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ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATION OF CYTOTOXIC AND APOPTOTIC EFFECTS OF *PRANGOS HEYNIAE* H. DUMAN & M. F. WATSON EXTRACTS ON HEPG2 CELLS

PRANGOS HEYNIAE H. DUMAN & M. F. WATSON EKSTRELERİNİN HEPG2 HÜCRELERİNDEKİ SİTOTOKSİK VE APOPTOTİK ETKİLERİNİN ARAŞTIRILMASI

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ABSTRACT

Objective: *This study aims to investigate the anticancer potential of Prangos Heyniae H. Duman & M. F. Watson root extracts against human hepatoma cells, and examine the molecular mechanisms potentially involved in extract-induced cytotoxicity.*
Material and Method: *HepG2 cells were treated with chloroform, n-hexane, or methanol extracts from roots of P. heyniae to investigate the possible effects on cell viability. Following the determination of IC_{50} values by the MTT test, n-hexane, and methanol extracts were excluded because of their selectivity indices. The chemical characterization of chloroform extract was performed by HPLC to understand the chemical composition-bioactivity relationship. Alterations induced by chloroform extract on mitochondrial membrane potential and caspase-3 activation were further investigated. In addition, cell viability was measured in the presence of different selective inhibitors of pathways to define the type of cell death pathway contributing to cytotoxicity.*
Result and Discussion: *Chloroform extract but not n-hexane or methanol extracts led to strong and selective inhibition of cell viability on HepG2 cells. In addition, cytotoxicity increased by chloroform extract was only restored in the presence of a pan-caspase apoptosis inhibitor. Also, treatment of HepG2 cells with chloroform extract impaired mitochondrial membrane potential and led to significant caspase-3 activation. Oxypeucedanin, isoimperatorin, and osthole were detected as the major components of the chloroform extract. These results represent that apoptosis may be involved in the anticancer effect of coumarin and furanocoumarin derivatives in chloroform extract.*
Keywords: *Anticancer effect, apoptosis, liver cancer, Prangos heyniae*

ÖZ

Amaç: *Bu çalışmanın amacı; Prangos Heyniae H. Duman & M. F. Watson kök ekstrelerinin insan*

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karaciğer kanseri hücrelerindeki antikanser potansiyellerini araştırmak ve ekstre ile indüklenen sitotoksisitede rol oynayan moleküler mekanizmaları değerlendirmektir.
Gereç ve Yöntem: *P. heyniae kök ekstrelerin HepG2 hücrelerinin canlılığına olan olası etkilerini araştırmak amacıyla hücreler kloroform, n-hekzan ya da metanol ekstreleri ile inkübe edildi. MTT testi ile IC_{50} değerlerinin belirlenmesi sonrası, selektivite indeksleri nedeniyle n-hekzan ve metanol ekstreleri ile çalışmaya devam edilmedi. Yapı-biyolojik aktivite ilişkisini kurabilmek amacıyla kloroform ekstresinin kimyasal karakterizasyonu HPLC analizi ile gerçekleştirildi. Kloroform ekstresinin mitokondriyal membran potansiyeli ve kaspaz-3 aktivasyonu üzerindeki etkileri ileri deneylerle araştırıldı. Ayrıca, sitotoksisitede rol oynayan hücre ölüm yolunun belirlenmesi amacıyla selektif inhibitörler varlığında hücre canlılığı ölçüldü.*
Sonuç ve Tartışma: *Kloroform ekstresi, HepG2 hücrelerinde canlılığın güçlü ve selektif inhibisyonuna neden oldu. Benzer sitotoksik etki n-hekzan ya da metanol ekstreleri ile saptanmadı. Kloroform ekstresi tarafından indüklenen sitotoksisite pan-kaspaz apoptoz inhibitörü varlığında önlendi. Ayrıca, HepG2 hücrelerinin kloroform ekstresi ile inkübasyonu, mitokondriyal membran potansiyelinde hasara ve kaspaz-3 aktivasyonuna neden oldu. Kloroform ekstresinin ana bileşenleri olarak oxypeucedanin, isoimperatorin ve osthole tespit edildi. Bu sonuçlar, kloroform ekstresindeki kumarin ve furanokumarin türevlerinin antikanser etki mekanizmasında apoptozun rol oynayabileceğini göstermektedir.*
Anahtar Kelimeler: *Antikanser etki, apoptoz, karaciğer kanseri, Prangos heyniae*

