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The Apoptotic Effect of Methanolic Extract of Galium verum on HT29 Cell Line

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Abstract

Galium verum has been reported to have cytotoxic effects, however, the mechanism of Galium verum extract action on cancer cells is unclear in many cases. This study aimed to evaluate the cytotoxic of the whole plant methanolic extract on colon cancer (HT29) cells. colon cancer cells were randomly divided into control group and groups treated with 12.5, 25, 50, 100, 200 1nd 400 μg/mL of the extract for 72h. Plant extract was obtained through maceration. MTT assay was applied to test viability of cells.Apoptosis and cell-cycle, and intracellular levels of ROS were determined by flow cytometry. Bax and Bcl-2 expression levels were evaluated using RT-PCR. Treatment of HT29 cells with 400 μg/mL of whole plant extract resulted in significant decreased viability and increased late apoptosis ($P<0.0001$), and a notable increase in cell number stoped in G0/G1 phase ($P<0.05$) and a notable decrease in cell number in S phase ($P<0.05$), and increased intracellular ROS levels ($P<0.01$). In conclusion, the high concentration of methanolic whole plant extract of Galium verum induces apoptosis through Bax-dependent pathway and increases intracellular levels of ROS in colon cancer (HT29) cells in vitro suggesting that the whole plant extract of Galium verum can play a role in treatment of colon cancer.

Keywords: Galium verum, HT29, Viability, Apoptosis, Cell cycle, ROS.

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1 Introduction

Colon cancer is the most common cause of death in the world and the most malignant disease (Siegel et al., 2020). The mortality rates caused by this disease in the world in 2020 was calculated 881,000 deaths(Siegel et al., 2020, Mattiuzzi; Sanchis-Gomar and Lippi, 2019).Currently the treatment for this tumor is surgery and other treatments are complex and aggressive (Siegel et al., 2020, Arnold et al., 2017).Many studies have been conducted to investigate the inhibitory effects of plants of the Rubiaceae family on cancer cells, however, the findings are still controversial (Schmidt et al., 2014a; Schmidt et al., 2014). Galium verum is applied to cure cancers (Farcas et al., 2018; Bradic; Petkovic and Tomovic, 2018).Many types of research demonstrated that G. verum included flavonoids with inhibitory effects on cancer cells (Schmidt et al., 2014; Schmidt et al., 2014) . Studies have shown that plants in this family can play a role in inducing apoptosis in cancer cells, including colon cancer cells (Atmaca et al., 2016).Although studies have revealed that G. verum extract has anticancer effects on several types of cancer cells similar to Gastrointestinal and reproductive cancers (Schmidt et al. 2014; Schmidt et al. 2014), there are findings showing that the extract of medicinal plants of the Rubiaceae family have no significant cytotoxic effects on colon cancer cells (Itharat et al., 2004).Despite significant findings on the inhibitory effects of G. verum on inhibiting the cancer cells growth and proliferation (Schmidt et al., 2014; Schmidt et al., 2014), the results of some studies in this area are disappointing (Itharat et al., 2004).According to our information, there is no conclusive evidence on anticancer and apoptotic effects of whole plant methanolic extract on HT29 cell line in vitro or in vivo. Therefore, considering that common treatments for colon cancer are very complex and aggressive with serious side effects (Chakrabarti et al., 2021), in this research, we studied the anticancer effects of whole plant methanolic extract of G. verum on HT29 cell line in vitro and evaluating the apoptosis, cell cycle distribution, and intracellular levels of ROS in HT29 cell line.

2 Materials and Methods

Study Area

This study was performed in Tehran Pharmaceutical Sciences Branch, IAU, Iran. This study was approved by the Ethics Committee of IAU (code: IR.IAUU.PS.REC.1399.148). G. verum (herbarium code: 4521) was collected and identified by the botanists of the Shahid Beheshti University of Medical Sciences, Tehran Iran. colon cancer cells were prepared from the Pasteur Institute of Iran.

Extraction

After drying the whole plant, it was powdered. Extraction was performed using methanol solvent and maceration method. 10 g of the plant powder was weighed, and 100 mL of methanol solvent was added and placed on a shaker (Faraz Teb Azuma Company, COMBI) for 24 hours. Then the filtered extract was concentrated by a rotary evaporator (Heidolph) and dried at ambient temperature(Norouzi et al., 2021).

