

## In Vitro Evaluation of Anticancer Potential of *Echispyramidum* Venom (*Viperidae*) and Related Genetic and Apoptotic Profile Alterations

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### ABSTRACT

**Background:** cancer is basically a disease of uncontrolled cell division. Its development and progression are usually linked to a series of changes in the activity of cell cycle regulators.

**Objective:** the present study aimed to investigate the anticancer potential of *Echispyramidum* venom against HepG2 and HCT 116 cancer cell lines in comparison with the Cisplatin as positive control.

**Patients and Methods:** cell viability performed using MTT assay was cell type and concentration dependent was inversely proportional with the tow substances (*Echispyramidum*) venom as well as Cisplatin had IC<sub>50</sub> of 11.8 and 112.7 µg/mL and 13.4 and 71.2 µg/mL developed in HCT116 and HepG2 cell lines respectively.

**Results:** both of *Echispyramidum* venom and Cisplatin showed DNA accumulation at the Pre-G1 and G2/ M Phases of cell cycle. Apoptotic profile of treated cells showed up regulation of apoptotic genes (P53, Bax and Casp-3) and down regulation of anti-apoptotic gene (Bcl-2) in a significant way compared with untreated cell control. Also, apoptotic profile was assured via cellular changes detected microscopically in H&E stained cells. Microscopic examination showed apoptotic and slightly necrotic features in case of venoms while Cisplatin induced a number of apoptotic bodies and necrotic cell colonies.

**Conclusion:** it can be concluded that *Echispyramidum* venom has a clear anticancer potential to human liver and colorectal cancer cells that was proven through the cell cycle arrest profile, induced apoptosis detected via detection of the levels of expression of pro and anti-apoptotic genes, as well as histological alteration detected.

**Keywords:** *Echispyramidum* venom, liver cancer, colorectal cancer, Cisplatin, cytotoxicity, apoptosis, anticancer.

### INTRODUCTION

Cancer is a disease arises from single abnormal cell with mutated DNA<sup>(1)</sup>. It is caused by disturbance in the balance between the growth promoting and growth inhibitors leading to progressive and uncontrolled cell proliferation disregarding the normal process of cell division and absence of the programmed cell death process "apoptosis"<sup>(2)</sup>. According to WHO<sup>(3)</sup>, the global cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018. One in 5 men and one in 6 women worldwide develop cancer during their lifetime, and one in 8 men and one in 11 women die from the disease. Worldwide, the total number of people who are alive within 5 years of a cancer diagnosis, called the 5-year prevalence, is estimated to be 43.8 million.

Liver cancer (841,084 cases, 5% of the total) is the sixth most common cancer worldwide and the fourth most common cause of cancer death (782,000 deaths, 8.2%). Colorectal cancer (1.8 million cases, 10.2% of the total) is the third most commonly diagnosed cancer and the second most common cause of cancer death (881,000 deaths, 9.2%). In Egypt, the cancer burden is estimated to have risen to 128,892 new cases and 85432 deaths in 2018, while the number of prevalent cases (5-year) is 257,288 cases. Also, Liver cancer (25,399 cases, 19.7% of the total) is the first most common cancer in Egypt and the first most common cause of cancer death (25,084 deaths, 29.4%), while colon cancer (3,477 cases, 2.7% of the total) is the eighth most commonly diagnosed cancer and the ninth most common cause of cancer death (2,051 deaths, 2.4%).

Current traditional treatments include; chemotherapy, radiotherapy, gene therapy and hormone therapy which were found to affect both cancerous cells and intact cells as well as causing severe side effects. As a result, the use of naturally extracted drugs is becoming a more attractive alternative as opposed to traditional drugs. Snake venom is a complex mixture of bioactive peptides, proteins, enzymes, and toxins including cytotoxins, cardiotoxins and neurotoxins, which exhibits cytotoxic activities. Snake venom targets cellular metabolism alterations rendering it a potential anticancer and anti-oxidizing complex. Thus, snake venoms can induce the blockage of some specific ion channels, inhibiting angiogenesis and subsequently suppressing metastasis, and activating the intracellular pathways causing apoptosis<sup>(4,5)</sup>.

One of the most dangerous snakes in the world and lives in Egypt is *E. pyramidum*. *E. pyramidum* venom consist of specific enzymes like **PLA2**, phospholipase A2; **SVSP**, snake venom serine protease; **SVMP**, snake venom metalloprotease; **LAAO**, L-amino acid oxidase; **CRISP**, Cysteine-Rich Secretory Protein; **CTL/SNACLEC**, C-type lectins and C-type lectin like; **DIS**, disintegrin and **VEGF**, vascular endothelial growth factor<sup>(6)</sup>.

PLA2 is considered the main component of the vipers venom which causes hydrolysis of membrane phospholipids which induces the release of lysophospholipids and fatty acids resulting in the pharmacological effects within cells such as membrane damage, disruption of membrane-bound protein and functional disturbances of the cellular cascade, and

liberation of some bioactive products<sup>(7)</sup>. LAAO in Echis venom show an accumulation of tumor cells at the sub-G1 phase of the cell cycle. It also induced apoptosis via the Fas pathway<sup>(8,9,10)</sup>.

