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Extraction and Pharmacological Validation of Ursolic Acid from *Hedyotis diffusa*: A Molecular Docking Approach against Inflammation and Colon Cancer

KANIGA PANDI*, BINOY VARGHESE CHERIYAN, ABISHEKA SREE ANBURAJ, LINI PRIYADHARSHINI CHRISTOPHER JEBARAJ, LAVANYA MURUGESAN, KAVIYASHRI RAMESH and SAM JEBARAJ

Department of Pharmaceutical Chemistry, Saveetha College of Pharmacy, SIMATS, Chennai-602105, India

*Corresponding author: E-mail: kanigapharma@gmail.com

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In traditional Chinese and Indian medical systems, *Hedyotis diffusa* is a well-known medicinal plant that is renowned for its wide range of pharmacological properties. The extraction, identification and pharmacological confirmation of ursolic acid a significant bioactive triterpenoid from *H. diffusa* are the main objectives of this investigation. The ethanolic extract was subjected to Soxhlet extraction, followed by column chromatography for the isolation of ursolic acid, which was subsequently confirmed by GC-MS analysis. A major peak at retention time 38.25 min and a molecular ion peak at *m/z* 456 confirmed its identity. The anti-inflammatory properties of ursolic acid were assessed using protein denaturation and membrane stabilization tests, showing notable suppression in a dose-dependent manner that was on par with that of regular diclofenac. Moreover, the MTT assay was used to evaluate its anticancer effectiveness against colon cancer cell lines (HT-29), where ursolic acid exhibited distinguished cytotoxicity with an IC_{50} value suggestive of strong anti-proliferative effects. To gain mechanistic insights, molecular docking studies were performed using AutoDock, targeting COX-2 (PDB ID: 1CX2) for inflammation and TNIK (PDB ID: 6GUE) for colon cancer. Ursolic acid displayed high binding affinities and stable interactions with both targets, indicating its potential dual inhibitory action. These results support the therapeutic relevance of ursolic acid from *H. diffusa* as a promising natural compound for managing inflammation and colon cancer.

Keywords: Ursolic acid, *Hedyotis diffusa*, Anti-inflammatory activity, Colon cancer, Molecular docking.

INTRODUCTION

Increased consumption of fruits, vegetables and certain spices has been strongly correlated with a reduced risk of cancer, as supported by numerous epidemiological and experimental studies [1-4]. Bioactive molecules present in these foods can protect against carcinogenesis induced by endogenous (physiological) and exogenous (environmental or pathogenic) agents, as well as radiation. Cancer development is now recognized as a dynamic, multi-factorial and long-term process involveing a complex network of signaling pathways. Traditionally, cancer progression has been divided into distinct stages, initiation, promotion and metastasis each contributing to uncontrolled cellular proliferation and tumor spread. While the initiation and promotion phases have long been emphasized, increasing evidence highlights inflammation as a critical driver of tumor development. Sites of chronic infection, irritation, and inflammation often serve as the origin of many malignancies. The tumor microenvironment, largely regulated by inflammatory cells, promotes cancer cell survival, proliferation, and migration [5-7].

Bioactive compounds derived from plants, fungi, marine organisms and microbes have demonstrated potent antitumor and anti-inflammatory effects by targeting key signaling pathways such as NF-κB, STAT3, PI3K/Akt and MAPK [8-10]. These agents modulate oxidative stress, suppress pro-inflammatory cytokines (*e.g.* TNF-α, IL-6) and inhibit enzymes like COX-2 and iNOS [11-14]. In cancer prevention and therapy, natural compounds function as blocking agents, which prevent carcinogen activation during tumor initiation or as suppressing agents, which inhibit tumor progression and metastasis [15,16]. Consistent with this, numerous epidemiological and preclinical studies have shown that diets rich in fruits, vegetables, grains, and spices can reduce cancer incidence and progression by modulating immune responses and inflammatory signaling networks [17-19].