MTT assay test

9×103 HT29 cells were seeded in a 96-well plate with DMEM medium . After 1 one, the cells were treated with 12.5, 25, 50, 100, 200 and 400 μg/ ml of the sample(Pashapour et al., 2021). After 72h incubation, the supernatant was removed and 80 μL was added culture medium.20 μL

of tetrazolium bromide (MTT) 3- (4,5-dimethylthiazol-2-yl) - 2,5-diphenyl solution was added at a concentration of 0.5 mg/mL. After 4 hours, 200 μL of DMSO were added and the absorbance of the sample was determined at 570 nm by ELISA Reader (epoch-BioTek) (Ali et al. 2021; Nandhini, Ezhilarasan and Rajeshkumar 2021; Noroozi, Ahmadi and Pashapour 2020).

Measurement apoptosis

1×105 HT29 cells were seeded in a T25 flask in DMEM medium. HT29 cells were exposed with 400 μg/ml of the extract for 72h and control cells were left untreated. The cells were washed twice with Phosphate Buffered Saline and centrifuged at 1500 rpm for 5 minutes. Annexin-V-FLUOS labeled reagent solution, incubation buffer, and PI were added to the cells and incubated for 0.5 h at room temperature. Finally, 600 to 700 μl of the buffer was added to the cells and measured using a flow cytometery . (BD biosciences, San Jose, CA, USA) 09:22 PM.

Measurement Cell cycle

The cells were trypsinized, and after washing with Phosphate Buffered Saline was fixed with 70% ethanol. Next, after adding PI MASTER MIX to the cells, they were then incubated at an ambient temperature for 0.5h and measured applying a flow cytometery (Samad et al., 2021; Lobo et al., 2021).

Measurement of reactive oxygen species (ROS)

After washing the cells with Phosphate Buffered Saline, 2'-7'dichlorofluorescin diacetate was added to the cells and incubated at 370° C for 45 minutes. After centrifuging, the MFI was tested applying a flow cytometery (Mbaveng et al., 2021; Chhipa, Baksi and Nivsarkar, 2021).

Measurement of expression of BAX and BCL2 genes

After seeding 3×106 HT29 cells in a T75 flask, they were treated with selective concentration of the sample for 3 day. The total RNA (Super RNA extraction kit for tissues and cells was extracted and transcribed into cDNA (cat cDNA synthesis kit) (Pashapour et al., 2021).
Then the primers were designed.A Fluorescent reporter molecule (cyber green) was used to observe the progress of RT-PCR. Gene expression level was evaluated using an AMPILICON master mix by RT-PCR (Step One ABI.The expression level was calculated applying 2-Δ Δ ct method (Kazemi, Soltanzadeh and Shahsavari, 2021; Ebadollahi et al., 2021).

Data analysis

Data analysis was performed with Graph Pad Prism software. Differences between cell viability and apoptosis in groups were tested using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. Independent samples t-test was used to detect differences in gene expression, cell cycle distribution, and ROS production between treated and control groups. All experiments were performed thrice. All data were expressed as the mean ± standard deviation (S.D.). Value differences were considered significant if the P value was <0.05.

3 Results and Discussion

viability of G. verum extract on HT29 cell line

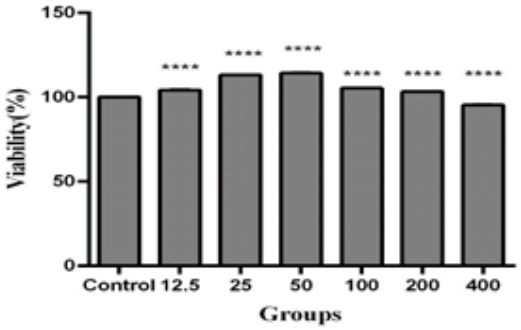

Figure 1. Viability of HT29 cells exposed with 12.5, 25, 50, 100, 200 and 400 μ g/mL of G. verum. * Shows significant difference proportion to control group (* ∗ ∗∗: P<0.0001).

