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The Cytotoxicity of the Chloroform and Petroleum Ether Fractional Extracts of *Galium verum* L. in HepG2 and HT29 Cell Lines

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Abstract

Background: Studies on the fractional extracts of *Galium verum* L. have confirmed their cytotoxicity and anticancer effects on various cancer cell lines.
Objectives: The present study aimed to investigate the cytotoxic effects of *Galium verum* extracts on liver and colon cancer cell lines.
Methods: Colon cancer (HT29) and liver (HepG2) cell lines were randomly divided into the test and control groups and exposed to 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL of the extract. MTT assay was used to evaluate the viability of the cells, and the groups were compared in the GraphPad Prism software using Tukey's post-hoc test.
Results: The chloroform fractional extract of *Galium verum* exerted cytotoxic effects on HT29 at the concentrations of 100 and 50 μg/mL and increased the cell viability of HepG2 cancer cells. On the other hand, the fraction of petroleum ether had cytotoxic effects on HT29 at all the determined concentrations, as well as on HepG2 at the concentration of 3.125 μg/mL. In addition, the treatment of HT29 with various concentrations of the petroleum ether fractional extract and HepG2 treatment with the same extract at the concentration of 3.125 μg/mL significantly decreased cell viability compared to the control group. The IC_{50} concentration was determined at > 100 μg/mL.
Conclusions: According to the results, the fractional extract of petroleum ether had cytotoxic effects on HT29 cancer cells at all the concentrations, while it affected HepG2 cancer cells only at the concentration of 3.125 μg/mL.

Keywords: *Galium verum*, MTT Assay, HT29, HepG2, Viability

1. Background

Liver and colon cancers are prevalent cancers and respectively the second and third leading causes of mortality across the world. Approximately 700,000 deaths due to colon cancer were reported in the United States in 2019, while liver cancer led to the death of 30,000 individuals in 2018 (1-6).

Reports have indicated that numerous plants have anticancer effects. *Galium verum* belongs to Rubiaceae family and is also known as 'Lady's Bedstraw' or 'Yellow Bedstraw' (7, 8). *Galium verum* contains anthraquinone compounds, tannins, phenolic compounds, and saponins. In Iran, the genus Galium is represented by almost 50 species, one of which is *Galium verum* (known as Shir Panir in Persian) (9). *Galium verum* is used for various purposes, including kidney and bladder swelling treatment (1), wound healing, psoriasis and rheumatism treatment, and as a sedative. According to the literature, this plant may help the improvement of various cancers, including gastrointestinal, liver, cardiovascular, kidney, and stomach cancer (10-13). In addition, other findings have shown that the methanolic extract of *Galium verum* has cytotoxic effects on several cancers, such as breast, larynx (14), head and neck, liver (14), and blood cancer (14). Several studies have also indicated the toxicity of the methanolic, chloroform, petroleum ether, and aqueous extracts of 25 plants of the Rubiaceae family on colon cancer (HT29) and fibroblast (HSF) cell lines. However, no antiproliferative effects have been observed in some of these plants (5). In another study, the cytotoxic effects of the ethanolic, methanolic, and aqueous extracts of some medicinal plants of the Rubiaceae family were confirmed on colon (HCT116) and liver (HepG2) cancer cell lines (15). In contrast, previous studies have revealed that the aqueous and ethanolic extracts of some members of the Rubiaceae family have no effects on

colon adenocarcinoma (LS/174 T) (16).

Several studies have confirmed the non-toxicity of the ethanolic extract *of Argemone mexicana terminalia bellerica* on the liver cell line, as well as the effects of the methanolic, chloroform, and aqueous extracts of *Dicranopteris linearis* on the colon cancer cell line (HT29) (17). Moreover, it has been reported that *Galium verum* could heal wounds and inflammations in non-cancerous cells. Several studies have also indicated that some medicinal plants (e.g., Rubiaceae family) have significant effects on the proliferation of fibroblasts and wound healing (11).

2. Objectives

Given the contradictory results regarding the effects of *Galium verum* on colon and liver cancer (14-18) and limitations of the previous studies, the present study aimed to investigate the cytotoxicity of two fractional extracts that differed in polarity on liver and colon cancer cell lines.

