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Traditional Medicine Research 2025;10(7):42. https://doi.org/10.53388/TMR20241106001

Investigation of ROS-mediated apoptotic and anti-metastatic effects of *Hydrastis canadensis* in lung adenocarcinoma cells: an in vitro and in silico study

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Author contributions

Chandramohan S, Pajaniradje S, and Rajagopalan R contributed to the conceptualization of the study. Chandramohan S, Pajaniradje S, and Mishra S contributed to the experimental design and data collection. Chandramohan S and Pajaniradje S performed the experiments. Chandramohan S, Subramanian S, and Bhat SA were involved in data analysis and interpretation. Chandramohan S and Subramanian S prepared the original draft, while Chandramohan S, Francis AP, and Rajagopalan R reviewed and edited the manuscript. Rajagopalan R supervised the overall project and managed project administration. All authors reviewed and approved the final version of the manuscript for publication.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

The authors thank DST-FIST-II, UGC-SAP and DBT-IPLS for providing infrastructural support in Pondicherry University.

Peer review information

Traditional Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations

HC-MT, *Hydrastis canadensis* mother tincture; EGFR, epidermal growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; DCFDA, 2′,7′-dicholorofluorescin diacetate; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate buffered saline; MMPs, matrix metalloproteinases; LDH, lactate dehydrogenase; AO/EB, acridine orange/ethidium bromide.

Citation

Chandramohan S, Pajaniradje S, Francis AP, et al. Investigation of ROS-mediated apoptotic and anti-metastatic effects of *Hydrastis canadensis* in lung adenocarcinoma cells: an in vitro and in silico study. *Tradit Med Res.* 2025;10(7):42. doi: 10.53388/TMR20241106001.

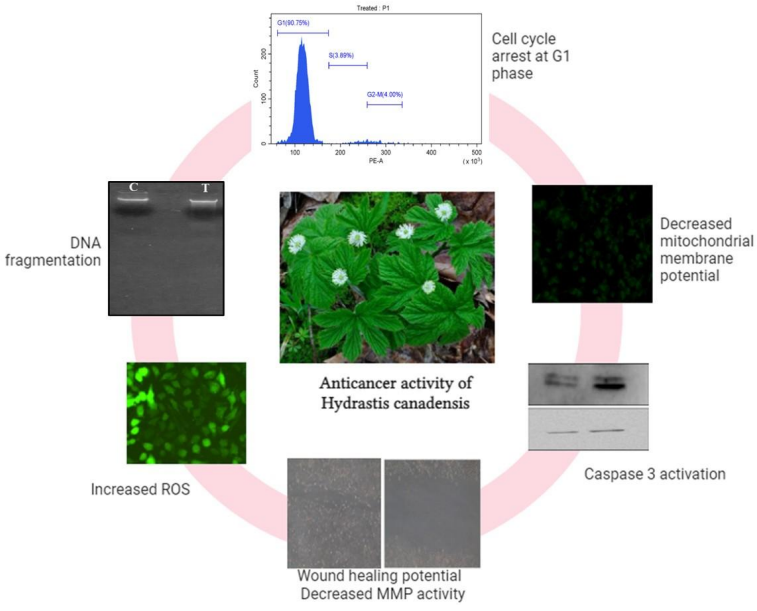
Executive editor: Jing-Yi Wang.

Received: 06 November 2024; **Revised:** 21 December 2024; **Accepted:** 06 February 2025; **Available online:** 10 February 2025.

Abstract

Background: *Hydrastis canadensis* mother tincture (HC-MT) is a preparation derived from the roots of the *Hydrastis canadensis* plant, commonly known as goldenseal. Despite its widespread use in homeopathic clinics for cancer treatment, there has been a lack of molecular studies assessing its efficacy against cancer. The aim of this study is to investigate the apoptotic and anti-metastatic properties of HC-MT in human lung adenocarcinoma cells (A549). **Methods:** MTT and LDH assays were conducted to assess the cytotoxic potential of HC-MT on tumor cells. Various staining techniques were employed to investigate the apoptotic potential, which was then confirmed through DNA fragmentation assay and western blotting. **Results:** The results showed that the HC-MT exhibited anti-proliferative effects with an IC_{50} concentration of 13.16 µL/mL with apoptosis as the predominant mode of cell death. Treatment with HC-MT at IC_{50} concentration resulted in cell cycle arrest at the G1 phase. Additionally, HC-MT exhibited anti-migratory properties. **Conclusion:** In summary, HC-MT demonstrated significant anti-cancer effects in lung cancer cells, inducing apoptosis primarily and arresting cell cycle progression at G1 phase mediated by ROS. It also exhibited anti-metastatic potential, with its active compound berberine showing interaction with key proteins involved in cancer progression. These findings suggest a promising therapeutic potential of HC-MT against lung cancer.

Keywords: *Hydrastis canadensis*; metastasis; apoptosis; lung cancer; A549



Highlights

This is the first study to provide molecular insights into the action of *Hydrastis canadensis* mother tincture (HC-MT) against lung cancer.

HC-MT showed significant anti-proliferative effects, with an IC_{50} concentration of 13.16 µL/mL.

Apoptosis was identified as the predominant mode of cell death induced by HC-MT.

IC_{50} concentration of HC-MT led to cell cycle arrest in the G1 stage. This effect is significant because dysregulation of the cell cycle is a characteristic feature of cancer cells and arresting it can inhibit cancer progression.

Collectively, these findings provide substantive evidence supporting the apoptotic and anti-metastatic effects of HC-MT in lung cancer cells.

Medical history of objective

In the first edition of *Collections for an Essay Toward a Materia Medica of the United States* (1798 C.E.), B.S. Barton documented the Cherokee use of Hydrastis as a cancer remedy, marking one of the earliest recorded uses of berberine-containing botanical drugs for cancer treatment. Goldenseal (*Hydrastis canadensis*) is a medicinal plant renowned for its extensive history of use in traditional medicine and as a dietary supplement. Native Americans employed goldenseal for its coloring properties and to treat various health issues, such as wounds, digestive problems, ulcers, skin and eye ailments, as well as cancer. In recent years, goldenseal has gained widespread popularity as a dietary supplement in the United States and other countries. Traditionally, people have utilized the rhizome of this plant to tackle various health concerns, such as gastrointestinal troubles, ulcers, muscle weakness, fatigue, constipation, skin, and eye infections. One of the primary bioactive compounds found in goldenseal is berberine, which has been associated with a wide range of therapeutic benefits. Extracts containing berberine demonstrate a range of health benefits, including hypoglycemic, antimicrobial, antioxidant, anti-inflammatory, hypolipidemic, cardioprotective, neuroprotective (specifically against Alzheimer's disease), and gastrointestinal protective properties.

