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The Potential Anticancer Action of Lectin Extracted from *Pisum sativum* Against Human Hepatocellular Carcinoma Cell Lines

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ABSTRACT

Blackgrond:plant lectins, carbohydrate binding proteins, are distributed in many species of medicinal plants mainly those belong to *legumonase* family. This study aimed to investigate the anticancer activity of the lectin extracted from *Pisum sativum* on Human Hepatocellular carcinoma (HepG2). **Materials and Methods**:the morphological signs of apoptosis were examined and the ability of lectin to induce alteration in both pro-apoptotic and anti-apoptotic such as Bax, IκBα, P53 and Bcl2 genes were analyzed using RT-QPCR method. The safety usage pattern of *P. sativum* lectin was evaluated on human HepG2 cell lines. Results: lectin displayed high antioxidant activity on the Hep2 cells when compared with 5FU (anti-cancer drug as a control). Lectin dilution that exhibits LC50 on HepG2 cells was found to touch 25% from the original concentration (100%). The extracted lectin was found with inhibition percentage in cellular viability touching 80.5% with 67.6% inhibits the integration of BrdU in the HepG2 proliferated cells. HepG2 treated cells showed apparent nuclear condensation after 16 h of treatment as lectin had the ability to upregulate the gene expression of P53 and IκBα and down regulates Bax and Bcl2. Conclusion: lectin may have a vigorous role in achievement of hepatocarcinoma therapy plan.

Keywords: Lectin, hepatocarcinoma, real time PCR, antioxidants, anticancer activity.

INTRODUCTION

The pharmacological importance of plant lectins has been provoked since 100 years ago [1]. Lectin is a non-immune carbohydrate-binding protein. Although glycoproteins are present in fungi, bacteria, invertebrates and vertebrates [2], they are mainly existing in plants i.e. in leaves, barks, seeds [3,4,5]. Plant lectins (Phytolectins) are considerably contributed in cancer therapy studies since they have vital roles in many processes of the cell biology [6]. Lectins were classified based on their carbohydrate types into: mannose binding [7], glucose binding [8], galactose binding [9] but a new taxonomy systems was introduced depending on the lectins sources (animals or plants) and on their structures [10,11,12]. Plant lectins were classified into 12 different types according to their structural and evolutionary relations when compared with legume lectins, jacalins and amaranthins [2,13].

Lectins have been extensively used in many purposes [1]. They are widely involved in many cellular progressions such as cell immune response, cell–cell and host–pathogen interaction, in addition to their positive actions on tumor growth and metastatic extent [14].

Furthermore, 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. Lectins have been shown to have a positive action on the interleukins and down-regulate telomerase activity [1]. Other studies concluded that lectins have several applications such as therapeutic mediators by binding these compounds to cancer cell membranes and/or their receptors, which regulate tumor growth, anticancer agents and potential cancer markers [1]. Pea (*Pissum sativum*) lectin has been shown to induce apoptosis in Ehrlich ascites carcinoma (EAC) cells in mice. The inhibition of cancer growth in mice was shown to involve apoptosis by cell cycle seizure at G2 /M phase by enhanced expression of proapoptotic Bax and reduced expression of anti-apoptotic Bcl-2 and Bcl-XL [15]. To our knowledge, there are no studies have investigated the anti-cancer effect of lectin extracted from the seeds of the wild medicinal plant *P. sativum* (non-edible plant which belong to *legumonase* plant family). Therefore, the current study will address this issue and investigate the response of human hepatocarcinoma cell line (HepG2)

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toward the lectins and its mode of action on the treated cell lines compared to non-treated ones.

MATERIALS AND METHODS

Plant materials

Plant seeds of wild medicinal plant *P. sativum* (non-edible plant, *family legumonase*) were collected from naturally grown plants in gardens around Borg-El Arab city, Alexandria, Egypt. The plant was identified by the faculty members of botany department, Faculty of Science, Mansoura University.

Total phenolic compounds in *P. sativum* hot water extract

The concentration of total soluble phenolics in *P. sativum* extract was estimated according to Nishimura *et al.* [16] using Gallic acid as a standard. The absorbance was measured at $O.D._{760}$ nm and the total phenolics were calculated in microgram in comparing to gallic acid equivalent using the next equation which was obtained from the standard gallic acid graph: Absorbance = 0.0028 x Gallic acid (mg).