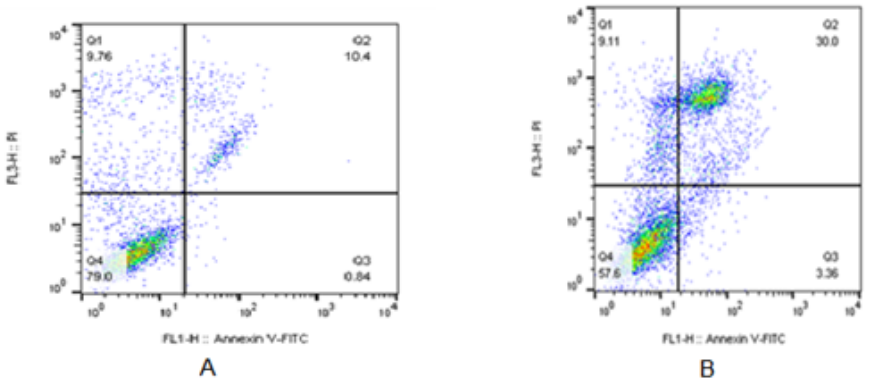

Figure 2. Necrosis, early and late apoptosis in control (A) and HT29 cells treated with G. verum (400 μg/mL) (B): Q1: Necrosis; Q2: Late Apoptosis; Q3: Early Apoptosis; Q4: Viable cells.

The cytotoxic effect of G. verum on HT29 cells was assessed using MTT assay with 3 replications to determine the cell viability. Viability of HT29 cells significantly increased when treated with 12.5, 25, 50, 100 and 200 μg/mL of G. verum compared to control group (P<0.0001); however, treatment of HT29 cells with 400 μg/mL of G. verum resulted in significant decreased cell viability (P<0.0001) (Figure 1).

Apoptosis inducing effect of G. verum on HT29 cells

The flow cytometry results reported no significant increase in the early apoptosis; although, a significant increase was reported in late apoptosis (2.87 times) in HT29 cells treated with selective concentration of G. verum compared to control group (P<0.0001) (Figure 2 and 3).

Effects of G. verum on cell cycle distribution in HT29 cells

The flow cytometry results revealed a significant increase in the number of cells stoped in the G0/G1 phase and a significant decrease in the S phase in HT29 cells treated 400 μg/mL of G. verum extract compared to control group (P<0.05) (Figure 4 and 5).

Effects of G. verum on reactive oxygen species (ROS) production in HT29 cells

A notable increase was shown in the MFI in HT29 cells treated with 400µg/mL of G. verum extract ratio to control group (P<0.01) (Figure 5 and 6).

Effects of G. verum on expression level of BAX and BCL2 in HT29 cells

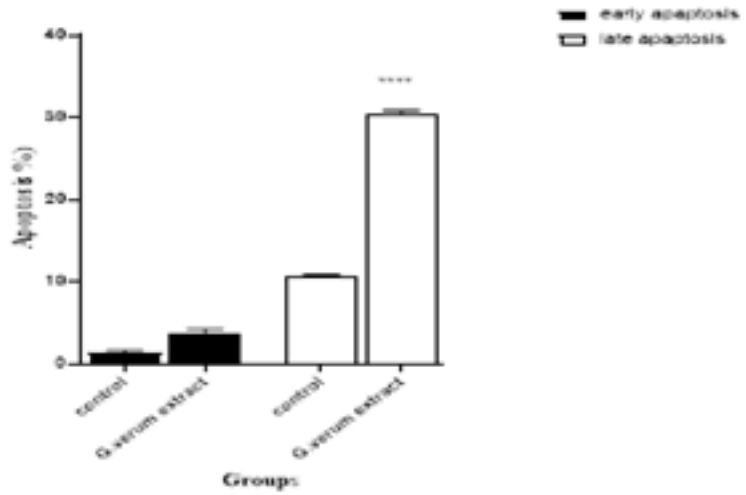

Figure 3. Early and late apoptosis in control and HT29 cells treated with G. verum (400 μg/mL) .* Shows significant difference proportion to control group (****: P<0.0001).

