RESEARCH ARTICLE

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Sida rhombifolia Exerts Anti-Proliferative and Pro-Apoptotic Effects in Human Liver Cancer HepG2 Cells *in Vitro*

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Abstract

Purpose: Modern research revealed that plants belonging to the Sida rhombifolia family (Malvaceae) contain biologically active compounds that make them prone to discovering and developing anticancer drugs. This study aimed to evaluate the apoptosis effects of S. rhombifolia extracts in HepG2 Cell Line was performed. **Methods:** The extractions were prepared, and an MTT assay was applied to evaluate its role in decreasing the viability of HepG2 and HFF cells. Phenolic compounds were analyzed using High-performance liquid chromatography (HPLC). FlowCytometry and RT-qPCR evaluated apoptosis was performed to measure the mRNA expression of pro-and anti-apoptotic mediators. **Results:** The results can be summarized as EtOAc extract was more cytotoxic against the HepG2 cells (IC_{50}= 364.3 µg/mL) compared to MeOH and HEX extracts (720.2 µg/mL) (560.4 µg/mL) with less cytotoxicity in HFF cells (353.2 µg/mL). The HPLC analysis results revealed most phenolic compounds, such as Epicatechin(1.3 mg/g). The EtOAc extract (300 µg/mL) induced 34% apoptosis in HepG2 cells. RT-qPCR data showed upregulation of the proapoptotic gene (Bax) and increased Bax/BCL-2 ratio by S. rhombifolia EtOAc extract (300 µg/mL). **Conclusion:** In conclusion, the EtOAc extract of S. rhombifolia is capable of inducing apoptosis in HepG2 cells through modulation of the mitochondrial pathway, which explains their antitumor activity.

Keywords: Apoptosis- Cancer- cytotoxic activity- Sida rhombifolia

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Introduction

Globally, liver cancer is the most frequent fatal malignancy with more than 840,000 new cases in 2018, including primary hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (iCCA), and secondary liver cancer (liver metastases from breast, lung, esophagus, pancreas, and stomach). Two major risk factors of liver cancer are nonalcoholic fatty liver disease and chronic infection with hepatitis B virus. The average age of patients diagnosed with liver cancer has risen, and women have a lower chance of developing the disease than men. The disease is diagnosed at an advanced stage due to having no obvious symptoms(Anwanwan et al., 2020; Jin et al., 2020; Rahmani et al., 2020).

Current cancer treatments include being with chemotherapeutic drugs, radiation, and surgical intervention, which can damage healthy cells and cause toxicity in patients. Therefore, researchers are looking for more tolerable anticancer therapies and ways to eliminate just cancerous cells. The use of natural compounds may be used as an approach to have better outcomes with fewer side effects and lower systemic toxicity (Anwanwan et al., 2020; Zaimy et al., 2017).

Eventually, herbal drugs may be turned into chemotherapy drugs. Herbal compound plays a role in cancer treatment by interfering in the cell cycle, activating apoptosis, or scavenging free radicals (Wu et al., 2020). Sida rhombifolia, a permanent and predominantly annual plant and native in tropical and subtropical areas. It is a medical herb that finds in different regions of the world especially, in the north of Iran (Sebastian et al., 2012).

The basic chemical constituents of Sida rhombifolia are glycosides, steroids, and alkaloids. The whole plant is a product to remedy tuberculosis and rheumatism. The stem and root of the plant are beneficial for the remedy of heart disease, inflammation, and fever (Ekramul et al., 2000). This plant has been used widely as a traditional treatment of gastrointestinal dysentery, asthma, anti-inflammatory, and antimalarial agent (Mahet al., 2017). Based on scientific reports, this plant has antioxidant, cytotoxicity, anti-obesity, cardioprotective, nephroprotective, and antibacterial activities (Peter et al., 2014; Thounaojam et

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al., 2011). In this study, the cytotoxic effect on whole parts and the apoptotic effect of its leaves were reported for the first time. This study was written based on CONSORT standard (Gagnier et al., 2006), due to addressed in CRIS Guidelines; the methodological structure of in vitro studies and clinical trial studies is the same (Krithikadatta et al., 2014).

Materials and Methods

Cell Culture

The liver cancer cell line of HepG2, and the normal cell lines of HFF were purchased from the pasture Institute (Tehran, Iran). These cell lines were grown in RPMI-1640 medium (Biowest, France) including 5% FBS (Biowest, France), 100 U/mL Penicillin (Biowest, France), and 100 µg/mL Streptomycin (Biowest, France) at 25 cm^2 flasks that were put in an incubator with temperature condition 37^0C and 5% CO_2 humidified atmosphere. The cells were treated with 0 -600 µg/mL concentrations of extracts. They were then analyzed after a 24-hour incubation period. The zero concentration of extracts were considered the control group, and the experiments were triplicated to increase the accuracy of comparison and process efficiency.