Phytochemical determination in *P. sativum* extract

The phytochemical compounds; tannins, phenols, flavonoids, alkaloids, reducing sugars, volatile oils, glycosides, amino acids and proteins, saponins and terpenoids were determined in the *P. sativum* extract according to Trowbridge [17].

Extraction of lectins from *P. sativum* seeds

Extraction and purification of lectins from *P. sativum* seeds was performed according to Al-Sohaimy *et al* [18].

Mammalian cell line

Human Hepatocellular carcinoma (HepG2) cells were cultured in RPM11640 media complemented with 200 µM l-glutamine and 25 µM HEPES buffer, N-[2-hydroxyethyl] piperazine-N-[2-ethanesulphonic acid] (all chemicals and media, Cambrex). Cells were suspended at 2×10^5 cells/ml in RPM1 culture media according to El-Aassar *et al* [19].

Total antioxidants of the extracted lectin

Total antioxidants were estimated in HepG2 treated and untreated cells. At the end of incubation time, cells were homogenized and total antioxidants were measured using Biodiagnostic kit according to the manufacture instructions.

Lectin cytotoxicity

The safe dose of the extracted lectin was tested on a normal HepG2 cell model. The cytotoxicity assay was approved according to the Borenfreund and Puerner [20]. Briefly, The HepG2 cells suspension (6×10^4 cell/ml) was planted in 96-well plates and after obtaining a semi confluent cell layer, a 100 µl of different plant extract concentrations was added. After 3 days of dose addition, cells were stained with 100 µl of neutral red stain (100 µg /ml) for 3 h. Only living cells are permeable to neutral red and assimilated it into liposomes providing a quantitative assay to the cytotoxic effects. The stain intensity was assayed at $O.D._{540}$ nm using automated ELIZA microplate reader (reference filters 620 nm).

Proliferation assay

The anti-proliferation activity of Lectin on Human Hepatocellular carcinoma (HPG2) cells was assessed using neutral red dye as referenced by El-Assar *et al* [21]. Proliferation was examined using neutral red staining depending on the evaluation of cell membrane integrity. After incubation, the stain intensity was assayed using automated ELIZA microplate reader at 540 nm (reference filters 620 nm).

Detection of apoptosis by Acridine Orange/Ethidium Bromide (A.O. / E.B.) stain

Apoptosis was detected by acridine orange and ethidium bromide according to Coligan *et al* [22]. At the end of treatment, about 25 µl of cell suspension (0.5×10^6 cells/ml) was incubated with 1µl of A.O. / E.B. solution. Mix gently, each sample should be mixed. Using a fluorescence Microscope, 10 µl of cell suspension onto a microscopic slide was surveyed. Acridine orange is a vital dye; it could stain both live and dead cells while, ethidium bromide will stain only cells that have lost membrane integrity.

Lectin and cancer marker gene expression

Lectin anticancer activity profiling was carried out and its activity was examined on the expression of P53, Bax, Bcl2 and IκBα genes in the treated HepG2 cells. HepG2 cells were culture in 12 well plates (6×10^3 cell/ml) for 2 days with the recorded nontoxic concentration of lectin. After incubation, cells were subjected to RNA extraction and RT-q PCR for quantification of the P53, Bax, Bcl2 and IκBα gene expression after and before treatment and the results were normalized using the beta actin as reference gene.

RNA extraction and cDNA synthesis

RNA extraction in treated and non-treated HepG2 cells was carried out using RNA extraction kit (Qiagene, Germany) according to the manufacture procedures. First-strand cDNA was synthesized using oligo (dT) primer and the AMV reverse transcriptase (Promega Corp., Madison, WI). The house-keeping gene β-Actin was used as internal controls for standardization of the PCR product. The first strand of cDNA synthesis was achieved in a total reaction volume of 25 uL. The reaction mixture consists of; 2.5uL of (5× MgCl2) buffer, 2.5 uL of 2.5 mM dNTPs, 4 uL of oligo (dT) primer (20 pmol), 2 ug RNA, and 200 units of reverse transcriptase enzyme (M-MLV, Fermentas, USA) and the reverse transcriptase reaction conditions were; 42°C for 1 h and 72°C for 10min. The reaction was performed in a thermal cycler (Eppendorf, Germany).

