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Apoptotic and Anti-Proliferative Effects of *Rhazya Stricta* Nanoparticles against Hepatocellular Carcinoma (Hep G -2 And Huh-7) Cell Lines

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Keywords

- *R. stricta* Nanoparticles
- Hepatocellular carcinoma
- Apoptotic cell death
- Gene expression.

Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most serious cancers worldwide. Even with recent developments, there has been slight progress in improving the survival rate of HCC patients. Current therapies are accompanied by marked side effects beside incomplete recovery. Therapy by Herbal products proved effective anticancer. **Aim:** Screening of the apoptotic and anti-proliferative effects of *Rhazya stricta* (*R. stricta*)**,** nanoparticles which locally known as “ Harmal” against highly aggressive HCC *in vitro* study. **Procedures:** *R. Stricta* Nano particles was gained by exposing *R. Stricta* sheets to ball milling process to convert particles into Nano scale diameter which examined by scanning electron microscopy (SEM) . Cytotoxicity of different concentrations of *R.stricta* nanoparticles (0.0, 100 and 500 µg/ml) was evaluated via its effects on the mRNA expression of Bax and Bcl-2 apoptosis related genes in Hep G-2 and Huh-7cell lines using real-time quantitative PCR analysis, MTT assay and FACS analysis

Results: It is clear from SEM images that *R.Stricta* nanoparticles are predominantly spherical in shape and polydispersity with particle size around 100 nm. After 24 and 48 hours, *R.stricta* nanoparticles showed a significant cytotoxic effect on all cell lines at 500 µg/ml ($p<0.05$) but has a high significant effect on decreasing Hep G-2 cell viability with 100 and 500 µg/ml of *R.stricta* nanoparticles Hr($p<0.001$) compared with other cell lines. **Conclusion and clinical relevance:** Moving on gene expression,MTT assay and FACS analysis, it could concluded that the *R. stricta,* nanoparticles produced apoptosis in Hep G-2 and Huh-7 cell lines

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INTRODUCTION

Hepatocellular carcinoma (HCC) was considered the ninth cause of death around the world [1]. Around 30,640 new cases of liver and intrahepatic bile duct tumors were recorded in 2013 with 21,670 deaths [2]. HCC contributes mainly in males than females (2.4:1), with a higher rate in Eastern and Southern Asia, Melanesia, Micronesia/Polynesia and Middle and Western Africa [3]. Both prolonged liver disease and cirrhosis are the major factors for HCC initiation and progression. In the same context, viral hepatitis infection and too much alcohol drinking are the foremost risk factors [4].Unluckily, the diagnosis of HCC is too often done at late disease stage. Unfortunately, there is no effective treatment at this stage and the morbidity rate become high and irrespective of the recovery therapy, HCC patients require a multidisciplinary therapy to achieve the optimal outcome [5].

In the last years, Herbal medicine was used as alternative therapies by many peoples suffered cancer [6, 7]. *R. stricta* is important medicinal plants grown in the desert and distributed widely in Saudi Arabia and throughout the Middle East[8, 9]. It contains flavonoids glycosides, alkaloids and tannins [10]. *R. stricta* alkaloid acts as antioxidant, antifungal, , keep glucose homeostasis and improve the liver functions [11, 12]. It increases insulin levels and reduce the levels of triglyceride [13]. It was proved to have anticancer effect where in human lung cancer cells(NSCLC line A549) it induces apoptotic (NSCLC line A549) cell death [14] and it also induces apoptotic cell death of MCF-7 and MDA-MB-231 breast cancer cells [15]. In addition, *R.stricta* isolated alkaloids have been shown to exhibit inhibition of growth and metastasis of many tumors in vitro and in vivo [16]. Although the activity of *R.stricta* extracts anti -proliferation effects against different cancer cells were investigated by many authors [17-20] however, The present study could be the first that focused on screening of apoptotic and anti-proliferative effects of *R.stricta* nanoparticles against highly aggressive hepatic cancer cells (in vitro) with focusing and explaining the mechanism of cell deaths and its anti-migratory activity

2. Materials and Methods

2.1. Plant Sample Collection and Extract Preparation

R. stricta leaves, were brought and dried as previously described by [17]. Once dried Nano particles was gained by exposing *R. stricta* sheets to ball milling process to convert particles into Nano scale diameter. Scanning electron microscope (SEM) was used. to determines the size and morphology of the formed nanoparticles.