Plant Materials

Sidarhombifolia plants were collected in July 2018 and confirmed by an experienced botanist (DR Amini Noshahr, Exobiology and Botanical garden, Iran). The voucher specimen was deposited in the School of Pharmacy herbarium (No.1428).

Preparation of Extracts

Arial parts and roots of S. rhombifolia were dried under dark conditions at room temperature. The dry materials were coarsely ground to obtain pieces up to 2-3 mm long. Each plant part was extracted separately by HEX (Merck, Germany), EtOAc (Merck, Germany), and MeOH (Merck, Germany) for 24 h at room temperature (EtOAc extract was prepared as defatty-EtAoc by HEX washing). The extracts were then separated from the sample residues by filtration through Whitman No.1 filter paper and repeated three times. The resulting extracts were concentrated over a rotary evaporator at 35-40°C until solid crude extracts were obtained. EtOAc extract of the leaf (EtL), EtOAc extract of the stem (EtS) and root (EtR), HEX extract of the leaf (HL), and MeOH extract of the leaf (ML) were obtained. Extracts were dissolvent in PBS and Tween 80 (0.025%) (Hamzeloo et al., 2014). The concentrations of 100, 200, 300, 400, and 600 µg/mL were prepared.

High-Pressure Liquid Chromatography

The EtOAc extract was quantified using a method adapted from Khalili et al., (2015) for screening flavonoid or phenolic acid contents. The HPLC apparatus consisted of a model K- 1001 solvent delivery system equipped with a Rheodyne injection valve (20 µL sample loop inserted) and a UV-vis spectrophotometric detector model K-2600 set at 275 nm.

Cytotoxicity examination of Sida extract

The proliferation of HepG2 and HFF cells was investigated by MTT colorimetric assay. Cells were separately seeded in two 96-well plates (6×10^3 cells mL1 in 200 µl medium in each well) for 24 h. The cells (with old media) were treated with a variety of designated concentrations of extracts (600, 400, 300, 200, 100, 0 µg/mL), but no sida extract was used either as control or vehicle (tween 80, 0.025%), in any experiment of any case study. On the next day, 20 µl of tetrazolium dye (MTT) solution (1mg mL^{-1} in PBS) was added to each well, and the cells were incubated for 4 hours at 37^oC in a 5% CO_2 humidified atmosphere. The mediums were discarded, and DMSO (200µl) was added to each well. The plates were checked for 20 min and read at 570 nm wavelength by a microplate reader (Synergy-USA). All Samples were conducted in triplicate. To determine the cytotoxicity of the extracts, IC_{50} was calculated by Graph Pad Prism software (version 6).

Apoptosis assay by Flow Cytometry

Apoptosis effects of S.rhombifolia extract were detected by flow cytometry analysis. For this assay, EtOAc extract in two concentrations (250 and 300 µg/mL) was selected based on the optimum cytotoxic effects on cells in the MTT assay performed. Cells from each cell line were seeded in 6-well plates (15×10^4 cells mL-1 in 2mL medium in each well), and after resting overnight, the cells were treated with Sida's extract. And then were incubated for 24h; with 2.5% trypsin, cells having been harvested according to the manufacturer's instructions (eBioscience, US, cat.no: BMS500FI-20 (the pellet was washed using a binding buffer, 5 µl Annexin-V was added to the pellet, and the solution was put in dark condition for 5 min. Afterward, 10 µl of PI dye was added to the pellet; after 10 minutes, they were analyzed by flowmax software (version 2.3) and flow cytometry device (partec, PAS).

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Apoptotic effects of Sida, based on the efficacy and expression of BAX and BCL2 Genes were assessed by Real-Time PCR assay. In short, 2×10^5 cells from each cell line were seeded in 6-well plates and based on the above explanation of sample preparation (250 and 300 µg/mL of extract, 24 H), samples were prepared. The evaluation of total RNA was performed using the manufacturer's instructions; Isolation of RNA from cells (Cinnagen, Cat.NO PR89162, Iran). To assess the quality of the synthesized RNA, Electrophoresis was performed on 1.5% agarose gel. The cDNA of Samples was prepared using a cDNA synthesis Kit (Parstous Biotech, Cat.NO A101162, Iran). In this protocol, we used Random Hexamer and Oligo dT primers. Moreover, to assess the quantitative synthesis of cDNA and the PCR value of the cDNA sample, qualitative PCR was conducted. QRT PCR was administrated in triplicate with Bio-Rad Real-time PCR system. This evaluation was performed using SYBR green. The GAPDH gene was used as a Housekeeping Gene. For calculating mRNA relative expression of the targeting genes, the $2^{-\Delta\Delta CT}$ method and Bio-Rad IQ5 Software were

used. The sequences of primers are presented in Table 1.

Statistical Analysis

The statistical analysis of the data in this study was performed using t-Test and one-way ANOVA in SPSS.23 (statistical package for the Social sciences) statistical software. IC_{50} was performed using GraphPad Prism 6. The results are conveyed in mean± standard deviation (SD) for four independent determinations. The p-value of 0.05 was considered statistically significant.

