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In Vitro Evaluation of Anticancer Activity and Phenolic Compounds of *Hypericum olympicum* in Human Lung Cancer Cells

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HIGHLIGHTS

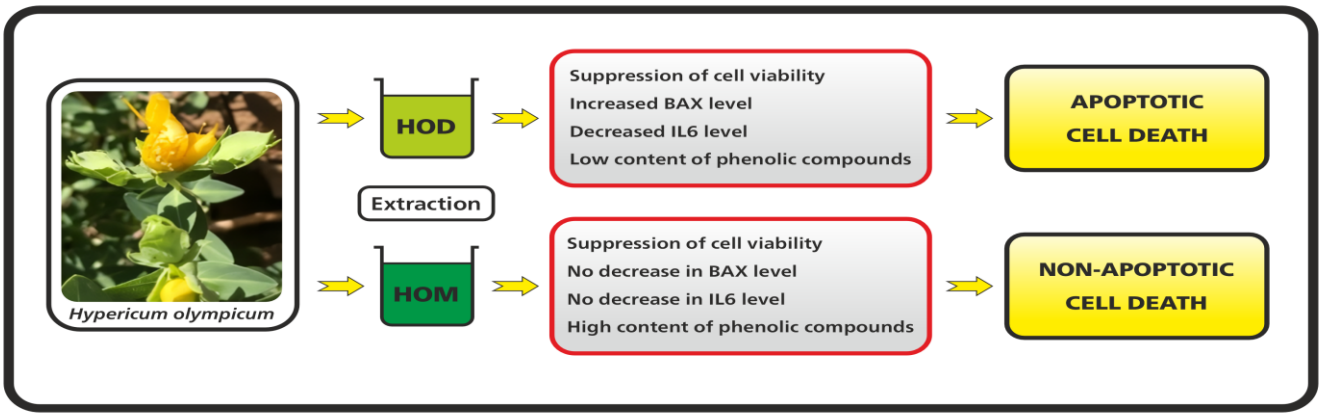
- Dichloromethane extract of *H. olympicum* (HO_D) has a cytotoxic effect on A549 cells
- HO_D increases BAX level and reduces pro-Caspase 3 level in A549 cells
- HO_D reduces the level of IL6, which can suppress apoptosis
- *H. olympicum* exhibited anticancer activity on lung cancer cells in vitro

Abstract: Lung cancer is the leading cause of cancer-related death and it is crucial to identify new sources for its treatment and suppression. Medicinal plants represent one of the most important resources in challenge against the cancer. *Hypericum* species have been shown in previous studies to suppress viability of various cancer cells. However, studies investigating the anticancer activities of *Hypericum olympicum* specifically on lung cancer cells remain quite limited. In this study, the anticancer activity of sequential dichloromethane (HO_D) and methanol (HO_M) extracts of *H. olympicum* on lung cancer cells (A549) was investigated, focusing on the reduction of cell viability, induction of apoptotic cell death, and suppression of proinflammatory cytokines. In this context, the effects of HO_D and HO_M on A549 cell viability were assessed using MTT method. The percentage of apoptotic and non-apoptotic cells was determined by AnnexinV/PI staining. The levels of Interleukin 6 (IL6) and 8 (IL8), as well as the apoptotic marker caspase-3, were measured using ELISA method. The mRNA levels of proapoptotic BAX and antiapoptotic BCL-2 were measured by Real-Time PCR method. The viability of A549 cells decreased following treatment with both

HO_D and HO_M. In the HO_D group, BAX level increased, while pro-caspase-3 and IL6 levels decreased. Additionally, percentage of apoptotic cells increased in HO_D group, whereas the percentage of non-apoptotic cells increased in HO_M group. Consequently, it is suggested that the anticancer activity of *H. olympicum* is associated with apoptotic pathway in the case of HO_D, and a non-apoptotic pathway in the case of HO_M.

Keywords: Apoptosis; *Hypericum olympicum*; Lung cancer; Proinflammatory cytokines; Phenolics.

GRAPHICAL ABSTRACT



INTRODUCTION

The cancer is the second leading cause of death worldwide following cardiovascular disease. Factors such as stress, malnutrition, occupational exposure, air pollution, heavy metals and especially smoking significantly increase the risk of developing cancer. Although incidence rates vary by countries, the most commonly diagnosed types of cancer are generally breast, lung, colon, prostate, cervix, skin and stomach. Among these, the lung cancer is the most prevalent in both men and women. Every year, the hundreds of thousands of people pass away from the lung cancer [1]. The lung cancer remains the leading cause of cancer-related death in the developed country such as United States and worldwide [2, 3]. It has been estimated that in 2023, approximately 1,950,000 new cases will be diagnosed in the United States alone, with around 610,000 cancer-related deaths [1].

The cancer has a process that generally occurs with an initial mutation. During this process, cancer cells accumulate many mutations that enable them to survive and proliferate. Under normal conditions, apoptosis (programmed cell death pathway) is activated to eliminate such abnormal cells. However, cancer cells often developed mechanisms to evade the apoptosis [4]. Disruptions in the apoptotic mechanism can lead to the transformation of cells into a malignant state, enable their spread, and contribute to the development of drug resistance [5]. When the apoptosis mechanism is impaired, various diseases may arise [6]. Apoptosis generally occurs through two distinct processes: the extrinsic and intrinsic pathways. At the onset of apoptosis, the balance between pro-apoptotic and anti-apoptotic proteins is altered. Alterations that lead to in the upregulation of apoptotic proteins promote the progression of the programmed cell death pathway. In the intrinsic apoptotic pathway, anti-apoptotic proteins such as BCL2, BCL-XL and MCL1, located in the mitochondrial membrane, are suppressed by increased levels of the proapoptotic protein BAX [5]. As Bax level rise, pores form in the mitochondrial membrane, allowing the release of cytochrome c, caspase inhibitor suppressors (SMAC/DIABLO). Cytochrome-c then binds with APAF1 (Apoptotic protease activating factor 1) to activate caspase 9, which subsequently cleaves procaspase-3 into active caspase-3. The caspase 9 is involved in the cleavage of procaspase 3 to form active caspase 3 that cleaves ICAD, leading to CAD (Caspase-activated DNase) formation. Subsequently, CAD initiates fragmentation of cellular DNA, a characteristic feature of apoptosis [7]. Therefore, many studies have aimed to activate the apoptotic pathway to eliminate of cancer cells using both synthetic and natural agents. [8-10]. However, due to the side effects associated with synthetic drugs, there is growing interest in herbal compounds that are believed to have fewer or no side effects. Medicinal plants play an important role among natural resources due to their unique phytochemicals, many of which have demonstrated the ability to induce apoptosis and suppress various types of cancer cells [8-11].