2.2. **Chemicals and reagents:**

Roswell Park Memorial Institute (RPMI) 1640 growth medium, fetal bovine serum (FBS) and anti-biotic mix (10.000 u penicillin/ml, and 10.000 u streptomycin/ml) were purchased from Gibco, (Invitrogen, CA, USA). Cell culture flasks, dishes, plates, falcon tubes, cryopreservation vials, disposable pipettes, sering filter (0.22 µm pore size) and glassware were manufactured by Greiner Bio-One, (Germany) and were purchased from Lonza. MicroAmpR optical 96- well reaction plates for real time PCR, microAmpR fast reaction 0.1 tubes for PCR were purchased from (applied

biosystem). MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H tetrazolium bromide),c and i, were purchased from Sigma (St. Louis, MO, USA). Triazol was purchased from Qiagen (Valencia, CA, USA), and ABoslute SYBR Green RoX mix cat.no AB-1163/A was purchased from Advanced Biotechnologies ltd (AB gene) UK. Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies Strata-gene USA).

2.3. Tissue culture:

MDA-MB-231 human breast cancer cells, Hep G-2 and HuH-7 human hepatocellular carcinoma cells, Caco-2 human colorectal adenocarcinoma cells, Hep-2 human laryngeal carcinoma, A549human lung carcinoma and Wi-38 human normal fibroblast cells were obtained from VACSERA - Cell Culture Unit, Cairo, Egypt. The originally cell lines were obtained from the American Tissue Culture Collection (ATCC). The HePG-2, HuH-7, WI-38, Caco-2 and MDA-MB-231 cell lines were cultured in RPMI-1640 medium supplemented with 10% inactivate fetal bovine serum (FBS) and 1%penicilin/streptomycin while Hep-2 and A 549 cells were cultured in DMEM medium supplemented with 10% inactivate fetal bovine serum (FBS) and 1%penicilin/streptomycin.

2.4. Cytotoxicity Assays

Cytotoxicity Determination (after a 48 h exposure period) was performed by the two following colorimetric methods

2.4.1. Assessment of cell viability using MTT colorimetry assay:

MTT (3-[4, 5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) cell viability assay was based on the cleavage of the insoluble tetrazolium salt to a detectable colored formazan product by succinate-tetrazolium reductase enzyme in the mitochondria of living cells. The produced insoluble formazan is directly proportional to the activity of the mitochondrial dehydrogenase enzyme and consequently to the portion of the viable cells. Briefly, Cells were cultured at initial concentration of $1*10^4$ cell/ well in 96-well plates and 24 hrs later, cells were treated with (0.0, 0.1, 1, 10, 100 and 500 µg/ml *R.stricta* nanoparticles for 24 and 48 hrs, then Hep G2 cells were treated with (0.0, 6.25, 12.5, 25, 50 and 100 µg/ml taxol as a positive control for 24 hrs. 10 µl/ well of MTT solution 5 mg/ml were added and incubated for 4 hrs and then intracellular formazan dye resulting from the metabolically active cells can be solubilized and quantified by measuring its absorbance using ELISA reader.

2.4.2 Assessment of Cell death by FACS analysis:

The induced apoptotic and necrotic cell death by *R. stricta* Decne and taxol were measured in Hep G2 hepatocellular carcinoma cells and WI-38 normal lung fibroblast cells using Annexin V and propidium iodide/ in Hep G2 hepatocellular carcinoma cells and WI-38 normal lung fibroblast cells. Briefly, treated cells were washed once with PBS and dispersed with trypsin and then suspend in 5 ml growth media and finally washed with 1 X binding buffer and stained for 15 min at room temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated annexin V (1µg/mL) and PI (0.5µg/mL) in a Ca2+- enriched binding buffer and analyzed by flow cytometry. Annexin V and PI emissions were detected in the FL1 and FL2 channels of a FACS Caliber flow cytometer, using emission filters of 525 and 610

nm, respectively. Apoptosis analysis was performed using Becton Dickinson FACS Caliber.