Results

Extraction Efficiency

The extraction efficiency of MeOH(Methanol), EtOAc(EthylAcetat), and HEX(Hexan) extracts were 7%, 3%, and 3.7%, respectively.

Plant extraction efficiency= (Dry extract weight/initial dry weight of the extract) ×100

Phytochemical Characterization

The analysis was performed using an ODS (C18) column (250 mm×4.6 mm I.D., 5 µm particle size, Shim-pack VP-ODS). All solvents were filtered and degassed before entering the column. A gradient solvent system consisting of solvent A (MeOH) and solvent B (H2O with 9% glacial acetic acid) (conditions: 5% A from 0 to 5 min and kept at 10% A from 5 to 15 min, kept at 30% A from 15 to 27 min and kept at 80% A from 27 to 45 min) were used for separation. All separations were performed at 25 °C with a flow rate of 1 mL/min. The mentioned assay was applied for the determination of the extract retention time and UV spectral peaks of the sample with authentic standards (Figure 1).

The standards were described as epicatechin, gallic acid, caffeic acid, p-coumaric acid, ferulic acid, and rutin from Sigma-Aldrich. The phenolic acids were separated via the current method during the time of 0 up to 45 min and determined based on the peak area of specific concentrations of the sample and standard. According to standards, the extract contains five phenolic compounds tested including: epicatechin(1.3 mg g^{-1} of powder), gallic acid (0.057 mg g^{-1} of powder), caffeic acid (0.04 mg g^{-1} of powder), ferulic acid (0.22 mg g^{-1} of powder), rutin(0.094 mg g^{-1} of powder) (Table 2). In this regard, most of the biological activity of extracts and the ability to synthesize nanoparticles can be attributed to these phenolic acids.

Effect of S.rhombifolia Extracts on Cell Viability

First, the cytotoxicity effects of different parts EtOAc extract of S. rhomobifolia and leaf extracts of MeOH & HEX were evaluated by MTT assay. Flowcharts of the effects on metabolic activity are shown in Figure 2. By increasing the concentration of EtOAc extract of the leaf (EtL) from 300 µg/mL to up to 600 µg/mL, the viability of the cells decreased significantly ($p<0.001$). There was significant different viability of HepG2 between 300 vs. 400 ($p<0.001$), 400 vs. 600 ($p<0.01$) in HepG2. Moreover, the viability between HepG2 and HFF was significantly different in 300 µg/mL ($p<0.001$) (Figure 2A). The viability of HepG2 cells in the presence of MeOH extract of the leaf (ML) was decreased 200 to 600 µg/mL, and there was significantly different viability

Table 1. Oligonucleotide Primers Used for Transcriptional Analysis of Target Genes

Name of The Gene		Sequence Of Primer	Type of The Gene
Bax	F	5'-CCAAGAAGCTGAGCGAGTGTCTC-3'	Pro-Apoptotic
	R	5'-AGTTGCCATCAGCAAACATGTCA-3'	
Bcl2	F	5'-ATCGCTCTGTGGATGACTGAGTAC-3'	Anti-Apoptotic
	R	5'-AGAGACAGCCAGGAGAAATCAAAC-3'	
GAPDH	F	5'-GGTGGTCTCCTCTGACTTCAACA-3'	Housekeeping Gene
	R	5'-GTTGCTGTAGCAAATTCGTTGT-3'	

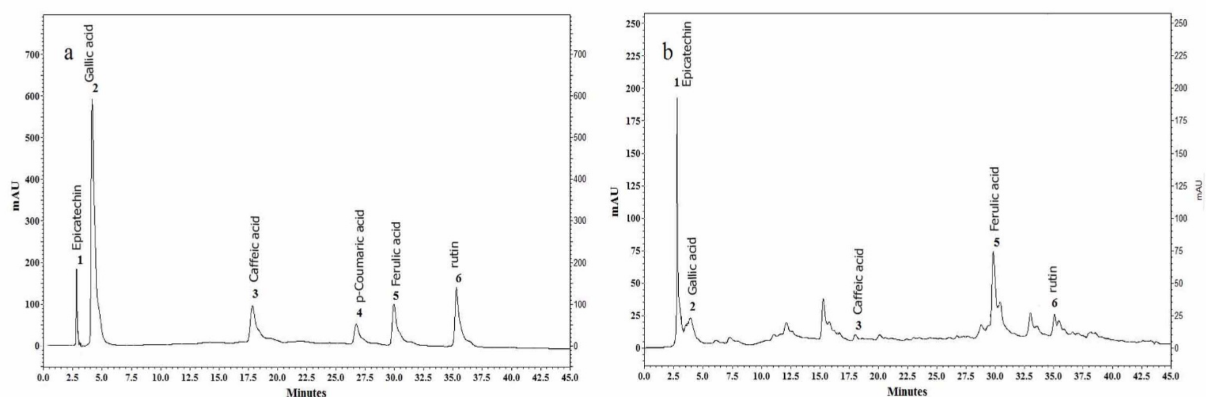

Figure 1. HPLC Profiles of the Extract were Analyzed at 275 nm. Extract; (peak1), Epicatechin;(peak 2), Gallic acid;(peak 3), caffeic acid; (peak 4), p-coumaric acid; (peak 5), ferulic acid;(peak 6), rutin