The genus *Hypericum* L. contain a total of 484 taxa that are naturally distributed across the world [12]. Bioactive compounds derived from *Hypericum* species exhibit a wide range biological activities including

antitumor, anti-inflammatory, antimicrobial, antidepressant and antioxidant effects [13-16]. Especially, *Hypericum* species have demonstrated significant anti-cancer activities, such as anti-proliferative effects and inhibition of cancer cell migration and invasion [17, 18]. Moreover, several *Hypericum* species have been reported to exert anticancer effect by inducing apoptosis in cancer cells [17, 19]. On the other hand, other studies have indicated that cancer cell death is not solely due to apoptosis [20] and that non-apoptotic pathways, such as necrosis or autophagy, may also be involved [21].

H. olympicum is a deciduous shrub species native to West Asia and Southeast Europe [22]. Previous studies have reported that various extracts and compounds derived from *H. olympicum* exhibit cytotoxic effects against different cancer cell lines [20, 23]. However, studies investigating the apoptotic and non-apoptotic effects of sequential dichloromethane and methanol extracts of *H. olympicum* on A549 lung cancer cells are quite limited. In particular, the dichloromethane extract of *H. olympicum* has not been thoroughly examined in relation to apoptosis in cancer cells. Therefore, this study was designed to evaluate the anticancer potential of the dichloromethane (HO_D) and methanol (HO_M) extracts of *H. olympicum* on A549 lung cancer cells. Specifically, the study investigated the effects of HO_D and HO_M on cell proliferation inhibition, suppression of inflammatory cytokines, and induction of apoptosis in A549 cells.

MATERIAL AND METHODS

Collection of *H. olympicum* and preparation of dichloromethane and methanol extracts

H. olympicum was collected from its natural habitat in Huzurlu Plateau (Gaziantep, Turkiye) on 02.07.2023 and identified by Dr. Mustafa PEHLIVAN (individually herbarium voucher number: MPH2023-2). *H. olympicum* was cleaned and dried on blotting paper away from sunlight, then ground into a fine powder using a morter. For extraction, 200 mL of dichloromethane was first applied to the powdered plant material using a Soxhlet apparatus at 40 °C for 6 hours. After the dichloromethane extraction was completed, the solvent was removed, and the extraction process was continued with 200 mL of methanol at 50 °C for another 6 hours. The resulting dichloromethane (HO_D) and methanol (HO_M) extracts were filtered and concentrated using a rotary evaporator under reduced pressure. The HO_D and HO_M extracts were then stored at +4 °C until further use in assays.

Maintenance and Growth of Lung cancer cells (A549) and BEAS2B cells

A549 cells (ATCC, human, epithelial non-small-cell lung cancer) and human lung epithelial cell (BEAS-2B as control cells) were obtained from Advanced Cell Analysis Laboratory of Gaziantep University-Turkiye. A549 and BEAS-2B cells were grown in RPMI supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic (Gibco, USA). A549 and BEAS-2B cells were cultured in an incubator at 37 °C in 5% CO_2 medium. The cells were seeded into 96-well plates at a density of 5×10^6 cells/mL and maintained in RPMI medium for 24 hours. Following this incubation period, the cells were treated with various concentrations (25, 50, 100, 200 µg/mL) of HO_D and HO_M for 24 hours. The extracts were prepared by dissolving in 10% dimethyl sulfoxide (DMSO).

Determination of cell viability of A549 and BEAS-2B cells following HO_D and HO_M treatment

The cells in 96-well plates, at 70–80% confluency, were treated with varying concentrations (25, 50, 100, and 200 µg/mL) of HO_D and HO_M extracts for 24 hours. A positive control group consisted of cells cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) without extract treatment. The viability of cells was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. After treatment, the culture medium was removed, and cells were incubated with MTT solution (1 mg/mL in saline) at 37 °C for 60 minutes. Following incubation, the MTT solution was discarded, and the resulting formazan crystals were dissolved in DMSO. Absorbance was measured at 550 nm using a microplate spectrophotometer. Cell viability was expressed as a percentage of the absorbance relative to untreated control cells.

Determination of mRNA expressions of proapoptotic BAX and antiapoptotic BCL2 in A549 cells

The A549 cells (5×10^6 cells/mL) were treated with 25 and 200 µg/mL concentrations of HO_D and HO_M for 24 hours. After incubation, the supernatant was discarded, and 700 µl of QIAzol Lysis Reagent was added to lyse the cells. Total RNA was extracted using TRIpure Total RNA Extraction Reagent (ELK Biotechnology, Wuhan, China) according to the manufacturer's instructions. The extracted RNA was then reverse transcribed into complementary (cDNA) by using reverse transcriptase kit following the manufacturer's protocol.

Quantitative real-time PCR (RT-PCR) was performed using a Rotor-Gene Q system (Qiagen, Germany). cDNA template for each primer with SYBR green master mix reacted at 95 °C for 15 min, 40 cycles 95 °C for 20 s,60 °C for 30s, 72 °C for 30 s. For the following PCR reaction was prepared; 10 µL SYBR Green Master Mix, 1 µL forward primer, 1 µL reverse Primer, 5 µL RNase-free water and 3 µL cDNA. Reactions were carried out on a Rotor-Gene Q instrument using the following cycling conditions: initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. After the reaction, Ct (cycling threshold) values were taken at an appropriate threshold. Relative gene expression for BAX (forward: GCCCTTTTGCTTCAGGGTTT, reverse: TCCAATGTCCAGCCTTTG) and BCL-2 (F: CGGAGGCTGGGATGCCTTTG, reverse: TCACTTGTGGCCCAGATAGG) genes was determined with normalization to GAPDH (forward: GATCATCAGCAATGCCTCCT, reverse: TGTGGTCATGAGTCCTTCCA). The mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Determination of Apoptotic and Non-Apoptotic Cell Percentages by Annexin V and Propidium Iodide (PI) Staining

To evaluate the effects of HO_D and HO_M on apoptosis and cell viability, A549 cells were seeded at a density of 5×10^6 cells/mL. The cells were treated with the minimum (25 µg/mL) and maximum (200 µg/mL) concentrations of the extracts for 24 hours. Following treatment, the percentages of apoptotic and non-apoptotic cells were determined using flow cytometry (Becton-Dickinson) with an Annexin V/PI staining kit, according to the manufacturer's instructions.