2.5. Assessment of BAX and bcl-2: *gene expression with real time PCR:*

Different concentrations of *R.stricta* (0.0, 100 and 500 μg/ml) effects on the mRNA expression of Bax and Bcl-2 apoptosis related genes in Hep G-2 and WI-38 cells were investigated using real-time quantitative PCR analysis. Using BAX: forward, 5' GGTGCCTCAGGATGCG -3', reverse, 5'-GGAGTCTGTGTCCACG-3';and for bcl-2: forward, 5'- TCGATGTGATGCCTCTGCGAA GAAC-3'; reverse, 5'-ATTGCACTGCCAAACGGAGCTG-3'; β-Actin (F) 5` - ATCCGCAAAG ACCTGT-3` and β-Actin (R) 5`- GGGTGTAACGCAACTAAG-3`. After performing the indicated treatments, Total RNA was prepared with Trizol and then the corresponding cDNA was prepared. The total reaction volume was 20μl volume mix containing 10μl SYBR Green Real time PCR Master Mix, 2μl of forward and reverse primer, 2μl cDNA template and 6μl sterile, distilled-deionized water. Cycling condition was: 95°C for 3 min, followed by 40 cycles at 95°C for 30s and 60°C for 1min. Each sample was run in three tubes. Relative gene expression was quantified using the comparative threshold cycle method and β-actin as an internal standard [21, 22].

2.6. Statistical analysis

Results were acquired using one-way ANOVA with SPSS 13.0 software. Data were presented as means ± SD. Statistical significance for all tests was judged at a probability level of 0.05.

3. Results:

3.1. **SCM of *R.stricta* nanoparticles**: It is clear from SEM images that the *R.stricta* nanoparticles are predominantly spherical in shape and polydispersity. Based on these images, the particle size was found to be around 100 nm figure1.

3.2. ***Assessment of inhibition of cell growth and proliferation of Hep G-2 hepatocellular carcinoma by R.stricta nanoparticles***

Screening of Anti-proliferative effect of different concentration (0.0, 0.1, 1, 10, 100 and 500 μg/ml)of R.STRICTA against MDA-MB-231, Hep G-2 and HuH-7, Caco-2, Hep-2 and A549cancer cell lines, and Wi-38 human normal fibroblast cells using MTT assay after 24 and 48 Hrs showed that the *R.stricta* nanoparticles have a significant cytotoxic effect on all cell lines at 500 μg/ml ($p<0.05$) after 48 Hr treatment but produced a **high significant** decrease in Hep G-2 cell viability at 100 and 500 μg/ml of *R.stricta* nanoparticles after 24 and 48 hrs ($p<0.001$) of treatment compared with normal cells as shown in figure 2.

3.3. ***Assessment* of the *R.stricta* induced apoptotic cell death in Hep G-2 cells.**

The mode of apoptotic and necrotic cell death was analyzed by flow cytometry with annexin V and PI. For WI-38 normal cells No markedly observed cell death was observed after 24 Hr treatment with 100 and 500 μg/ml of *R.Stricta* nanoparticles comparing with the 10 μg/ml taxol as a positive control. In the other hand, Hep G-2 cells treated with the same concentrations (100 and 500 μg/ml of *R.Stricta* nanoparticles for 24 hrs) showed a

high significant apoptotic cell death compared with the 10 µg/ml taxol as shown in figure 3.

3.4. Assessment of *R.Stricta* nanoparticles positive impact on apoptotic cell death markers (Bax and bcl2) of Hep G-2 hepatocellular carcinoma:

The effect of *R.Stricta* on the apoptotic markers; Bax and bcl-2 mRNA expression levels in hepatocellular carcinoma cells; Hep G2 cells and WI-38 normal cells were investigated using quantitative real time PCR analyses. In Hep G-2 cells, real time PCR results showed high significant increase in Bax mRNA expression (3.5 and 6-fold) with 100 and 500 µg/ml of *R.Stricta* nanoparticles treatment for 24 Hrs; respectively, while WI-38 cells showed a significant increase in Bax mRNA expression 2.5-fold only with 500 µg/ml of *R.Stricta* nanoparticles treatment. On the other hand, Hep G-2 cells show of markedly down regulation of Bcl-2 mRNA expression (0.4 and 0.2 fold) with 100 and 500 µg/ml of *R.Stricta* nanoparticles treatment for 24 Hrs comparing with (0.8 and 0.7 fold) in WI-38 normal cells as shown in figure 4.