INTRODUCTION

Hepatocellular carcinoma is one of the most serious primary malignant tumors [1]. The limitations of current therapeutic approaches highlight the necessity of discovering more effective, selective, and less toxic novel drugs for cancer therapy. In recent years, the investigation of new and effective therapeutic strategies, including detecting molecular targets and searching for anticancer drugs, is one of the most essential types of research areas [2]. Based on this strategy, natural compounds are seemingly promising remarkable anticancer drug candidates. Depending on their various chemical structures, natural compounds may exert cytotoxic and apoptotic activities on different cancer cell lines through various molecular mechanisms. Numerous studies investigating natural compounds and the molecular mechanisms of their anticancer effects contribute to understanding carcinogenesis pathways and provide less toxic, more effective treatment protocols [3]. Especially, the secondary metabolites were isolated from the plant extracts, and further studies were carried out to identify novel therapeutic agents for cancer therapy [4].

The genus *Prangos* Lindl., a significant genus of the family Apiaceae, is widely used traditionally for various diseases, including hemorrhoids, intestinal diseases, dyspepsia, diabetes, and hypertension. The distribution of *Prangos* species has been reported from Europe to Tibet, mainly located in Iran and Türkiye [5-7]. Coumarin glycosides, simple phenolics, flavonoids, terpenic compounds, γ-pyron derivatives, fatty acids, phytosterols, and essential oils were isolated from *Prangos* species [7], and several pharmacological activities of *Prangos* species including antidiabetic [8], anticholinesterase, antityrosinase [9], anti-inflammatory and anti-microbial [10] activities were evaluated via especially *in vitro* assessments.

In recent years, the cytotoxicity potential of *Prangos* species is gaining more attention owing to their marker compounds, coumarin derivatives. The cytotoxic and cytostatic activities of *P. asperula, P. uloptera*, *P. turcica*, and *P. ferulacea* extracts containing coumarin derivatives were demonstrated in Vero [11], Hela [12], PC-3 [13], and HT29 [14] cell models, respectively. Thus, it is valuable to investigate and reveal the cytotoxicity and anti-proliferative profile of other species of the genus.

Prangos heyniae H. Duman & M. F. Watson is an endemic plant of Türkiye, and the investigations on the anticancer potential of this species are extremely limited. The biological activities of *P. heyniae,* including antityrosinase, anticholinesterase, antioxidant, and cytotoxic activities, were reported previously [9,15,16], however, the cytotoxicity against hepatocellular carcinoma and the response of liver cancer cells to different extracts of this plant have not been studied yet. In our previous study, the cytotoxicity of the extracts of the plant and the isolated coumarin derivatives on different cells were evaluated [16]. However, molecular mechanisms of observed cytotoxic effects and anticancer

pathways induced by *P. heyniae* root extracts have not been studied yet. For that reason, the present study aimed to discover the cytotoxic and apoptotic potential of different extracts prepared from *P. heyniae* H. Duman & M.F. Watson on HepG2 cells. Moreover, we aimed to clarify the chemical characterization of the extract in order to establish a correlation between the chemical composition and its pro-apoptotic activities.

MATERIAL AND METHOD

Preparation of Root Extracts

The plant roots were dug up from Hadim-Korualan road, roadside, Konya province, Türkiye, in June 2016. The roots of *Prangos heyniae* H. Duman & M. F. Watson were authenticated and deposited at the Herbarium of Ege University, Faculty of Pharmacy. The plant material was air-dried and grinded before the extraction process. The extracts were prepared with one of the three solvents; *n*-hexane, chloroform ($CHCl_3$), or methanol. The process was repeated three times at room temperature. After filtration, the yielded extracts were prepared for bioactivity studies. The preparation of extracts and detailed description of methods were mentioned in our previous study [16].