Natural compounds exhibit diverse pharmacological activities that depend on dosage, target tissue and duration of exposure [20-22]. Their differential effects on tumor *versus* normal cells are attributed to their ability to activate specific apoptotic pathways, modulate key metabolic enzymes and induce detoxifying enzymes or tumor suppressor genes in a cell type dependent manner [23]. Several clinically used chemotherapeutic agents such as vincristine, camptothecin and paclitaxel originate from natural sources, highlighting their significance in oncology. Similarly, bioactive phytochemicals including ursolic acid, resveratrol and curcumin exhibit dual anticancer and anti-inflammatory properties, making them promising candidates for integrated cancer therapy. Their multitargeted mechanisms and synergy with existing drugs position natural products as effective agents for managing both cancer and chronic inflammatory disorders [24,25].

Traditional medicine, especially Traditional Chinese Medicine (TCM), uses herbal formulations to treat diseases such as cancer and infections by restoring balance and strengthening the body's natural defenses. TCM targets both tumors and their microenvironment, reducing inflammation, modulating immunity and easing side effects of conventional therapies [26,27]. This holistic approach is increasingly recognized for enhancing treatment outcomes and patient quality of life. Among TCM herbs, *Hedyotis diffusa* (Bai Hua She She Cao, Oldenlandia diffusa (Willd) Roxb.) is well known for its detoxifying, anti-inflammatory and anticancer effects [28-32]. Traditionally used to treat infections and abscesses, its applications have expanded to cancers of the digestive tract, liver, and lung. The pharmacological activity of herb is attributed to flavonoids, polysaccharides and terpenoids, which induce apoptosis, inhibit cell-cycle progression, and suppress metastasis and angiogenesis [33,34]. By modulating the tumor microenvironment reducing inflammation and enhancing immune responses *H. diffusa* serves as a valuable adjunct to conventional cancer therapies [35-41].

A key bioactive constituent of *H. diffusa* is ursolic acid (3β-hydroxy-urs-12-ene-28-oic acid), a pentacyclic triterpenoid abundant in the leaves, bark, and fruit peels of plants such as *Rosmarinus officinalis* and *Ocimum sanctum*. Ursolic acid exhibits anti-inflammatory, anticancer, antioxidant and hepatoprotective effects with minimal toxicity [42]. In cancer cells, it induces apoptosis *via* both intrinsic and extrinsic pathways and interferes with STAT3, PI3K/Akt, and MMPs, thereby reducing tumor proliferation, angiogenesis, and metastasis. Of particular interest, COX-2 (PDB ID: 1CX2) and TNIK (PDB ID: 6GUE) represent critical molecular targets linked to inflammation and colorectal cancer, respectively. Dual inhibition of these targets offers a promising strategy for managing inflammation-associated colon cancer.

Thus, the present study investigates ursolic acid as a multitarget natural compound using molecular docking to evaluate its binding affinities with COX-2 and TNIK. This integrated in silico and pharmacological approach bridges traditional knowledge of *H. diffusa* with modern molecular insights, validating its therapeutic relevance. By correlating docking results with known pharmacological effects, the study aims to support the development of ursolic acid and related derivatives as potential dual-action agents for inflammation-associated cancer therapy.

EXPERIMENTAL

Plant material collection and authentication: The whole plant of *Hedyotis Diffusa* was collected from a local medicinal herb habitat during its flowering season in early year 2025. The plant material was cleaned, shade-dried and coarsely powdered for further analysis. Botanical identification and authentication were carried out by a qualified taxonomist at Research Officer-Botany, Central Council for Research in Ayurvedic Sciences (CCRAS), Government of India.

Extraction and purification of ursolic acid: Ursolic acid was extracted using the Soxhlet apparatus with ethanol (95%) as the solvent. The extraction was carried out for 8-10 h until the solvent in the siphon tube appeared colourless. To obtain a thick semi-solid mass, the extracted material was concentrated using a rotary evaporator at a lower pressure. Silica gel (60-120 mesh size) was used for column chromatography of the concentrated extract. A gradient of hexane:ethyl acetate (9:1 to 1:1) was used for the elution process. Chloroform:methanol (9:1) was used as the solvent system for the collection and monitoring of the fractions using thin layer chromatography (TLC). By comparing R_f values with the standard, the spot corresponding to ursolic acid was verified [43].