Lectins triggers apoptosis accompanied by downregulation of Bcl-2 gene expression (anti-apoptotic) and upregulation of Bax gene expression (pro-apoptotic). Once Bax is activated, it's inserted into the mitochondria causing changes in the mitochondrial membrane permeability leading to release of cytochrome C, and activation of caspase cascade pathway that results in cell apoptosis<sup>(11)</sup>. Also, lectin inhibited the proliferation of hepatoma HepG2 cells<sup>(12)</sup>. Additionally, a lectin a dose-dependent antiproliferative effect on HCT-116 cells from human colonic carcinoma by apoptosis induction<sup>(13)</sup>. Metallproteases, phospholipase A2, and disintegrins from *E. pyramidum* venom promote apoptosis as a part of their pathological mechanisms. For example, *In vitro* studies using adherent cell cultures have shown exposure to purified disintegrins or SVMP induce cellular detachment<sup>(14,15)</sup>. So, this work aims to evaluate the possible anticancer effects of some natural components such as snake (*E. pyramidum*) venom that compared to a synthetic anticancer drug (**Cisplatin**). The evaluation parameters include cytotoxicity, cell cycle, and the expression of some genes related to apoptosis by semi-quantitative RT-PCR technique to show the effect of combination on human cell line. We can expect the improvement of a new agent from snake venoms in the future which will be useful in cancer therapy.

## AIM OF THE WORK

The present study aimed to investigate the anticancer potential of Echispyramidum venom against HepG2 and HCT 116 cancer cell lines in comparison with the Cisplatin as positive control.

## MATERIALS AND METHODS

### Materials:

Liver and colorectal cancer cell lines (**HepG2** and **HCT<sub>116</sub>**) as well as *E. pyramidum* venom were purchased from VACSERA EGYPT. Cisplatin (**Cis**) Produced by Technopharma Egypt. Total RNA isolation System, Reverse Transcription System, dNTPs Mixture, Taq DNA polymerase, BenchTop 1kb DNA Ladder and DNA loading buffer were all from Promega. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (**MTT**) and RPMI media were purchased from Sigma-Aldrich.

### Methods:

#### Cytotoxicity / IC<sub>50</sub> calculation:

*E. pyramidum* venom and **Cisplatin (Cis)** were prepared as 2 mg/ ml in sterile phosphate buffer saline (**PBS**) and **DMSO** respectively. Maintenance of cell lines was performed according to manufacturer instructions, where exhausted growth medium was decanted and cells were washed using sterile **PBS** then

treated with 5 ml of 0.25 % trypsin solution (Sigma – Aldrich ,USA) for 5 minutes at 37°C. Detached cells were re-suspended in 10 ml of fresh growth medium and counted according to **Soliman, et al**<sup>(16)</sup>. Cytotoxicity Assay of - *E. pyramidum* venom and **Cis** using MTT was performed via dispensing 2-fold serially diluted venom and **Cis** on HepG2 and **HCT<sub>116</sub>** pre-cultured 96 well plates (TPP-Swiss). Morphologic changes were detected using inverted microscope (Hund –Germany). Detached cells were washed out using sterile **PBS** and residual live cells were stained with MTT stain (0.5 mg/ ml) for 4 hrs at 37°C developed purple crystals were dissolved using DMSO as 50 µL / well. Plates were incubated for 30 minutes (Jouan-France). The IC<sub>50</sub> was calculated using **Masterplex-2010** software according to **El-Garhy et al**<sup>(17)</sup>. **The study was approved by the Ethics Board of Al-Azhar University.**

### Apoptosis:

The expression of both pro and anti-apoptotic gene (**P<sup>53</sup>, Bax, Casp-3** and **Bcl-2**) were examined using rt-PCR using the following primer sequences (**p53 F 5'-CCCCCTCTGGCCCTGTCATCTTC-3'**, **p53 R 5'-GCAGCGCCTCACAACTCCGTCAT-3'**, **Bax F 5'-GTTTCA TCC AGG ATC GAG CAG-3'**, **Bax R 5'-CATCTT CTT CCA GAT GGT GA-3'**, **Casp-3-F 5'-TTC ATTATTCAGGCC TGCCGA GG-3'**, **Casp-3-R 5'-TTCTGACAG GCCATG TCA TCC TCA-3'**, **Bcl-2 F 5'-CCTGTG GAT GAC TGA GTA CC-3'**, **Bcl-2 R 5'-GAGACA GCC AGG AGA AAT-3'**, **β-actin F 5'-GTGACATCCACACCCAGAGG-3'**, **β-actin R 5'-ACAGGATGTCAAACCTGCCC-3'**)

In both cell lines; **HepG2** and **HCT<sub>116</sub>** either treated and negative control 24 hr post treatment with test products, where RNA extraction was performed according to **Dinicola et al**<sup>(18)</sup>.

### Histopathological examination:

Histopathological alterations were detected using light microscope (LICA- Japan). Detached cells were dispensed on a clean ethanol washed glass slides, kept at 37°C to dry then fixed using methanol and stained it Hematoxylin and Eosin stain.

### Statistical Analysis

The obtained results were processed statistically according to **Snedecor and Cochran**<sup>(19)</sup>, where minimum, maximum, mean value, standard deviation, standard error, and range were presented. Comparison between groups for significance was done using t-test "Difference between two means".