Background

Cancer is a cluster of more than 100 diseases that develop over time. Cancer involves the uncontrolled division of cells [1]. As of 2020, lung cancer has become the primary cause of cancer deaths globally, with an estimated death of 1.8 million cases (18% of cancer deaths) [2]. Lung cancer shows highly variable symptoms and signs based on its anatomic location as it can arise from different sites of the bronchial tree and due to its heterogeneous nature. About 70% of diagnosed patients with lung cancer were in the advanced stages [3]. Unlike normal cells, cancer cells lose most of their regulatory functions and continue to divide abnormally, making them susceptible to chemotherapeutic medication more than normal cells. Advances in drug discovery and the development of new drugs resulted in an extensive collection of helpful chemotherapeutic agents routinely used in cancer treatment. However, chemotherapeutic therapies also have their intrinsic problems. Toxic side effects are of significant concern in many chemotherapeutic drugs [4].

Several drugs are either currently in clinical use or in development for the treatment of lung cancer. Erlotinib, Gefitinib, and Osimertinib are targeted therapies that inhibit epidermal growth factor receptor (EGFR) signaling, which is often overactive in many lung cancers. These drugs show high specificity for EGFR-mutated lung cancers and have clinically proven efficient in non-small cell lung cancer. However, their effectiveness can be compromised by secondary mutations that lead to resistance, and they are associated with significant side effects, including rash and diarrhea [5]. Pembrolizumab and Nivolumab, immune checkpoint inhibitors, block the PD-1/PD-L1 pathway, thereby enhancing T-cell-mediated anti-tumor immunity. These therapies can have long-lasting effects in patients who respond, but they are effective primarily in tumors with high PD-L1 expression or microsatellite instability. The high cost and potential for severe immune-related side effects limit their broader applicability. Cisplatin and Paclitaxel, non-specific cytotoxic agents, kill rapidly dividing cells and are widely used across various lung cancer subtypes. While they are applicable to all lung cancer types, they come with severe side effects such as nephrotoxicity, neuropathy, and myelosuppression, and there is a risk of developing resistance over time [6].

Most of the alternative therapies proposed for cancer treatment use plant-derived products. Over 3,000 plant species have demonstrated anti-cancer properties. Plants, the reservoir of phytochemicals still potentially able to provide newer drugs with a chemoprotective nature against cancer [7]. Medicinal plants are increasingly recognized as a valuable resource for cancer treatment, given their antioxidant and anti-mutagenic activities, along with their low side effects, affordability, and accessibility. *Hydrastis canadensis* mother tincture (HC-MT) presents several unique advantages over current lung cancer treatments. HC-MT works independently of the immune system by targeting intrinsic cellular pathways such as ROS generation, apoptosis, and cell cycle arrest. Additionally, HC-MT is not limited by specific mutations, unlike EGFR TKIs, and also could provide an option for patients who are non-responsive to immunotherapies. As a plant-based treatment, HC-MT may also be more accessible and affordable, particularly in resource-limited settings. Moreover, its natural origin may result in reduced systemic toxicity compared to conventional chemotherapeutics, such as cisplatin and paclitaxel. By addressing current treatment challenges such as resistance, toxicity, and specificity, HC-MT holds promise as a complementary or alternative approach to lung cancer treatment. However, further clinical validation is needed to establish its full efficacy and safety profile.

Hydrastis canadensis, commonly known as goldenseal, is a perennial herb native to North America. Goldenseal contains a diverse array of components, including amino acids, carbohydrates, and isoquinoline alkaloids. The primary active compounds of goldenseal are berberine, hydrastine and canadine. This plant is used both internally and externally to treat a variety of infections affecting the skin, mouth, and eyes, as well as gastrointestinal and urinary disorders [8]. Over recent years, pharmacological research has focused on the alkaloid berberine, which is known for its antiamoebic, antifungal, antibacterial, antiparasitic, and antituberculosis properties. Berberine has also garnered attention for its potential to lower cholesterol. Clinical studies have shown that berberine supplementation reduced cholesterol and triglyceride levels in both hypercholesterolemic patients and hamsters, likely by increasing the expression of low-density lipoprotein receptors in the liver [9]. Additionally, berberine has demonstrated efficacy in lowering blood glucose levels, glycated hemoglobin (HbA1c), triglycerides, total cholesterol, and low-density lipoprotein cholesterol in individuals with type 2 diabetes [10].

Recent studies have investigated the anticancer effects of berberine, revealing its ability to suppress cancer cell proliferation and promote apoptosis. For instance, in cervical cancer cells, berberine triggered cell cycle arrest and apoptosis by regulating activator protein-1 (AP-1) activity [11]. In cases of hepatocellular carcinoma, berberine suppressed Id-1 expression, leading to inhibited cell growth and metastasis [12]. Furthermore, β-hydrastine, another significant active compound in goldenseal, is known for its potential to trigger early apoptosis and suppress the proliferation and invasion of lung adenocarcinoma cells. Another in vitro study demonstrated that hydrastine decreased the viability of PC12 cells by promoting apoptotic cell death [8]. Mother tinctures are extremely concentrated herbal extracts used in homeopathy that are diluted to provide a variety of treatments [8]. Most existing studies have primarily focused

on isolated compounds like berberine, with limited information available on the therapeutic potential of HC-MT as a whole. While goldenseal has long been used in alternative medicine, its potential in modern oncology requires further evidence-based validation. This study aims to explore whether the synergy of multiple bioactive compounds in HC-MT enhances its anti-cancer efficacy compared to individual components. Additionally, it seeks to bridge the gap between traditional medicine and scientific validation by targeting specific molecular mechanisms and cancer-related pathways. By emphasizing its apoptotic and anti-metastatic properties, this study lays the foundation for the development of innovative, plant-based therapies for cancer treatment.

Methods

Chemicals

Human lung cancer (A549) cell line was obtained from National Centre for Cell Sciences (Pune, India). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, streptomycin, amphotericin B, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Himedia (Mumbai, India). Fluorescent stains such as 4',6-diamidino-2-phenylindole (DAPI), 2',7'-dicholorofluorescin diacetate (DCFDA), and Rhodamine 123 were acquired from Sigma (Bengaluru, India). Acridine orange, ethidium bromide, and propidium iodide were procured from Himedia (Mumbai, India). Primary antibodies (Caspase-3 and β-actin) and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Luminol and Coumaric acid were procured from Sigma (Bengaluru, India). All other lab chemicals are of analytical grade and were purchased from Himedia (Mumbai, India).

Cell lines and culture conditions

A549 cells were maintained and grown optimally in DMEM medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in a 5% CO_2 atmosphere. The cells were grown in sterile tissue culture flasks through routine sub-culturing, with the medium being replaced every 48 h. Cells were passaged once they reached 85–90% confluency.

In vitro cytotoxicity studies

In vitro cytotoxicity of A549 cells was studied using the MTT assay [13]. The principle behind this test is the reduction of yellow MTT soluble salt into purple-blue insoluble formazan crystals by the mitochondrial dehydrogenase of living cells. All tests were carried out in three replicates using 96-well culture plates. MTT solution was prepared by dissolving 5 mg of the salt in 1 mL of phosphate buffered saline (PBS). A549 cells were treated with varying doses of HC-MT along with plain DMEM medium as a control and were incubated for 24 h. Followed by 24 h incubation, the medium was replaced with MTT (5 mg/mL) solution. The plate was incubated for four more hours, and the resulting formazan crystals were dissolved in DMSO. The absorbance of the formazan solution was measured at 570 nm using a microplate Reader (Versamax, Molecular Devices Inc., Sunnyvale, CA, USA).