Quantitative PCR analysis for P53, Bax, Bcl2 and IκBα genes expression

RT-PCR was performed using the SYBR Green PCR Master Mix (Fermentas, USA). The 25 uL reactions consist of; 1μL of 10 pmol of each primer, 1uL of template cDNA (50 ng), 12.5 uL of 2×SYBR Green PCR Master Mix, and 9.5 μL of nuclease free water. Each sample was run in triplicate. The amplification reaction conditions were; 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Data acquisition was performed during the extension step. The reaction was performed using a Rotor-Gene 6000 (QIAGEN, ABI System, USA). After the 40 cycles, the melting curves were obtained to eliminate the inclusion of non-specific products. Sequences of forward and reverse primers used in the study are presented in Table 1.

The Bromodeoxyuridine (BrdU) proliferation assay

Bromodeoxyuridine (5-bromo-2'-deoxyuridine) is a synthetic nucleoside that is similar to thymidine. BrdU is generally used in the recognition of proliferating cells in living tissues. In this study, the BrdU assay was performed on HepG2 cells according to the manufacturer's protocol (BrdU proliferation assay; Roche Diagnostics, Mannheim, Germany).

RESULTS

Determination and measurement of total phenolic

Our results showed that *P. sativum* lectin had 900 mg Gallic acid/ 100 g total phenolics and these compounds include: falvones, flavanones, chalcones and isoflavone.

Determination of antioxidant activity

Results presented in **Figure 1** showed the total antioxidant activities of the lectin in treated and untreated HepG2 cells. The activity was scanned at wide wave length range and compared with 5FU as a standard anticancer drug. The obtained data indicated that the highest antioxidant activity was recorded in lectin-treated cells followed by the negative control cells. On the other hand, the lowest antioxidant activity was detected with 5FU treated cells.

Cytotoxicity of the lectin on human HepG2 cells

In this study, cytotoxicity of the fractional purified lectin of the wild *P. sativum* was examined on human HepG2 cells as non-cancerous cell line using neutral red assay protocol **Figure 2**. The obtained data revealed that the proper extracted lectin showed non-toxic effect and the 25% is the recommended dilution from the proper stock extracted lectin.

Lectin anticancer activity on HepG2 cell line

The anticancer activities of the extracted lectin were tested against HepG2 cells. The results indicated that the extracted lectin with the non-toxic dose was superior in its action against HepG2 cells with inhibition percentage 80.5% (**Figure 3).** At the end of treatment, cells undergoing apoptosis and after treatment are remarked by rounding up, shrinkage, membrane blebbing and loss of cell adhesion.

Lectin and cancer marker gene expression

In **Table 2**, HepG2 treated cells showed apparent nuclear condensation after 16 h of treatment as lectin had the ability to upregulate the gene expression of P53 and IκBα genes and down regulates Bax and Bcl2 genes.

The Bromodeoxyuridine (BrDU) assay and inhibition of HepG2 cell division

Figure 4 shows that the safe dose of lectin had a high inhibition percentage in BrDU hepatocellular carcinoma incorporation 67.6% (**Figure 4A&B).** The morphological changes

were verified in the treated hepatocarcinoma cells with the lectin safe dose (**Figure 4B**) compared with the control non-treated hepatocarcinoma cells (**Figure 4A**). The treated cells showed low granulation and cell desiccation which reflect the anti-cancer activity of the lectin.

Apoptosis and necrosis detection using dual AO/EB staining

The ability of lectin to convince apoptosis was visualized by acridine orange/ethidium bromide fluorescent stains (**Figure 5**). The obtained results indicated that lectin has the ability to induce all stages of apoptosis (both early and late) by converting the nucleus appearance forming multinucleated cells, condensing nucleated cells and chromatin fragmented cells that visualized under the fluorescent microscope. Cells were impermeable to ethidium bromide during early apoptotic stages and their nuclei stained green. However, during late stages, this ability was lasted and cell nuclei stained red. While, in necrotic staged cells, cellular membrane permeability was interrupted. Cells became permeable to ethidium bromide that stained nucleus with a red stain but without nuclear condensation exhibited either normal nuclear structure or without nuclear staining.