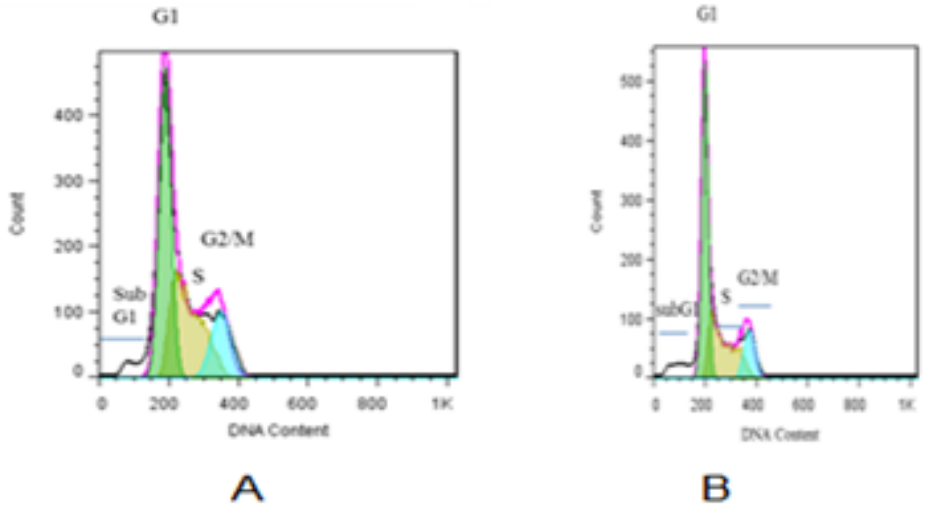

Figure 4. Flow cytometry results showing the cell cycle distribution in control (A) and HT29 cells treated with selective concentration of G. verum (B).

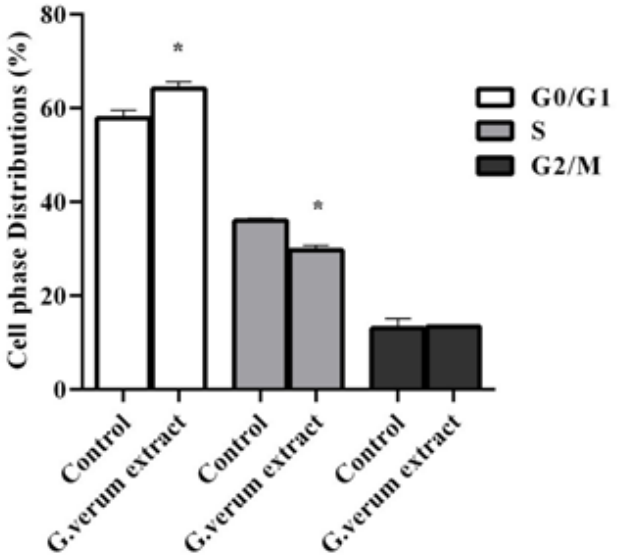

Figure 5. Cell cycle distribution in control cells, and HT29 cells treated with selective concentration of G. verum.* Shows significant difference proportion to control group (*: $P<0.05$).

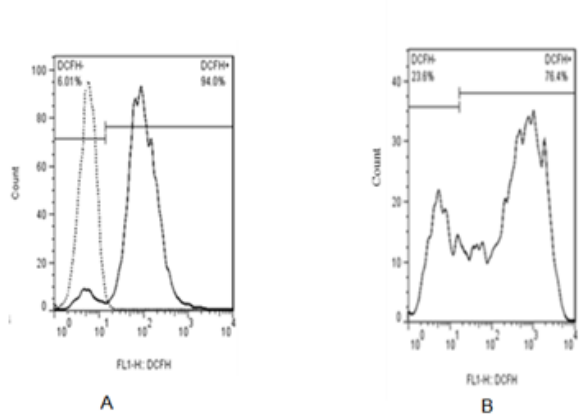

Figure 6. CFlow cytometry results showing the ROS production in control (A) and HT29 cells treated with selective concentration of G. verum extract (B).

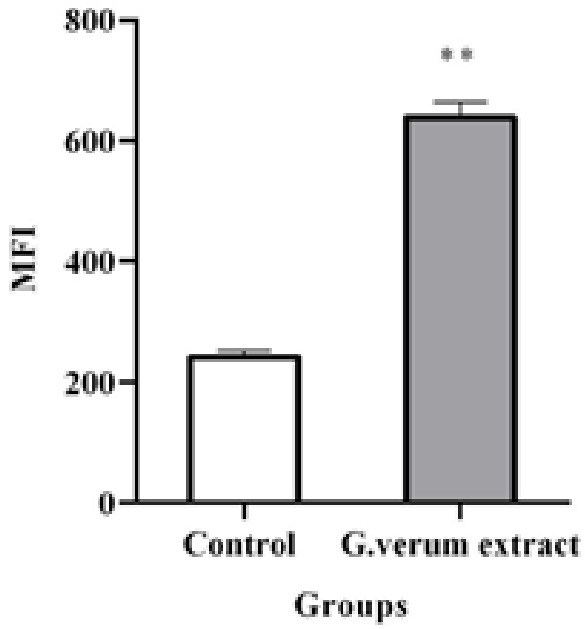

Figure 7. Fold change in BAX and BCL2 genes (indicating the expression level) in control and HT29 cell line treated with selective concentration of G. verum extract. * * Shows significant difference proportion to control group (**:P<0.01) .

