabolites eluted between 1 and 35 min (Fig. 2A and B; Table 1). The majority of these compounds belonged to significant classes such as fatty acids, tannins, flavonoids, phenolic acid derivatives, terpenes, and terpenoids. Terpenes and terpenoid derivatives found in TdRM included Gibberellin A91, Taraxacolide 1-O-b-D-glucopyranoside, Yucalexin P15, Limonexic acid, Eremosulphoxinolide A, Rutaevin, Austin, Nigakihemiacetal A, Cascarillin, and Pleuromutilin. In addition to terpenes, several flavonoid compounds were detected, such as Nb-trans-Feruloylserotonin glucoside, Kaempferol 3-sophoroside 7- glucoside, Gossypetin 8-glucoside, Myricetin 7-rhamnoside, Kaempferol 3-O-β-D-galactoside, 8-(1,1-dimethylallyl) galangin and Rutin. Phenolic compounds like ellagic acid, curcumin diglucoside, Kelampayoside A, synergic acid, *trans*-chloregenic acid and glycosides, Linusitamarin, Perilloside E, Nb-trans-Feruloylserotonin glucoside, Glucosylgalactosyl hydroxylysine, Astragalin 7-rhamnoside, and Kaempferol 3-(2''- rhamnosylgalactoside) 7- rhamnoside were also found.

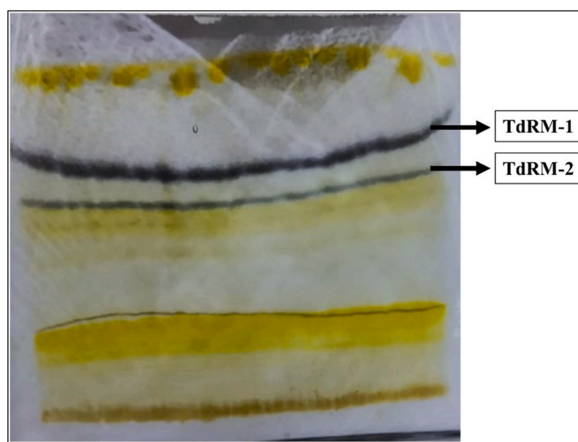

Fig. 1. Thin layer chromatogram showing the separation of methanolic root extract of *Tragopogon dubius* into separate bands.

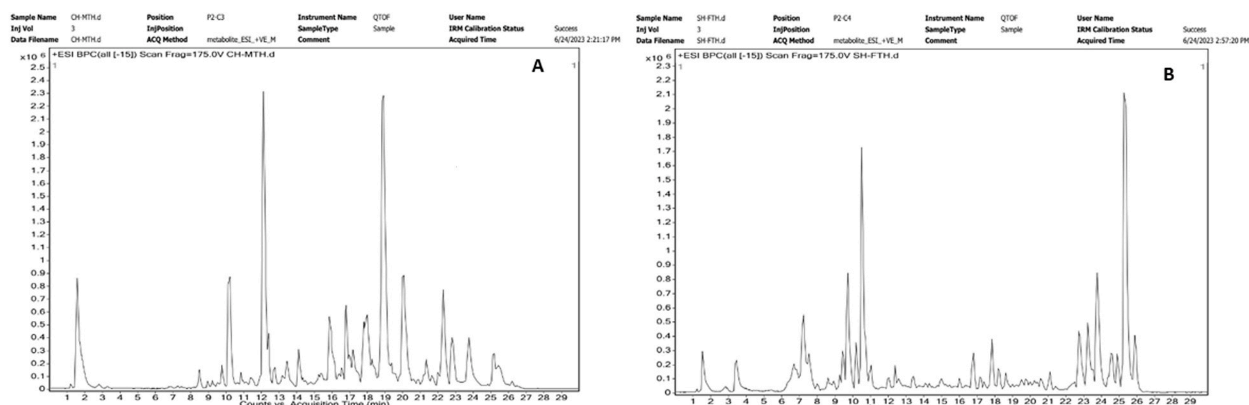

Fig. 2. UPLC-ESI-QTOF-MS chromatograph of methanolic root fraction of *Tragopogon dubius* (A) Positive ionization (B) Negative ionization.

3.4. GC-MS profiling of T. dubius methanolic root extract

GC-MS analysis unveiled a total of 17 compounds in the methanolic extract of the root of *T. dubius*. Fig. 3A illustrates the GC-MS chromatogram, and while Table 2 delineates the chemical components of the methanolic root extract alongside their concentration, molecular formula, peak area, and retention time. Several bioactive molecules were identified, including Undecane, 2,5-O-Methylene-D-mannitol, 1-Decanol, Cycloheptasiloxane tetradecamethyl, 2,4-Di-tert-butylphenol, 1-Heptadecene, Cyclooctasiloxane, octadecamethyl, 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol, (Z)-9-Tricosene,(z), Eicosane,1-iodo, Cyclononasiloxane, octadecamethyl-, Neophytadiene, Methyl 14-methyl-eicosanoate, D:A-Friedooleanan-7-ol, (7. alpha), n-Hexadecane, Pentasiloxane, 1,1,3,3,5,5,7,7,9,9-decamethyl-, and 1-Nonadecene. The GC-MS chromatograms of TdRM-1 fractions are illustrated in Fig. 3B, and those of TdRM-2 fractions are shown in Fig. 3C. Additionally, two compounds, Cyclooctasiloxane hexadecamethyl-, and n-Hexadecanoic acid, were detected in the TdRM-1 fraction, while Hydrazinecarboxamide and Neophytadiene were detected in TdRM-2 fraction (Tables 3 and 4).

3.5. FTIR analysis

Fig. 4 presents the absorption spectra of the methanolic root extract and isolated TdRM-1 and TdRM-2 of *T. dubius*. Various functional groups, including phenols, aliphatic primary amines, alkanes, carboxylic acids, halogen compounds, aromatics, alcohols, aliphatic amines, alkyl halides, and alkynes, were identified within the spectra, spanning the range of 400–4000 cm^{-1}. Notable peaks include a broad band at 3304 cm^{-1} assigned to OH stretching in the alcohol and phenol groups, 2997.38 cm^{-1} to 2887.44 cm^{-1} attributed to C–H stretching vibration in the alkane and carboxylic acid groups, and 2125.56 cm^{-1} to alkynes. Peaks around 1627.92 cm^{-1} are due to the presence of alkenes, while small bands at 1251.60 cm^{-1} represent C═O stretching vibrations of carboxylic acids. Additionally, the spectrum displays at 1408.04 cm^{-1} for C–H stretching in the alkanes group, weak bands at 1182.36 to 1132.21 cm^{-1}, 1049.28 cm^{-1} attributed to the sulphates, sulfoxide and ester bonds, and at 586.36 to 511.14 cm^{-1} corresponding to alkyl, halogen and hydroxyl groups.