Determination of Pro-Caspase-3 Levels

After treatment with HO_D and HO_M, the supernatants were discarded to assess apoptosis in A549 cells (5×10^6 cells/mL). The A549 cells were then washed with PBS and lysed using a lysis solution. Pro-caspase-3 level in the cell lysates were measured using a ELISA kit following the manufacturer's instructions. Absorbance for pro-caspase-3 levels were read at 450 nm using a ELISA reader (BioTek instrument, USA).

Determination of interleukin 6 (IL6) and interleukin 8 (IL8) levels

After 24 hours of treatment with HO_D and HO_M, the supernatants from A549 lung cancer cells (5×10^6 cells/mL) were collected. To determine the levels of IL-6 and IL-8 in the supernatants were used a ELISA kits, according to the manufacturer's instructions. Absorbance for IL-6 and IL-8 was recorded at 450 nm using an ELISA reader (BioTek Instruments, USA).

Screening of phenolic compounds in HO_D and HO_M by LC-MS-MS

For the screening of phenolic compounds by LC-MS-MS, HO_D and HO_M were dissolved in methanol and subsequently filtered through a 0.22 µM filter. LC-20AD two-pump Nexera Ultra High Performance Liquid Chromatography (UHPLC) (Shimadzu) Liquid chromatography–mass spectrometry (LC-MS-MS) instrument, DGU-20A3R degasser, CTO-10ASVP column oven and SIL-20AC autosampler were utilized. For this screening, the analysis was run with a C18 Intersil ODS-4 analytical column (3.0mm x 100mm, 2µm). Injection volume of the samples was 2 µL and flow rate was 0.3 ml/min. In linear gradient flow, it was used mobile phase A (Water and 0.1% Formic acid) and mobile phase B (Methanol and 0.1% Formic acid). The column temperature was initially set to 40 °C.

Statistical Analysis

To evaluate statistically the results of MTT, apoptosis, BAX, BCL2, IL6, IL8 and pro-caspase3 assay, Dunnett's test of one-way ANOVA were used. The results of all the assays were presented as mean ± SD, and the level of significance was accepted as "*" $p<0.05$, "**" $p<0.01$ and "***" $p<0.001$.

RESULTS

Effects of HO_D and HO_M on viability of A549 and BEAS2B cells

To determine the effect of HO_D and HO_M on the viability of A549 and BEAS-2B cells, MTT assay was used. As shown in Figure 1, the viability of A549 cells decreased in concentration-dependent manner at concentrations of 25, 50, 100, and 200 µg/mL of HO_D. All concentrations of HO_D significantly reduced cell viability compared to the control group ($p < 0.05$ for 25 and 50 µg/mL; $p < 0.01$ for 100 and 200 µg/mL). On

the other hand, the viability of A549 cells was significantly reduced only at a 200 µg/ml concentration of HO_M, whereas no change was observed at the other concentrations when compared to the controls. In addition, HO_D and HO_M were also tested on the viability of BEAS2B cells for 24 hours. Although there was a decrease in the viability of the cells, especially at concentrations of 100 and 200 µg/ml, these changes were not statistically significant. Therefore, further anticancer activity assays were conducted exclusively with A549 cells.