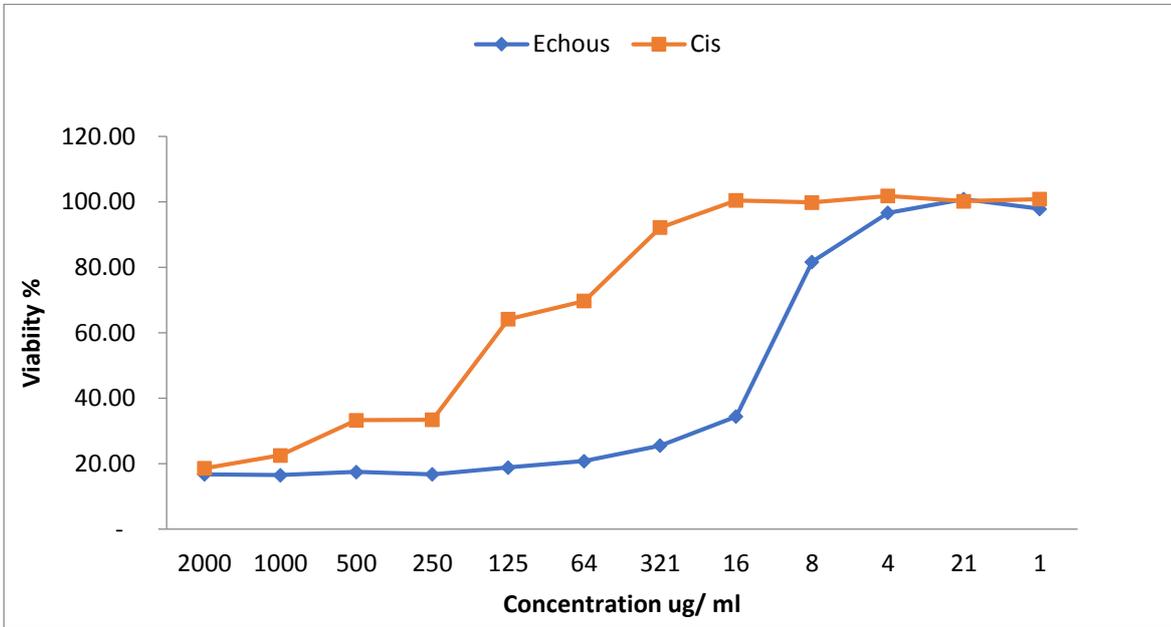
## RESULT

### I. Cytotoxicity

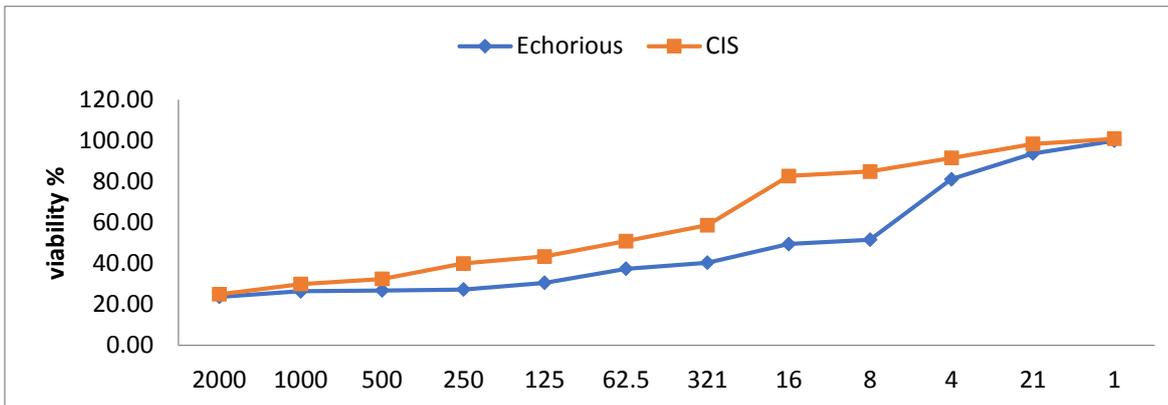
Viability of HepG2 and HCT<sub>116</sub> cells treated with both Cisplatin and *E. pyramidum* venom showed a variable

% of viability that was product, cell line and concentration dependent. Cisplatin showed a significantly ( $P < 0.05$ ) reduced viability (higher toxicity) compared with *E. pyramidum* venom on HCT<sub>116</sub> and HepG2 cell lines (Fig. 1-2). Also, data recorded revealed that the IC<sub>50</sub> of *E. pyramidum* on HCT<sub>116</sub> was significantly reduced ( $P < 0.05$ ) compared with

that induced by Cisplatin recording 11.7 µg/ml and 13.4 µg/ml for HCT<sub>116</sub> and HepG2 respectively (Fig. 3-4). While, **Cisplatin** showed a significantly higher IC<sub>50</sub> values in case of HCT<sub>116</sub> treatment than in case of HepG2. This means that Cisplatin was significantly ( $P < 0.05$ ) toxic to HepG2 than HCT<sub>116</sub> (Fig. 3-4).



**Figure (1): Evaluation of Viability % of HCT<sub>116</sub> cell line under the effect of *E. pyramidum* venom using MTT assay**



**Figure (2): Evaluation of Viability % of HepG2 cell line under the effect of *E. pyramidum* venom using MTT assay**

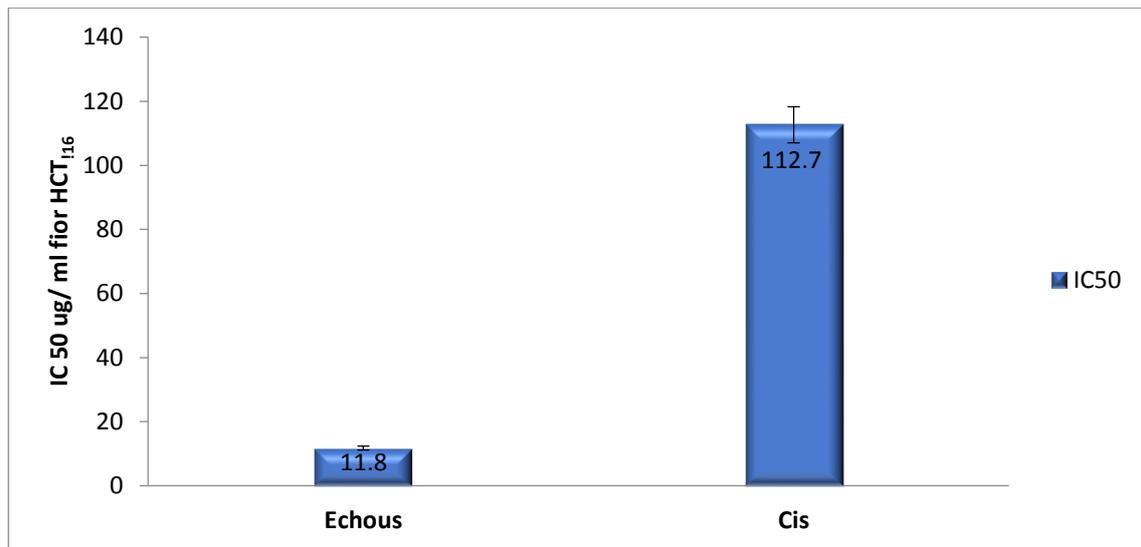


Figure (3): Evaluation of IC<sub>50</sub> of *E. pyramidum* venoms and Cis on HCT<sub>116</sub> cell line