DISCUSSION

Our results showed that *P. sativum* lectin had falvones, flavanones, chalcones and isoflavone. This indicates that lectin has the phenolics bioactive compounds formed inside the plant cells and play an important role as antioxidant materials [23].

The obtained data indicated that the highest antioxidant activity was recorded in lectin-treated cells followed by the negative control cells. On the other hand, the lowest antioxidant activity was detected with 5FU treated cells. Ghasemzadeh *et al.* [24]. indicated that the antioxidants are the metabolites which inhibit the oxidation of any oxidant substrates in the cells. The antioxidants are regularly added to food to counterbalance its oxidation through the evolved free radicals that result in exposure to different environmental factors [25].

The obtained data revealed that the proper extracted lectin showed non-toxic effect and the 25% is the recommended dilution from the proper stock extracted lectin. Lectins have the propensity to differentiate between the normal and malignant cells [26, 27, 28]. Moreover, not all lectins are potent toxins and most toxic lectins kill the animal cells by striking the protein synthesis reversing their specific cytotoxic chemotherapeutic agents [29]. The plant protein ricin has emerged as the toxin of choice for such constructs. 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 [15, 30]. Furthermore, lectins from different sources inhibit cancer growth with different manner based on their concentration [31]. Furthermore, lectins have the ability to promote apoptosis and to activate the cell proliferation via immune system motivation [32]. In addition, food lectins can induce differentiation, proliferation and apoptosis of many cancer cells such as colon cancer cells [30, 33].

The lectin extracted from *P. sativum* has anticancer activities against HepG2 cells. This is in line with other studies performed on lectin extracted from different plants on different cell lines. Lin *et al.* [34] reported that lectin extracted from the glossy black soybean (Glycine max) inhibited the proliferation of both breast cancer MCF7 cells and hepatoma HepG2 cells. Moreover, Del Monte banana lectin showed activity as proliferative leukemia (L1210) cells and hepatoma (HepG2) cells [35] and the extra-long autumn purple bean extracted lectin on hepatoma HepG2 cells by prompting the production of apoptotic bodies [36].

lectin had the ability to upregulate the gene expression of P53 and IκBα genes and down regulates Bax and Bcl2 genes. Many studies have reported that Lectins stimulate apoptosis in different cancer cell lines such as B16-BL6 melanoma cells [37], human A253 cancer cells [38], HeLa cells [39], Dalton's lymphoma cells [40] murine fibrosarcoma L929 cells [41], breast cancer cells (MDA-MB-468, and MDA-MB-231HM cells [41], cancer MCF-7 cells [42]. Caspase-3 shows a vital role in apoptosis and interacts with both of caspase-8 and -9 [42]. It has been reported that apoptosis can be facilitated by passing receptors induced by lectins. This kind of reaction may be protein–protein interaction [41, 42]. On the other hand, Wang *et al.* [43] described that proteins stimulation resulted in convincing apoptosis genes such as Bax and Puma and inhibition of Bcl2 genes. In this study, the expression of the Bcl2 was down regulated in the treated cells

with the P. sativum lectin. These results were in agreement with other previous studies [40, 41, 42, 43]. Hadari *et al*. [44] stated that cell adhesion based on the interactions between proteins and carbohydrates have negative effect on the human carcinoma cells adhesive ability and meanwhile induce p53-independent cell apoptosis. This is in agreement with our obtained results where the expression of P53 gene was upregulated in cells treated with lectin. Tammy *et al*. [45] documented that lectin even more promising, augment the effects of IκBα gene. This is in agreement with our obtained results where the expression of IκBα gene was upregulated in cells treated with lectin.

The safe dose of lectin used in this study had a high inhibition percentage in BrDU hepatocellular carcinoma incorporation 67.6%. The thymidine analog (BrDU) is mutagen to target rapidly dividing cancer cells [46,47]. Therefore, it is used entirely to birth-date dividing cells because they incorporated into DNA chains [48] and sustain reasonably normal function at least in the short term [49]. It was suggested that BrDU play a role in induction of the premature senescence in a wide range types of cells [50, 51]. It has been observed that BrDU increase the sensitivity of the cancer cell to the treatment by ionizing radiation, which explained the high percentage of inhibition in the treated hepatocarcinoma cells with *Pissum* lectin [52]. It enables real-time quantification of DNA replication and it could be used as proliferation marker in the cancer cell. The morphological changes were verified in the treated hepatocarcinoma cells with the lectin safe dose compared with the control non-treated hepatocarcinoma cells. The treated cells showed low granulation and cell desiccation which reflect the anti-cancer activity of the lectin.