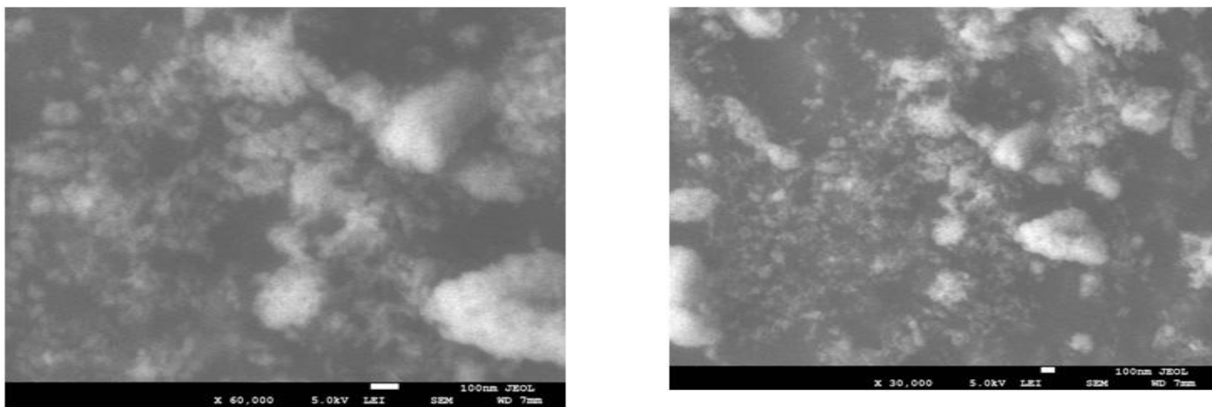

Figure 1: Scanning electron microscope of individual *R.stricta* nanoparticles.

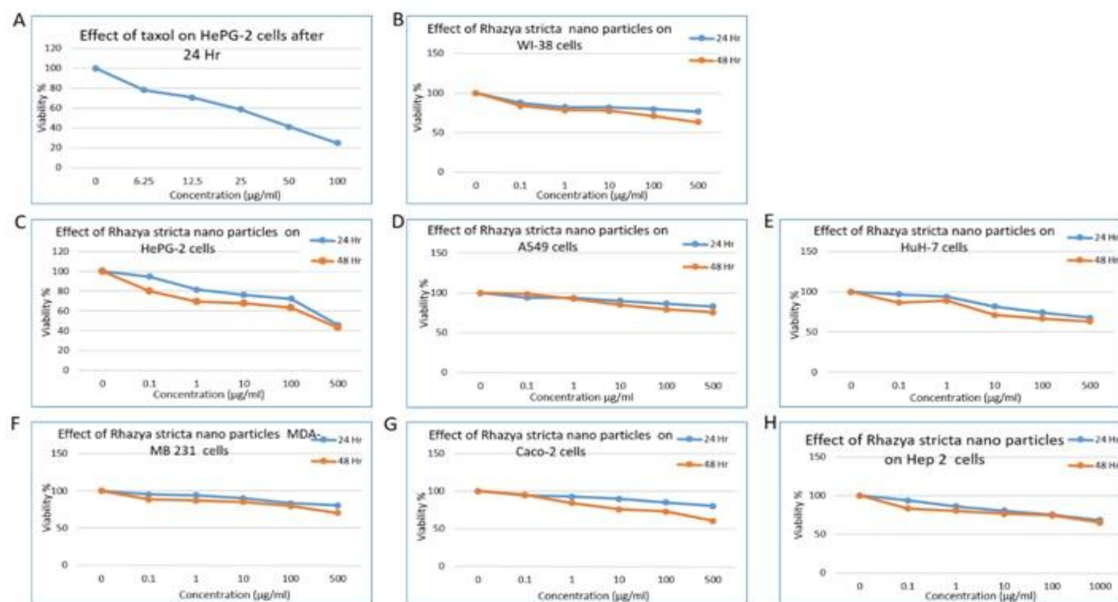

Fig.2. MTT assay shows the cytotoxic effect of different concentrations of R.STRICTA against different cell lines, (A) effect of paclitaxel on Hep G2 cells after 24 Hrs, (BWI-38 cells after 24 and 48 Hrs, (C) Hep G-2 after 24 and 48 Hrs, (D) A549 cells after 24 and 48 Hrs, (E) HuH-7 cells after 24 and 48 Hrs, (F) MDA-MB-231 cells after 24 and 48 Hrs, (G) Caco-2 cells after 24 and 48 Hrs, (H) Hep-2cells after 24 and 48 Hrs.