3.6. Total phenolic and flavonoid content

Table 5 presents the total phenolic and flavonoid contents of TdRM extract, TdRM-1, and TdRM-2 fractions. The TdRM-2 fraction exhibited the highest phenolic content (35.2 ± 0.01 mg GAE/g dry weight (DW)), while the TdRM-1 fraction demonstrated the lowest phenolic content (7.2 ± 1.76 mg GAE/g dry weight). Similarly, the TdRM-2 fraction displayed the highest total flavonoid content (28.77 mg RE/g DW) compared to TdRM-1 (7.66 ± 1.92 mg RE/g DW). Thus, the TdRM-2 fraction exhibited higher phenolic and flavonoid contents than TdRM-1 fraction.

3.7. Antioxidant activity

The antioxidant activity of the methanolic root extract/fractions obtained from *T. dubius* was evaluated using *in vitro* assays, including ABTS, DPPH and superoxide radical scavenging assays. The TdRM-2 fraction exhibited the highest radical scavenging activity, with percentages of 88.21 %, 82 % and 79.43 % at a concentration of 400 μg/mL in the DPPH, ABTS and superoxide radical scavenging assays, respectively (Fig. 5A–C). The corresponding IC_{50} values were 51.29 μg/mL, 55.10 μg/mL, 60.03 μg/mL. It was noted that the radical scavenging activity showed a concentration-dependent trend. In comparison to the TdRM-2 fraction, TdRM-1 and TdRM fractions displayed lower antioxidant activities (Fig. 5A–C, Table 6).

Table 1
List of major metabolites identified from methanolic extract of root of *Tragopogon dubius* using UPLC-ESI-QTOF-MS analysis.

Name of metabolite	Formula	Precursor *m/z*	Accurate mass (Da)	RT (min)	Diff (ppm)	Nature of compound
Maritimetin	$C_{15}H_{10}O_6$	286.0454	287.0527	9.773	8	Phenol
Gravacridonediol	$C_{19}H_{19}NO_5$	341.1247	342.1309	10.006	4.87	Alkaloid
Gibberellin A102	$C_{20}H_{26}O_7$	378.1678	401.1568	12.099	0.03	Phenolic glycosides
Gibberellin A91	$C_{19}H_{24}O_7$	364.1525	387.1415	12.6	−0.83	Diterpenoids
Taraxacolide 1-O-b-D-glucopyranoside	$C_{21}H_{32}O_9$	428.2045	451.1938	12.829	0.23	Terpenoids
Rugosinone	$C_{19}H_{15}NO_6$	353.0889	354.0949	13.171	2.84	Alkaloid
Methyl 6-O-digalloyl-beta-D-glucopyranoside	$C_{17}H_{18}N_4O_2$	310.1419	333.131	13.599	3.35	Tannins
Yucalexin P15	$C_{20}H_{26}O_4$	330.1827	353.1719	14.042	1.15	Diterpenoid
Limonexic acid	$C_{26}H_{30}O_{10}$	502.1836	525.1726	14.538	0.59	Triterpenoids
Eremosulphoxinolide A	$C_{24}H_{32}O_7S$	464.1841	465.1914	14.772	5.93	Terpene
Dorsteniol	$C_{14}H_{14}O_5$	262.0843	285.0732	15.893	−0.52	Flavonoid
Rutaevin	$C_{26}H_{30}O_9$	486.1884	509.1776	16.625	1.23	Terpenoid
Nb-trans-Feruloylserotonin glucoside	$C_{26}H_{30}N_2O_9$	514.1967	515.204	17.553	−1.09	Flavonoid
3-Methylbutyl 2-furanbutanoate	$C_{13}H_{20}O_3$	224.1419	247.1311	17.875	−2.73	Fatty acid
Cimifugin	$C_{16}H_{18}O_6$	306.1105	329.0996	19.619	−0.38	Coumarin
Austin	$C_{27}H_{32}O_9$	500.204	523.1931	19.692	1.35	Terpenoid
12S,15S)-15-O-Demethyl-10,29-dideoxy-11,12-dihydro-striatin C	$C_{25}H_{38}O_6$	434.267	457.2557	21.627	−0.39	Diterpenoid
2-Tridecanone	$C_{13}H_{26}O$	198.1991	221.1884	22.414	−3.82	Alkane
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.1203	523.1184	7.205	−12.48	Calcium ionophores
Kaempferol 3-sophoroside 7- glucoside	$C_{33}H_{40}O_{21}$	772.2045	771.1973	8.532	2.2	Flavonoids
Manghaslin	$C_{33}H_{40}O_{20}$	756.2095	755.2012	8.824	2.37	Glycoside and member of flavonoids
Trans-chlorogenic acid	$C_{16}H_{18}O_9$	354.095	413.1091	8.966	0.31	Phenolic
Syringic acid	$C_9H_{10}O_5$	198.052	197.0448	9.077	4.11	Phenolic
Kaempferol 3-(2''- rhamnosylgalactoside) 7-rhamnoside	$C_{33}H_{40}O_{19}$	740.2145	739.2077	9.204	2.47	Glycoside and member of flavonoids.
Gossypetin 8-glucoside	$C_{21}H_{20}O_{13}$	480.0905	479.0835	9.218	−0.15	Flavonoids
Rutin	$C_{27}H_{30}O_{16}$	610.1526	609.1456	9.305	1.35	Flavonoid glycoside
2-O-*p*-Coumaroyl-1,6- digalloyl-beta-D-glucopyranoside	$C_{29}H_{26}O_{16}$	630.1311	629.1254	9.565	−14.26	Tannins
Astragalin 7-rhamnoside	$C_{27}H_{30}O_{15}$	594.1575	593.1505	9.614	1.71	Flavonoid-7-o-glycosides
Myricetin 7-rhamnosid	$C_{21}H_{20}O_{12}$	464.0944	463.0871	9.756	2.4	Flavonoid
Xanthoxylin	$C_{10}H_{12}O_4$	196.0724	195.0653	10.116	5.67	Alkaloid
Kaempferol 3-O-β-Dgalactoside	$C_{21}H_{20}O_{11}$	448.0968	447.0896	10.327	8.41	Flavonoid
Nigakihemiacetal A	$C_{22}H_{34}O_7$	410.2287	409.2213	11.493	4.32	Triterpenes
(±)14,15-DHET	$C_{20}H_{34}O_4$	338.2432	337.2357	11.501	7.34	Eicosanoid
Cascarillin	$C_{22}H_{32}O_7$	408.2105	407.2017	11.518	10.43	Diterpenoid
Vanillin acetate	$C_{10}H_{10}O_4$	194.058	193.0509	12.446	−0.68	Phenol esters
Corchorifatty acid	$C_{18}H_{32}O_5$	328.2222	327.2147	12.704	8.44	Fatty acid
3-α(S)-Strictosidine	$C_{27}H_{34}N_2O_9$	530.2269	575.2253	14.144	−0.98	Alkaloid ester
Kelampayoside A	$C_{20}H_{30}O_{13}$	478.1602	523.1578	14.408	17.74	Phenolic glycosides
Glucosylgalactosyl hydroxylysine	$C_{18}H_{34}N_2O_{13}$	486.2029	545.2167	14.778	6.59	Glycosides
Xylopinine	$C_{21}H_{25}NO_4$	355.1772	400.1756	15.33	3.25	Alkaloid
Notoginsenoside R10	$C_{30}H_{50}O_9$	554.3434	553.3362	15.952	3.83	Steroidal glycosides
8-(1,1-Dimethylallyl) galangin	$C_{20}H_{18}O_5$	338.1134	337.1065	16.382	5.86	Flavonoid
Pleuromutilin	$C_{22}H_{34}O_5$	378.2365	437.2502	16.444	10.86	Diterpenoid
(Z)-Resveratrol 3,4'- diglucoside	$C_{26}H_{32}O_{13}$	552.1859	551.1801	16.504	−2.91	Stilbene glycosides
4,7-Didehydroneophysalin B	$C_{28}H_{28}O_9$	508.1713	507.1644	16.513	4.06	Steriods
Pleuromutilin						Diterpenoid
Nb-trans-Feruloylserotonin glucoside	$C_{26}H_{30}N_2O_9$	514.1968	513.1885	17.471	−3.3	Glycosides
Strictosamide	$C_{26}H_{30}N_2O_8$	498.2004	543.1993	17.683	−0.28	Monoterpene
Karwinskione	$C_{32}H_{32}O_7$	528.2128	573.2105	17.81	3.76	Lignan
Curcumin diglucoside	$C_{33}H_{40}O_{16}$	692.2341	751.2497	18.171	−3.62	Polyphenol
Edulisin I	$C_{28}H_{26}O_8$	490.1626	535.1592	19.099	0.33	Furanocoumarins
Ellagic acid	$C_{14}H_6O_8$	302.0058	300.9983	22.107	1.52	Polyphenol
Perilloside E	$C_{17}H_{22}O_9$	370.1292	369.1246	24.366	−7.63	Glycosides
Melleolide H	$C_{24}H_{30}O_7$	430.1995	429.1929	24.589	−0.89	Sesquiterpenoid
Linusitamarin	$C_{17}H_{22}O_9$	370.1302	369.1257	24.722	−10.24	Glycosides