There was notable increase in BAX and significant decrease in BCL2 expression level in HT29 cells treated with a selective concentration of G. verum extract. The BAX/BCL2 ratio significantly increased (30 times) in HT29 cells treated with selective concentration of G. verum extract compared to control group (P<0.01) (Figure 7).

Although many types of research have shown that *G. verum* can be used to inhibit cancer cells growth and proliferation (Farcas et al. 2018; Bradic et al., 2018; Mitic et al., 2014), the anticancer effect of G. verum extract, particularly on HT29 cell line, is still one of the most challenging research topics. In this research, we studied the rate of apoptosis of methanolic extract on HT29 cell line to demonstrate whether the methanolic extract can inhibit the proliferation of HT29 cell line at the appropriate time. The results of this study report that the high concentration (and no lower concentrations) of methanolic extract can reduces the viability of HT29 cell line. In consistent with our findings it has been shown that the chloroform and petroleum ether G. verum could decrease the cell viability of colon and HepG2 cell line in vitro (Pashapour et al., 2020). *G. verum* extract also has been reported to increase the expression level of tumor suppressor genes and to decrease the expression level of oncogenes in HepG2, HLaC79, and FADU cell lines(Schmidt et al. 2014; Schmidt et al. 2014).It has been reported that *G. aparin* methanolic extract had cytotoxic effects on breast cancer cells assessed by MTT assay test (Atmaca et al., 2016). In contrast to the findings of this study on the inhibitory effect s of *G.verum* extracts on reducing the proliferation of colon cancer cells, a study has shown that *G. veum* chloroform extract at concentrations of 25, 12.5, 6.25, and 3.125 μg/mL could not reduce the proliferation of cancer cells (Pashapour et al. 2020).

The results of the cell cycle distribution in HT29 treated with *G. veum* methanolic extract revealed that the extract at high concentration had the ability to increase the G0 /G1 phase and to reduce the cells in the S phase. Consistent with this finding, other studies have shown that 250 μg/ mL of methanolic extract of *Galium aparine* from Rubiaceae family could increase the

cell distribution in G0/G1 phase along with a decrease in S1 phase in breast cancer (MCF7) cells (Atmaca et al. 2016).The ethanolic extract of Hedyotis diffusa (from Rubiaceae family) has been reported to induce inhibitory effects on HT29 cell line through cell distribution at the G1 phase (Lin et al., 2015; Lin et al., 2012).

We have shown that high concentration of *G. verum* methanolic extract induces apoptosis through increasing the ROS production and BAX/BCL2 ratio. In consistent with this finding, it has been demonstrated that methanolic extract of *G. Aparin* (from Rubiaceae family) could induce apoptosis in breast cancer cells (Atmaca et al., 2016).Induction of apoptosis with ethanolic extract Hedyotis diffusa Willd (from Rubiaceae family) has shown that the extract induces apoptosis in colon cancer cells by increasing the expression level of BAX gene (Lin et al., 2015; Lin et al., 2012). Treatment of colon cancer (HT29 and HCT116) cells with aqueous extract of *Oldenlandia diffusa* (from Rubiaceae family) has been reported to induce early and late apoptosis in colon cancer cells (Lu et al., 2016). The effects of *Uncaria rhynchophylla* aqueous extract on apoptosis, ROS production, aqueous has been reported by Anita Ko´smider et al (Kośmider et al., 2017). In contrast, 100, 50, 10 μg/mL of *Galium mollugo* (from Rubiaceae family) have been reported to increase cell viability by inhibiting ROS production in fibroblastic cell line (Chaher et al., 2016). It seems that flavonoids as well as phenolic compounds in *G. verum* methanolic extract (Demirezer et al., 2006)played a significant role in induction of apoptosis in HT29 cell line. Further research are required to precisely reveal that what compounds in methanolic extract of G. verum could induce apoptosis in cancer cells.

4 Conclusion

The results show that high concentration of whole plant methanolic extract can reduce the proliferation of colon cancer cells through inducing BAX dependent apoptosis, increasing the ROS production, and stopping the cells in G0/G1 phase.

Conflict of interests

The authors declare that they have no conflict of interest.

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