Cell Culture and Chemicals

Human liver cancer cell line HepG2 (ATCC, HB-8065, USA) and mouse embryonic fibroblast cell line NIH/3T3 (ATCC, CRL-1658, USA) cells were proliferated in DMEM (Thermo Scientific, Waltham, USA) enriched with 10% FBS and 1% pen/strep (Santa Cruz Texas, USA). Cells were kept and grown in the required conditions (37°C, 5% CO_2). JC-1 dye and caspase-3 activity kit, MTT, Q-VD-Oph, and other chemicals were provided by Sigma-Aldrich (Darmstadt, Germany).

MTT Assay

For *in vitro* incubations, 10 mg/ml solutions of extracts were prepared in dimethyl sulfoxide (DMSO). Then, stock solutions were diluted with appropriate amounts of medium to incubate the cells with desired final concentrations (0-600 µg/ml) for 48h. The maximum concentration of DMSO was 1% (v/v). Effects of *n*-hexane, $CHCl_3$, and methanol root extracts on the viability of HepG2 and NIH/3T3 cell lines were evaluated by MTT assay [17].

The cells (6×10^3 cells/well) were exposed to the extracts at increasing concentrations (0-600 µg/ml). The treatment period was 48 hours. Cells were maintained at the required conditions, as mentioned before. Subsequently, MTT solution was added to the wells. Before the measurement, crystals were dissolved using 120µL DMSO. The absorbance values of purple crystals were measured by a microplate reader at 540 nm [17]. Cells exposed to DMSO (1%, v/v), and Triton-X (1%, v/v) were taken as positive and solvent control, respectively [17].

"% cell viability" was determined by calculating the ratio of the average absorbance of the treated cells to that of the solvent control (the cell viability of the solvent control was considered 100%). The cytotoxic concentration values that killed cells by 50% (IC_{50}) were calculated from the response versus concentration curve. In addition, we have determined the IC_{10} and IC_{75} values of the $CHCl_3$ extract. The selectivity index (SI), indicating cytotoxic selectivity, was calculated using the ratio of IC_{50} in NIH/3T3 cells to IC_{50} in HepG2 cells.

Chemical Characterization of the $CHCl_3$ Extract Using HPLC-DAD

$CHCl_3$ was selected for HPLC analysis due to its selective cytotoxic effect. HPLC studies were conducted in order to assess whether the activity of $CHCl_3$ extract depends on the presence of major compounds, including oxypeucedanin (OXY), isoimperatorin (ISO), and osthole (OST), which are the most common bioactive molecules isolated and characterized from *P. heyniae*. HPLC analyses were applied as described previously [18]. In brief, separations of 10 µl of 7.5 ppm $CHCl_3$ extract or 100 ppm standard molecules (OXY, ISO, and OST) were performed by using ACE 5-C18 column (250x4.6 mm; particle size 5 µm) with gradient elution of A (Water, 0.5% acetic acid) and B (Methanol). The running time was 8.35 min, and the flow rate was 1 ml/min in the wavelength range of 200-400 nm [18].

MTT Assay in the Presence of Selective Inhibitors of Different Cell Death Pathways

$CHCl_3$ extract was evaluated for the potential molecular and apoptotic effects through cell death analysis, mitochondrial membrane potential (MMP), and caspase-3 activity.

Selective inhibitors can be used to determine the role of different types of cell death pathways in the anticancer effect of drugs/drug candidates [19]. Therefore, the cell viability was measured in the presence or absence of selective inhibitors of apoptosis, necroptosis, autophagy, or ferroptosis to assess the form of the cell death pathway induced by $CHCl_3$ extract in HepG2 cells. In this experiment, cells ($6x10^3$ cell/well) were pre-incubated with selective inhibitors of apoptosis (Q-VD-Oph; final concentration: 25 µM), necroptosis (necrostatin-1; final concentration: 20 µM), autophagy (chloroquine; final concentration: 12.5 µM), and ferroptosis (ferrostatin-1; final concentration: 2.5 µM) for 1 h [19]. Then, $CHCl_3$ extract was added into wells at IC_{50} concentration. In parallel experiments, cells were treated with the indicated final concentrations of inhibitors without extract to assess their cytotoxic effects. Following the incubation period (48 h), MTT assay was performed, as mentioned previously.