3.8. Anticancer studies

The *in vitro* anticancer effects of the TdRM extract and its isolated fractions, TdRM-1and TdRM-2 from *T. dubius* were evaluated across various concentrations (31.25–1000 μg/mL) on the cancer cell lines, A549 (human lung carcinoma), LN-18 (human brain glioblastoma), and MCF-7 (human breast cancer). The aim was to assess their antiproliferative potential and observe selective effects on these distinct cancer cell lines, given their diverse cellular behaviors and origins. This approach aids in understanding specificity

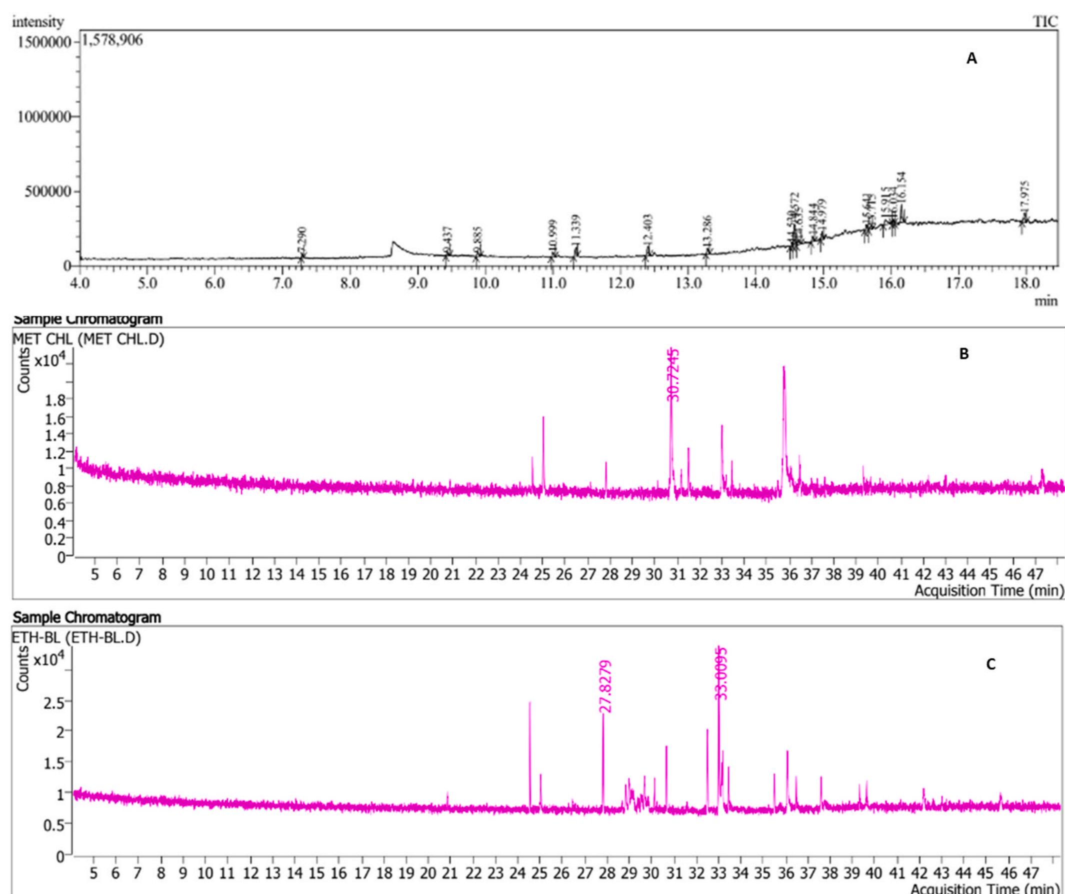

Fig. 3. GC-MS analysis of TdRM, TdRM-1 and TdRM-2 fractions of root *Tragopogon dubius* (A) GC-MS chromatogram of crude methanolic root extract of *T. dubius* (B) Chromatogram of TrRM-1 fraction, (C) Chromatogram of TdRM-2 fraction.