Characterization: The purified compound was further confirmed as ursolic acid by GC-MS analysis and comparison with standard spectral data from NIST libraries [44]. Thermo MS DSQ II and Thermo GC-Trace Ultra Version: 5.0 were used for GC-MS analysis of the fruit extract that contained cayenne pepper. The DB 35-MS Capillary Standard, a non-polar column that measures 30 mm × 0.25 mm ID × 0.25 μm film, was included with the instrument. Helium, the carrier gas, travels slowly at a rate of 1.0 mL/min. As the oven was set to function at 250 ºC, the injector was run at that temperature for 15 min. Then, during the next 3 min, the temperature increased gradually to 280 ºC. For component identification, Wiley and NIST libraries were utilized and their retention indices were contrasted. The results were consistent once the components of the GC-MS instrument were identified and compared with those in the computer library (Wiley and NIST) [44].

In silico docking studies

Docking study: ChemDraw ultra 8.0, AutoDock vina and the molecular graphics laboratory (MGL) tools were downloaded from Pyrx Virtual Screening Tools. It was possible to download the Biovia Discovery studio visualizer. Swiss ADME was used to translate proteins into PDB format, whereas Chem 3D Pro 8.0 was used to translate ligand Mol files into PDB format [45].

Protein required: The target proteins selected for this study were cyclooxygenase-2 (COX-2, PDB ID: 1CX2) (Fig. 1a) and Traf2- and Nck-interacting kinase (TNIK, PDB ID: 6GUE) (Fig. 1b), representing inflammation and colon cancer pathways, respectively. Using AutoDock Tools, these protein structures were processed by eliminating water molecules and non-essential heteroatoms after being retrieved from the RCSB