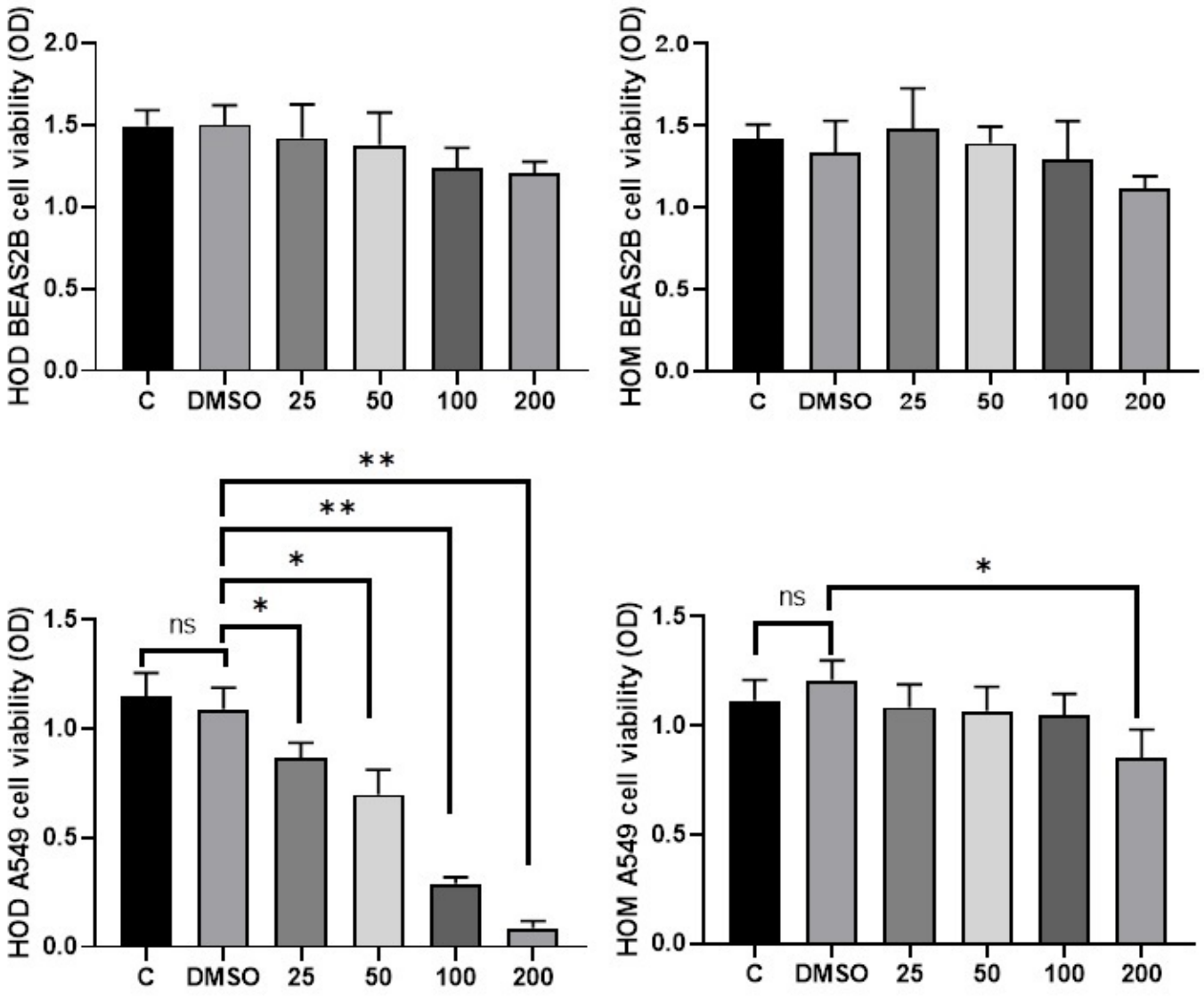


Figure 1. Cytotoxic effect of HO_D and HO_M on viability of lung cancer cells. Statistical analyzes were performed using the One-way Anova Dunnett' test. C: Control, OD: optic density, HO_D: Dichloromethane extract of H. *olympicum*, HO_M: Methanol extract of H. *olympicum*, DMSO: dimethyl sulfoxide. "*" and "**" mean $p<0.05$ and $p<0.01$, respectively

Determination of apoptotic cells percentages by Annexin-V/PI staining

In this study, apoptosis assay was performed using the lowest and highest concentrations of HO_D and HO_M. To assess the percentage of early apoptotic, late apoptotic and non-apoptotic, A549 cells were treated with HO_D and HO_M for 24 hours. Apoptotic cell populations were then quantified using flow cytometry (FACS, fluorescence-activated cell sorting). The apoptotic effects of HO_D and HO_M were shown in Figure 2a. At 200 µg/ml concentration of HO_D, 65.7% of A549 cells were found to be in the late apoptotic stage. This increase in late apoptotic cells was statistically significant compared to the control group ($p < 0.001$) (Figure 2b). Additionally, as shown in Figure 2b, the percentages of non-apoptotic cells following HO_M treatment were 79% and 98.7% at 25 and 200 µg/mL concentrations, respectively ($p < 0.001$). On the other hand, no significant change was observed in the percentage of non-apoptotic cells after HO_D treatment.

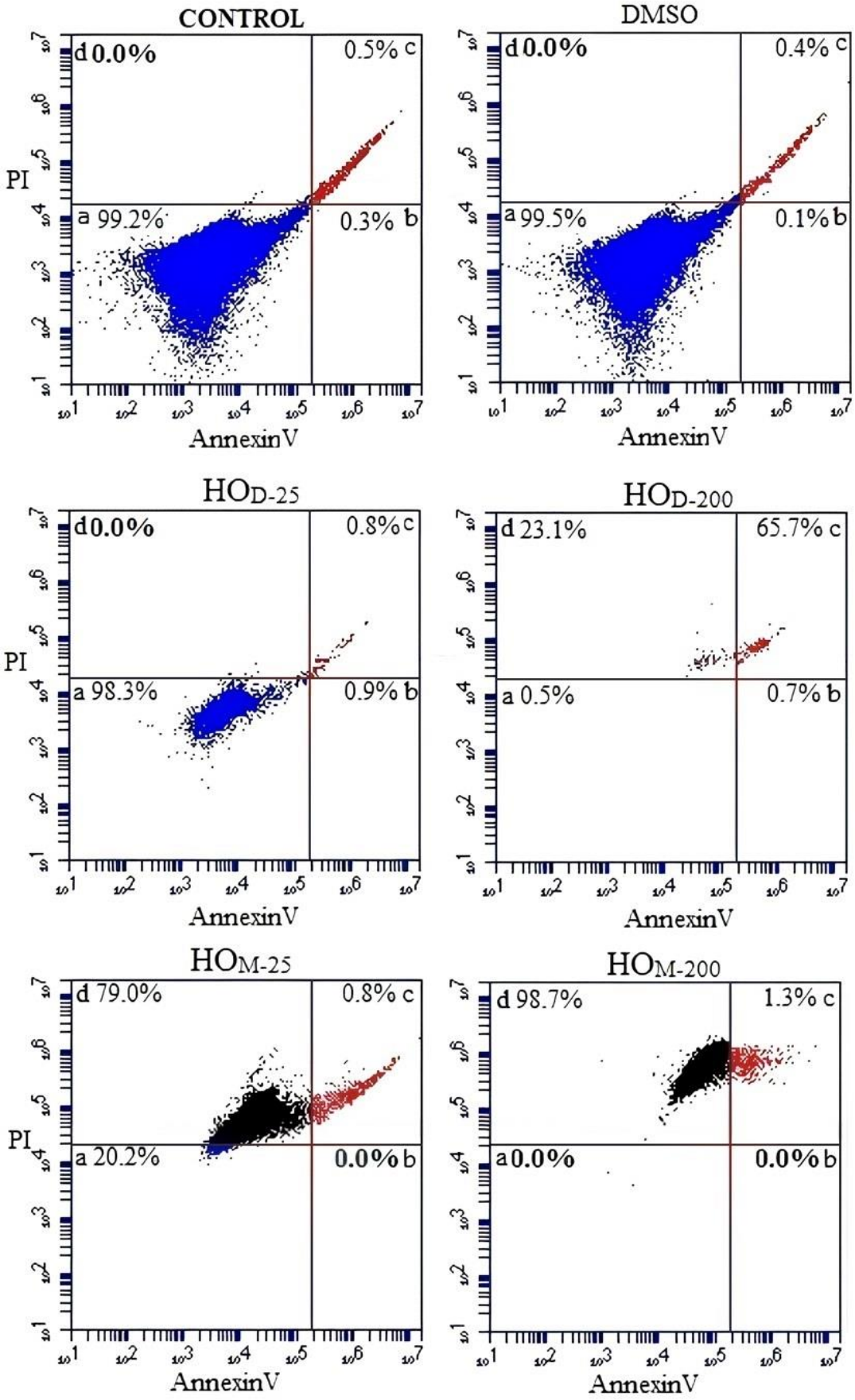


Figure 2a. Percentages of the apoptotic and non-apoptotic A549 cells after HO_D and HO_M treatment. HO_D: Dichloromethane extract of H. *olympicum*, HO_M: Methanol extract of H. *olympicum*, DMSO: dimethyl sulfoxide.