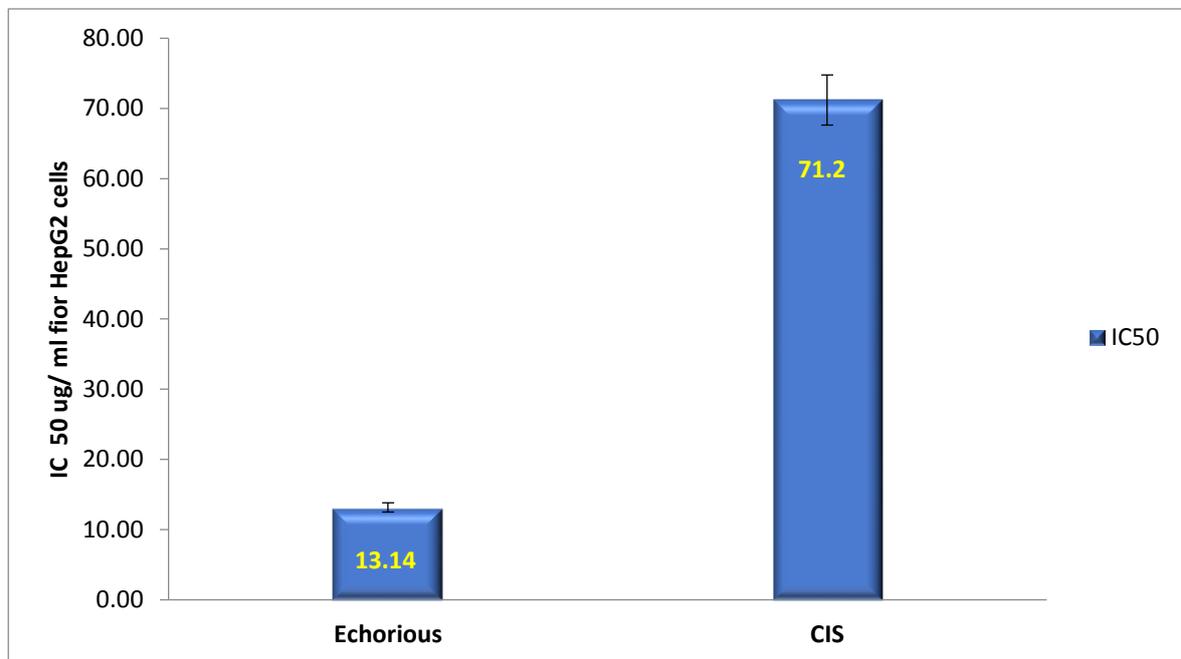


Figure (4): Evaluation of IC<sub>50</sub> of *E. pyramidum* venoms and Cis on HepG2 cell line

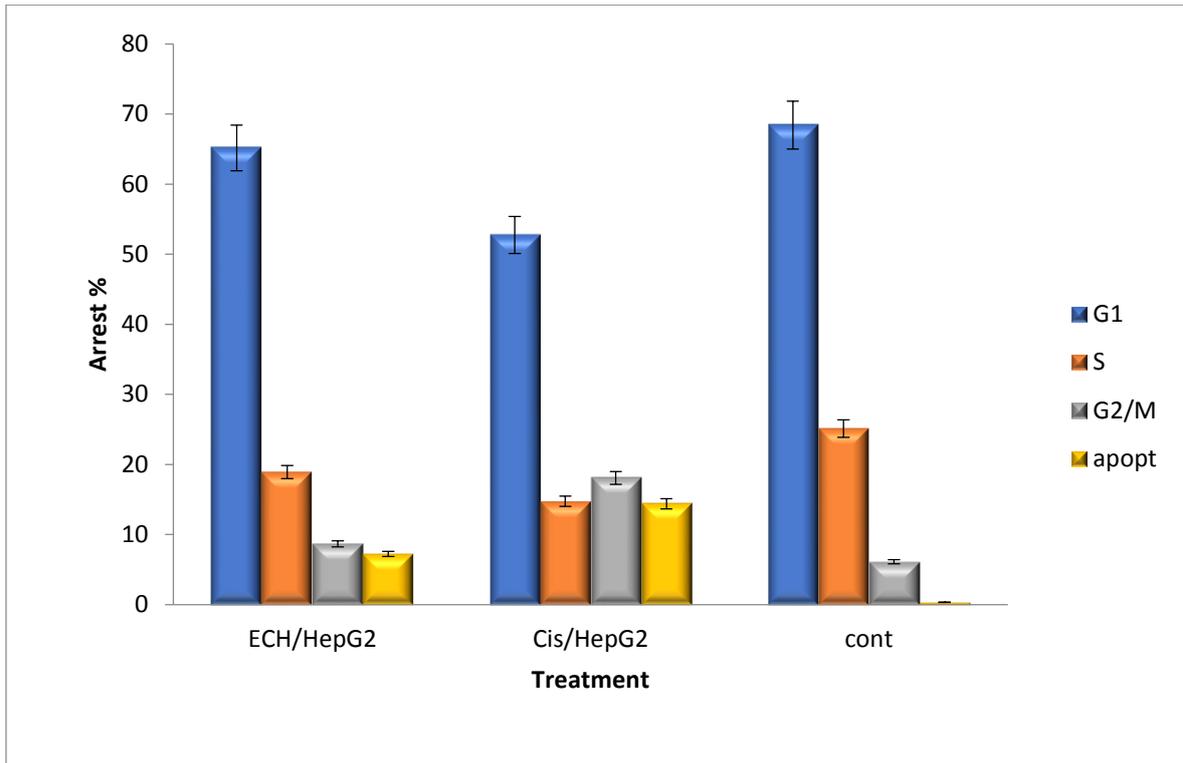
Regarding the IC<sub>50</sub> dose measured for **HepG2**, it was determined that the IC<sub>50</sub> for *E. pyramidum* was 13.14 µg/ml and Cis was 71.2 µg/ml. Thus, *E. pyramidum* venom was selected for determining their effect on the pro and anti-apoptotic gene expression level after the treatment.