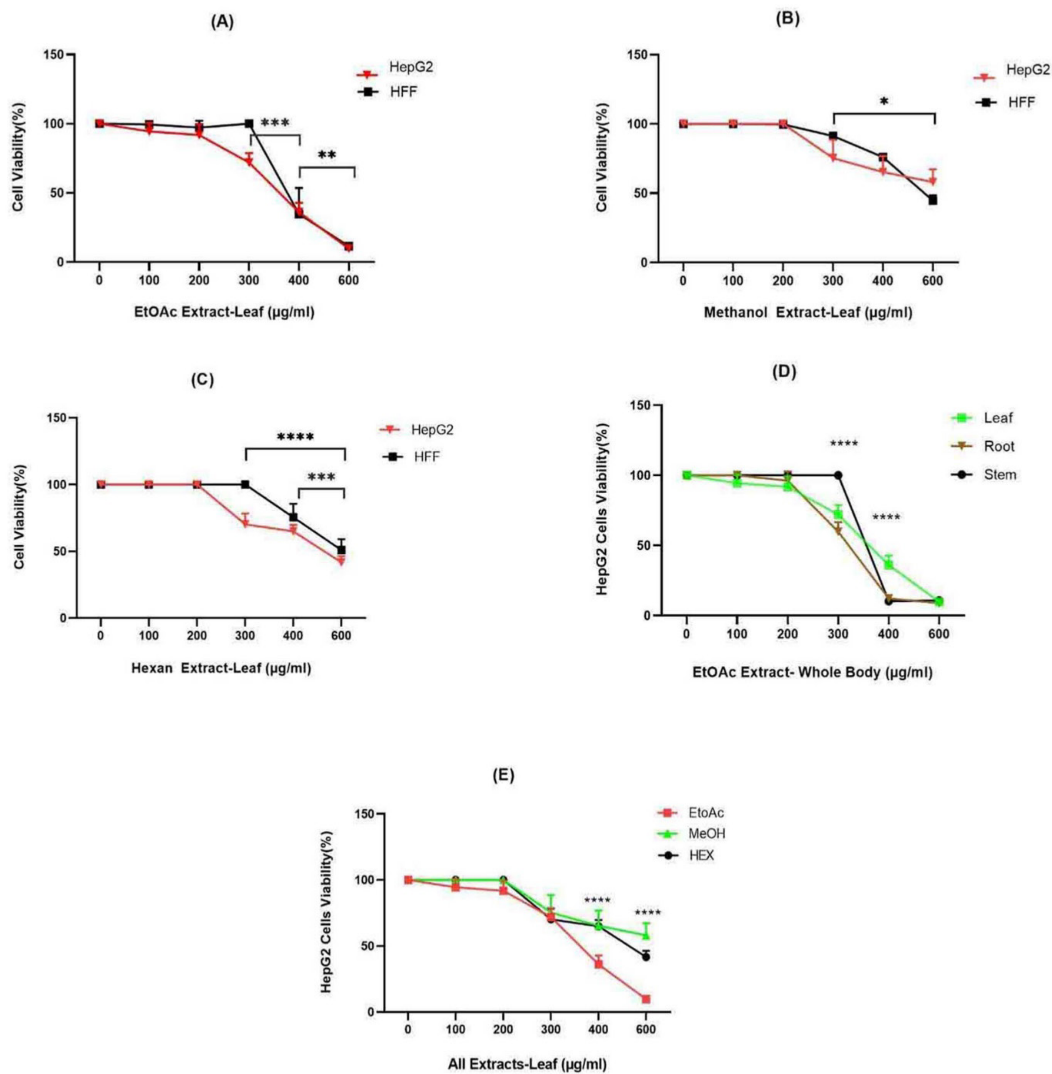

Figure 2. The Viability of Cells was Evaluated Following 24 h exposure to Different Extracts of S. rhombifolia. (A) by EtL, significantly different between 300 Vs 400 ($p<0.001$),400 Vs 600 ($p<0.01$) in HepG2, (B) by ML, significantly different between 300 Vs 600 ($p<0.001$) in HepG2, (C) by HL, significantly different between 300 vs 600($p<0.0001$), 400 Vs 600 ($p<0.001$) in HepG2,(D) by EtL in whole body, significantly different between leaf vs stem, root vs stem and leaf vs root(300µg/ml), leaf vs stem(400µg/ml) ($p<0.001$), (E) by all extracts, significantly different between all extracts (600µg/ml) and EtOAc vs HEX, EtOAc vs MeOH(400µg/ml) ($p<0.001$). Results represent the mean ± SD of four replicates.. Note: HepG2 and HFF cells were treated with various doses of S. rhombifolia Extracts ranging from 0 µg/mL (untreated) to 600 µg/mL. The comparisons were independently conducted several times.