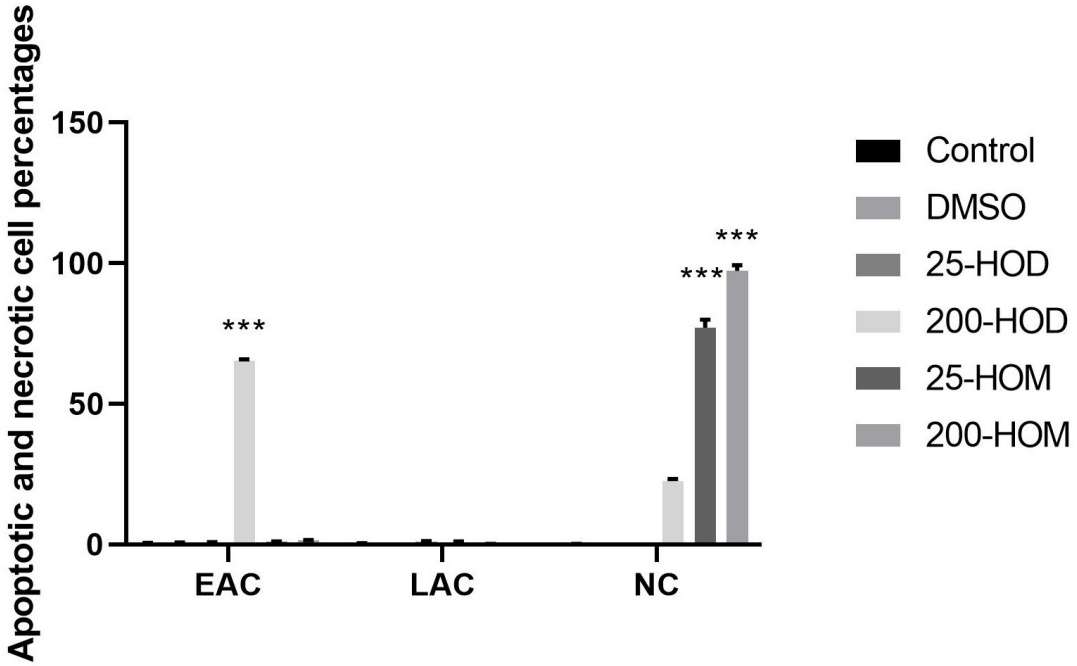


Figure 2b. Statistical evaluation of percentages of the apoptotic and non-apoptotic A549 cells. HO_D: Dichloromethane extract of H. *olympicum*, HO_M: Methanol extract of H. *olympicum*, DMSO: dimethyl sulfoxide, EAC: Early apoptotic cells , LAC: Late apoptotic cells , NC: Non apoptotic cells.

Determination of mRNA levels of BAX and BCL-2 in A549 cells

Proapoptotic BAX and antiapoptotic BCL-2 mRNA expressions were determined in A549 cells after treatment of HO_D and HO_M, and the results were shown in Figure 3. As shown in Figure 3, BAX expression increased approximately 4.53 fold at 200 µg/ml HO_D concentration compared to the control ($p<0.05$). Furthermore, BCL-2 level increased approximately 7.12-folt at 200 µg/ml HO_M concentration ($p<0.05$), while BAX mRNA level did not change.

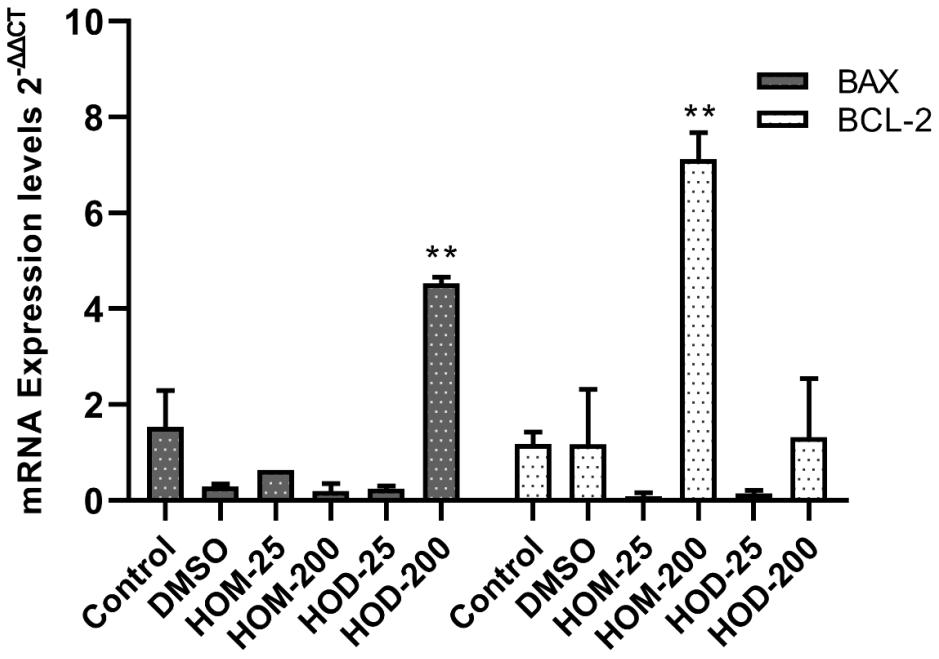


Figure 3. BAX and BCL2 expressions in A549 cells after treatment of HO_D and HO_M. Statistical analyzes were performed using the One-way Anova Dunnett's test. C: Control, HO_D: Dichloromethane extract of H. *olympicum*, HO_M: Methanol extract of H. *olympicum*, DMSO: dimethyl sulfoxide, BAX: BCL2 Associated X, BCL2: B-Cell Lymphoma 2 "*" and "***" mean $p<0.05$ and $p<0.001$, respectively.