Lactate dehydrogenase (LDH) assay

Cells were exposed to the IC_{50} concentration of HC-MT and incubated for 24 h. Afterward, the media from both control and treated wells were collected. Each sample was then mixed with 2 mL of sodium pyruvate solution (1.22 mM) and 40 µL of NADH solution (12.4 mg/mL), followed by incubation at room temperature for 30 minutes. Subsequently, their absorbance was obtained at 340 nm in a VersaMAx ELISA Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA) [14].

DNA fragmentation assay

About 2×10^5 A549 cells were seeded in 6-well plates and treated with IC_{50} concentration of HC-MT which was incubated for 24 h. The samples were collected, rinsed with PBS, and centrifuged for 3 min. The pellets were lysed using lysis buffer and centrifuged again. The resulting supernatant was extracted and treated with RNase A (5 µg/mL), followed by incubation at 60 °C for 20 min. Proteinase K (2.5 µg/mL) was then added, and the mixture was incubated for an additional hour at 60 °C. DNA was precipitated using 100% chilled ethanol, and the fragments were separated using gel electrophoresis and visualized using a gel documentation system [15].

Evaluation of nuclear morphology

A549 cells (0.1×10^6) treated with HC-MT (IC_{50} concentration) were incubated for 24 h. Cells were thoroughly washed with PBS and fixed in 70% ethanol. They were then re-suspended in DAPI solution (5 µg/mL) and incubated in the dark at 37 °C for 5 min. Afterward, the cells were rinsed multiple times with PBS, and images were acquired using a Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a digital imaging system [14].

Evaluation of the mode of cell death by acridine orange/ethidium bromide (AO/EB) staining

AO/EB staining was carried out to detect the mode of cell death. Briefly, 0.1×10^6 A549 cells were treated with an IC_{50} dose of HC-MT and incubated for 24 h. They were then stained with an AO/EB solution (equal parts acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) in PBS) for 5 min in the dark. Afterward, the cells were washed twice with PBS and examined using a fluorescence microscope (Olympus IX71, Tokyo, Japan) equipped with a camera [14].

Mitochondrial membrane potential (ΔΨm) analysis with Rhodamine 123 (Rh123)

Briefly, 0.1×10^6 A549 cells were pre-cultured in a 6-well plate for 24 hours in DMEM. The cells were exposed to the IC_{50} dose of HC-MT and incubated for an additional 24 h. Rh123 (10 µg/mL) was added to the cells and incubated at 37 °C for 30 min in the dark. Then the cells were washed repeatedly with PBS and were visualized using the blue filter of a fluorescence microscope (Olympus IX71, Tokyo, Japan) equipped with a digital imaging system [14].

Intracellular ROS detection and evaluation

Intracellular production of reactive oxygen species (ROS) was evaluated using the oxidative-sensitive fluorescent probe DCFDA staining. About 0.2×10^6 A549 cells were cultured in 6 well plates, treated with IC_{50} concentration of HC-MT, and incubated for 24 h. Following incubation, the cells were stained with 10 µM DCFDA for 30 min at 37 °C in the dark. Cells were then washed twice with PBS and visualized under a fluorescence microscope [16].

Cell cycle analysis

A549 cells were treated with HC-MT and incubated for 24 h. After treatment, the cells were collected, washed thoroughly with PBS, fixed with 70% ethanol, and incubated at 4 °C for 4 h. Following incubation, the cells were separated by centrifugation and further incubated with a propidium iodide (10 µg/mL) solution, prepared in a buffer containing 0.1% Triton-X-100 and 20 µg/mL RNase A in PBS, for 30 minutes. Finally, the cells were analyzed using a flow cytometer (BD FACSAria, BD Biosciences, Milpitas, CA, USA) [15].

Protein expression studies

Briefly, protein lysates prepared from cells treated with HC-MT and untreated control were quantified through Bradford assay (MM 1976). The protein was separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane using a semi-dry blotting unit (Hoefer, Holliston, MA, USA). The membrane was then blocked by incubating with 5% skim milk in TBST (Tris-buffered saline with 1% Tween 20) for 40 min. Later, the membrane was exposed to primary antibodies and left to incubate overnight at 4 °C. Following this, the membrane was rinsed with TBST and incubated with a secondary antibody conjugated to horseradish peroxidase. The blot was then developed using the enhanced chemiluminescence method

[15].

Migration assay

A549 cells (0.2×10^6) were cultured in a 6-well plate until they reached full confluence, and scratches were made perpendicular to each other, extending from one end to the other. The cells were then treated with the IC_{50} concentration of HC-MT for 24 h. Images were captured before and after incubation using a phase contrast microscope (Olympus CX41, Tokyo, Japan) [16].

Gelatin zymography

Gelatin zymography was employed to assess the activity of matrix metalloproteinase. A549 cells were exposed to the IC_{50} concentration of HC-MT and allowed to incubate for 24 h. After the incubation period, the culture medium was collected and run on a native polyacrylamide gel containing gelatin for 2½ h. The gel was then immersed in a calcium buffer solution and incubated for 12 h. Subsequently, the gel was stained with Coomassie blue and destained until clear bands appeared [15].

In silico studies

In silico studies were carried out to analyze the docking of active compounds, hydrastine, and berberine, with proteins associated with apoptosis and metastasis. Molecular docking was performed using Schrödinger software, while the ligand structures were prepared using ChemDraw and processed with Open Babel. Protein structures, including Cox-2, MMP-9, Bcl-2, Bax, Cyclin D1, and EGFR, were retrieved from the Protein Data Bank. The ligands were docked to these proteins, and their binding energies along with hydrogen bond interactions were evaluated.

Statistical analysis

Statistical analysis was performed using a t-test, and values with $P \leq 0.05$ were considered statistically significant.

Results

Cytotoxicity of HC-MT against lung cancer cell line A549

MTT assay and LDH assay were performed to evaluate the cytotoxicity. Treatment with HC-MT reduced the viability of A549 lung adenocarcinoma cells in a dose-dependent manner. The cytotoxic effect of HC-MT on the A549 cell line increased with increasing concentrations, with an IC_{50} determined at 13.16 µL/mL (Figure 1a). The LDH assay results indicated a significant increase in LDH activity, corroborating the cell death induced by HC-MT (Figure 1b).