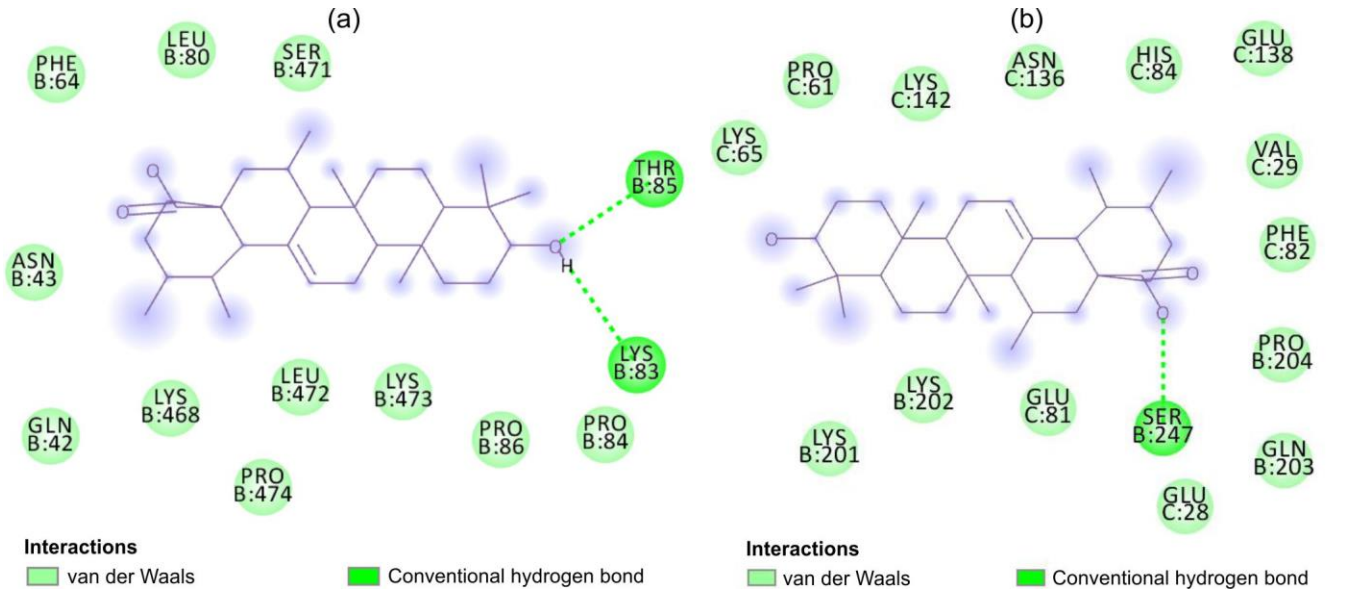

Fig. 1. (a) 1CX2 ligand and (b) 6GUE ligand

protein data bank. To prepare the receptor molecules, Kollman charges were assigned and polar hydrogens were added [46,47].

Anti-inflammatory activity: Utilizing the human red blood cell (HRBC) membrane stabilization technique, the anti-inflammatory qualities of ursolic acid were evaluated. To stop clotting, fresh human blood was drawn and combined with Alsever's solution. Three isotonic saline rinses were performed on the red blood cells (RBCs) following a 10 min centrifugation at 3000 rpm. In regular saline, a 10% v/v RBC suspension was made. 0.5 mL of the 10% RBC suspension, 1 mL of plant extract in varying concentrations, 2 mL of hypotonic solution (distilled water) and 1 mL of phosphate buffer (pH 7.4) were all included in the reaction mixture for each test. A control sample without extract and a standard drug sample containing diclofenac sodium were also prepared. Each sample was centrifuged for 10 min at 3000 rpm following 30 min of incubation at 37 ºC. The absorbance of the supernatant was measured at 560 nm. The absorbance of the test and control samples was compared in order to determine the percentage inhibition of haemolysis. A higher inhibition percentage indicated greater membrane stabilization and hence better anti-inflammatory activity. This assay simulates lysosomal membrane stability under inflammatory conditions, supporting the anti-inflammatory potential of the chemical constituent [48].

Anti-colon cancer activity: HT-29 colon cancer cells were pre-coated with 4% gelatin and then plated at a density of 2×10^4 cells per 35 × 10 mm tissue culture dish. After that, the cells were kept in a humidified atmosphere with 5% CO_2 for 24 h at 37 ºC. The medium was aspirated after 24 h and replaced with new culture medium that contained 10% foetal bovine serum (FBS) and different amounts of ursolic acid. To guarantee total detachment, cells were harvested using 0.25% trypsin-EDTA after 48 h of treatment and incubated for 2-3 min at 37 ºC. Only medium, devoid of the test extract, was given to the control wells. Cell viability was assessed by trypan blue exclusion. Equal parts of cell solution and 0.4% trypan blue dye were mixed and the number of viable (unstained) and non-viable (stained) cells was counted using a hemocytometer.

In comparison to the control, the growth inhibition% was computed. Every therapy was carried out three times. To visually evaluate the cytotoxic effects, morphological changes in treated cancer cells, including cell detachment, rounding and shrinkage, were detected under a phase-contrast microscope. This assay provided both quantitative and qualitative insights into the cytotoxic potential of the chemical constituent against colon cancer cells [49].

RESULTS AND DISCUSSION

Based on GC–MS chromatogram, ursolic acid ($C_{30}H_{48}O_3$) exhibited a molecular ion peak at *m/z* 456, corresponding to its molecular weight. This peak was the major signal observed at a retention time (RT) of 38.25 min (Fig. 2). Fragment ions corresponding to the characteristic fragmentation pattern of ursolic acid were detected at *m/z* 438, 410, 392, 203, 189 and 121. Ursolic acid accounted for 24.56% of the total ion chromatogram (TIC) area, indicating that it is one of the major constituents and is present in significant amounts in the *Hedyotis diffusa* ethanol extract. Comparison of the compound's mass spectrum with the NIST Mass Spectral Library yielded a 97% similarity index, confirming its identification as ursolic acid.