Effects of HO_D and HO_M on IL6 and IL8 levels in A549 cells

After A549 cells were treated with HO_D and HO_M for 24 hours, the culture medium was collected, and IL6 and IL8 levels were measured from the supernatants. As shown in Figure 4, IL6 level did not change significantly at concentrations of 25 and 50 µg/ml of HO_D, but decreased at 100 and 200 µg/mL, with only the decrease at 200 µg/mL being statistically significant ($p < 0.05$). IL-8 levels, also presented in Figure 4, were not affected by any concentration of HO_D. In contrast, IL-8 levels increased at 25, 50, and 100 µg/mL concentrations of HO_M, while the 200 µg/mL concentration of HO_M caused a strong decrease in IL-8 levels.

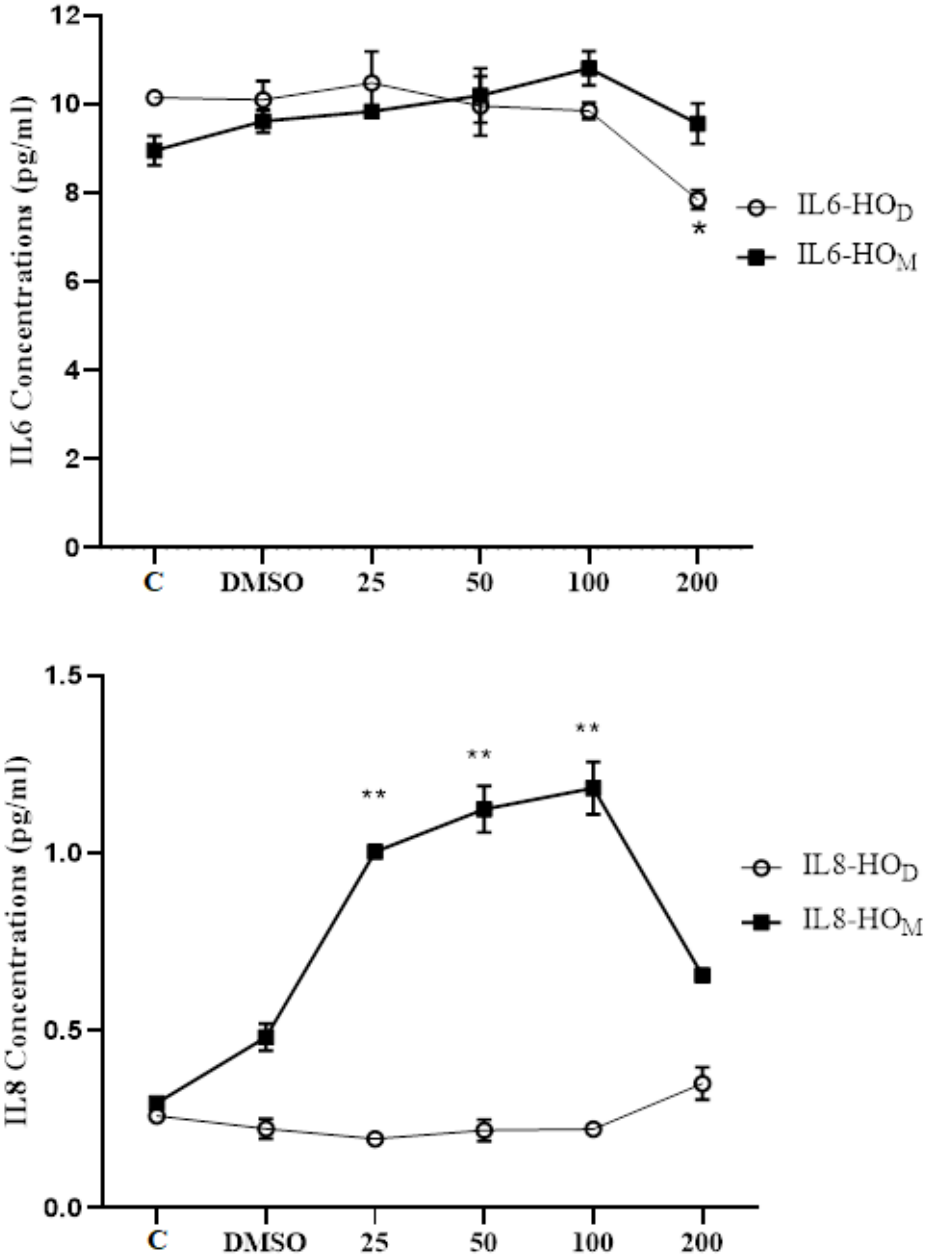


Figure 4. Change of IL6 and IL8 levels in A549 cells after HO_D and HO_M treatment. Statistical analyzes were performed using the One-way Anova Dunnett's test. C: Control, HO_D: Dichloromethane extract of H. *olympicum*, HO_M: Methanol extract of H. *olympicum*, DMSO: dimethyl sulfoxide, "**" mean $p<0.01$.

Determination of levels of pro-caspase 3

Figure 5 illustrates the change in pro-caspase 3 levels in A549 cells after exposure to various concentrations of HO_D and HO_M for 24 hours. As shown in Figure 5, pro-caspase-3 levels in A549 cells decreased at all concentrations of HO_D, with the most significant reductions observed at 100 and 200 µg/mL ($p < 0.001$). On the other hand, none of HO_M concentrations caused a significant change in pro-caspase-3 levels ($p>0.05$).