Evaluation of morphological features associated with apoptosis

DNA cleavage at the inter-nucleosomal linker regions, resulting in the formation of DNA fragments, is recognized as a distinctive biochemical indicator of apoptosis. In the DNA fragmentation assay, we readily observed a ladder formation in the HC-MT treated group indicating the involvement of apoptosis in mediating cell death rather than necrosis (Figure 1c). In DAPI staining, the treated cells exhibited distinct signs of apoptosis in their nuclear morphology, including chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies when compared to untreated cells (Figure 2a). AO/EB staining on the other hand showed green-fluorescent cells in the untreated group indicating live cells and yellow to orange-fluorescent cells in the treated group indicating cells in mid-late stages of apoptosis (Figure 2b). Rh123 staining revealed a decrease in the green-fluorescent intensity in the treatment group confirming a decrease in the mitochondrial membrane potential (MMP) and further validating the involvement of apoptosis (Figure 3a).

Intracellular ROS detection and evaluation

Non-fluorescent DCFDA is transformed into the highly fluorescent DCF inside cells due to the action of oxidative free radicals produced within the cells. In this study, the treated cells exhibited enhanced green fluorescence, indicating a higher level of ROS production in the treatment group relative to the untreated group (Figure 3b).

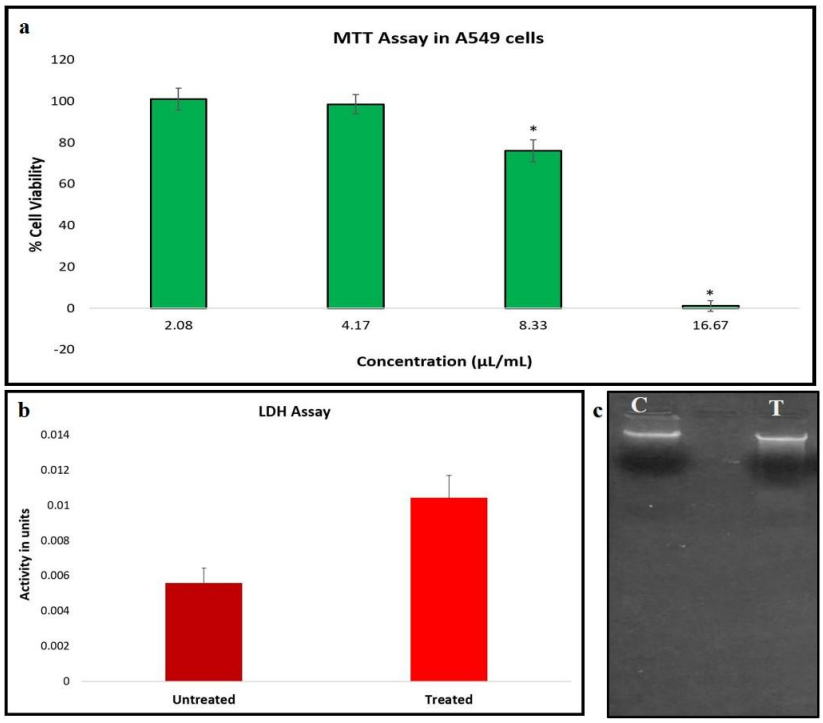


Figure 1 HC-MT-induced cytotoxicity and DNA fragmentation in A549 cells. (a) Graphical representation of anti-proliferative effects of HC-MT on A549 cell line through MTT assay. Bars denote % cell viability of A549 cell line. Values are represented as Mean ± Standard deviation. * indicates significance when compared to control. $P < 0.05$ was considered to be statistically significant. (b) Graphical representation of LDH activity in untreated and cells treated with HC-MT. Values are represented as Mean ± Standard deviation. (c) DNA fragmentation analysis for human lung cancer cells A549 in the presence or absence of HC-MT. Lane 1: "C" is untreated A549 cells. Lane 2: "T" is treated A549 cells with HC-MT for 24 h. MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.

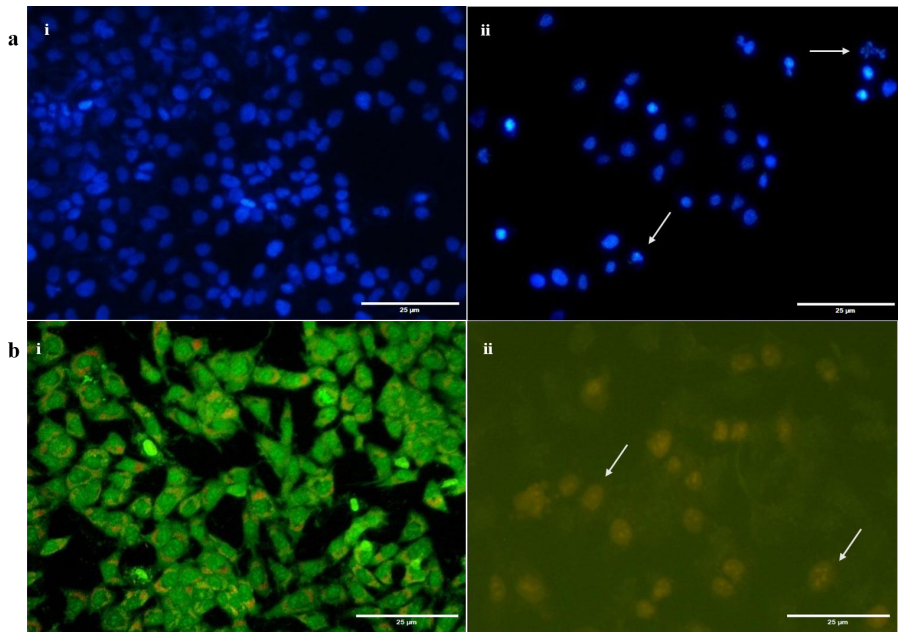


Figure 2 Nuclear and cellular staining analysis. (a) Nuclear staining using DAPI of human lung cancer cells A549 in the presence or absence of HC-MT. (i) Untreated A549 cells. (ii) A549 cells treated with IC_{50} dose of HC-MT for 24 h. Arrows indicate nuclear changes, including DNA fragmentation and chromatin condensation. (b) Cellular staining using AO/EB of human lung cancer cells A549 in the presence or absence of HC-MT. (i) Untreated A549 cells. (ii) A549 cells treated with IC_{50} dose of HC-MT for 24 h. Arrows point to cells undergoing late apoptosis in the treated group. Scale bar: 25 μm.