Table 2
Chemical constituents detected in the Methanolic root extract TdRM of *Tragopogon dubius* by GC-MS analysis.

Peak	R.T (min)	% Area	Compound name	Molecular formula	Nature of compound
1	7.29	1.98	Undecane	$C_{11}H_{24}$	Alkane
2	9.437	2.2	2,5-O-Methylene-D-mannitol	$C_7H_{14}O_6$	Sugar Alcohol
3	9.885	3.03	1-Dodecanol	$C_{12}H_{26}O$	Fatty Alcohol
4	10.999	3.46	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	Terpenoid
5	11.339	7.17	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	phenol
6	12.403	7.78	1-Heptadecene	$C_{17}H_{34}$	Alkane
7	13.286	3.79	Cyclooctasiloxane hexadecamethyl	$C_{16}H_{48}O_8Si_8$	Phenolic
8	14.53	2.96	17,17-Dimethyl-18-nor-5.beta.-androsta-1,13-diene-3.alpha-ol	$C_{19}H_{26}O_2$	Terpenoid
9	14.572	15.26	9-Tricosene, (Z)-	$C_{23}H_{46}$	Fatty acid
10	14.635	4.67	Eicosane, 1-iodo-	$C_{20}H_{41}I$	Alkane
11	14.884	3.7	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	Phenolic
12	14.979	4.78	Neophytadiene	$C_{20}H_{38}$	Diterpene
13	15.641	4.01	Methyl 14-methyl-eicosanoate	$C_{22}H_{44}O_2$	Omega 6 fatty acid
14	15.715	3.17	D: A-Friedooleanan-7-ol, (7. alpha.)-	$C_{30}H_{52}O$	Triterpene
15	15.915	8.26	n- hexadecanoic acid	$C_{16}H_{32}O_2$	Fatty acid
16	16.034	2.97	Decamethylcyclopentasiloxane	$C_{10}H_{30}O_4Si_5$	Organosilicon compound
17	17.975	8.87	1-Nonadecene	$C_{19}H_{38}$	Alkene

and optimizing the therapeutic potential of drug. The results revealed a concentration-dependent increase in antiproliferative activity. Among the tested extracts, TdRM-2 exhibited the highest antiproliferative activity against A549 (Fig. 6A), LN-18 (Fig. 6B), and MCF-7 cells (Fig. 6C), with GI_{50} values of 31.62 μg/mL, 35.68 μg/mL, and 48.66 μg/mL, respectively. This was followed by TdRM-1, which had GI_{50} values of 78.03 μg/mL, 69.65 μg/mL,96.05 μg/mL, respectively. The TdRM extract showed the lowest antiproliferative activity compared to the isolated fractions, with GI_{50} values of 136.59 μg/mL, 80.06 μg/mL, and 190.49 μg/mL, respectively (Table 7,

Table 3
Bioactive compounds found in TdRM-1 fraction of *Tragopogon dubius* by GC-MS analysis.

Peak	R.T (min)	Area%	Compound name	Molecular formula	Nature of Compound
1	27.8279	6.446	Cyclooctasiloxane hexadecamethyl	$C_{16}H_{48}O_8Si_8$	Phenol
2	33.0095	16.57	n- hexadecanoic acid	$C_{16}H_{32}O_2$	Fatty acid

Table 4
Bioactive compounds found in TdRM-2 fraction of *Tragopogon dubius* by GC-MS analysis.

Peak	R.T(min)	Area %	Compound name	Molecular formula	Nature of Compound
1	4.15	2.134	Hydrazinecarboxamide	CH_5N_{30}	Monocarboxylic acid amide
2	30.72	20.21	Neophytadiene	$C_{20}H_{38}$	Diterpene.

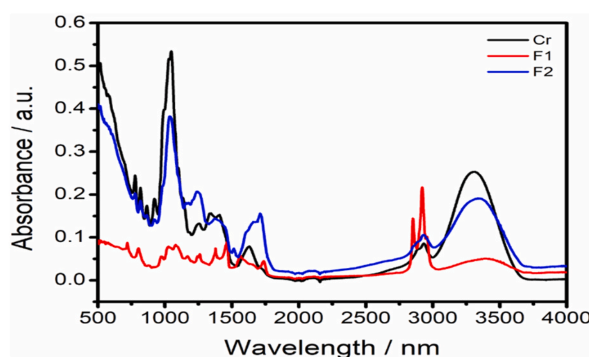

Fig. 4. FT-IR spectrum of *Tragopogon dubius* methanolic root extract TdRM (black spectrum), TdRM-1 (red spectrum), TdRM-2 (blue spectrum).

Table 5
Total phenolic and flavonoid content of Extract/fractions of *Tragopogon dubius*.

Extract	TPC mgGAE/g DW	TFC mgRE/g DW
TdRM	35.2 ± 0.01^a	28.77 ± 1.11^a
TdRM-1	7.2 ± 1.76^b	7.66 ± 1.92^b
TdRM- 2	10.53 ± 2.40^b	12.11 ± 2.93^b

Fig. 6A–C).

3.9. Cytomorphological changes in A549, LN-18 and MCF-7 cancer cells

The TdRM-2 fraction, identified as the most effective against the tested cancer cell lines, was further assessed for cytomorphological changes. Following the treatment of A549, LN-18 and MCF-7 cells with the TdRM-2 fraction, alterations in morphology were observed under a fluorescence microscope (Fig. 7).

Fluorescence microscopy of TdRM-2 treated cells stained with the DNA-binding dye DAPI revealed an increase in the frequency of cells displaying bright blue, condensed, and fragmented nuclei, contrasting with the uniformly blue-stained nuclei found in the untreated control. TdRM-2 treated cells stained with a combination of ethidium bromide (EB) and acridine orange (AO) also showed a distinctive staining pattern, indicative of apoptosis induction. Ethidium bromide, capable of entering cells with compromised membrane integrity, interacts with DNA, producing yellow to orange fluorescence depending on the stage of apoptosis, while AO, permeable across intact cell membranes, stains nuclei green. Rhodamine-123, a mitochondria-specific, voltage-dependent fluorescent probe, was employed to study changes in mitochondrial membrane potential, which are associated with the intrinsic cell death pathway. Fluorescence microscopy revealed TdRM-2-induced loss of mitochondrial membrane potential (Fig. 7).