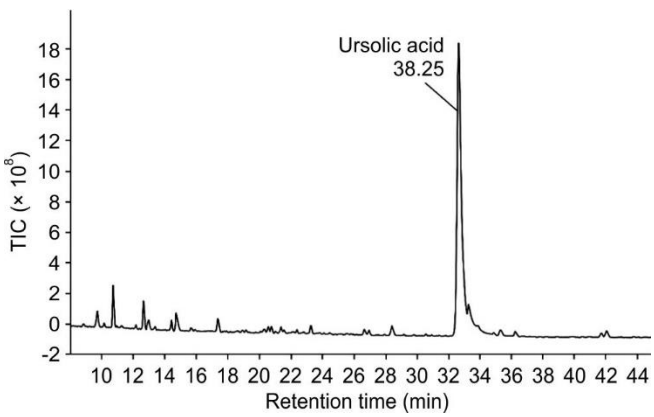

Fig. 2. GC-MS chromatogram

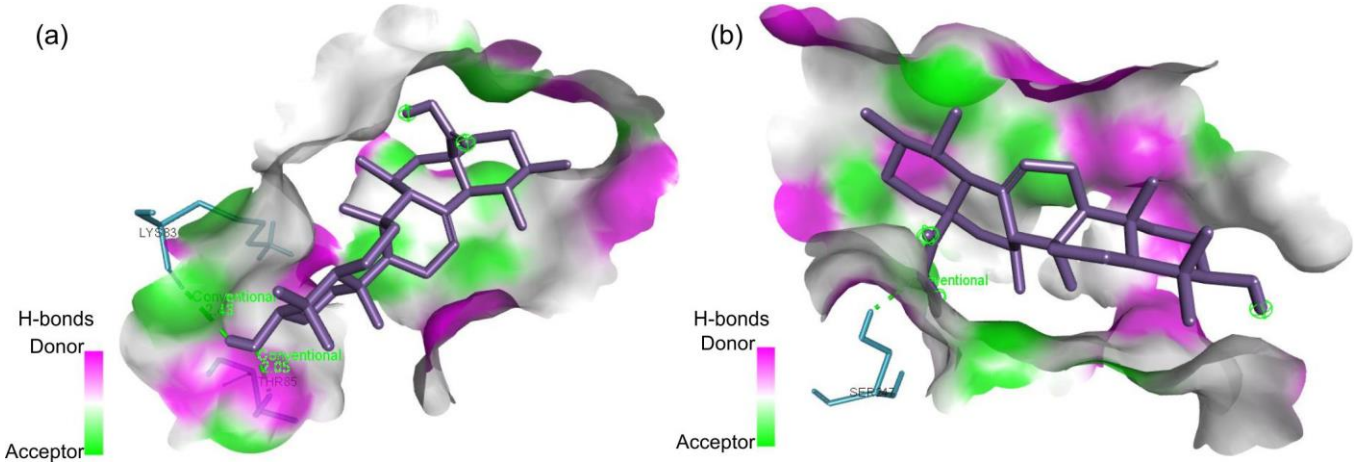

Fig. 3. (a) 1CX2 3D UA and (b) 6GUE 3D UA

Docking results: The docking study of ursolic acid against the inflammation-associated enzyme COX-2 (PDB ID: 1CX2) and colon cancer-related kinase TNIK (PDB ID: 6GUE) revealed strong binding interactions. The structure of the tested compound confirmed the characteristic pentacyclic triterpenoid scaffold of ursolic acid. The binding score obtained from AutoDock Vina indicated a high binding affinity of ursolic acid towards both targets. Against COX-2 (1CX2), ursolic acid displayed a binding energy of –9.5 kcal/mol, suggesting potential inhibitory interaction with the catalytic site of the inflammatory enzyme (Fig. 3a). In case of TNIK (6GUE), which is involved in Wnt signaling and tumor proliferation, ursolic acid showed a binding score of –9.0 kcal/mol, indicating good interaction stability and possible anti-proliferative potential (Fig. 3b).