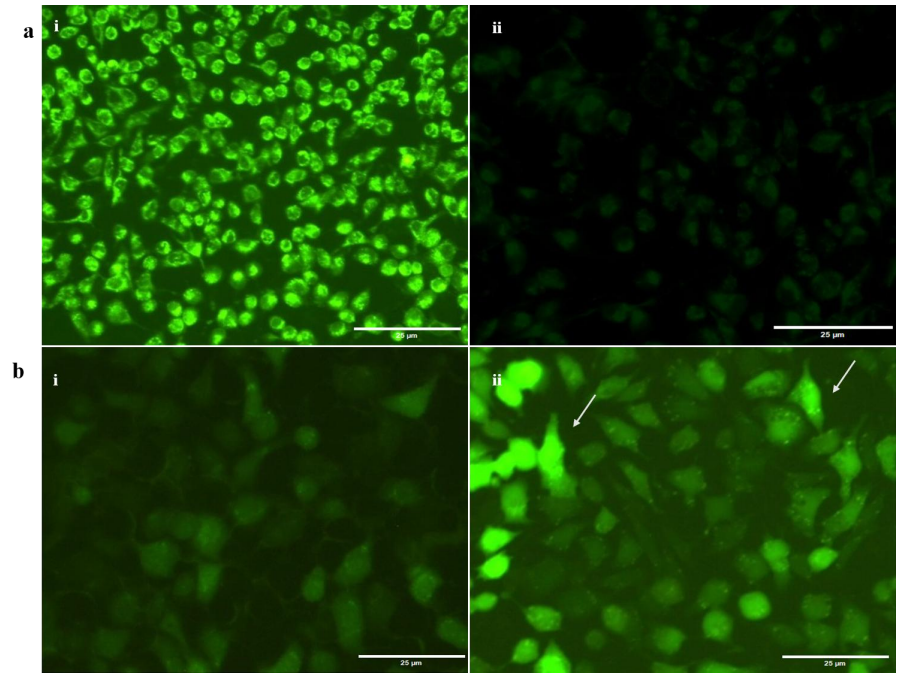


Figure 3 Analysis of MMP and ROS generation. (a) Mitochondrial staining using Rh123 of human lung cancer cells A549 in the presence or absence of HC-MT. (i) Untreated A549 cells. (ii) A549 cells treated with IC_{50} dose of HC-MT for 24 h showing decreased MMP. (b) DCFDA staining for ROS generation of human lung cancer cells A549 in the presence or absence of HC-MT. (i) Untreated A549 cells. (ii) A549 cells treated with IC_{50} dose of HC-MT for 24 h. The arrows indicate ROS generation, signifying apoptosis. Scale bar: 25 μm.

Cell cycle analysis

Cell cycle analysis clearly demonstrated significant differences between the control and treatment groups. Cell cycle analysis of the control group revealed that 70.70% of cells were in the G1 phase, 20.89% in the S phase, and 5.45% in the G2-M phase (Figure 4a). In the treatment group, 90.75% of cells were in G1, 3.89% in S phase, and 4% in G2-M phase (Figure 4b). This data strongly indicates that the cells treated with HC-MT were effectively arrested in the G1 phase, as evidenced by the substantial reduction in cell populations advancing to the S and G2-M phases.

Western blot analysis

To confirm the mode of apoptosis, the protein expression of caspase 3, an effector caspase in both intrinsic and extrinsic pathways, was assessed using western blotting. The results indicated higher levels of caspase 3 in the experimental group compared to the control group, confirming that HC-MT induces apoptotic cell death (Figure 5).

Wound healing assay

The ability of cells to migrate is essential in the process of metastasis. In this study, the migratory ability of A549 cells, with or without HC-MT treatment, was assessed using scratch assay. The results demonstrated that treatment significantly inhibited wound healing and migration of A549 cells compared to untreated cells (Figure 6). This suggests that HC-MT has the potential to inhibit cancer cell migration and could therefore play a role in preventing metastasis.

Gelatin zymography

Gelatin zymography was carried out to evaluate the activity of matrix metalloproteinases (MMPs) enzymes in both treated and untreated A549 cells. Our study revealed a decrease in MMP activity (Figure 4c), evidenced by the reduced intensity of MMP bands in the experimental group relative to the control group.

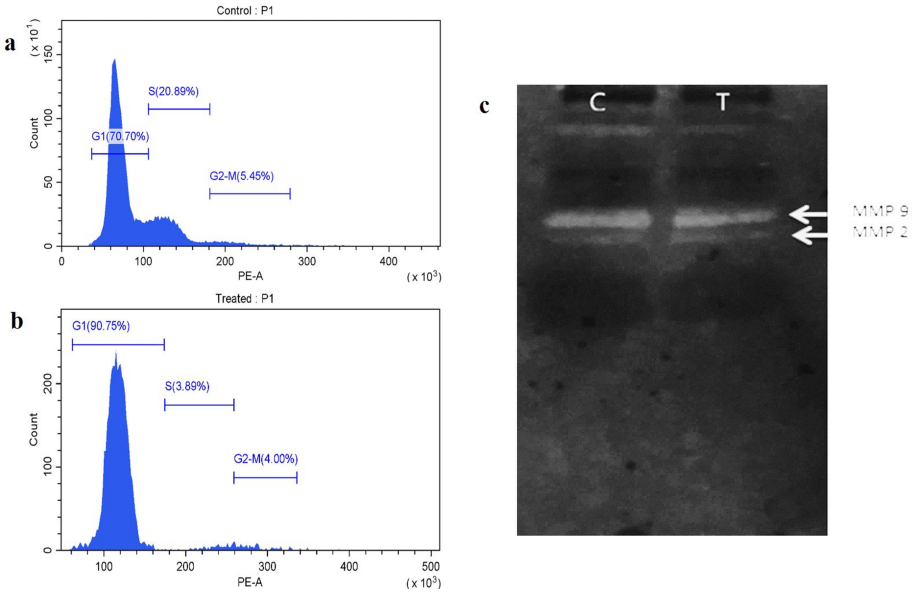


Figure 4 Cell cycle analysis of (a) Untreated A549 cells, (b) A549 cells treated with IC_{50} dose of HC-MT and (c) Evaluation of MMP activity in untreated and treated A549 cells. Lane 1: "C" is untreated A549 cells. Lane 2: "T" is A549 cells treated with IC_{50} dose of HC-MT.

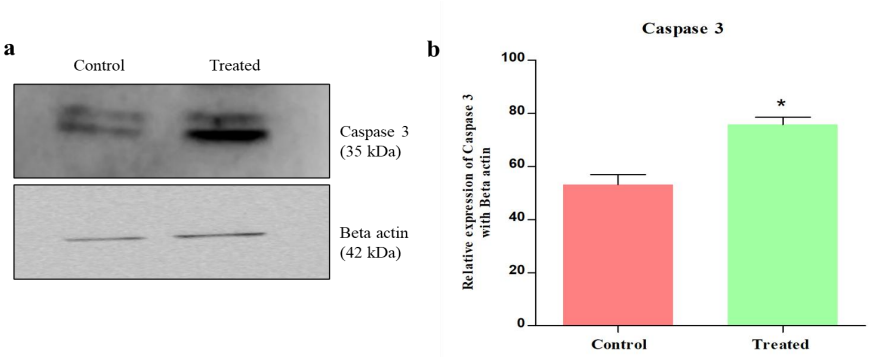


Figure 5 Expression and quantification of caspase-3. (a) Western blot analysis for protein expression of caspase 3. (b) Densitometry for protein abundance of caspase 3 in untreated and treated A549 cells. The *P*-value of ≤ 0.05 were considered statistically significant and are indicated by asterisks (*).