Measurement of MMP

JC-1 was used to determine the effect of $CHCl_3$ extract on MMP in HepG2 cells. Briefly, HepG2 cells were seeded on a black 96-well plate. $CHCl_3$ extract was incubated with cells for 48 h at three concentration levels (IC_{10}, IC_{50}, and IC_{75}). Cells that were incubated with 25 µM rotenone [20] were utilized as a positive control. After the incubation time, cells were exposed to 5 µg/ml dye for 10 min. MMP was measured at ex: 490 nm, em: 520 nm [21].

Measurement of Caspase-3 Activity

In HepG2 cells, caspase-3 activity induced by $CHCl_3$ extract was measured using a commercial kit (Thermo Fisher Scientific, E13183). Cells ($6x10^3$ cells/well) were incubated with $CHCl_3$ extract at various concentrations (IC_{10}, IC_{50}, and IC_{75}). After 48 h, the reaction mix (50 µM) was added into wells. Then, 2mM DEVD-pNA (10 µl, 200µM final concentration) was added to each control and sample well. Absorbance was measured at 405nm by a multi-plate reader. The activity of caspase-3 in each well was presented as the fold of the solvent control group (DMSO %1) [22].

Statistical Analysis

GraphPad Prism® version 8 (GraphPad Software, San Diego California, USA) was assessed for statistical analyses. Experiments were conducted three times, and performed in triplicate. All data were expressed as the mean± standard deviation (SD). Following the evaluation normality of the data by Kolmogorov-Smirnov, statistical differences were determined by one-way ANOVA and then Dunnett's post hoc test. $p < 0.05$ was accepted as statistically significant for differences.

RESULT AND DISCUSSION

The mortality rate of patients diagnosed with hepatocellular carcinoma remains at very high levels due to poor selectivity and adverse effects of chemotherapeutic treatments, or drug resistance [23]. Therefore, numerous studies have been conducted to develop more effective and safe therapeutic strategies for liver cancer treatment [24]. Within the concept of these potential strategies, natural products have also been recommended as valuable anticancer drug candidates because of their anti-proliferative properties [25]. Thus, continuing investigations for anticancer agents from natural sources play a fundamental role in drug discovery [26].

Cytotoxicity by MTT Assay

Isolated compounds from different *Prangos* species, such as *P. turcica, P. ferulacea, and P. pabularia* were shown to exhibit strong anti-proliferative effects on various cancer cells [11-14]. Although studies investigating *P. heyniae* are limited, we previously reported that $CHCl_3$ extract of *P. heyniae* selectively reduced the viability of A549, HK-2, and SH-SY-5Y cells [16]. In that study, isolation and characterization steps were explained in detail; however, the mechanisms of cytotoxic and anti-proliferative activities were not revealed.

In the present study, we assessed the cytotoxic effects of *P. heyniae* root extracts on HepG2 and NIH/3T3 cells by MTT assay (Fig. 1A-F). In accordance with the results, $CHCl_3$ and *n*-hexane extracts were shown to be potent against liver cancer cell viability. IC_{50} values of $CHCl_3$ and *n*-hexane extracts on HepG2 cells were 15.50±1.632 µg/ml (Fig. 1A) and 34.09 ± 1.114 µg/ml (Fig. 1C), respectively. As revealed in Fig. 1D, methanol fraction exhibited weaker cytotoxic activity (IC_{50}=188.3 ± 1.214 µg/ml) against HepG2 cells in contrast to other extracts.

It is also critical whether the extract or compound is selectively toxic to cancer cells [26]. Hence, MTT assay was also performed on NIH/3T3 cells, and selectivity indices were calculated for each extract. As can be seen in Fig. 1B, 1D, and 1E, IC_{50} values of $CHCl_3$, *n*-hexane, and methanol extracts on NIH/3T3 cells were 198.9±1.607, 37.41±1.431, and 180.7±1.479 µg/ml, respectively. $CHCl_3$ extract demonstrated higher selectivity to HepG2 cells than non-malignant cell lines (SI=12.8). Nonetheless, the same selective toxic effect was not found in the results of *n*-hexane (SI=1.05) and methanol (SI=0.95) fractions. Therefore, *n*-hexane and methanol extracts were excluded from further evaluation. The IC_{10} (2.045 µg/ml) and IC_{75} (36.564 µg/ml) values of $CHCl_3$ extract were also calculated for further experiments.