3. Methods

3.1. Extraction

In this study, *Galium verum* with the herbarium code of 4521 TMRC for the entire plant was collected and identified by Traditional Medicine and Materia Medica Research Center of Shahid Beheshti University of Medical Sciences in Tehran, Iran. After drying and grinding all the plant parts, fractionation was performed using chloroform and/or petroleum ether solvents (Merck, Germany) and the maceration method. Afterwards, two portions of the obtained powder (30 g) were separated and mixed with 300 milliliters of petroleum ether and 300 milliliters of chloroform and placed on a shaker (Faraz Teb Azma Company, COMBI) for 24 hours. Following that, the obtained solutions were filtered and concentrated using a rotary evaporator (Heidolph Company), and the concentrated samples were dried at room temperature.

3.2. Cell Culture and Cell Treatment

Human hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma cell lines (HT29) were purchased from the Iranian Genetic Resource Center and stored frozen in a nitrogen tank. The HepG2 and HT29 cell lines were divided into the control and treatment groups (concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL).

3.3. MTT Assay

In order to perform the MTT assay, 100,000 cells were obtained from the HepG2 and HT29 cancer cells separately and initially cultured in 96-well plates on the DMEM culture medium containing 10% FBS for 24 hours. The treatments were performed with concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL. After 72 hours of exposing the cells to the fractional extract, the supernatant was removed, and 80 microliters of the fresh culture medium and 20 microliters of MTT were added to each well. At the next stage, the plates were incubated for four hours, the culture medium was completely evacuated, and 200 microliters of the DMSO solution was added to each well. After the complete dissolution of the purple formazan crystals by the DMSO solvent, the absorbance rate of the samples was measured at 570 nanometers using the ELISA reader (Viragen Co., Epoch).

3.4. Statistical Analysis

Data analysis was performed using the analysis of variance and Tukey's range test in the GraphPad Prism software version 8 at the significance level of $P \leq 0.05$.

4. Results

As is shown in Figure 1, cell viability increased in the HT29 cancer cells treated with the plant chloroform fractional extract at the concentrations of 25, 12.5, 6.25, and 3.125 μg/mL. In contrast, treatment with the concentrations of 100 and 50 μg/mL significantly decreased cell viability compared to the control group ($IC_{50} > 100$ μg/mL). In the treatment with the petroleum ether fractional extract (100, 50, 12, 25.5, 6.25, and 3.125 μg/mL), cell viability significantly decreased compared to the control group ($IC_{50} > 100$ μg/mL).

Figure 2 depicts the viability of the HepG2 cancer cells treated with the chloroform and petroleum ether fractional extracts. Accordingly, the cell viability increased in the HepG2 cancer cells treated with the chloroform fractional extract at all the selected concentrations compared to the control group. On the other hand, treatment with the petroleum ether fractional extract significantly decreased the cell viability only at the concentration of 3.125 μg/mL.

5. Discussion

According to the results of the present study, the fractional petroleum ether extract of *Galium verum* had cytotoxic effects on HT29 at all the administered doses, while it affected the HepG2 cancer cells only at the concentration of 3.125 μg/mL. Although the chloroform fractional extract

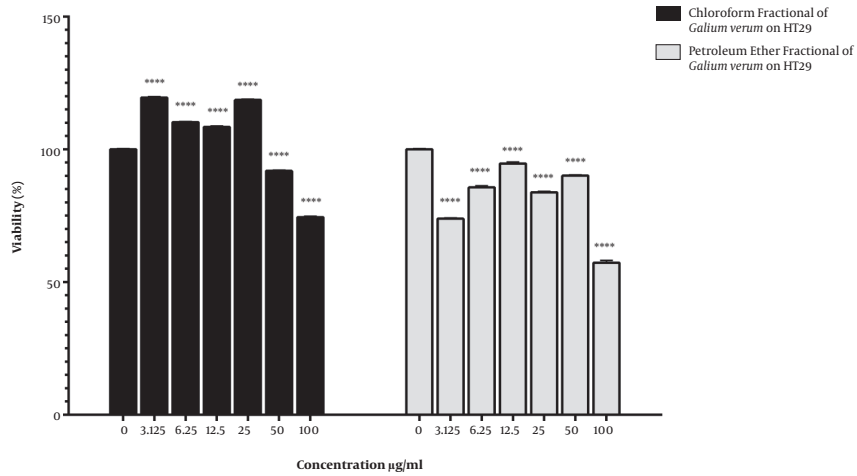

Figure 1. Comparison of Effects of Various Concentrations of Entire Plant Chloroform and Petroleum Ether Fractional Extracts on HT29 Cell Viability over 72 Hours (Results expressed as mean ± SD; ****P < 0.0001 compared to control)