The current results of cancer cell apoptosis in agreement with the results were obtained by Hostanska *et al*. [53]**.** These results are in agreement with the results obtained by Faheina-Martins *et al*. [54] which postulated that lectins from *Canavalia ensiformis* repressed cell proliferation in tumor cells and that apoptosis was the main death mechanism. Meanwhile, lectins molecules have a high potentiality as antitumor. Many studies studied the anti-proliferative activity of lectins against the cancer cells both *in vivo* and *in vitro* [41, 55, 56]. It was reported that legume lectins among the seven known classes of lectin have received more attention in cancer treatment due to their significant anti-tumor properties compared to the other lectin families [54]. In Addition, both *in vitro* and *in vivo* cell death were studied (apoptosis and autophagy) in cancer cells by concanavalin A [41, 58, 59, 60, 61].

CONCLUSION

In conclusion, the findings of the current study indicate that lectin extracted from wild *P. sativum* promotes both apoptosis and autophagy in HepG2 cancer cells. Therefore, lectin may have a vigorous role in achievement of hepatocarcinoma therapy plan.

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Table 1. List of sequences of forward and reverse primers used in the study

Primer's name	SEQUENCE FROM 5`-3`
β-Actin	Forward: 5'-GGCGGCACCACCATGTACCCT-3' Reverse: 5'-AGGGGCCGGACTCGTCATACT-3'
Bcl2	Forward: 5'-TTGTGGCCTTCTTTGAGTTCGGTG-3' Reverse: 5'-GGTGCCGGTTCAGGTACTCAGTCA-3'
P53	Forward: 5'-TAACAGTTCCTGCATGGGCGGC 3' Reverse: 5' -AGGACAGGCACAAACACGCACC 3'
Bax	Forward: 5'-CCTGTGCACCAAGGTGCCGGAACT-3' Reverse: 5'-CCACCCTGGTCTTGGATCCAGCCC-3'
IκBα	Forward: 5'-GATCCGCCAGGTGAAGGG-3' Reverse: 5'-GCAATTTCTGGCTGGTTGG-3'

Table 2. Gene expression in HepG2 cells treated with lectin compared with the non-treated cells.

Target	Sample	Mean Cq	Mean Efficiency corrected Cq	Normalised Expression	Relative Normalised Expression	Regulation	Compared to Regulation Threshold
Actin	Lectin	17.52	17.52				No change
Actin	Control	34.59	34.59				No change
Bax	Lectin	39.21	39.21	0.00	0.00	-966131.9	Down regulated
Bax	Control	36.39	36.39	0.26	1.00	1.00	No change
P53	Lectin	3.20	3.20	36621.6	113.9	113.94	Up regulated
P53	Control	34032	34032	767.53	1.00	1.00	No change
BCl2	Lectin	27.01	27.01	0.001	0.002	-49.29	Down regulated
BCl2	Control	35.45	35.45	0.006	1.00	1.00	No change
IκBα	Lectin	2.76	2.76	27751.7	42.25	42.29	Up regulated
IκBα	Control	25.23	25.23	656.23	1.00	1.00	No change