3.10. Annexin V-FITC/PI double staining method

A-549, LN-18, and MCF-7 cells were treated with TdRM-2 fractions for 24 h and then stained with Annexin V-FITC and PI to determine the proportion of cells undergoing early apoptosis (EA), late apoptosis (LA) and necrosis. The findings showed a significant increase in the number of apoptotic cells in the treated groups, compared to the untreated controls. Specifically, A-549 cells treated with 31.62 μg/mL of TdRM-2 fraction exhibited 53.3 % early apoptotic cells, in contrast to 5.9 % in the control group. Similarly, the

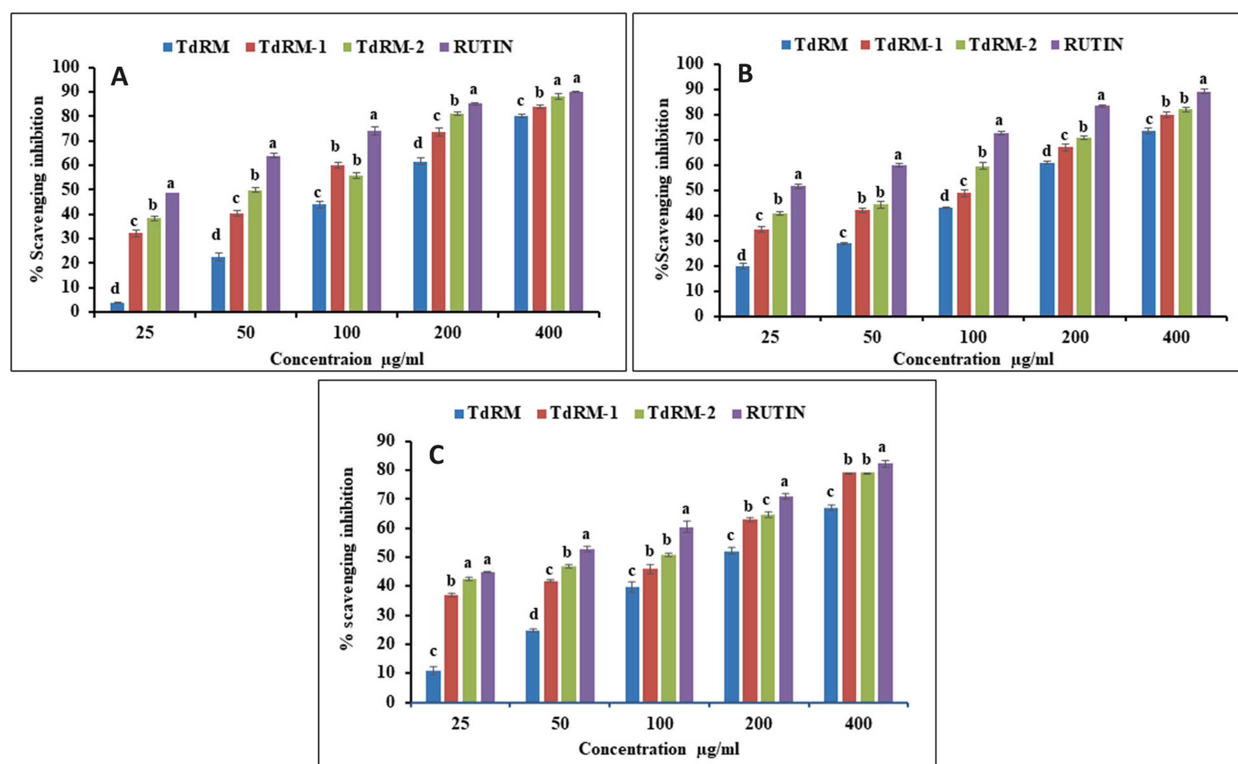

Fig. 5. Antioxidant activity of *Tragopogon dubius* crude methanolic root extract TdRM, TdRM-1 and TdRM-2 fractions. (A) DPPH radical scavenging assay (B) ABTS (C) Superoxide radical assay radical scavenging assay. Error bars are representative of ±SE. Different letters denote significant difference ($p < 0.05$) between different fractions at different concentrations.

Table 6
IC50 values of Methanolic root extract/fractions of *Tragopogon dubius* fractions obtained in different antioxidant assays.

Antioxidant Assays	IC_{50} (µg/mL)			
	TdRM	TdRM-1	TdRM-2	Rutin
DPPH radical scavenging activity	131.9	66.73	51.29	19.5
($ABTS^{\bullet +}$) radical scavenging activity	128.3	77.24	55.1	22.5
Super oxide radical scavenging assay	173.9	80.14	59.92	40.2

(IC_{50}- 50 % inhibitory concentration).

treatment with TdRM-2 lead to an increase in early apoptosis in LN-18 and MCF-7 by approximately 36 % and 11 %, respectively, after 24 h (Fig. 8). These results indicate that the TdRM-2 fractions from *T. dubius* can significantly inhibit the growth of A-549, LN-18, and MCF-7 cancer cell lines by inducing apoptosis.

4. Discussion

In recent years, there has been a surge in research on the drug discovery, particularly focusing on the health benefits of plant-derived secondary metabolites [39,40]. A qualitative preliminary phytochemical analysis of the methanolic root extract of *T. dubius* unveiled a spectrum of phytoconstituents, including carbohydrates, glycosides, alkaloids, flavonoids, steroids, tannins, saponins, resins, phenols, and terpenoids. Among these polyphenols, triterpenes, tannins, and glycosides exhibit promising pharmacological characteristics such as anti-inflammatory, antimutagenic, antigenotoxic, antioxidant, antidiabetic, cardioprotective, neuroprotective, and anti-allergic properties [41,42]. Further analysis through UPLC-ESI-QTOF-MS identified numerous flavonoids, phenolics, glycosides, terpenes and terpenoid derivatives in the methanolic root extract. Notable compounds like rutin, *trans*--chlorogenic acid, and synergic acid have demonstrated medicinal qualities, including antimicrobial, antioxidant, anticancer, anti-hepatocarcinogenic, anti-inflammatory and antidiabetic effects, consistent with findings from other *Tragopogon* [43,44]. This study presents a comprehensive list of chemicals found in the methanolic fraction of the *T. dubius* roots, corroborating earlier research [45]. Compounds like Kaempferol 3-sophoroside 7-glucoside, Kaempferol 3-O-β-D galactoside, Gossypetin 8-glucoside, and Curcumin diglucoside exhibit diverse pharmacological properties, suggesting their potentially utility in developing a chemo-axonomical library