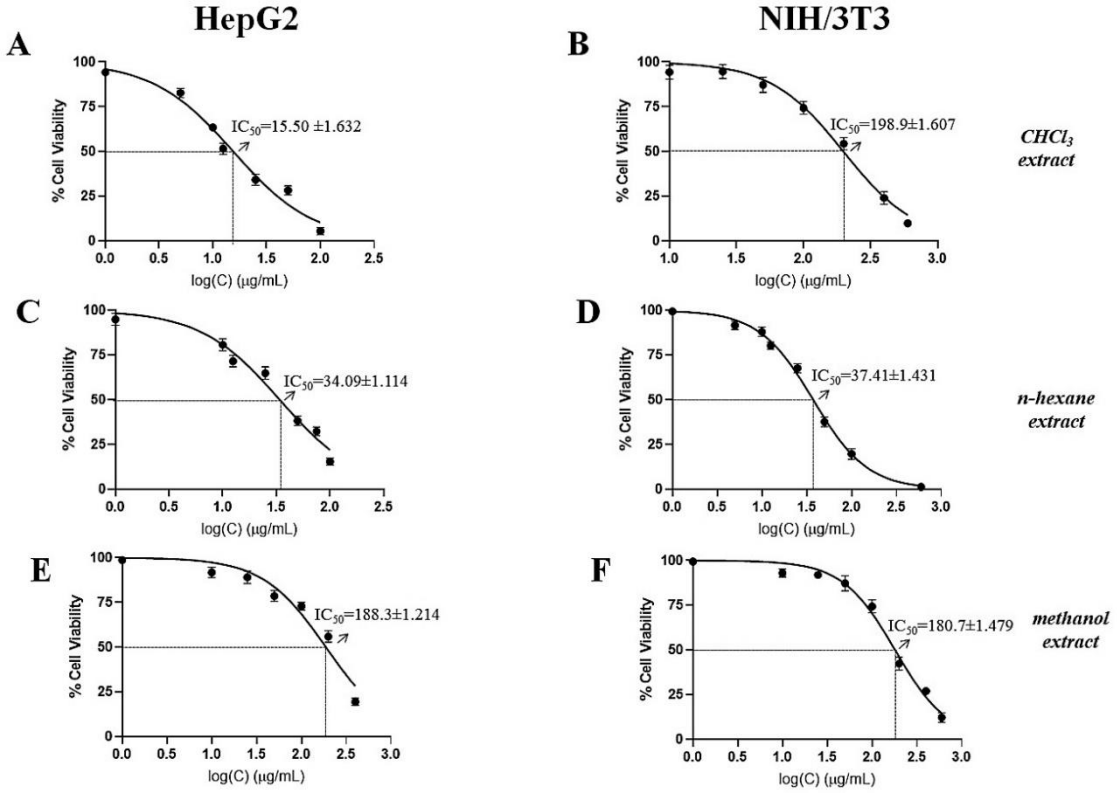

Figure 1. Cytotoxicity of $CHCl_3$, *n*-hexane, and methanol extracts on HepG2 and NIH/3T3 cells for 48 h. "% Cell viability" was calculated as a percentage of solvent control (DMSO-1%, v/v). Graphs represent % cell viability versus log (concentration). Values are represented as mean ± SD. **A,** Cytotoxicity of $CHCl_3$ extract on HepG2 cells; **B,** Cytotoxicity of $CHCl_3$ extract on NIH/3T3 cells, **C,** Cytotoxicity of *n*-hexane extract on HepG2 cells; **D,** Cytotoxicity of *n*-hexane extract on NIH/3T3 cells, **E,** Cytotoxicity of methanol extract on HepG2 cells; **F,** Cytotoxicity of methanol extract on NIH/3T3 cells

HPLC-DAD Analysis of $CHCl_3$ Extract

The chemical characterization of the $CHCl_3$ extract was determined by HPLC-DAD system. OXY, ISO, and OST were chosen as standards in accordance with our previous study [16].