between 300 vs. 600 µg/mL. Moreover, significantly different viability between HepG2 and HFF was observed in 300 µg/mL ($p<0.01$). Besides, the effect of ML on HFF cells as control was more than HepG2 cells in 600 µg/mL (Figure 2B). As seen in chart C, the viability of HepG2 cells was initiated to decrease from 200 µg/mL due to HEX extract of the leaf (HL) treatment, but this trend in HFF cells was initiated from 300 µg/mL; that is a sign of the negligible effect of HL up to 300 µg/mL on HFF cells. There was significantly different viability of HepG2 between 300 vs. 600 ($p<0.0001$), 400 vs. 600 µg/mL ($p<0.01$) in HepG2, also such a significant difference was observed between HepG2 and HFF in 300 µg/mL

Table 2. Phytochemical Analysis of S. rhombifolia (Leaf Extract)

Phenolic compounds	Retention time (min)	Amount (mg/g of powder)
Epicatechin	2.5	1.3
Gallic Acid	4	0.057
Caffeic Acid	18	0.04
p-coumaric acid	NA	NA
Ferulic Acid	30	0.22
Rutin	36	0.094

ND, not detected

Table 3. IC_{50} Values Calculated for Extracts on HepG2 Cells

Plant Extract	Concentration(µg/mL)
HEX	560.4
EtOAc	364.3
MeOH	720.2

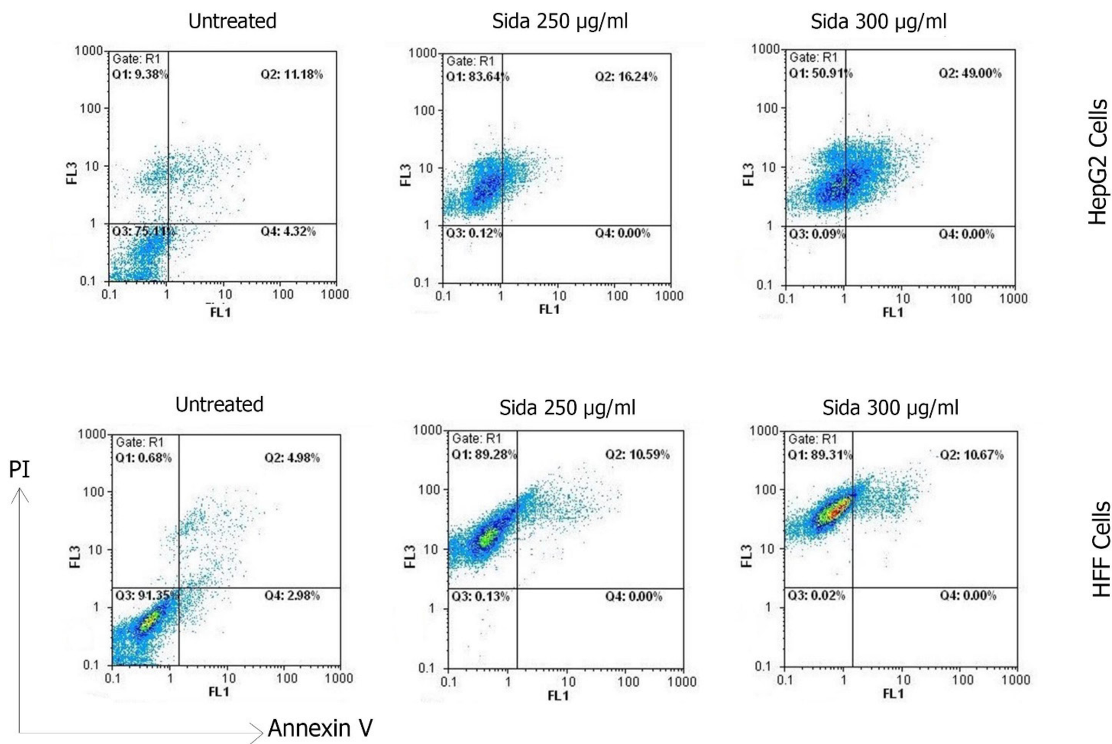

Figure 3. FACS Analyses of Annexin V/PI to Evaluate the Apoptotic Effect of EtL on HepG2 and HFF Cells for 24h. Untreated groups were considered as controls.

(p<0.0001) (Figure.2C). The trend of reducing the viability of cells in EtOAc extract of leaf (EtL) was more logical than other parts. Moreover, there was significant different viability of HepG2 between leaf vs. root (p<0.01), root vs. stem (p<0.0001) (Figure 2D). As seen in Figure 2E, what can be deduced is that the process of viability reduction of HepG2 cells caused by EtL, has logical and principled inclinations as dose-response criteria. Besides, all extracts in 600 µg/mL to one another had a significant difference in viability (p<0.0001) and EtL vs. ML and Etl vs. HL in 400 µg/mL (p<0.001) (Figure 2E).