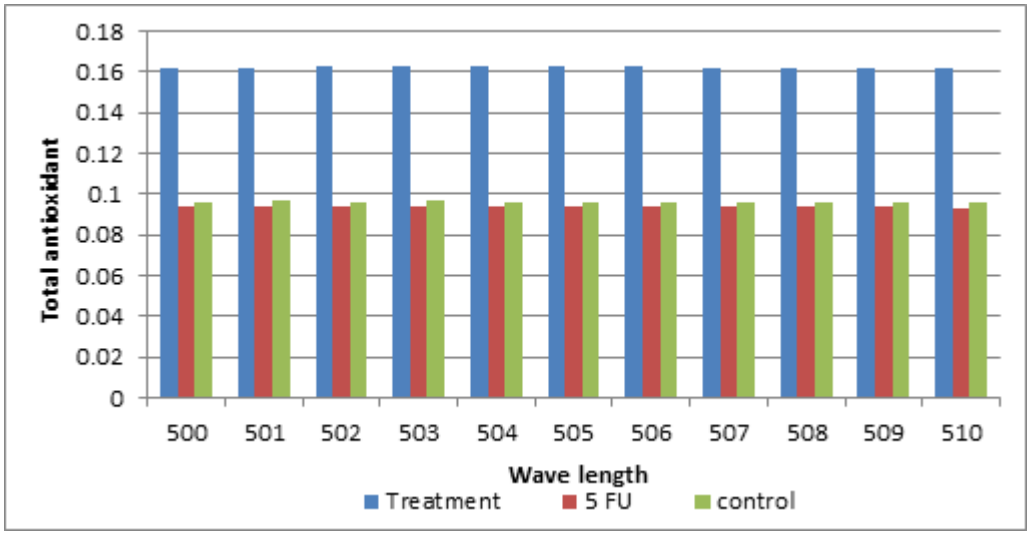

Figure 1. The antioxidant activity of the *P. sativum* lectin on the HepG2 cancer cell line