The lower chromatogram indicates the retention times for standard molecules. Retention times for OXY, ISO, and OST at 100 ppm were 19.94, 13.82, and 14.23 min, respectively. The higher chromatogram displays the major peaks of molecules in 7.5 ppm $CHCl_3$ extract. Retention times for major peaks were 10.99, 13.82, and 14.24 min, respectively. A comparison of both chromatograms showed that $CHCl_3$ extract contained all three molecules, and these molecules were the major compounds in the wavelength range of 200-400 nm (Fig. 2).

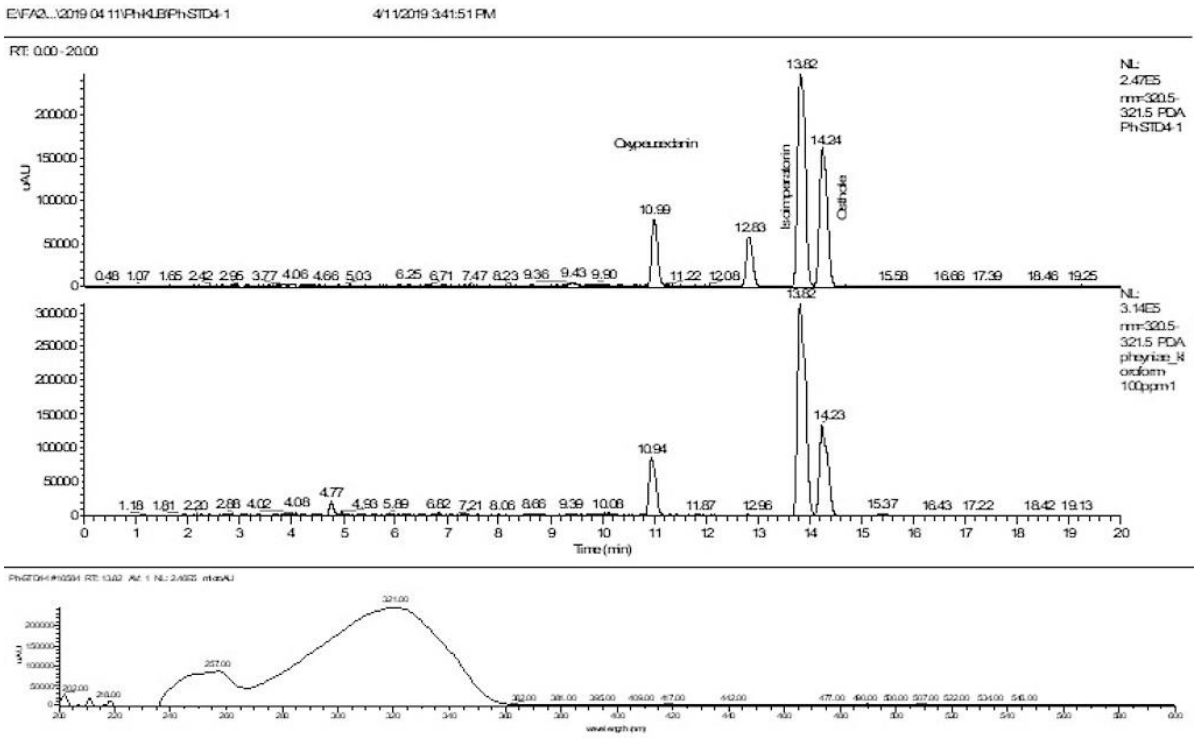

Figure 2. Chemical characterization of $CHCl_3$ extract by HPLC-DAD

Evaluation of Cell Death Pathway Potentially Involved in $CHCl_3$ Extract-Induced Cytotoxicity

According to the MTT assay, $CHCl_3$ extract significantly diminished the number of viable HepG2 cells (Fig. 1A). However, this assay alone is inadequate to reveal data about cell death induction. Therefore, the results need to be supported by additional experiments [19].

Selective inhibitors of apoptosis, necroptosis, ferroptosis, and autophagy were used to assess which mode of cell death pathway was induced by $CHCl_3$ extract [19]. HepG2 cells were treated with $CHCl_3$ extract with or without cell death pathway inhibitors. Additionally, cells were exposed solely to selective inhibitors in order to determine their own cytotoxicity. However, no significant decrease was observed (data not shown).