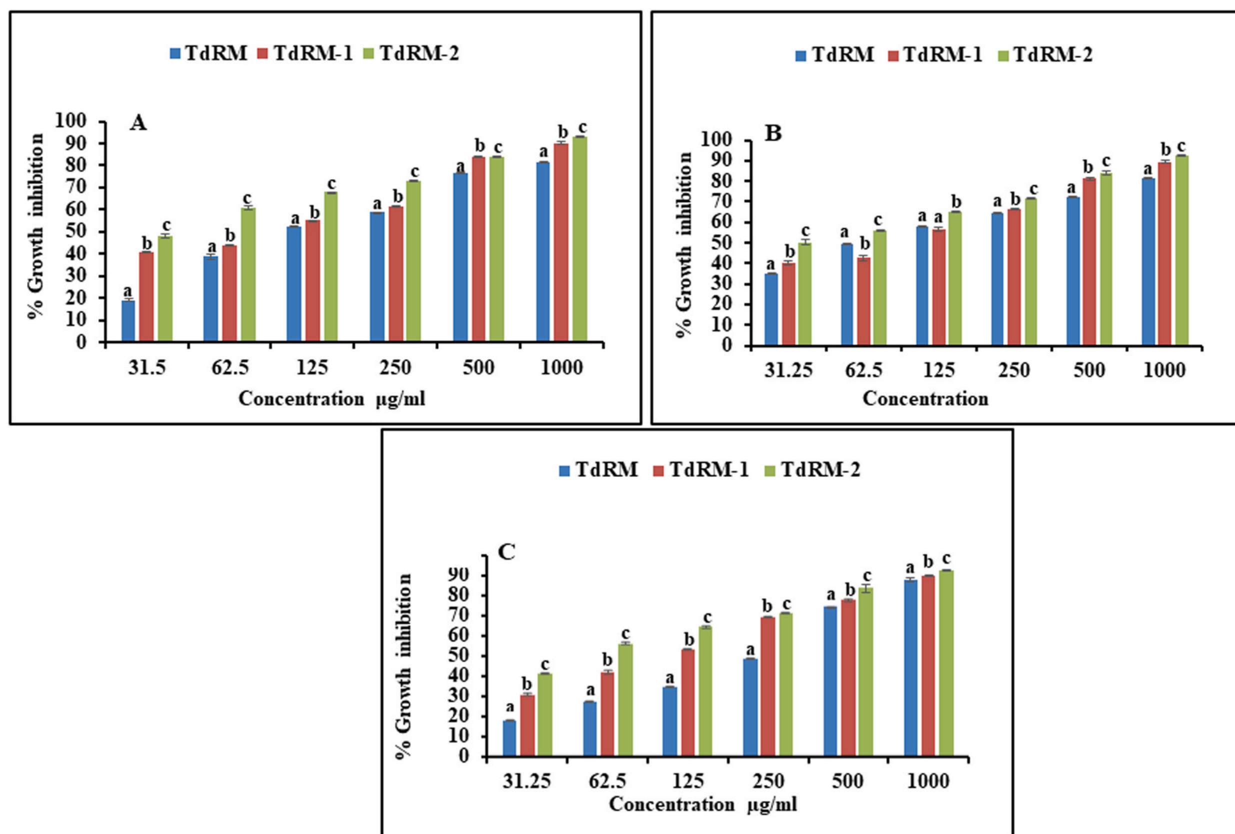

Fig. 6. Antiproliferative and apoptotic potential of *Tragopogon dubius*, TdRM, TdRM-1 and TdRM-2 fractions against (A) A549, (B) HeLa and (C) MCF-7 cell line after 24 h treatment. Values are represented as Mean ± S.E ($p \leq 0.05$). Data labels with different letters represents significant difference among the values.

Table 7
GI_{50} values Extract/fractions against the A549, MCF-7 and LN-18 cell line.

Extract/Fractions	GI_{50} (μg/mL)		
	A-549	LN-18	MCF-7
TdRM	136.6	80.06	190.49
TdRM-1	78.03	69.65	96.05
TdRM-2	31.62	35.68	48.66

(GI_{50}-50 % inhibitory concentration).

for plant identification of this plant as reported previously [46,47]. GC/MS and HRLC/MS analyses revealed 17 compounds in TdRM, and 2 compounds each in TdRM-1 and TdRM-2 fractions. These compounds include as fatty acid esters, alkanes, alkenes, diterpenes, triterpenes, phenols, and sugar alcohols. Previous studies have associated compounds such as 2,4-Di-tert-butylphenol, Hexadecanoic acid, Neophytadiene, Tetradecamethylcycloheptasiloxane detected in the GC-MS analysis with antimicrobial, antioxidant, antiseptic, hair conditioning, skin conditioning, anticancer, and anti-inflammatory properties [47,48]. These phytoconstituents likely contribute to the observed antioxidant and anticancer activities, supporting the pharmacognostic potential of *T. dubius*. FT-IR analysis of the methanolic root extract and isolated fractions of *T. dubius* revealed functional groups corresponding to components identified in the GS-MS analysis. These compounds include alcohols, phenols, alkenes, alkanes, and esters consistent with previous studies [46,48]. The methanolic root extract of TdRM was found to possess a high total phenolic content (35.2 ± 0.01 mg GAE/g DW) and total flavonoid content (28.77 ± 1.11 mg RE/g DW) compared to its isolated fractions. Previous studies on total phenolic content in methanolic, ethyl acetate and aqueous leaf extracts of *T. dubius* reported lower values compared to TdRM [8,12] Tawaha et al. [48] considered any value exceeding 20 mg GAE/g DW of plant extract exceptionally high, indicating the abundance of polyphenols in *T. dubius* methanolic root extract. Flavonoids are well-known for their ability to inhibit various diseases, including cancer and cardiovascular disorders [21,49]. Compared to other Mediterranean dietary plants, *T. dubius* is believed to have a relatively high flavonoid content, suggesting potential medicinal benefits of the plant [50–52]. In all three antioxidant tests of the present study, the TdRM-2 fraction exhibited the highest