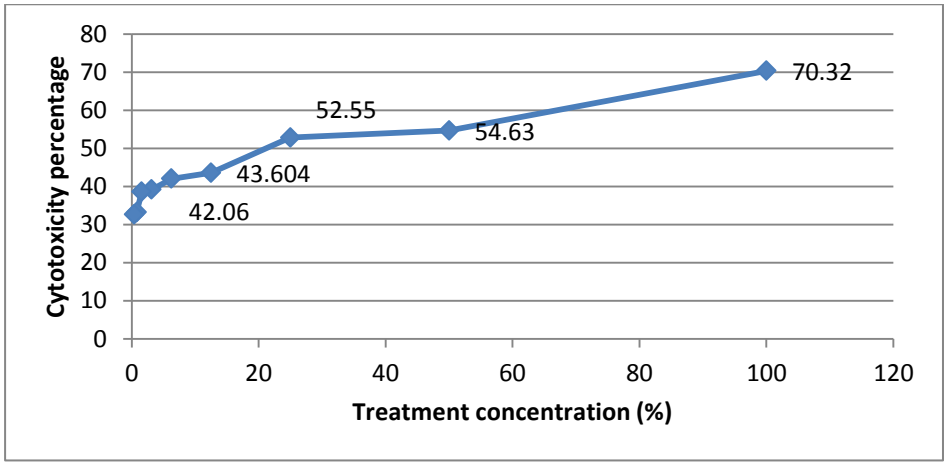

Figure 2. The safety pattern of lectin on human HepG2 cells

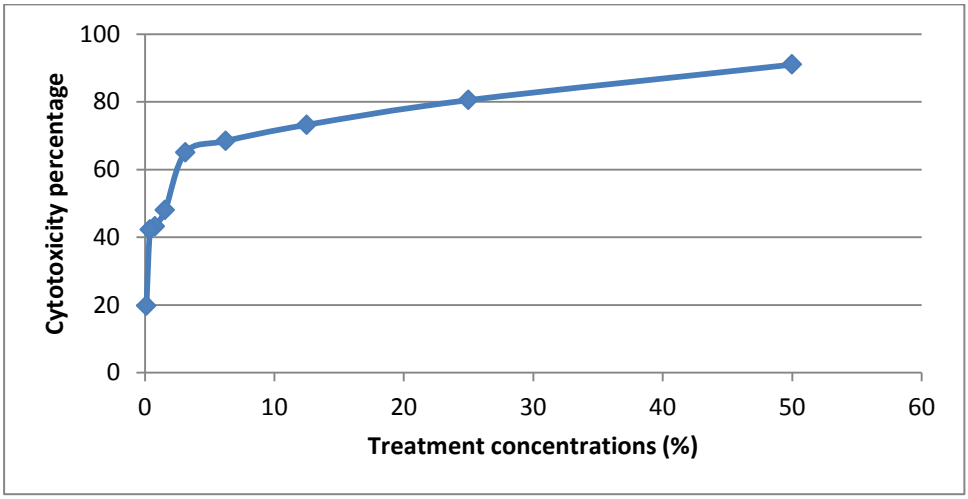

Figure 3. Anticancer activity of lectin on HepG2 carcinoma

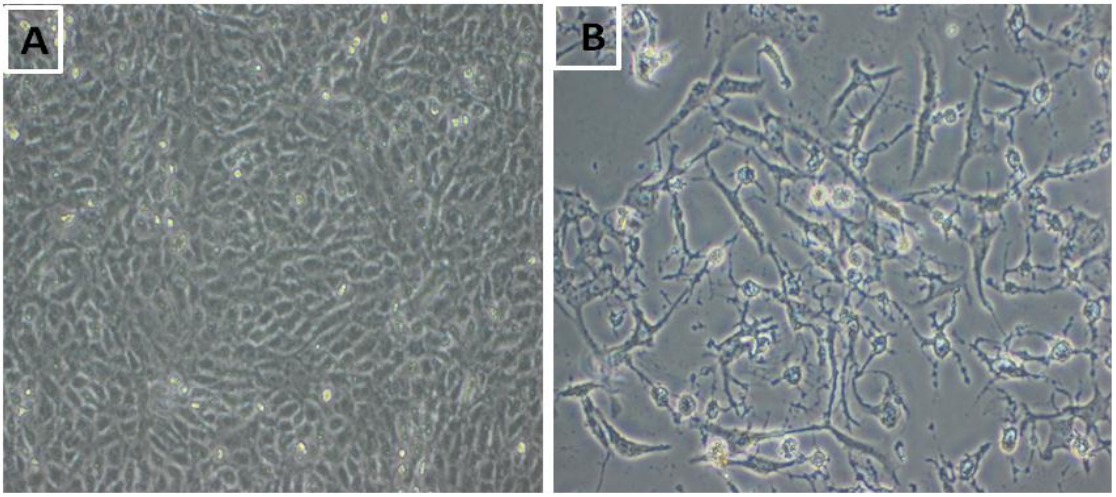

Figure 4. The effect of lectin dose on BrDU hepatocellular carcinoma, A: control cells, B: cells treated with lectin after 12 h.

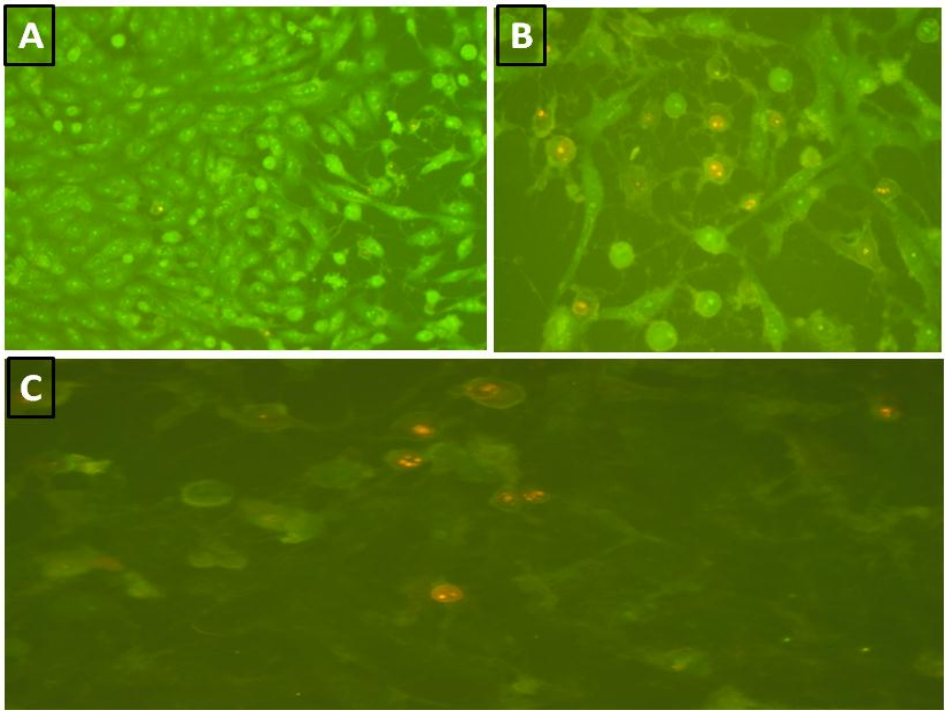

Figure 5. Effect of lectin on cell apoptosis; A: control cells; B: treated cells in early stage of apoptosis. C: treated cells in late stage of apoptosis.