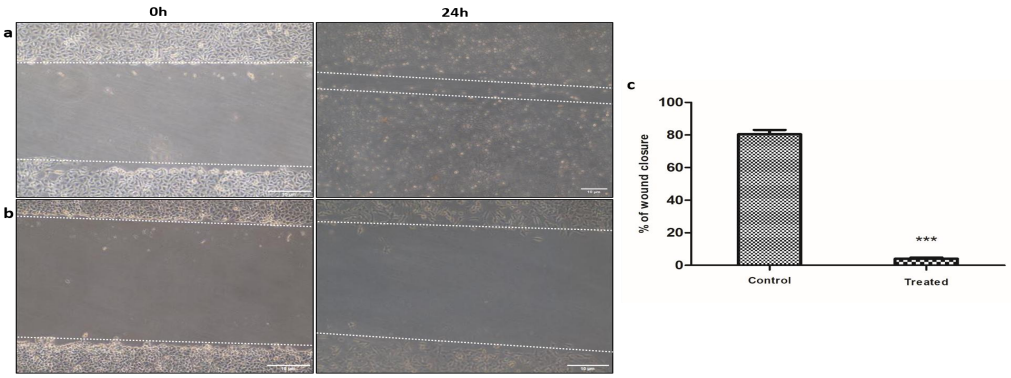


Figure 6 Wound healing assay to evaluate the migratory potential of the (a) Untreated, (b) Treated A549 cells and (c) Bar graph illustrating percentage of wound closure. $^{***}P < 0.001$ compared to Control. Scale bar: 10 µm.

Traditional Medicine Research 2025;10(7):42. https://doi.org/10.53388/TMR20241106001

Molecular docking studies

The molecular docking studies between Cox-2, Bcl-2, Bax, MMP-9, Cyclin D1, and EGFR proteins with the active compounds of HC-MT were assessed through molecular docking studies (Figure 7, 8), along with their respective standard compounds Etoricoxib, Navitoclax, Bax inhibitory peptide, Mariamstat, Cyclin D1 inhibitor, and Erlotinib as shown in Figure 9. The glide score of Berberine is observed to be higher in comparison to the standards Etoricoxib and Cyclin D1 protein (Table 1, 2). These results align with the biological testing, which verified that HC-MT is a potent cytotoxic and anti-metastatic agent.

Discussion

Chemotherapy usually alone or together with radiotherapy still plays a critical role in the treatment of many cancers. Lung cancer is a leading cause of high mortality rates and relatively poor clinical prognosis, which shows the need for effective therapeutic options [17]. Since long-term complications and serious side effects are related to conventional chemotherapy, investigation for natural agents that can minimize such untoward effects is presently gaining interest. It has been estimated that more than 60% of chemotherapeutic agents in clinical use are directly or indirectly obtained from natural products, and plants have lately become a critical resource for many anticancer drugs [18]. The current study reported the cytotoxic activity of HC-MT against lung cancer cell line (A549) and also elucidated the underlying molecular mechanisms.

The cytotoxic potential of HC-MT was evaluated using the MTT assay. MTT, initially yellow in color, is converted into a purple compound by NADPH-dependent oxido-reductase enzymes present in active mitochondria of live cells. The results indicate that HC-MT exhibits cytotoxic properties, with an IC_{50} observed at 13.16 µL/mL. In this regard, Khan et al. showed the cytotoxic effect of homeopathic medicine *Hydrastis canadensis* on hormone-dependent (MCF 7) and hormone-independent (MDA-MB-468) breast cancer cells [19].

LDH is a cytosolic enzyme catalyzing the interconversion of L-lactate and pyruvate. It is regarded as a stable marker in medium for the onset of cell death. When LDH leaks from the cytoplasm into the surrounding medium, it signals a disruption in plasma membrane integrity or the initiation of apoptosis or necrosis [20]. In this study, it was noted that LDH levels in the treated group were significantly higher compared to the control group, indicating cytotoxicity resulting from HC-MT.

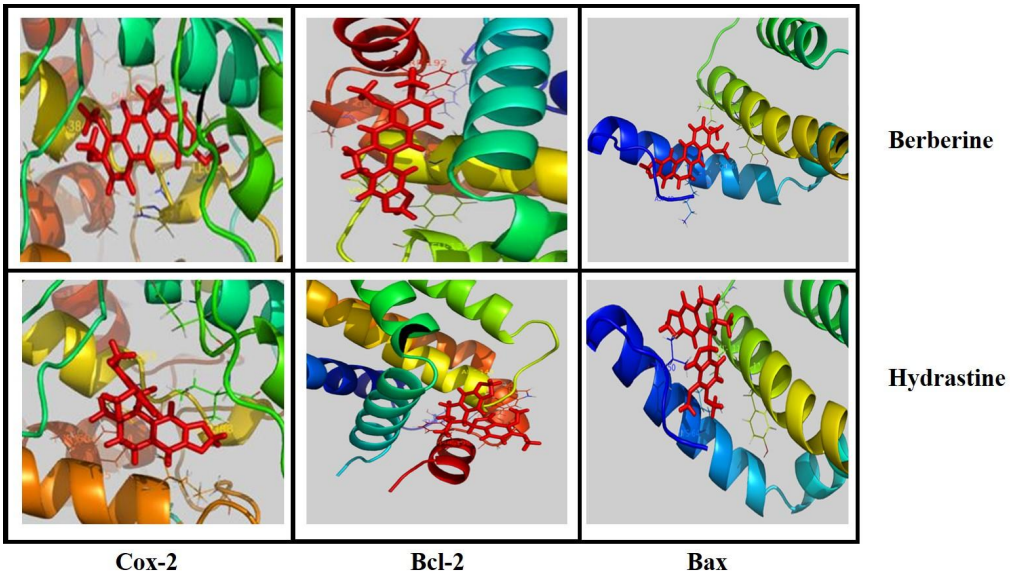


Figure 7 Docking poses of Berberine and Hydrastine with Cox-2, Bcl-2 and Bax proteins

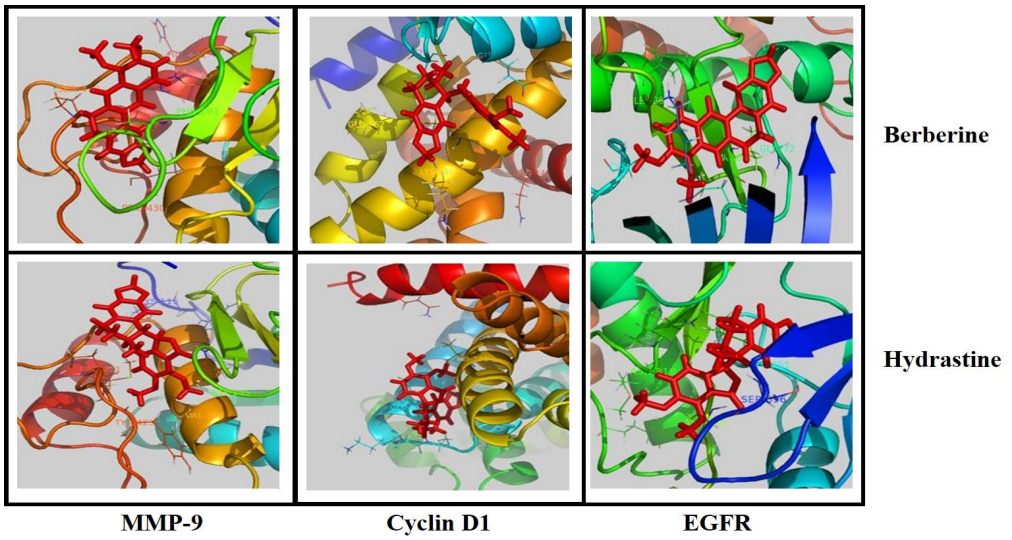


Figure 8 Docking poses of Berberine and Hydrastine with MMP-9, Cyclin D1 and EGFR proteins