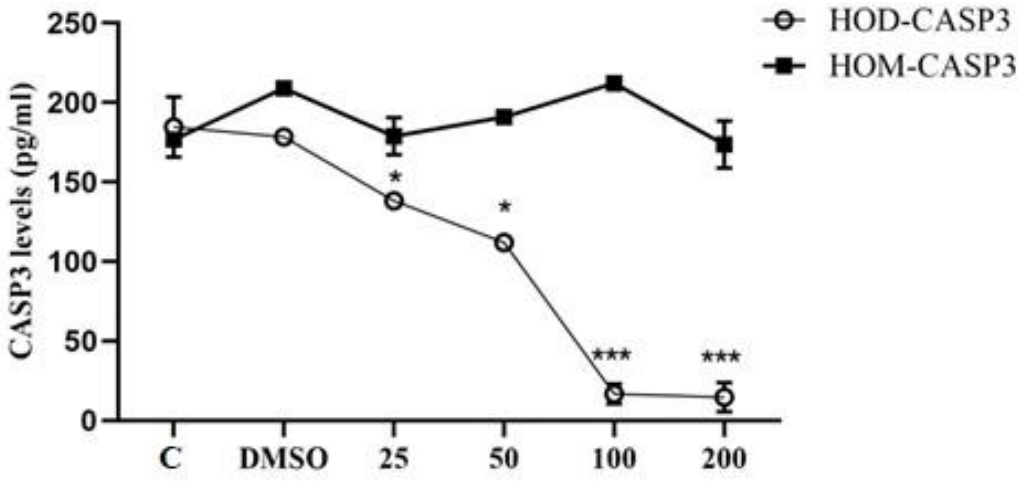


Figure 5. Change of pro-caspase 3 level in A549 cells after treatment of HO_D and HO_M. HO_D: Dichloromethane extract of H. *olympicum*, HO_M: Methanol extract of H. *olympicum*, C: Control, DMSO: dimethyl sulfoxide, CASP3: Caspase 3

Determination of phenolic acid and flavonoids of HO_D and HO_M by LC-MS-MS

The phenolic acid and flavonoids of HO_D and HO_M was screened for 22 compounds by LC-MS-MS and the results were given in Table 1. As a result of LC-MS-MS analysis, while 15 compounds in HO_M were found, only five phenolic compounds were determined in HO_D. As a result of the screening performed by LC-MS-MS, phenolic acids ferulic acid and protocatechuic acid were determined in HO_D, while flavonoids myricetin and phenolic acids fumaric, gallic and protocatechuic acid were the most abundant phenolic compounds in HO_M.

Table 1. Phenolic compounds of HO_D and HO_M by LC-MS-MS

No	Compounds	HO_M (µg/mL)	HO_D (µg/mL)
1	Acetohydroxamic Acid	3.37	2.01
2	Catechinhyrate	nd.	nd.
3	Vanilic Acid	12.79	nd.
4	Syringic Acid	1.52	nd.
7	Myricetin	3.043.77	3.456
8	Kaempferol	34.624	nd.
9	Fumaric Acid	126.206	10.88
10	Gallic Acid	176.72	nd.
11	Protocatechuic Acid	180.19	0.87
12	4-Hydroxybenzoic Acid	13.47	nd.
13	Caffeic Acid	37.43	nd.
14	Salicylic Acid	0.64	nd.
15	Phloridzindyhrate	124.55	2.37
16	2-Hydoxycinamic Acid	nd.	nd.
17	Oleuropein	nd.	nd.
18	2-hyroxy1,4 naphthaquinone	nd.	nd.
19	Naringenin	nd.	nd.
20	Silymarin	0.34	nd.
21	Quercetin	93.77	nd.
22	Luteolin	17.66	nd.
23	Alizarin	nd.	nd.
24	Curmin	nd.	nd.

Nd: not determined.

DISCUSSION

Medicinal plants have been used since ancient times for the treatment of various diseases. Moreover, the extracts and compounds derived from certain medicinal plants have been reported to exhibit anticancer activity in previous studies [19, 22, 23]. These natural products may influence multiple anticancer mechanisms, including suppression of cancer cell viability [23], induction of cell death pathways such as apoptosis and necrosis [22], cell cycle arrest [24], inhibition of migration and invasion [25], modulation of multidrug resistance [26] and suppression of epithelial-mesenchymal transition [27]. The ability of extracts containing diverse phytocompounds to target several of these mechanisms simultaneously may result in stronger anticancer effects compared to single isolated compounds [28]. Additionally, cytotoxic studies on *H. olympicum* have generally focused on the methanol extract. Sequential extractions with solvents of different polarity such as dichloromethane and methanol, may yield fractions containing distinct compounds. This approach can help clarify the relationship between the observed anticancer activity and the specific extract fraction. Therefore, this study investigated the potential anticancer effects of HO_D and HO_M, including suppression of cell viability, induction of apoptosis, and inhibition of proinflammatory cytokines. The cytotoxic effects of HO_D and HO_M on viability of lung cancer cells was evaluated using the MTT assay. It was determined that HO_D extract exhibited cytotoxic activity a concentration-dependent manner on A549 cells, while HO_M reduced only at higher concentrations. Previous studies have reported the cytotoxic activities of *Hypericum* species against several cancer cell lines, including lung, prostate, breast, liver, glioma. It has reported that methanol extracts of *H. adenotrichum* and *H. olympicum* had cytotoxic effects on A549 and PC3 (prostate cancer) cells in a dose-dependent manner [20]. Additionally, *H. olympicum* subsp. *olympicum* has demonstrated strong cytotoxic effects on breast cancer cell lines MCF7 and MDA-MB-231 [29]. Methanol extracts of *H. olympicum* and *H. adenotrichum* also reduced viability in human Hep3B (heap tocarcinoma) and C6 (rat glioma) cell lines [21]. Considering these previous findings alongside our study results, it can be concluded that both HO_D and HO_M possess in vitro cytotoxic activity against lung cancer cells.