#### Cell cycle profile:

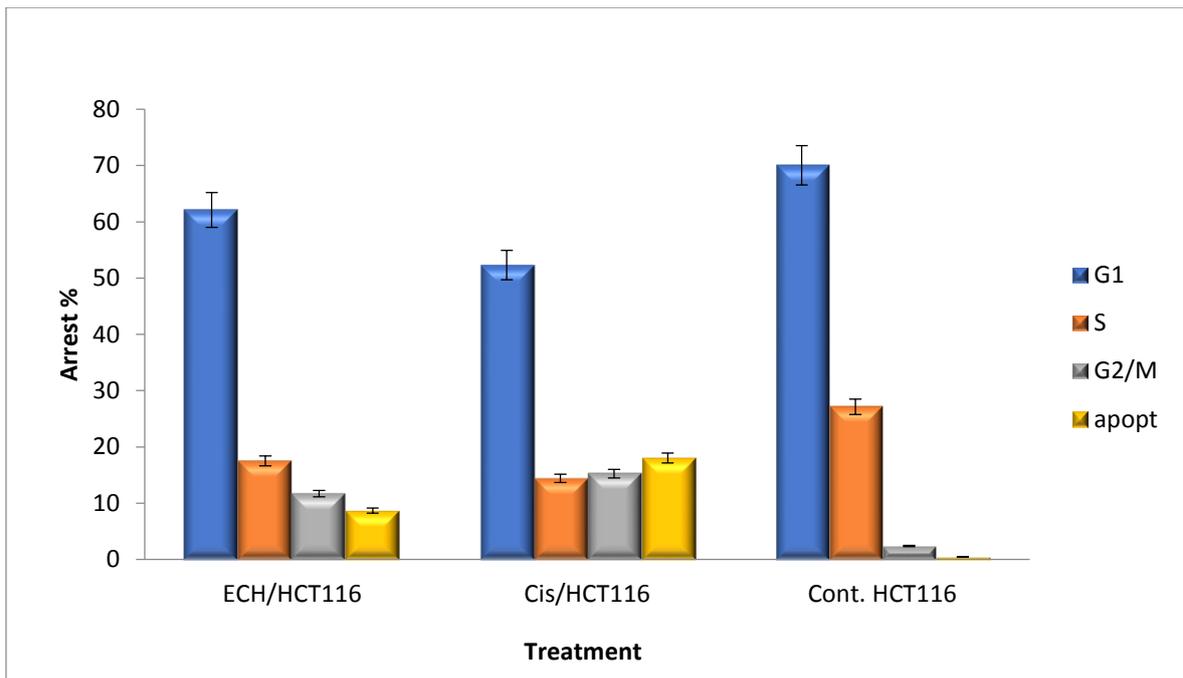
*E. pyramidum* venom affected cell viability inducing cell DNA accumulation (cell cycle arrest; CCA). It was noticed that there was a significant accumulation of cellular DNA induced during the G<sub>2</sub>/M phase in a significantly compared

with the arrest induced in untreated cell control (P<0.05).

Also, it was noticed that arrest of cells at the G<sub>2</sub>/M phase in case of Cis/HepG2 and Cis/HCT<sub>116</sub> was significantly elevated than in case of *E. pyramidum*/HepG2 and *E. pyramidum*/HCT<sub>116</sub> (P<0.05). Concurrently the total cell apoptosis showed the same pattern as cell arrest in case of comparing the effect of Cisplatin and *E. pyramidum* on both HCT<sub>116</sub> and HepG2, and treatment with both Cisplatin and *E. pyramidum* showed a significant elevated total apoptotic % than control untreated cells (P<0.05).