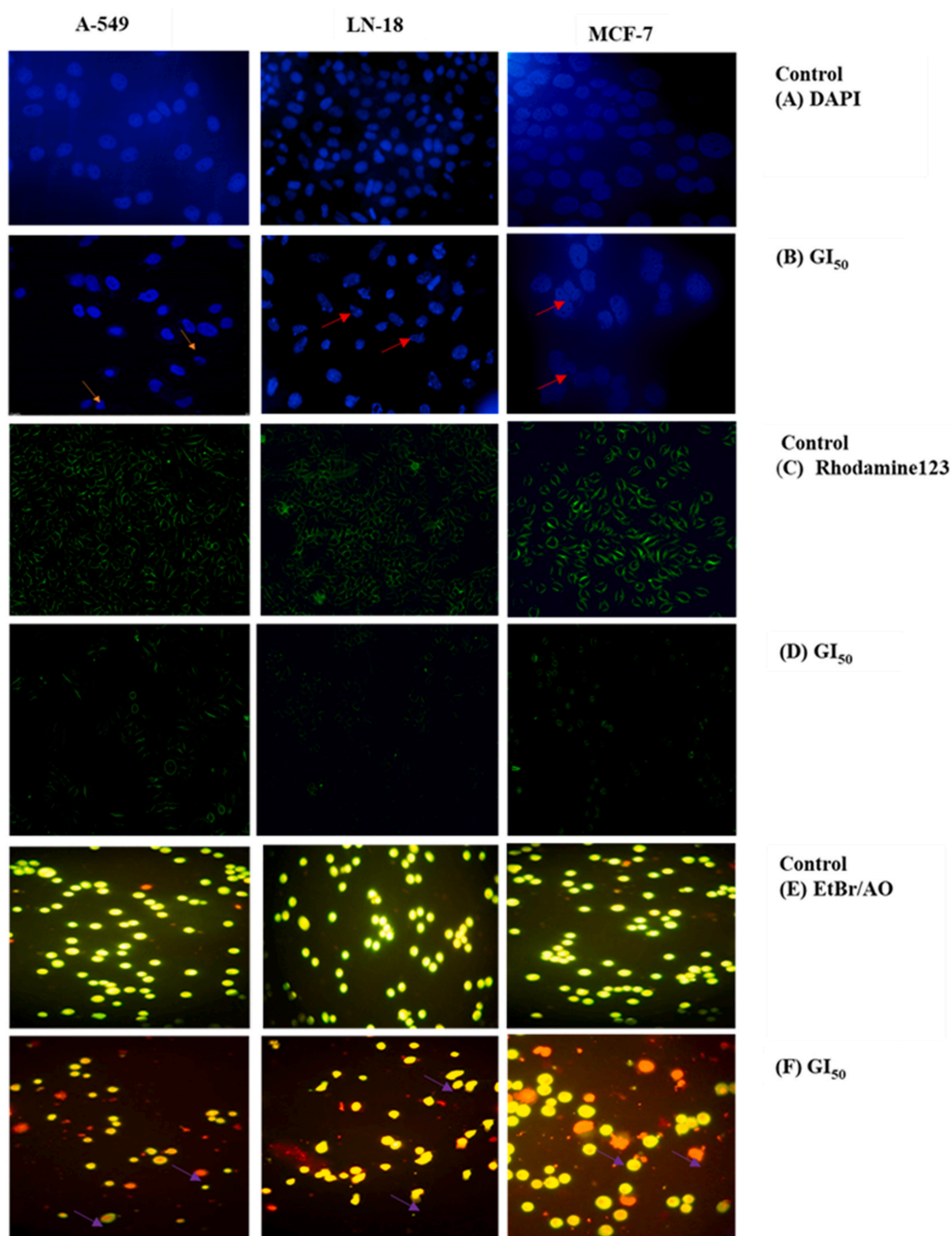

Fig. 7. Cytomorphological alterations induced by TdRM-2 fraction associated with apoptosis induction detected using fluorescence microscopy of A549, LN-18 and MCF-7 cells treated with GI_{50} of TdRM-2 fraction for 24 h, following observations under fluorescence microscopy (DAPI, Rhodamine 123, and AO/EtBr dual stained cells), for apoptosis-related cytomorphological changes (arrow heads indicate cells undergoing apoptosis).

radical scavenging activity, with IC_{50} values of 51.29 μg/mL, 60.03 μg/mL, and 55.10 μg/mL, respectively. Weak antioxidant was observed in the crude TdRM extract with IC_{50} of 132.52 μg/mL in DPPH, 128.28 μg/mL in superoxide assay and 173.87 μg/mL in the ABTS assay. The antioxidant potential of TdRM-2 fraction can be attributed to the presence of diterpene, Neophytadiene, as detected in GC-MS analysis. Previous studies support antioxidant potential of diterpene, Neophytadiene [12]. The antioxidant potential of *T. dubius* leaf extracts in water, methanol, and ethyl acetate was assessed by Uysal et al. [8], indicating that methanolic and ethyl

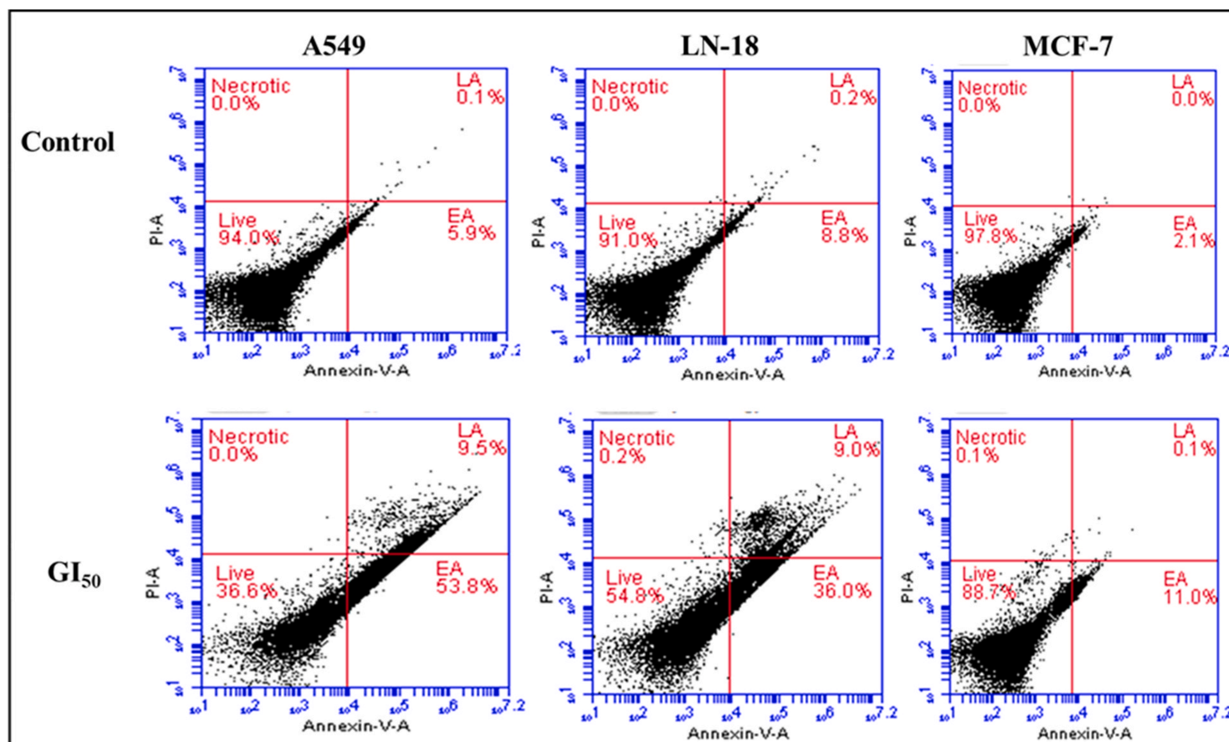

Fig. 8. Effect of the TdRM-2 fraction of *Tragopogon dubius* on apoptosis induction in A-549, LN-18, and MCF-7 cells by flow cytometry using the Annexin V-FITC/Propidium Iodide (PI) double staining technique. The percentages of live, early apoptotic (EA), late apoptotic (LA), and necrotic cell populations are reported within the quadrants. The cell lines were treated with GI_{50} values.

acetate extracts had promising antioxidant effects, with values of 205 and 159 mg/g extract and 1.56 and 2.26 nmol TE/g, respectively.