In-vitro Cytotoxic Activity of S. rhombifolia Extracts

The ability of cells for exposure to the extracts was determined by MTT assay. As shown in Table 3, the crude extract of EtL with an IC_{50} value of 364.3 µg/mL during 24 h treatment has the highest cytotoxic activity for HepG2.

Flow Cytometry Analysis for Apoptotic Effects of S. rhombifolia Extract

To determine the apoptotic effects of EtL on the HepG2 cell line, the cells were stained by FITC Annexin V/PI and were assessed by Flow Cytometry. Annexin V is commonly used to detect apoptotic cells because of its ability to bind to phosphatidylserine. It roles as a marker for apoptosis when it is on the outer leaflet of the plasma membrane, as well as propidium iodide (PI) for the identification of early and late apoptotic cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI.

Flowcytometric results are shown in Figure 3, where Q2 and Q4 represent the percentage of Late and early apoptotic cells, respectively. As the results show, in Figure 3, the EtL has to lead the HepG2 cells to 34% apoptosis in 300 µg/mL and to 1.24% in 250 µg/mL compared to the untreated group. Furthermore, the results showed that the EtL has to lead the HFF cells to. 6% apoptosis in 300 µg/mL and to 7.5 % in 250 µg/mL compared to the untreated group.

Effect of S.rhombifolia Extract on Genes Related to Mitochondrial-Mediated Apoptosis

The effect of EtL on the mRNA of Bax and Bcl2 gene expression was evaluated using quantitative real-time PCR. After completing the treatment of HepG2 cell line and HFF cells with EtL and extraction of RNA and cDNA synthesis, the relative expression of mRNA for each gene post-normalization with the GAPDH gene was calculated. Based on the MTT result, EtL extract was selected for its effect on apoptotic gene expression. In that assay, EtL extract (300 µg/mL) had the cytotoxic effect on cancer cells with Significantly lower toxicity on normal cells (P<0.01). The gene replication diagram of Bax, Bcl2, and Bax/bcl2 ratio after normalizing againstGAPDH gene by Real-Time PCR assay was shown in Figure 4. As shown in Figure 4A, 300 µg/mL of S. rhombifolia extract significantly increased the expression of the proapoptotic Bax gene and Bax to *bcl2* gene ratio in HepG2 treated compared to untreated cells(p<0.01), while it did not have a significant effect on the expression of the anti-apoptotic *Bcl2* gene in that comparison. On the other hand, the average of the Fold-change expression of the Bax gene in 300 µg/mL treatment was 7.69, while this value for the bcl2 gene was 0.8. In Figure 4B, there was no significant difference between treatment and gene expression in HFF cells as a control group (p>0.01). Moreover, the results