**Figure (5): Effect of Echis venom and Cis on HepG2 cell cycle stages**



**Figure (6): Effect of Echis venom and Cis on HCT<sub>116</sub> cell cycle stages**

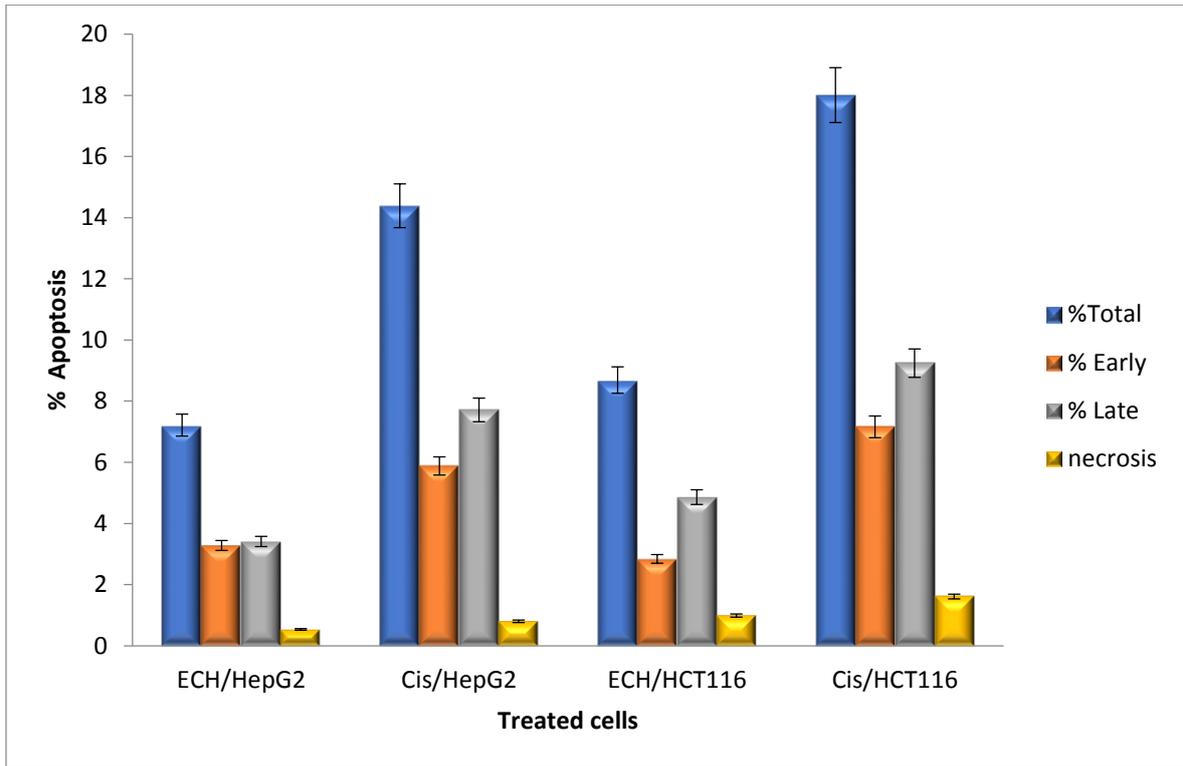


Figure (7): effect of *E. pyramidumvenom* and Cisplatinon HepG2 andHCT<sub>116</sub> cell apoptosis

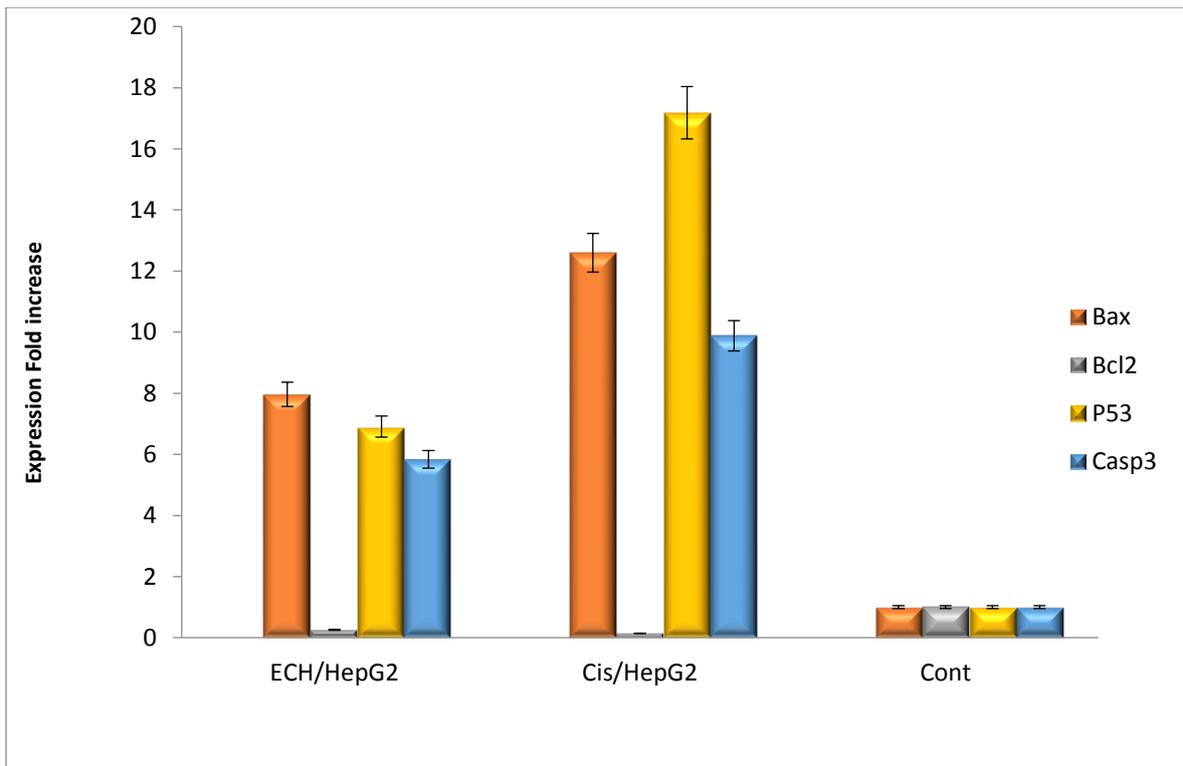
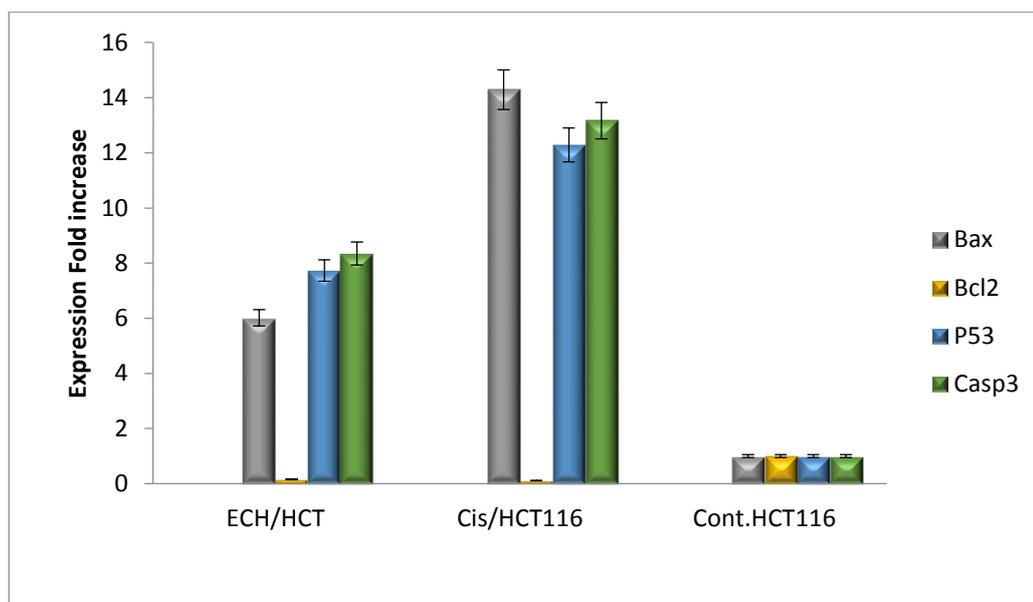