In addition to cytotoxic effect, identifying the cell death pathway responsible for this effect is important for determining anticancer activity. Generally, one of the most desired outcomes in suppressing of cancer cells in vitro is the induction of apoptosis. In order to determine the relationship between cytotoxic effect and apoptosis induction, Real Time PCR was performed to evaluate the mRNA levels of BAX and BCL2. Treatment of cancer cells with HO_D at a concentration of 200 µg/ml caused an increase in level of BAX, while HO_M at a concentration of 200 µg/ml had no effect in BAX expression, while HO_M at the same concentration had no effect on BAX levels. Conversely, BCL2 expression significantly increased after HO_M treatment but remained unchanged following HO_D treatment. Next, the effects of HO_D and HO_M on induction of apoptosis in lung cancer cells were evaluated using AnnexinV/PI staining. Phosphotidylserine is normally located in the inner layer of the cell membrane, but during apoptosis, it translocates to the outer leaflet, serving as an “eat me” signal for phagocytes [30, 31]. The phosphotidylserine in the outer layer of cell membrane could be stained with AnnexinV, allowing the percentage of apoptotic cells to be determined. Following AnnexinV/PI staining of A549 cells treated with the lowest and highest HO_D concentrations, a high percentage of lung cancer cells were found to be in the late apoptotic stage. When the BAX and BCL-2 mRNA expression levels were evaluated alongside the Annexin V/PI staining results, consistent evidence for apoptosis induction was observed. The increase in BAX mRNA levels, together with the elevated percentage of cells in the late apoptotic phase, suggests that HO_D has apoptosis-inducing activity. On the other hand, an increase in the percentage of non-apoptotic dead A549 cells was observed following HO_M treatment. There are a few reports on the cell death pathways activated by *Hypericum* species in cancer cells, and one of them is the necrotic pathway [21]. Previous studies have demonstrated that the methanol extract of *H. olympicum* reduced the viability of Hep3B and C6 cell lines, and this decrease attributed to the necrotic pathway rather than apoptosis [21]. Furthermore, the level of caspase 3 was determined as an indicator of apoptosis. Activation of caspase-cascade is a key step in the execution of apoptosis. Caspase 3, which is generated through the cleavage of procaspase3, functions as an effector caspase and plays a central role in both the intrinsic and extrinsic apoptosis pathways [32]. The present study showed that level of pro-caspase 3 decreased by HO_D treatment, whereas no change was observed with HO_M. Considering the reduction in cell viability, the increase in the percentage of cells in late apoptotic phase, the up-regulation of BAX mRNA level, and the decrease in pro-caspase-3 level, it can be concluded that HO_D exhibits its the anticancer activity on lung cancer cells by activating the apoptotic pathway. However, previous studies have reported no effect of HO_M on caspase 3 levels in various cancer cells such as breast and lung [20, 29]. Therefore, the unchanged levels of BAX and caspase 3, along with the increase in non-apoptotic cell death suggest that HO_M may triggers a death pathway other than apoptosis such as necrosis in lung cancer cells. However, a limitation of our study is that necrotic markers such as RIPK1**,** RIPK3 (Receptor-interacting serine/threonine-protein kinases 1 and 3), and MLKL

(Mixed Lineage Kinase Domain-Like protein) were not evaluated. Thus, it is recommended that future studies investigate the mRNA and protein expression levels of these necrotic markers to allow a clearer interpretation of the underlying cell death mechanisms.

It has been reported the up-regulation of IL6 and IL8 enhances proliferation, invasion and metastasis in many cancer cells [33-35], and also contributes to the suppression of the apoptosis process [36]. In this study, HO_D treatment led to a decrease in IL6 level, while IL8 levels remained unchanged. The IL6 promotes cancer cells proliferation and inhibits apoptosis by activating the STAT3 (Signal transducer and activator of transcription 3) signaling pathway, which frequently activated in cancer pathogenesis [37, 38]. Therefore, the reduction in IL6 levels following HO_D treatment is thought to contribute to decreased cell viability by promoting apoptosis in lung cancer cells. In contrast, no significant changes were observed in IL6 or IL8 levels after HO_M treatment.

In previous studies, the phytochemicals present in HO_D and HO_M have been identified using various methods, with hypericin being the most well-known among them [39]. In addition, (E)-anethole and β-farnesene were identified as the main compounds of *H. olympicum* essential oil using Gas Chromatography-Mass Spectrometry (GC-MS) [40]. Additionaly, Ilieva and coauthors [41] reported that the presence of olympiforin A and olympiforin B in *H. olympicum*. In a previous study, rutin, hyperoside, isoquercitrin and chlorogenic acid were determined as the major phenolic compounds in *H. olympicum* using HPTLC [42]. Ferulic acid and protocatechuic acid were the most abundant phenolic compounds in HO_D, while fumaric acid, gallic acid, protocatechuic acid, and myricetin were predominant in HO_M. Additionally, kaempferol, luteolin, and quercetin were also detected in HO_M. Saddiqe Ilieva and coauthors (2011) determined various amounts of quercetin, myricetin, luteolin, apigenin, rhamnetin, isorhamnetin and kaempferol in HPLC analysis of polar extracts of *H. androsaemum, H. ericoides, H. calycinum*, *H. patulum* and *H. olympicum* species. Moreover, the high amounts of rhamnetin and isorhamnetin were determined in *H. olympicum*, but myricetin was not detected [43]. In our study, the lack of apoptosis induction activity in HO_M, contrasted with the clear apoptosis induction by HO_D suggests that the apoptosis inducing effect of *H. olympicum* may not be related to the phenolic compounds identified.

CONCLUSION

This study aimed to determine the potential cytotoxic effects, apoptosis-inducing activity, and pro-inflammatory cytokine suppression of HO_D and HO_M extracts—with differing polarities—on A549 lung cancer cells, as well as to analyze their phenolic compositions. Based on the overall findings, it is suggested that both HO_D and HO_M exhibit cytotoxic effects on lung cancer cells, with the effect of HO_D likely associated with apoptosis and that of HO_M potentially linked to necrosis. Although HO_M contained a greater variety and quantity of phenolic compounds compared to HO_D, HO_D demonstrated stronger apoptotic activity. This suggests that the phenolic compounds identified may not be responsible for the apoptosis-related anticancer activity of *H. olympicum*.

Lastly, limitations of our study are that protein levels of apoptotic markers and necrotic markers such as RIPK1, RIPK3 and MLKL1 were not determined. Future studies should investigate the mRNA and protein levels of these markers to better understand the necrotic cell death observed following HO_M treatment in lung cancer cells. In addition, a single type of lung cancer cells was used in our study. It is recommended to perform apoptotic and necrotic cell death experiments with different lung cancer cells.

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