As seen in Fig. 3, remarkable cell viability recovery was only observed with Q-VD-Oph. Pre-treatment with a pan-caspase inhibitor dramatically reversed the cytotoxic effect of the extract. Cell viability was increased with Q-VD-Oph from 50% to 95%. In contrast, all other selective inhibitors were not able to restore the number of living HepG2 cells. Adding necrostatin-1, ferrostatin-1, or chloroquine did not lead to a significant change in the cytotoxicity (Fig. 3). These results suggest that apoptosis may contribute to cell death induced by $CHCl_3$ extract treatment. However, this conclusion should be supported by apoptosis determination assays or markers.

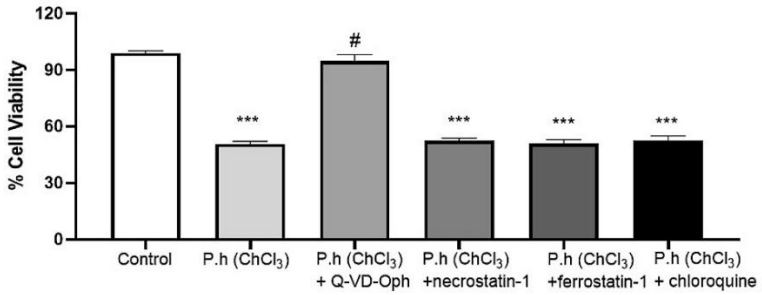

Figure 3. % Cell viability after $CHCl_3$ extract from roots of *P. heyniae* (P. h ($CHCl_3$)) treatment in combination with selective inhibitors of different cell death pathways. HepG2 cells were treated with P. h ($CHCl_3$) at IC_{50} concentration in the presence or absence of inhibitors for 48 h. Q-VD-Oph (25 µM, pan-caspase inhibitor), necrostatin-1 (20 µM, inhibitor of necroptosis), ferrostatin-1 (2.5 µM, inhibitor of ferroptosis), and chloroquine (12.5 µM, inhibitor of autophagy) were used as selective inhibitors. % viability results were normalized to solvent control (DMSO %1). The lines indicate mean ± SD. ***, significantly different ($p < 0.0001$) than control; #, significantly different ($p < 0.0001$) than P. h ($CHCl_3$); cells treated only with $CHCl_3$ extract

Determination of MMP

The intrinsic apoptosis pathway results in oxidative stress, mitochondrial dysfunction, and caspase activation [27]. Hence, MMP is an important aspect of the intrinsic apoptotic pathway [28]. We investigated whether $CHCl_3$ extract impairs the MMP of HepG2 cells and the role of mitochondrial dysfunction in cytotoxicity. Rotenone (positive control) decreased MMP by 63% as expected. IC_{1O} concentration of the $CHCl_3$ extract did not lead to impairment of the mitochondrial membrane. However, the higher doses of the extract significantly decreased the potential compared to the control. MMP was decreased by 16%, and 22% at IC_{5O} and IC_{75} concentrations, respectively (Fig. 4).

We demonstrated that $CHCl_3$ extract seems to impair mitochondrial structure and functions and induces intrinsic apoptosis in HepG2 cells. Previous studies showed that OST led to activation of Bax, and disruption of MMP in different cancer cell lines [29-31]. However, only a few studies have studied the effects of OXY and ISO on MMP. The present study revealed data about the mitochondrial effects of *P. heyniae*. In our further studies, we aimed to conduct incubations with three main compounds of $CHCl_3$ extract to reveal their individual contributions.

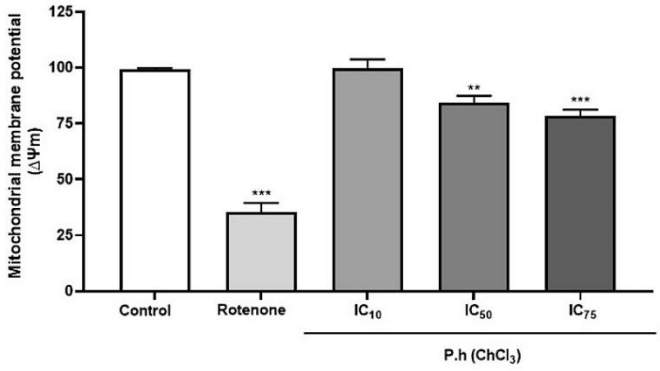

Figure 4. $CHCl_3$ extract decreased MMP in HepG2 cells. After the incubation with increasing doses of extract (IC_{10}: 2.045, IC_{50}: 15.50, IC_{75}: 36.564 mg/ml) for 48 h, the fluorescence of JC-1 dye was directly measured. Rotenone (25 µM) was used as a positive control. Results (mean ± SD) were calculated as the percent of the control signal (DMSO, 1%). ** $P < 0.001$, *** $P < 0.0001$ considered as significantly different from solvent control