The observed docking scores signify that ursolic acid has favourable binding affinity for both inflammation and cancer targets, supporting its dual therapeutic role. The biological data is supported by these computational results, which also highlight potential of ursolic acid as a natural therapeutic candidate for the treatment of colon cancer and inflammatory diseases. A comparison with the common drugs regorafenib (TNIK inhibitor) [50] and celecoxib (COX-2 inhibitor) [51] was done in order to confirm the docking efficacy of ursolic acid. Ursolic acid exhibited higher binding affinities with COX-2 (–9.5 kcal/mol) and TNIK (–9.0 kcal/mol) compared to celecoxib (–8.2 kcal/mol) and regorafenib (–8.7 kcal/mol), respectively. These results suggest that ursolic acid could be a powerful dual-acting substance that has anti-inflammatory and anticancer effects. Its stable interactions through hydrogen bonds and hydrophobic contacts with key active site residues further support the *in vitro* results and reinforce its potential as a natural therapeutic compound.

Anti-inflammatory activity: The anti-inflammatory potential of ursolic acid was assessed through a red blood cell (RBC) membrane stabilization assay. The compound exhibited a concentration-dependent increase in membrane protection activity. As shown Table-1, ursolic acid demonstrated substantial membrane stabilization effects across all tested concentrations, with the highest inhibition observed at 400 µg/mL. At 50 µg/mL, ursolic acid inhibited hemolysis by 39.8%, which increased

TABLE-1
MEMBRANE STABILIZATION ASSAY – ANTI-INFLAMMATORY ACTIVITY OF URSOLIC ACID

Concentration (µg/mL)	% Inhibition (membrane stabilization)
50	39.8
100	56.4
200	70.1
400	81.3
Diclofenac (100 µg/mL)	85.9

to 56.4% at 100 µg/mL and 70.1% at 200 µg/mL. The maximum activity of 81.3% was recorded at 400 µg/mL, closely approaching the standard diclofenac sodium (100 µg/mL), which showed 85.9% inhibition. These findings indicate that ursolic acid exhibits significant membrane stabilization potential, suggesting effective anti-inflammatory properties. The observed results support the hypothesis that ursolic acid stabilizes the lysosomal membrane, thereby preventing the release of inflammatory mediators and enzymes, similar to the mode of action of standard non-steroidal anti-inflammatory drugs (NSAIDs) (Fig. 4).

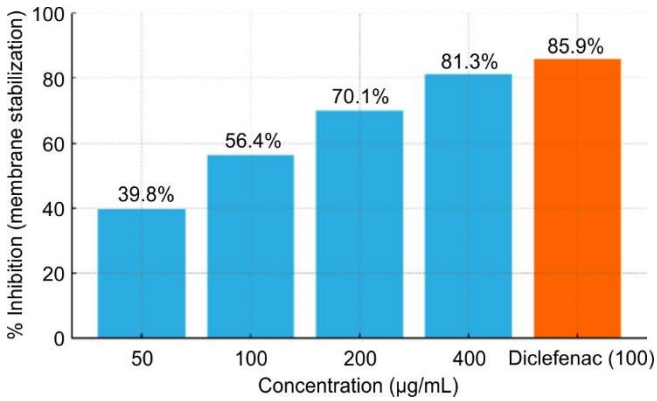

Fig. 4. The percentage inhibition of membrane stabilization by ursolic acid at various concentrations, compared with diclofenac (100 µg/mL)