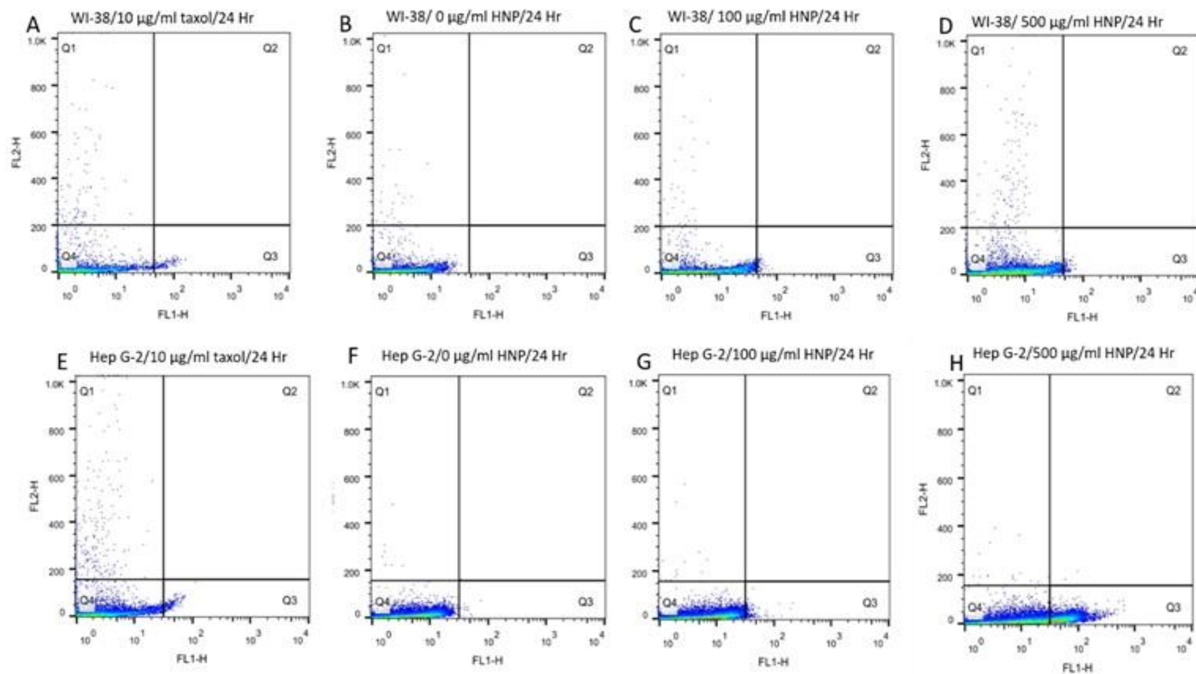

Fig. 3. Apoptotic and necrotic cell death analysis by FACS analysis using annxinV and PI, (A) WI-38 cells treated with 10 µg/ml taxol as a positive control, (B) WI-38 cells grow only in growth media as a negative control, (C) WI-38 cells were treated with 100µg/ml *R.Stricta* nanoparticles,(D) WI-38 cells were treated with 500µg/ml *R.Stricta* nanoparticles, (E) Hep G-2 cells treated with 10 µg/ml taxol as a positive control, (F) hep-G2 cells grow only in growth media as a negative control, (G) Hep G-2 cells treated with 100 µg/ml *R.Stricta* nanoparticles and (H) Hep G-2 cells treated with 500 µg/ml *R.Stricta* nanoparticles