Figure (8): Evaluation of Expression profile of Pro and anti-apoptotic genes using real time PCR in liver cancer cell lines treated with *E. pyramidumvenom* and Cis



**Figure (9): Evaluation of Expression profile of Pro and anti-apoptotic genes using real time PCR in colorectal cancer cell lines treated with *E. pyramidum* venom and Cis**

## DISCUSSION

The present study aimed to estimate the anti-cancer potentials of *E. pyramidum* venom against HepG2 cell line and HCT<sub>116</sub> cell lines. The results showed that the treatment with Echis venom reduced the cell viability relative to concentration and cell line. Moreover, they induced morphological changes and apoptotic profile compared with untreated cell control.

Regarding the previous results we found that The Echis venom resulted in inhibited cell proliferation and induced morphological changes are in agreement with the results of a study done by **Khunsapet et al.**<sup>(20)</sup>, they confirmed that the action of PLA2 damaged inducing cytotoxicity, apoptosis and inhibition of cell migration at 24 h and gene damage after 72 h of incubation by MTT assay. As same as the cell line HepG2, the Echis venom showed higher toxicity more than the Cis. At concentration 1000 µg/ml, the Echis venom showed cell low cell viability about 26.46%. However, at the same concentration the Cis showed high cell viability approximately 30%. Thus, this confirms that Echis venom has high toxicity and more effect on the cells than the CIS. From the previous result, IC<sub>50</sub> of Echis venom was 11.8 µg/ml on HCT<sub>116</sub> cell line while IC<sub>50</sub> of Echis venom on HepG2 cell line was 13.4 µg/ml. This value is approximately near to the value of **Bahadorani et al.**<sup>(21)</sup>, despite their use of different cell line and they recorded an IC<sub>50</sub> value in the order of where the IC<sub>50</sub> value of *E. pyramidum* snake venom on HEK 293 cell was (14.06 ± 3.17 µg/mL) 24 hours post cell treatment.

Also, our result of lower Toxicity of CIS was in agreement with **Khalid et al.**<sup>(22)</sup> recording an IC<sub>50</sub> Value of *E. ocellatus* snake venom on skeletal muscle

cell line (L6) cells as 10.33 µg/ml. This confirms the results mentioned before which showed **Echis** venom more toxic than Cis. This may be due to the presence of lectin, LAAO and phospholipase A2. These ingredients characterized by cytotoxicity.

**Conlon et al.**<sup>(23)</sup> investigated the cytotoxic activities of purified phospholipase A2 from the venom of the *E. pyramidum* lung adenocarcinoma A549 cells and human umbilical vein endothelial cells (HUVECs) and showed concentration dependent inhibition of cells. As well as, the action of PLA2 damaged cells by causing cytotoxicity, apoptosis and inhibition of cell migration at 24 h and gene damage after 72 h of incubation. On the contrary PLA2 showed no toxicity towards normal skin cells by MTT as **Khunsap et al.**<sup>(20)</sup>. Also, it was reported that the interaction of lectin with altered glycosylation patterns in malignant cell surfaces resulting in apoptosis, cytotoxicity, and inhibition of tumor growth<sup>(24)</sup>. Lectins exhibited cytotoxic effect on HCT-15 cells from human colorectal adenocarcinoma and an antiproliferative effect by apoptosis induction<sup>(25)</sup>.

The results suggested lectins have a selective cytotoxic effect, affecting HepG2 cells more than their non-carcinogenic counterparts, whose proliferation was not significantly affected<sup>(26)</sup>.

The anticancer activities of the extracted lectin were tested against HepG2 cells. The results histological alteration of treated cells indicated that the non-cytotoxic concentration of lectin was superior in its action against HepG2 cells with inhibition percentage 80.5%. At the end of treatment, cells undergoing apoptosis and after treatment are remarked by rounding up, shrinkage, membrane blebbing and loss of cell adhesion. It was reported that

the interaction with altered glycosylation patterns in malignant cell surfaces and the lectins resulted in apoptosis, cytotoxicity, and inhibition of tumor growth<sup>(24,27)</sup>.

Our explanation to the effect of test venom may be due to the presence LAAO in Echis venom which showed an accumulation of tumor cells at the sub-G1 phase of the cell cycle. It also induced apoptosis via the Fas pathway<sup>(8,9,10)</sup>.

Few studies have been conducted to assess the effects of LAAO on the cell cycle progression as **deMeloAlves-Paiva et al.**<sup>(28)</sup> evaluated the cycle modulation and the induction of apoptosis in cells treated with LAAO, reporting that this toxin induced a delay in the G0/G1 phase and this delay may prevent the initiation of DNA synthesis and, consequently, the replication of tumor cells, which could represent another possible mechanism by which LAAO display its antitumor effects.