Anti-colon cancer activity: The anticancer activity of ursolic acid was evaluated against a human colon cancer cell line using the MTT assay. A concentration range of 100 to 500 µg/mL was used for the experiment. Ursolic acid has strong

cytotoxic effects on colon cancer cells, as evidenced by the results, which showed a dose-dependent suppression of cell proliferation. At the lowest tested concentration of 100 μg/mL, ursolic acid inhibited approximately 42% of cancer cell proliferation. This effect progressively increased with higher concentrations: 58% at 200 μg/mL, 66% at 300 μg/mL and 87% at 400 μg/mL. The maximum inhibition of 98% was observed at 500 μg/mL, confirming strong anticancer efficacy at this concentration (Figs. 5 and 6). The observed dose-dependent cytotoxic response suggests that ursolic acid exerts significant antiproliferative activity on colon cancer cells, possibly through mechanisms such as apoptosis induction, cell cycle arrest and interference with oncogenic pathways (*e.g.*, TNIK/Wnt). These findings align with the *in silico* docking results, further supporting the potential of ursolic acid as a promising lead compound for colon cancer therapy.

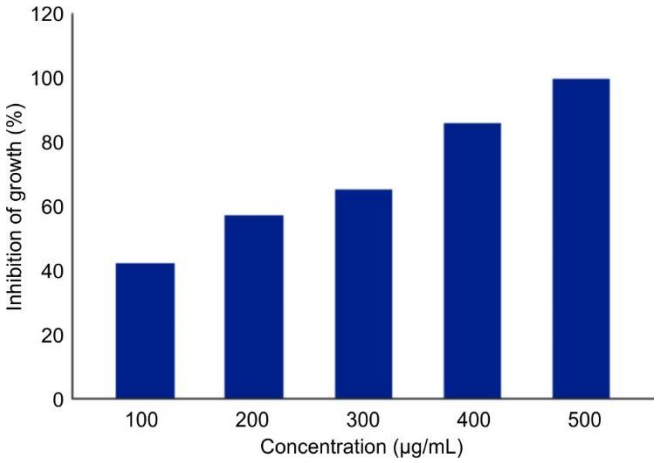

Fig. 5. Cytotoxic effect of ursolic acid on colon cancer cell line

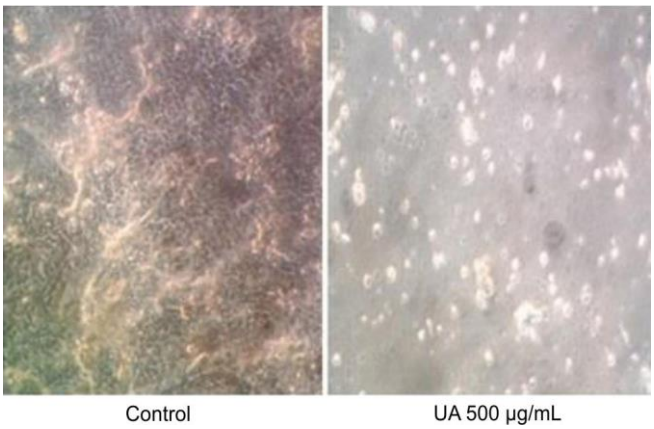

Fig. 6. Morphological changes in cancer cells induced by UA at 500 μg/mL

Conclusion

The present study successfully demonstrated the therapeutic potential of ursolic acid extracted from *Hedyotis diffusa* through both *in vitro* and *in silico* approaches. Ursolic acid demonstrated strong anticancer activities against colon cancer cells in a dose-dependent manner and shown noteworthy anti-inflammatory activity by stabilizing cell membranes. According to the results of molecular docking, ursolic acid has substantial binding affinities with TNIK (–9.0 kcal/mol) and COX-2 (–9.5 kcal/mol), surpassing those of standard drugs, indicating its ability to modulate both inflammatory and oncogenic pathways. These findings highlight ursolic acid as a promising dual-targeted natural therapeutic agent with potential applications in inflammation and colon cancer management. The results of this study offer a solid basis for additional preclinical and clinical research, opening the door for the creation of multifunctional therapeutic candidates based on ursolic acid.

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CONFLICT OF INTEREST

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

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