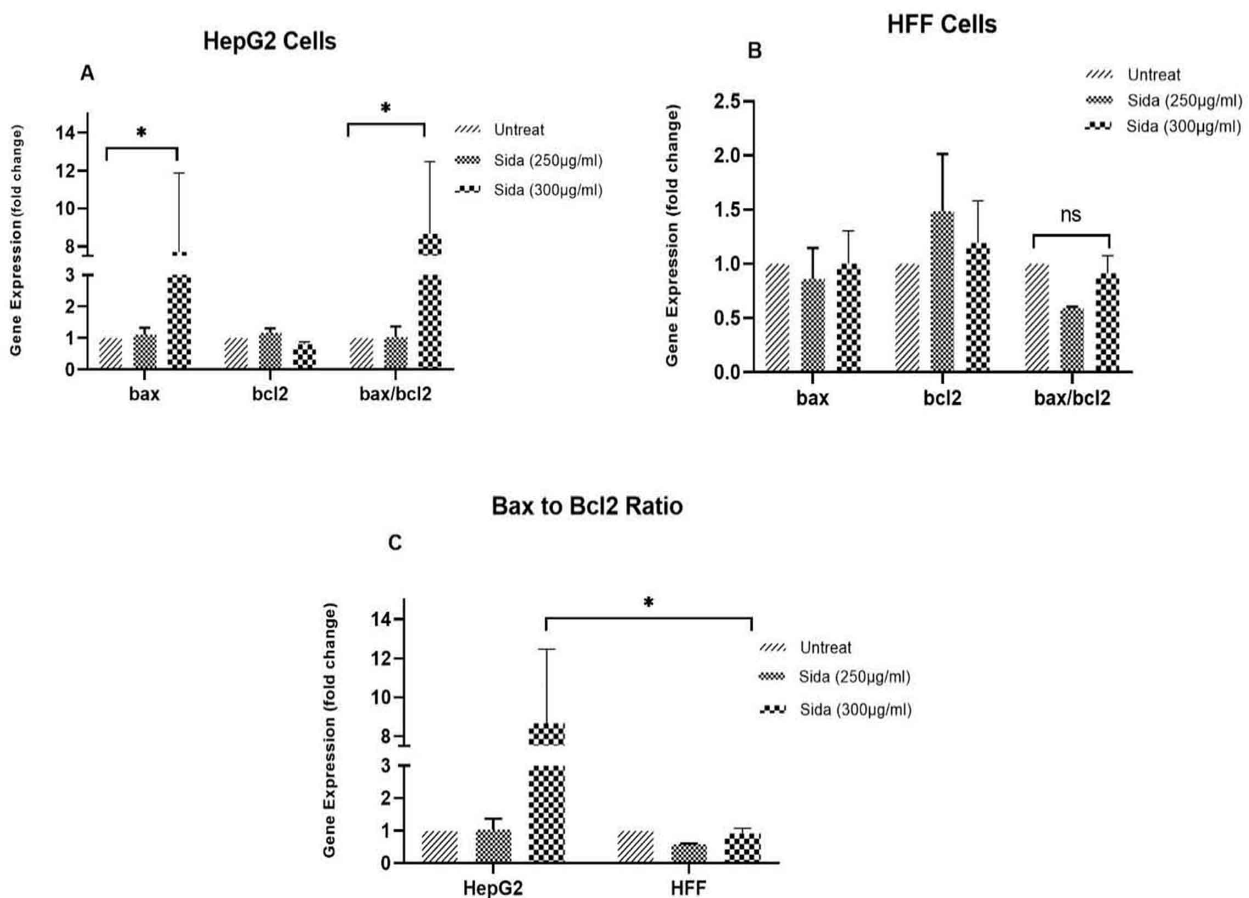

Figure 4. Relative Quantification of Bax, bcl2, and bax/bcl2 in HepG2 (A) and HFF (B) treated by S. rhombifolia (250,300 µg/ml), Bax to bcl2 gene ratio (C) in HepG2 and HFF. *: Significant difference between the groups was shown, $p < 0.05$.

of this assay showed a significantly higher expression of Bax to *bcl2* gene ratio in HepG2 cells as compared to HFF ($p<0.01$) (Figure 4C). EtL significantly increased the expression of the proapoptotic Bax gene, while it did not have much effect on the expression of the antiapoptotic *Bcl2* gene ($p<0.01$).

Discussion

In this study, the apoptosis effects of a Sida extract in the HepG2 Human Liver Cancer Cell Line were assessed. Three types of extraction (HEX, EtOAc, MeOH) and bodies (Leaf, Root, Stem) of S. rhombifolia were prepared, and MTT assay was applied to evaluate its role in decreasing the viability and proliferation of HepG2 and HFF cells, HPLC was performed to analyze phenolic compounds, Apoptosis was evaluated by FlowCytometry and RT-qPCR was conducted to measure mRNA expression of pro and anti-apoptotic mediators.

The results of this study showed EtOAc leaf extract of S. rhombifloia has more inhibitory effects and an apoptosis role on the HepG2 cancer cell line. As shown in the results regarding the MTT assay, S. rhombifolia extracts with a higher concentration than 300 µg/mL have a significant cytotoxic effect on HepG2 cell lines compared to untreated cells, and the maximum inhibitory effect was observed in 24-hour treatment with 600 µg/mL EtoAc extract. Several studies reported the cytotoxicity effect of S.rhombifolia extracts against cancer cell lines. Islam et al., (2003), showed EtoAc extract S.rhombifolia had the most cytotoxic effect on shrimp brain compared to MeoH extract, based on this note MeoH is more polar than EtoAc. In the other study, the antiproliferative effect of *S. rhobmifolia* on cervix cancer cells (HeLa) has been reported, confirming the results of our study. In this study, the effect of hot aqueous *S.rhombifolia* extract along with five other plants that are not present in the Malvacea family and named N031 was assessed on HeLa cancer cells. IC_{50} was calculated as 2.65 µg/mL for the antiproliferative effect of N031 on the HeLa cell line; which according to the classification of the National Cancer Institute is classified as active (IC_{50} value> 20 µg/mL). The low level of IC_{50} calculated in this study may result from the combination of S.rhombifolia with other plants from different families. In this study, it is stated that the antiproliferative effect of extracts on HeLa cell line maybe because of the phytochemical content in plants such as S.rhombifolia including alkaloids, flavonoids, tannins, and Xanthos (Manosroiet al., 2012; Siddhurajuet al., 2002). It is the strong antioxidant effects of these phytochemicals that can inhibit the proliferation of reported tumor cells (Oszmianski et al., 2007; Santanamet al., 2004; Zouet al., 2004).