Also, Lectins are capable to adapt the cell cycle by encouraging both cell cycle seizure and caspase cascade, and stimulation of the genes that play the main role in apoptotic cell death<sup>(29)</sup>. Regarding the apoptotic potential of test venom was explained by **Evellyne de Oliveira et al.**<sup>(11)</sup>, that lectins activates the intrinsic caspase cascade pathway.

Lectins triggers apoptosis accompanied by downregulation of Bcl-2 gene expression (anti-apoptotic) and upregulation of Bax gene expression (pro-apoptotic). Once Bax is activated, it's inserted into the mitochondria causing changes in the mitochondrial membrane permeability leading to release of cytochrome C, and activation of caspase cascade pathway that results in cell apoptosis<sup>(11)</sup>. Metallproteases (MMPs), phospholipase A2 (PLA2), and disintegrins from *E. pyramidum* venom promote apoptosis as a part of their pathological mechanisms. For example, *In vitro* studies using adherent cell cultures have shown exposure to purified disintegrins or SVMP induce cellular detachment<sup>(14, 15)</sup>. In addition, lectins can induce differentiation, proliferation and apoptosis of many cancer cells such as colon cancer cells<sup>(27)</sup>. It was reported that lectin inhibited the proliferation of hepatoma HepG2 cells<sup>(12)</sup>. Additionally, a lectin a dose-dependent antiproliferative effect on HCT<sub>116</sub> cells from human colonic carcinoma by apoptosis induction<sup>(13)</sup>. Also, Lectins exhibited cytotoxic effect on HCT-15 cells from human colorectal adenocarcinoma and an antiproliferative effect by apoptosis induction<sup>(25)</sup>. The results suggested lectins have a selective cytotoxic effect, affecting HepG2 cells more than their non-carcinogenic counterparts, whose proliferation was not significantly affected. It was also determined that the most prevalent type of cell death was apoptosis,

with the presence of DNA fragmentation, apoptotic bodies, chromatin condensation and membrane depolarization; however, necrosis was also found<sup>(26)</sup>.

The expression level of pro-apoptotic gene p53, Bax, Caspes3 and anti-apoptotic gene Bcl-2 was measured using PCR after 24 hr treatment with Echis venom and Cis for both cell lines. Regarding the apoptotic potential of snake venom in the present study was confirmed by **Dipietrantonio et al.**<sup>(30)</sup>, explained that apoptotic effect was due to the effect of H<sub>2</sub>O<sub>2</sub> which catalyze from LAAO increase Caspes3 activity. Caspases are proteases of the cysteine family that are commonly apoptosis markers. Also, few studies have been conducted to assess the effects of SV-LAAOs on the cell cycle progression. Another interesting finding was the observation of increased positivity of the pro-apoptotic protein, Bax following *E. Pyramidum* crude venom injection. The expression of Bax protein was increased significantly in rats injected with *E. Pyramidum* venom, which indicated that the cellular injury may be the result of the elevation of BAX expression<sup>(31)</sup>. While Bcl2 show significant increase in Echis than Cis in both cell lines. These mean venoms caused a significant down-regulation of Bcl-2 compared to control cells. The latter results agree with studies that have reported the down-regulating effect of some snake venoms on Bcl-2 gene expression<sup>(32)</sup>. Also, Lectins enhanced expression of proapoptotic genes (Bax and P<sup>53</sup>) and reduced expression of anti-apoptotic (Bcl-2)<sup>(24)</sup>.

Regarding the cytotoxic effects of lectins on CRC, *in vitro* studies conducted by **Khil et al.**<sup>(33)</sup> have shown that lectins exhibit a dose-dependent effect on a cell line of colon cancer (COLO). Approximately 65% of the treated cells showed apoptosis mediated by the activation of caspases-2, -3, -8, and -9 and the inhibition of anti-apoptotic proteins.

A lectin binds to sugar containing receptor(s) present at cell surface and trigger different metabolic pathways that induce imbalance in mitochondrial bioenergetic resulting in overproduction of reactive oxygen species (ROS) and release of pro-apoptotic factor. Lectins induce necrosis mainly *via* the mitochondrial pathway through an increase in cytosolic calcium and reactive oxygen species (ROS) in tumor cells. Debio 025 and bongkreikic acid are inhibitors of mitochondrial permeability transition (MPT) widely used to evaluate the role of MPT in cell death induced by a compound (such as lectin)<sup>(11)</sup>.

The previous results of the measured gene expression level indicate that the induction of apoptosis with *E. pyramidum* venom treatment was related to the regulation of p53, Bax, Caspes3 and Bcl-2 expression level. But by detection of the expression genes (**fig. 8-9**) we found that p53, Bax,

Caspases 3 show significant increase with Cis than Echis in both cell lines.

## CONCLUSION

Finally, it can be concluded that snake (*E. pyramidum*) venom has anticancer potentials on human liver and colorectal cancer in comparison with Cisplatin current marketed anticancer drug. Venom performance was assured via detection of pro/antiapoptotic gene up/down regulation, also, DNA accumulation and nuclear fragmentation of treated cells using H&E stain.

## RECOMMENDATIONS

- 1- More cancer cell lines could be tested for verification of anti-cancer potentials of target test materials (*E. pyramidum* venom).
- 2- In vivo application of anti-cancer potentials of tested materials using murine models.
- 3- Monitoring of biochemical changes pre and post treatment with anti-cancer agent regarding the antioxidant parameters (GTH, MDA, NO, ROS etc.).
- 4- Evaluation of biochemical, pathological drawbacks of test venom relative to time, dose and venom formulation and type of cancer.
- 5- Mode of action of *E. Pyramidum* on cancer cell apoptosis must be defined.

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