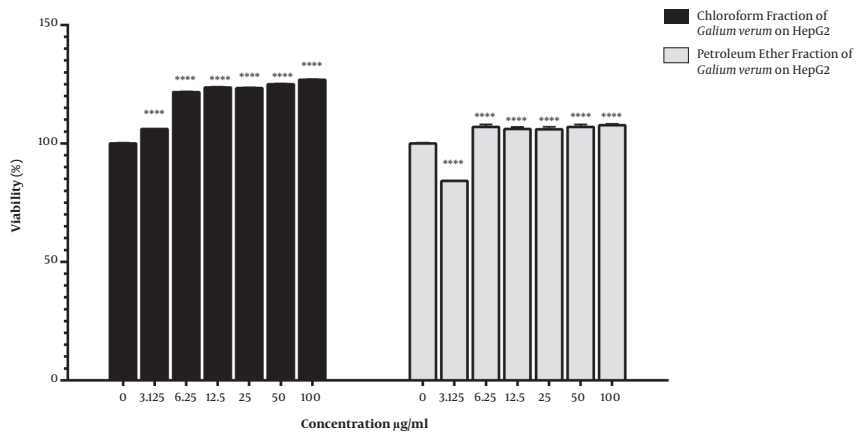

Figure 2. Comparison of Effects of Various Concentrations of Entire Plant Chloroform and Petroleum Ether Fractional Extracts on HepG2 Cell Line Viability over 72 Hours (Results expressed as mean ± SD; ****P < 0.0001 compared to control)

had cytotoxic effects on HT29 at the concentrations of 100 and 50 μg/mL, it increased the cell viability of the HepG2 cancer cells.

In line with our findings, Manosroi et al. investigated the effects of the methanolic, chloroform, petroleum ether, and aqueous extracts of 25 plants of the Rubiaceae family on colon cancer cell lines (HT29) and fibroblasts (HSF) using the MTT assay. According to the obtained results, some of the plants had anticancer effects, while no antiproliferative effects were observed in others. Furthermore, the petroleum ether and chloroform extracts were reported to be the best solvents for extraction (5). In another study, Itharat et al. assessed the effects of the ethanolic and aqueous extracts of 11 medicinal plants belonging to the Rubiaceae family with the concentrations of 100, 50, 25, 10, 1.5, 0.5, and 0.1 μg/mL on colon adenocarcinoma (LS/174 T) using the MTT assay. In the case of most of the plants, the aqueous extract with the concentration of 15.6 μg/mL as the IC_{50} was more toxic compared to the ethanolic extract. However, the aqueous extracts of *Dioscorea birmanica* and *Siphonodon celastrineus* had no effects on the colon cancer cell line (16).

In another research, Talib Hussain evaluated the effects of the ethanolic extract of *Terminalia bellerica* on the liver cancer cell line (Hep-3B), as well as the effects of the methanolic, chloroform, and aqueous extracts of *Dicra-*

nopteris linearis on the colon cancer cell line (HT29) using the MTT assay. The obtained results suggested that the aqueous extracts had no effects on the HT29 and HepG2 cancer cell lines (17).

In the present study, the petroleum ether fractional extract had cytotoxic effects on the HT29 cancer cells at all the determined concentrations, while it affected the HepG2 cancer cells only at the concentration of 3.125 μg/mL. Consistent with this finding, other studies have also indicated that *Galium verum* extracts have cytotoxic effects on several cancers, including breast, larynx (8), head and neck, liver (14), and blood cancer cell lines (18).