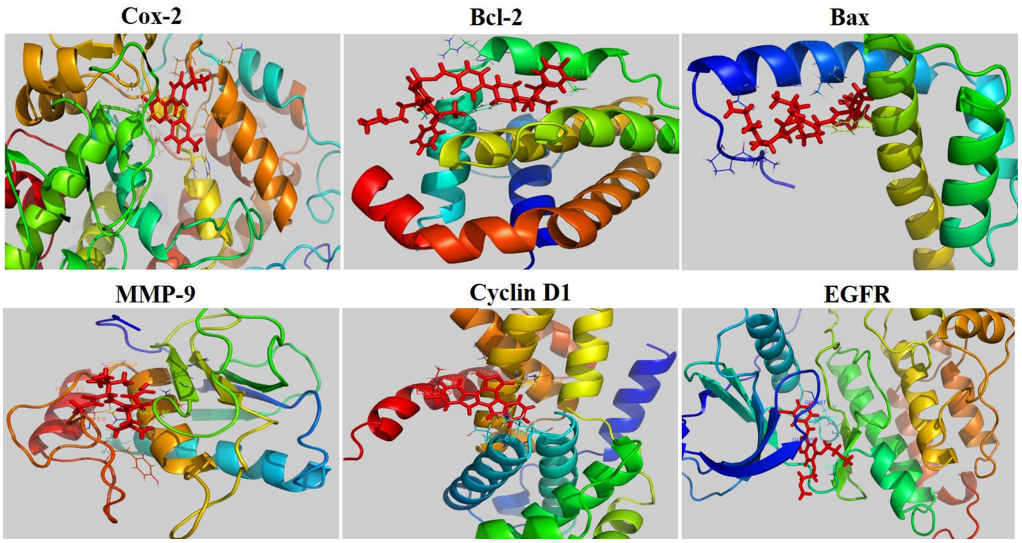


Figure 9 Docking poses of Cox-2, Bcl-2, Bax, MMP-9, Cyclin D1, and EGFR proteins with standard compounds Etoricoxib, Navitoclax, Bax inhibitory peptide, Mariamstat, Cyclin D1 inhibitor, and Erlotinib respectively

Table 1 Comparative illustration of the Glide scores and Glide energies between the target proteins and the ligands (Berberine and Hydrastine)

Targets	Glide g score		Glide energy (Kcal/mol)	
	Ligands			
	Berberine	Hydrastine	Berberine	Hydrastine
COX-2	−6.432	−5.559	−36.326	−33.585
BCL 2	−3.554	−4.66	−32.826	−34.559
BAX	−3.984	−3.470	−35.446	−31.924
MMP 9	−2.511	−3.728	−28.719	−26.376
Cyclin D1	−5.206	−3.022	−35.591	−25.227
EGFR	−5.544	−4.34	−37.153	−35.585

Table 2 Comparative illustration of the Glide scores and Glide energies between the target proteins and the standard drugs

Target receptors	Standard drug	Glide g score	Glide energy (Kcal/mol)
COX-2	Etoricoxib	−5.947	−37.868
BCL 2	Navitoclax	−5.872	−57.545
BAX	Bax inhibitory peptide	−5.51	−48.301
MMP 9	Mariamstat	−8.358	−43.894
Cyclin D1	Cyclin D1 inhibitor	−4.073	−38.649
EGFR	Erlotinib	−9.871	−60.601

It was evident that the cytotoxicity seen in cells was due to apoptotic induction but not necrosis, since typical apoptotic morphological changes were observed. Further studies confirmed that HC-MT does induce apoptosis in lung cancer cell lines through DNA fragmentation analysis, DAPI staining, Rhodamine 123 staining, dual acridine orange/ethidium bromide staining, DCFDA staining, cell cycle analysis, and caspase-3 immunoblotting.

Degradation of genomic DNA is one of the key characteristics associated with apoptosis, resulting in a ladder-like pattern upon electrophoresis [21]. It was found that the DNA isolated from the HC-MT treated cells showed the laddering pattern. This pattern results from the internucleosomal degradation of cellular DNA by an endonuclease, leading to the formation of DNA fragments [22].

Differentiating apoptosis and necrosis can be achieved through characteristic changes in the nuclear morphology of the cells [15]. The morphological changes observed in HC-MT treated cells align with traits of apoptotic cell death, such as reduction in cell size, membrane protrusions, and nuclear condensation. Similarly, staining the treated cells using AO/EB confirmed that the mode of cell death is predominantly apoptosis and not necrosis. Since apoptosis cannot be confirmed by just one or two experiments, we further tested the membrane potential of mitochondria using Rh123, and protein levels of caspase 3 in the presence or absence of the mother tincture. In the intrinsic apoptotic pathway, mitochondrial membrane depolarization increases permeability of the outer membrane, eventually forming pores to release pro-apoptotic molecules, such as cytochrome c. Rhodamine 123 assay is a commonly used technique to evaluate MMP in live cells. Rhodamine 123 is a cationic, lipophilic fluorescent dye that selectively accumulates in the mitochondria of cells with intact membrane potential [23]. The present results show that HC-MT significantly reduced the percentage of cells with an intact membrane potential and the disruption of $\Delta\Psi m$ (MMP) was relatively low in the control group. In the control group, cells with high MMP show significant accumulation of Rhodamine 123 in the mitochondria, driven by the electrochemical potential gradient across the inner mitochondrial membrane, resulting in strong fluorescence. In contrast, in the treated group, the loss of MMP, or mitochondrial depolarization, causes Rhodamine 123 to dissipate from the mitochondria, leading to a decrease in fluorescence intensity. This loss of MMP signifies mitochondrial dysfunction, a key indicator of

apoptosis. The disruption of MMP in the treated group promotes the release of cytochrome c and other pro-apoptotic factors into the cytosol, thereby activating the intrinsic apoptotic pathway [24].