Caspase-3 Activity

Caspase-3, the main effector caspase of apoptosis, is an effective marker to assess the mechanism of cell death. The result of significant cytotoxicity induced by $CHCl_3$ extract was prevented in the presence of Q-VD-Oph, which led us to confirm apoptotic death by caspase-3 activity [32]. It is unknown whether caspase-3 activation and apoptosis were induced by *P. heyniae* in HepG2 cells. Our findings indicate that caspase-3 activation was triggered by the treatment of $CHCl_3$ extract in a dose-dependent manner. The signals of control and cells treated with IC_{10} of $CHCl_3$ extract were similar. At the IC_{50} level, there was a significant increase in caspase-3 activity, which was three times higher than the control. This increase was gradually amplified at the IC_{75} level (3.6-fold over control) (Fig. 5).

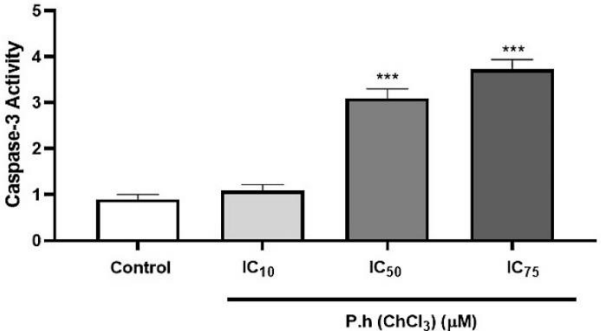

Figure 5. Caspase-3 activity induced $CHCl_3$ extract of *P. heyniae*. Values indicate the fold change relative to the solvent control (DMSO 1%). Lines were expressed as mean ± SD. *** P<0.001 vs. solvent control

Caspase-3 activity is one of the main hallmarks of apoptosis [32]. Previous studies suggested that OXY, OST, and ISO led to anticancer activities and induced apoptosis by activating caspase-3, -8, and -9 proteins in various types of human cancer cells, including DU145 [33], CD133 [34], PC-3, H1299 [35]. In contrast, one study revealed that OXY and ISO decrease caspase-3 activity, increase MMP, and demonstrate a protective effect against doxorubicin-induced apoptosis and neurotoxicity in PC-12 cells [36]. As seen in Fig. 5, $CHCl_3$ extract treatment induced caspase-3 activity in HepG2 cells. This result is consistent with our other findings (Fig 3-5).

The present study assessed the anticancer and apoptotic effects of $CHCl_3$ extract on HepG2 cells. According to our data, $CHCl_3$ extract treatment principally results in intrinsic apoptosis. Nevertheless, this conclusion should be supported by Annexin V, cleavage of PARP1, and other studies. In our laboratory, further mechanistic studies are planned to investigate the mechanism of the cell death pathway.

AUTHOR CONTRIBUTIONS

Concept: E.A.; Design: E.A., G.A., A.E.; Control: E.A., G.A., A.E., E.A., İ.T., Ş.B.; Sources: E.A., G.A., A.E., E.A., İ.T., Ş.B.; Materials: E.A., G.A., A.E., E.A., İ.T., Ş.B.; Data Collection and/or Processing: E.A., G.A., A.E., E.A., İ.T., Ş.B.; Analysis and/or Interpretation: E.A., G.A., A.E., E.A., İ.T., Ş.B.; Literature Review: E.A., G.A., E.A., A.E., İ.T., Ş.B.; Manuscript Writing: E.A., G.A., E.A., A.E., İ.T., Ş.B.; Critical Review: E.A., G.A., A.E., E.A., İ.T., Ş.B.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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