The TdRM fraction of the plant extract exhibited dose dependent reduction in cell survival percentages in MCF-7, A549, and LN-18 cancer cell lines. These findings are supported by several studies demonstrating the antiproliferative and apoptotic efficiencies of various *Tragopogon* species, such as *T. porrrifolius*, *T. pratensis, T. malicus,* and *T. gramnifolius,* and their potential anti-proliferative effects on the growth of cancer cells [8,43]. Notably, the TdRM-2 fraction showed better antiproliferative activity than the crude extract, marking this is the first report on the antiproliferative potential of root extract/fractions of *T. dubius*. The antiproliferative and apoptotic potential of TdRM and its isolated fractions against the three cancer cell lines, A549, LN-18, and MCF-7, was highest in the TdRM-2 fraction. GC-MC analysis identified two compounds in the TdRM-2 fraction—Neophytadene, a diterpene, and Hydrazine-carboxamide, a monocarboxylic acid amide. These compounds might be responsible for the fraction's antiproliferative and apoptotic activities. Neophytadiene has been shown to reduce skin damage caused by free radicals and possesses antimicrobial, anti-inflammatory, and analgesic properties [53,54]. Diterpenes and their derivatives are known to act as potential anticancer agents [55,56]. Similarly, derivatives of Hydrazinecarboxamide have demonstrated potent anticancer activities [57,58]. Cell death mechanisms caused by TdRM-2 fraction were observed through cell morphology assays, where the acute toxicity was accompanied by morphological change in A549, LN-18, and MCF-7 cells, particularly at the GI_{50} concentration. Fluorescence microscopy, providing exemplary images of apoptotic bodies and chromatin condensation compared to non-apoptotic control cells, is widely used to measure the amount of apoptosis in DAPI-stained nuclei. AO and EB dual-stained TdRM-2 treated cells also displayed the distinctive staining pattern, demonstrating the concentration-dependent induction of apoptosis. According to Garro et al. [59] EB can only enter cells with compromised membrane integrity to interact with DNA and produce yellow to orange fluorescence, depending on the stage of apoptosis. In contrast, AO can pass through intact cell membranes and stain nuclei green. The intrinsic cell death pathway is associated with changes in mitochondrial membrane potential, which were investigated using the voltage-dependent, mitochondria-specific fluorescent probe Rhodamine-123. Fluorescence microscopy revealed a TdRM-2-induced dose-dependent reduction in mitochondrial membrane potential.

Flow cytometry using annexin V/propidium iodide (PI) staining is widely employed to distinguish viable, apoptotic, and necrotic cells based on plasma membrane integrity and permeability [60,61]. PI enters cells with compromised membranes, staining necrotic or late apoptotic cells. In contrast, annexin V binds to phosphatidylserine exposed on the outer leaflet of the plasma membrane early in apoptosis, marking cells in early apoptosis [62]. Apoptotic cells are annexin V-positive and PI-negative, necrotic cells are positive for both annexin V and PI, and viable cells are negative for both stains. This dual staining method enables precise quantification and characterization of cell death mechanisms [63]. The data demonstrate that treatment with TdRM-2 increased the number of annexin V-positive cells (indicative of apoptosis) in A-549, LN-18, and MCF-7 cancer cells. This effect of TdRM-2 on apoptosis induction is

comparable to the growth inhibition and apoptosis induction observed in human cancer cells treated with tea polyphenols, as evidenced by annexin V staining [64].

Our anticancer results supported previous studies carried on *Tragopogon* taxa and further highlighted the importance of using *T. dubius* active fractions as a therapeutic agent. No report exists on HPLC, GC–MS analysis, antioxidant, or anticancer activities of methanolic root extract and isolated fractions of *T. dubius* root. Thus, TdRM deserves more in-depth research from a phytopharmacological point of view, and recommends more high-quality evidence-based research on *T. dubius* for further development of plant-derived remedies and compounds.

5. Conclusion

The investigation on the methanolic root extract of *T. dubius* elucidated its rich phytochemical composition and diverse bioactivities. Through a comprehensive array of analytical techniques, including PTLC, UPLC-ESI-QTOF-MS, GC-MS, and FTIR, the study identified numerous metabolites and functional groups present in the extract and its fractions. The phytochemical screening highlighted the presence of important secondary metabolites such as flavonoids, phenols, alkaloids, and terpenoids, which are known for their bioactive properties. Furthermore, the antioxidant and anticancer activities of the extract and its fractions were evaluated, showcasing remarkable radical scavenging potential and significant antiproliferative effects against A549, LN-18, and MCF-7 cancer cell lines. Among the fractions, TdRM-2 emerged as particularly potent, demonstrating superior antioxidant and anticancer activities compared to TdRM-1 and the crude extract. Cytomorphological studies revealed apoptosis induction and disruption of mitochondrial membrane potential in cancer cells treated with TdRM-2, further elucidating its mechanism of action. These findings underscore the therapeutic potential of *T. dubius* as a source of natural antioxidants and anticancer agents, warranting further exploration for pharmaceutical applications.

Ethics statement

Not applicable.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations consent to participate

All listed authors have been approved to participate in the manuscript.

CRediT authorship contribution statement

Sheikh Showkat Ahmad: Writing – original draft, Software, Investigation, Data curation. **Grag Chandni:** Software, Data curation. **Rasdeep Kour:** Writing – review & editing, Investigation, Conceptualization. **Aashaq Hussain Bhat:** Writing – review & editing, Visualization, Validation, Resources, Formal analysis, Funding acquisition, Project administration. **Vaseem Raja:** Validation, Resources. **Sumit G. Gandhi:** Investigation. **Farid S. Ataya:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Dalia Fouad:** Writing – review & editing, Resources, Funding acquisition. **Arunkumar Radhakrishnan:** Resources. **Satwinderjeet Kaur:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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