Several intracellular signalling pathways are also known targets of ROS, which act as secondary messengers. Nevertheless, in addition to normal cellular signalling, increased ROS production can result in oxidative stress, cellular dysfunction, and apoptosis [25]. Mitochondrial membrane potential (ΔΨm) loss is reported to be directly associated with ROS generation in several studies. Elevated ROS levels trigger lipid peroxidation, protein oxidation, and DNA damage, ultimately leading to apoptosis. Moreover, it has been demonstrated that ROS-induced loss of ΔΨm is coupled with the activation of caspase-3, an important enzyme that drives programmed cell death [26]. In this work, we measured the levels of ROS in lung cancer cells, assuming that HC-MT may cause induction in ROS production. Our results indicated that the level of ROS significantly increased after treatment with HC-MT. The fluorescence intensity of HC-MT-treated cells was markedly higher compared to the untreated controls. HC-MT, which is rich in bioactive compounds like berberine, disrupts cellular redox balance, resulting in elevated levels of ROS, including superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2). This ROS generation is primarily linked to mitochondrial electron transport chain dysfunction caused by HC-MT. Disruption of electron transport chain complexes causes electron leakage, which reacts with molecular oxygen to form ROS. HC-MT may also inhibit cellular antioxidant systems, including glutathione (GSH) and enzymes like superoxide dismutase (SOD) and catalase. This inhibition further exacerbates ROS buildup, resulting in oxidative stress. The elevated ROS levels activate stress-related pathways, such as the p38 MAPK and JNK pathways, which amplify apoptosis signalling [27]. This suggests that HC-MT induces ROS accumulation in cancer cells, potentially leading to nuclear DNA damage and cell death.

Cell cycle is a crucial regulatory process in cellular growth, and dysregulation at specific checkpoints is widely implicated in the pathogenesis of many malignancies [28]. To investigate this, we examined cell cycle phase distribution using flow cytometry. The cell cycle is composed of four consecutive phases, all required for cell division and proliferation. Uncontrolled proliferation and genomic instability result in the accumulation of tumor cells. Cell cycle checkpoints normally arrest the progression of cells when DNA damage is detected to ensure genetic fidelity [29]. Our data indicated that HC-MT arrested the cell cycle at the G1 phase in A549 cells. Cell-cycle arrest in the G1 phase prevents A549 cells from entering S and G2/M, thus stopping DNA synthesis and consequently inhibiting cell proliferation. Similarly, Sabiha Khan et al., reported that *Hydrastis canadensis* promotes the G0/G1-phase cell cycle arrest and the apoptosis induction in breast cancer cells [19].

Caspase-3, a key effector and central executor in both intrinsic and extrinsic apoptotic pathways, is essential for mediating apoptosis and modulating drug cytotoxicity [30]. Comparison of caspase-3 activity between treated and control cells showed a significant increase in activity in A549 cells exposed to HC-MT. This study demonstrated that HC-MT promotes apoptosis through a caspase-3 dependent mechanism in cancer cells.

Anti-metastatic potential of HC-MT was confirmed by wound healing and gelatin zymography experiments. Wound healing experiment results confirmed the inhibitory effect of the HC-MT in cancer cell migration. The reduced migratory ability of A549 cells indicates that HC-MT may have antimetastatic properties, likely through the downregulation of key pathways involved in cell migration. Specifically, HC-MT could influence the expression of Cox-2, an enzyme associated with inflammation and metastasis, as well as MMPs, a group of enzymes crucial for extracellular matrix remodeling during metastasis [31]. By modulating these pathways, HC-MT may impair the ability of cancer cells to migrate and invade, ultimately reducing metastasis. MMP are important enzymes for cancer cell migration. MMPs, like MMP-9 and MMP-2, are members of the zinc-dependent protease family [32]. Gelatin zymography is an assay to evaluate the activity of these MMPs. We found that MMP activity decreased in the treatment group when compared to untreated. This is probably due to the suppression of MMP by one of the constituents of HC, namely berberine. Thus, confirming the anti-metastatic potential of HC-MT [33].

The primary mechanisms of action of HC-MT on lung adenocarcinoma cells can be outlined as follows:

ROS production and cellular stress: The induction of intracellular ROS by HC-MT results in oxidative stress. Increased ROS levels result in disruption of DNA, proteins, and lipids, activating DNA damage response (DDR) pathways mediated by p53. This leads to the upregulation of p21 by p53, which halts the cell cycle at the G1 phase by inhibiting CDKs and reducing Cyclin D1 levels, ultimately stopping cell proliferation [34].

Mitochondrial dysfunction and the release of cytochrome c: ROS accumulation leads to mitochondrial dysfunction, including the loss of MMP. Damaged mitochondria release cytochrome c, which combines with Apaf-1 to form the apoptosome, subsequently activating caspase-9, an initiator caspase [35].

Execution of apoptosis and feedback loop: Caspase-9 activates caspase-3 (the executioner caspase), leading to the degradation of cellular components and the induction of apoptosis, characterized by nuclear condensation, membrane blebbing, and DNA laddering. Amplification of ROS by caspases reinforces the apoptotic cascade, creating a positive feedback loop that ensures effective cancer cell death [36].

The active compounds of *Hydrastis canadensis* are hydrastine and berberine. We performed molecular docking to explore the role of certain proteins involved in key regulatory and signalling pathways associated with cancer. We performed docking of hydrastine and berberine with Cox-2, Bcl-2, Bax, MMP-9, Cyclin D1, and EGFR proteins. Results showed that the interacting partners to these active compounds especially to berberine are most likely to be Cyclin D1 and Cox-2 proteins indicating that its mechanism of action could involve mainly cell cycle and metastatic pathways. Overall, our study provides evidence for the anti-cancer, induction of apoptosis, and anti-metastatic effects of HC-MT in A549 lung cancer cells.

Conclusion

In conclusion, our study investigated the effects of HC-MT on lung cancer cells. We found that the mother tincture exerts an anti-cancer effect, with an IC_{50} of 13.16 μL/mL, predominantly inducing apoptosis as evidenced by morphological changes, DNA laddering, and protein expression studies. Elevated levels of ROS contribute to apoptosis induction by the mother tincture and cause cell cycle arrest in the G1 phase. Additionally, HC-MT demonstrated anti-metastatic properties. The active compound berberine showed potential interaction with Cyclin D1 and Cox 2 proteins. These findings underscore the potential of HC-MT as an effective apoptotic and anti-metastatic agent for treating lung cancer.

Future perspective

The promising preclinical results for HC-MT lay the groundwork for its potential clinical application in lung cancer treatment. However, translating these laboratory findings to clinical settings requires a systematic approach to validate its efficacy, safety, and feasibility. If the efficacy and safety demonstrated in preclinical studies are confirmed in clinical trials, HC-MT could become a valuable addition to the repertoire of lung cancer treatments.

For patients with early-stage lung cancer, HC-MT may offer a potential treatment option or serve as an alternative for those who are intolerant to standard therapies.

HC-MT could be combined with chemotherapeutics or immune checkpoint inhibitors to enhance treatment efficacy and reduce the development of resistance, potentially improving patient outcomes.

It may also be used as a supportive treatment to alleviate side effects associated with conventional therapies.

Given its multi-targeted mechanism of action, HC-MT holds promise

for potential efficacy against other types of cancer, including breast and colon cancers.

Identifying biomarkers could help predict patient responses, allowing for more personalized treatment strategies.

As clinical trials progress, further research will be essential to validate these potential applications, optimize treatment regimens, and ensure the safety and effectiveness of HC-MT in cancer therapy.

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