Coumaric and ferulic acids can be mentioned as

the active substances present in S.rhombifolia whose their presence was proven by chromatography and spectroscopic techniques (Chaves et al., 2017). Coumaric and ferulic acid is an effective substance in treating cancer and the side effects of radiotherapy and primarily used in treating prostate cancer, renal cell carcinoma, and leukemia. Due to this, it may be possible to use S.rhombifolia to alleviate the side effects of routine cancer treatments. according to our chromatography results, *S. rhombifolia* contains these two substances (Finn et al., 2002; Loprinzi et al., 1999). Pieme et al., (2010) reported that the S.rhombifolia extract reducing cell viability of hepatic cancer cells in a dose-response-dependent manner. Four genera of Sida in the Malvaceae family (Sida cordifolium, Sida rhombifolia, Urena lobate, Sida acuta) which included in this study had cytotoxic effects on the HepG2 cell line and the genus *S. acuta* had the maximum inhibitory effect on cancerous cell growth.The inhibitory effective substances in S.rhombifolia on the HepG2 cell line were assessed in another study (Pieme et al., 2010) and the results showed an increase in the toxicity of S.rhombifolia by different solvents: MeOH, EtOAc, and HEX (From lowest to highest toxicity). Furthermore, based on published reports from Mah's study(Pieme et al., 2010), increasing in the plant's cytotoxic effect was associated with a decrease of polarity in the effective ingredients existing in the plants. It's possible that the reason behind the insignificant difference between MeOH and HEX extracts in the cytotoxic effect was the supply of primary defatty-EtAOc (with HEX washing) and the reduction of non-polarization of the obtained extract. Moreover, the reported IC_{50} in our study was leaf extract but in Mah's study, the total extract of the plant was evaluated. Of course, with the results obtained from the effect of the toxicity of extracts derived from the organs of the plant separately, in the present study, it may be argued that the toxic effect of the organs in total is more than the toxicity of each organ alone. Overall, a strongly inhibited HepG2 cell line confirms the natural cytotoxicity effect of S.rhombifolia.

In this study, the apoptotic effect of S.rombifolia's extract was investigated and reported, the results of flow cytometry assay were shown that 300 µg/mL concentration of EtL has an apoptotic effect (approximately five-fold compared to the untreated group) on HepG2 cell line that this apoptosis percentage corresponds to a significant decrease in the percentage of viability in the MTT assay. It should be noted that cell adhesion was increased, causing by treatment of the cells by EtL, and as a result, harvesting cells was difficult especially in HFF as a primary cell. It is not far from the expectation that it can affect the number of living cells. In the other study conducted by Ahmed, the apoptotic effect of other species of Sida (Sida acuta) was confirmed. They showed *S. acuta* induced cell death by TRAIL-induced apoptosis(Ahmed et al., 2011). Moreover, in this study, the expression of two apoptotic genes (Bax and BCL2) was evaluated by Real-Time PCR assay. Increasing the expression of Bax versus Bcl2 in cells treated with EtL (300 µg/mL) suggests an increase in the induction of apoptosis in the HepG2 cell line, while the Bax gene expression level in normal HFF cells did not increase significantly compared to the Bcl2 gene.

The results of this study showed 5 phenolic compounds exist in S. rhombifolia. The most important gradients and the highest amounts are epicatchin and ferulic acid. Catechin is a substance abundant in green tea and many studies approved its anticancer effects (Buttet al., 2015; Ohishi et al., 2016). These effects can be due to its role in apoptotic and signaling pathways (Kumar et al., 2019; Yang et al., 2019). The cytotoxic and apoptotic effects of S. rhombifolia extract may be attributed to the presence of epicatechin, why so it has also been present in *S. rhombifolia* to a great extent than other compounds and its apoptotic effects have been confirmed in other studies. On the other hand, the presence of ferulic acid, even to a small extent, can be effective in the anticancer role of *S. rhombifolia* (Damasceno et al., 2017).

In conclusions, our result was shown that the EtOAc extracts of S. rhombifolia are capable of inducing apoptosis in HepG2 cells through modulation of mitochondrial pathway, which explains their antitumor activity. After purification of the extract and determination of some bioactive components, epicatechin and ferulic acid are two abundant components that can be effective roles in the anticancer effects of S. rhombifolia. This plant may use as useful resources to develop effective treatment for cancer.

Limitation

Since the cells are highly adhering after treatment with the extract and flow cytometry tests, which require separation of cells, using trypsin at a high concentration and for a long time has damaged the cells, especially the normalized ones, and has resulted in apoptosis rate measurements being compromised.

Author Contribution Statement

Conceptualization, M.A. and A.R.; methodology, M.A., A.R., M.K and M.E.Z; software, M.A. and A.R; validation, A.R. and M.E.Z.; formal analysis, M.A.; investigation, A.R., M.E.Z and M.E; resources; M.A and A.R, writing-original draft preparation: M.A.; writing-review and editing, M.A., A.R and M.E.Z; visualization, M.A, project administration, A.R. All authors have read and agreed to the published version of the manuscript. The authors declare that all data were generated in house and that no paper mill was used.

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Ethical approval

Not applicable.

Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of conflict of interest

All the authors declare no conflict of interest.

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