Schmidt et al. (14) investigated the cytotoxic effects of the aqueous extract of *Galium verum* at the concentrations of 50 and 100 μg/mL on larynx, liver, and head and neck cancer cells (HLaC78, Hep2, and FADU) using the MTT assay, western blot, and real-time polymerase chain reaction (PCR). According to their findings, the plant could be used as a preventive measure for cancer treatment (8, 10-12, 14). In another study, Zhao et al. examined the cytotoxic effects of the effective substances of Diosmetin from *Galium verum* at the oral doses of 400, 200, and 100 mg/kg on mice with thymic cancer using western blot, MTT assay, and flow cytometry. No mortality was observed in the mice treated with Diosmetin, and Diosmetin was reported to exert protective effects on the thymus against tumor invasion, while reducing the proliferation of the thymic cells, increasing the thymus weight, and inhibiting tumor growth in the treated mice compared to the controls (19).

In another study, Siew et al. assessed the cytotoxic effects of the ethanolic, methanolic, and aqueous extracts of some medicinal plants of the Rubiaceae family on the colon (HCT116) and liver (HepG2) cancer cell lines using the MTT assay. The obtained results indicated that the methanolic extract of the leaves with the IC_{50} concentration of 31.5 μg/mL could significantly decrease cell viability (15). In another study, the effect of the methanolic extract of some medicinal herbs of the Rubiaceae family on HepG2 was investigated using the MTT assay, and the viability of the cancer cells treated with *Smallanthus sonchifolius* leaf at the concentration of 58.2 μg/mL as the IC_{50} was reported to decrease (1).

Chiranjeevi et al. investigated the effects of the ethanolic, ethyl acetate, and petroleum ether extracts of *Lindernia madayiparense* of the Linderniaceae family at the concentrations of 1,000, 500, 250, 125, and 62.5 μg/mL on the HepG2 cell line using the MTT assay, reporting that the petroleum ether extract with the concentration of 149 μg/mL as the IC_{50} exerted the most toxic effect (20). Similarly, Deivayanai et al. evaluated the effects of the ethanolic and chloroform extracts of various plants with the concentrations of 10, 20, 30, 40, and 50 μg/mL on the HepG2 cell line using the MTT assay, reporting the reduction of the viability of the cancer cells treated with both extracts (21).

The results of the present study indicated that the chloroform fractional extract of the entire *Galium verum* plant had no cytotoxic effects on the HepG2 and HT29 cancer cell lines, except at the concentrations of 50 and 100 μg/mL. However, the petroleum ether fractional extract exerted cytotoxic effects on the HT29 cancer cells at all the determined concentrations, while also affecting the HepG2 cancer cells at the concentration of 3.125 μg/mL.

In another research, Buk-Gu Heo examined the effects of the methanolic extract of *Uncaria rhynchophylla* of the Rubiaceae family at the concentration of 500 μg/mL on the HT29 cell line using the MTT assay, reporting that the presence of polyphenols and high biological activity of the extract led to cell growth inhibition in a dependent manner (22). Furthermore, Jaya Kumar investigated the inhibitory effects of the hexane extract of *Morinda pubescens* of the Rubiaceae family at the concentrations of 250, 100, 50, and 25 μg/mL on the HepG2 cell lines using the MTT assay. The results of the mentioned study demonstrated that the hexane extract of the leaves significantly decreased cell viability at all the concentrations (23). The other studies in this regard have also confirmed the effects of the *Galium verum* extract on wound healing and psoriasis (11).

According to the literature, compounds as anthraquinones, flavonoids, triterpenoid saponins, glycoside monoterpenes, erythroids, and phenols contain bioactive components with cytotoxic effects of variable grades (7, 23). The findings of the current research suggested that the petroleum ether extract had more significant cytotoxic effects than the chloroform extract due to the presence of non-polar compounds, such as triterpenoid saponins (24-27).

Further investigation is required in order to evaluate the effects of the fractional extracts of petroleum ether and chloroform of *Galium verum* on the liver cancer cells, especially in the fields of phytochemicals and instrumental analysis. The present study examined the effects of the petroleum ether and chloroform fractional extracts of *Galium verum* on the survival of liver and colon cancer cells in the cell culture medium, and the results indicated that the fractional petroleum ether extract had cytotoxic effects on the HT29 cancer cells at all the administered doses, while it affected the HepG2 cancer cells only at the concentration of 3.125 μg/mL.

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Footnotes

Authors' Contribution: Study concept and design: Z. M., and M. H.; analysis and interpretation of data: S. P., M.H; drafting of the manuscript: S. P.; critical revision of the manuscript for important intellectual content: S. E.; statistical analysis: Z. M.

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