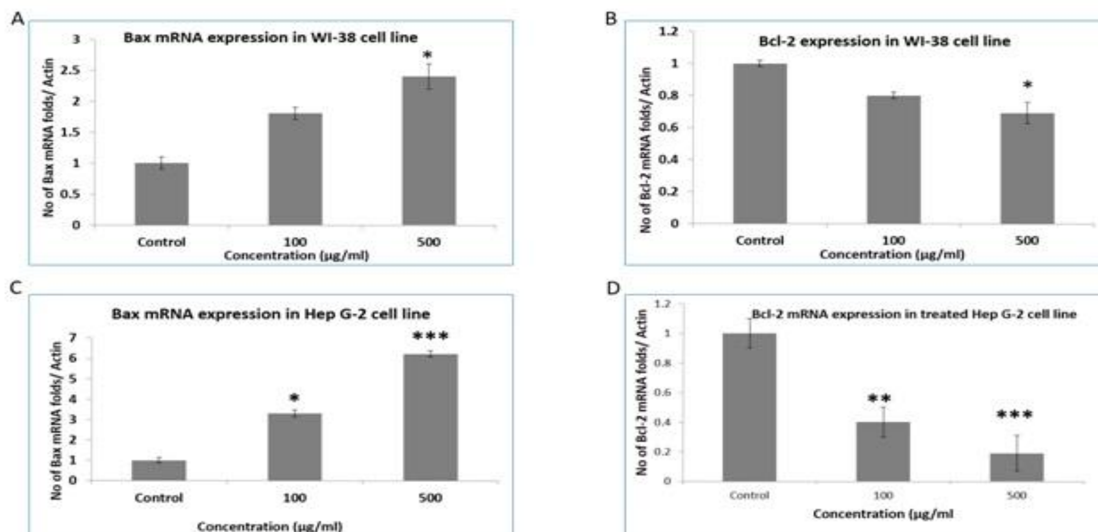

Fig. 4. Show the mRNA expression by qPCR. (A) And (C) Bax mRNA expression in WI-38 and Hep G-2 cells under indicated doses of *R.Stricta* nanoparticles, (B) And (D) Bcl-2 mRNA expression in WI-38 and Hep G-2 cells under the same experimental conditions. (*=p<0.05), (**=p<0.01) and (***=p<0.001).

4. Discussion

R. stricta is a medicinal popular plant present in many parts of Asia. . Previously, several studies investigated anti-proliferation activity of *R. stricta* extracts against different cancer cells [17-20]. However, the present study could be considered the first to evaluate R. stricta leave extracts in its nanoparticle form against different cancer cell lines ; using MTT assay that showed clear significant cytotoxic effect on all cell lines at 500 µg/ml however treatment,a high significant decrease in Hep G-2 cell viability with 100 and 500 µg/ml of *R.stricta* nanoparticles treatment compared with the other cell lines[17], [18],. To obtain a maximum amount and diversity of biologically active phytochemicals, we performed these extractions in *R. Stricta* nanoparticles techniques which needed no solvent during its applications on tissues culture. The present results were consistent with the findings of, [20] who reported that aqueous and ethanol extracts of *R. Stricta* had anti-proliferative effects in both MCF-7 and MDA-MB-231 cells. However, our results showed higher activity of the nanoparticle extracts compared to their results and proved the advantage of nanoparticles formulation in increasing antiproliferative effects of R. stricta.

The aqueous extract of *R. stricta* inhibited cell proliferation and induced apoptotic cell death of the cancer cell lines. Many researchers use these *R. stricta* compounds as a novel potential anti-tumor agent in different treatments regimens for cancer [19, 23, 24] who reported that Rhazimanine(a new indole alkaloid, rhazimanine, has been isolated from the fruits of *R. stricta*) hinders metastasis and encourages apoptosis by down regulating bcl-2 gene *in vitro* in breast cancer as well as in colon cancer, which is mediated by inhibition of NF-kB and **activator protein-1.** MCF-7 and MDA MB-231. Treatment of HCC groups with Teucrium oliverianum or R. stricta experienced significant improvement in the measured biochemical parameters as well as in the structural organization of the liver. Such effect could be attributed to having hepatoprotective properties, antiproliferative activity and antiangiogenic potential. They documented evidences for the antitumor potential against HCC induced in rats **[11- 13]**.

Moving on, gene expression and FACS analysis showed that *R.Stricta* nanoparticles significantly indued apoptotic cell death in Hep G-2 cells, real time PCR results showed high significant increase in Bax mRNA expression (3.5 and 6-fold) with 100 and 500 µg/ml of *R.Stricta* nanoparticles treatment for 24 hrs; respectively, while WI-38 cells showed a significant increase in Bax mRNA expression 2.5-fold only with 500 µg/ml *R.Stricta* nanoparticles treatment. In the other hand, Hep G-2 cells showed markedly down regulation of cl-2 mRNA expression (0.4 and 0.2 fold) with 100 and 500 µg/ml *R. Stricta* nanoparticles treatment for 24 hrs comparing with (0.8 and 0.7 fold) in WI-38 normal cells .Similar finding were reported by Al-Zharani et al. [20].

5. Conclusion:

The results of the present study indicated that the *R.stricta* nanoparticles significantly induce remarkable apoptotic cell death in **Hep G-2 and Huh-7** cancer cells

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Conflict of interest

The authors declare that there is no any conflict of interest in the current research work

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