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IN VITRO ASSESSMENT OF ANTICANCER ACTIVITY OF SHRIMP DERIVED CHITOSAN AND RELATED APOPTOTIC PROFILE ALTERATION

By
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Abstract

Liver breast cancer represents a major health problem worldwide. The present study evaluated the anti-cancer properties of Shrimp derived chitosan on human liver (HepG-2) and breast cancer (MCF7) cell lines. The cytotoxicity of chitosan was determined using MTT assay. Chitosan showed an anti-proliferative and cytotoxic effect against (HEPG-2) and (MCF7) cancer cell lines in a dose and cell type dependent manner. The IC_{50} value of chitosan was 600µg/ml and 348µg/ml for treated HepG-2 and MCF-7 respectively. Anticancer potential of chitosan was monitored via evaluation of cell cycle profile and pro- and anti-apoptotic genes. Cell cycle arrest profile was cell type dependent and occurred insignificantly ($P>0.05$) during S and G2-M phases in case of HepG2 cells, while it was significantly elevated during G2-M phase in case of MCF-7 treatment. Treated cell lines showed a significant apoptotic % during the Pre-G1 phase ($P<0.05$). ER and HER-2 as specific markers for MCF-7 and BCL-2 in treated HEPG-2 cells were significantly down regulated ($P<0.05$), while, p53 was significantly up regulated ($P<0.05$).

Key words: Shrimp derived chitosan, Anti-cancer activity, Apoptotic profile

Introduction

Cancer is still one of the world's major health problems, resulting in high mortality rates (Jitender *et al*, 2018). Mortality due to cancer accounted for about 15% of all death cases. Mortality rate due to cancer would continue to increase to 20% by 2030 (Chaturvedi, 2013). Chemotherapy is defined as a systemic cancer therapy in the form of direct intravenous injections or infusions, tablets, or capsules that contain anticancer or cytotoxic drugs, which disrupt the cancer cells growth and destroy them (Calastretti *et al*, 2018). Chemotherapy usually became a therapeutic modality for patients with advanced disease stages (III & IV) or in patients treated with surgery and radiation but have recurrences (Wu *et al*, 2018). The drugs damage the molecules that regulate cell division or inhibit the chemical processes that occur when the cells are proliferating (Huang and O'Sullivan, 2013). There are different sources of chitosan to obtain from crab- and shrimp shell wastes. Although from the fungi by using fermentation methods, insects, squid, prawn, lobster and Cray fish (TylIszczak *et al*, 2017). Chitosan is a polysaccharide of biological origin. The characteristics of chitosan have a degree of acetylation and molecular weight that may affect chitosan functional properties, from its solubility and materials-forming capacity to biodegradability (Bautista-Baños *et al*, 2016). The chitosan chemical structure is a straight-chain copolymer composed of D-glucosamine and Na-cetyl-D-glucosamine was obtained by the partial de-acetylation of chitin; a raw material for obtaining of chitosan. Chitosan is the most abundant basic biopolymer and its solubility, biodegradability, reactivity, and adsorption of many substrates depend on the amount of protonated amino groups in the polymeric chain (Balázs and Sipos, 2007). Number of N-acetyl groups determined the degree of de-acetylation of chitosan. Low-molecular-weight chitosan (LMWC) had selective cytotoxic effects on oral cancer cells compared to cisplatin (Wimardhani *et al*, 2012). Besides, observations of the cell death mechanisms of LMWC showed that apoptosis is not the death pathway of the primary cells involved, so remained to be study whether the other cell death mechanisms were the ones involved in

cytotoxic effects of LMWC on cancer cells (Wimardhani *et al*, 2014). The presence of histologic changes in the cells exposed to LMWC, compared to cisplatin, was interesting and needed to be more investigated, as it might provide an explanation of interaction between LMWC and cancer cells (Balázs and Sipos, 2007). More detailed changes in the cells, ultra structural and morphological analysis are required to have the information about what happens to these cells which expected to add to the knowledge of the anticancer activity of chitosan on the cancer cells (especially oral cancer cells).

The present work aimed to monitor the role of chitosan as anticancer agent against different cancer cells; liver & breast. Also, to monitor relation between cytotoxic effects of the chitosan and related morphological, genetic and cell cycle distribution profile.

Materials and Method

Cell culture and MTT staining: Low molecular weight chitosan (85% de-acetylation degree), heat inactivated fetal calf serum (FBS), L-glutamine, penicillin-streptomycin, 0.25% trypsin-EDTA were purchased (Sigma Chemicals Co, USA). Cell culture grade dimethyl-sulfoxide (DMSO), medium RPMI1640 Human liver (HEPG-2) and human breast cancer (MCF-7) cell lines were kindly supplied from R&D Sector, The International Center For Advanced Research HEPG-2 and MCF-7 cancer cells were propagated in $75cm^2$ cell culture flasks (Griner, Germany) using RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (LSP-UK) incubated in 5% (v/v) CO_2 incubator (Jouan, France) at 37°C. Confluent cells were detached using 0.25% (w/v) trypsin solution and 0.05% (v/v) Ethylene Di-amine-Tetra-Acetic acid (GIBCO-USA) for 5min. Detached cells were cold and centrifuged using (Jouan-Ki-22, France) cell pellets were re-suspended in growth media.

The cells were plated at a concentration of $2x10^5$cells/ml in 96-well cell culture plates (TPP Swiss), plated and incubated at 37°C for 24hrs to achieve confluence. Viability was assessed, as exhausted growth medium of 72hrs pre-cultured TC flasks was decanted and cells were dissociated as previous. Suspended cells were dispensed in TC plates as $2x10^5$/ml. Fresh medium containing various concentrations of 1% acetic acid dissolved chitosan was dispensed to 24hr pre-cultured cells post decanting exhausted growth medium. Dead cells were washed out 24hr post treatment using the phosphate-buffered saline (PBS) and 50µl of MTT (Serva, Swiss) stock solution (0.5mg/ml) were added/well. After 4hr incubation at 37°C, MTT solution was discarded and the developed MTT-Formazan crystals were solubilized using 50µl per well of dimethyl-sulfoxide (DMSO) supplied (Sigma Aldrich USA). Plates were incubated in the dark for 30min at 37°C and absorbance was determined at a wavelength of 570nm by using micro plate reader (Elx-800, Biotek, USA).

Cell viability percentage was calculated by using the following formula: Cell via-bility (%) =OD of treated wells x 100/OD of control:

The residual live cell viability% was blotted against the tested chitosan concentrations. IC_{50} value of test chitosan was determined using Masterplex-2010 software program. The effect of chitosan on morphological alterations of cells was analyzed using an inverted microscope (Nikon, Japan).

Cell cycle profiling: $25cm^2$ surface area pre-cultured HEPG-2 and MCF7 cells cell culture flasks were treated for 24hr with the chitosan cell line specific IC_{50} values. For cell cycle analyses, the detached and residual attached cells were harvested and fixed gently with 70% (v/v) methanol and kept at +4°C. Cells were re-suspended in PBS containing 40µg/ml PI and 0.1mg/ml RNAs and 0.1% (v/v) Triton X-100 in a dark room. After 30min at 37°C, the cells were analyzed using a flowcytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488nm. Cell cycle and sub-G1 group were determined and analyzed, as described previously.

Real time PCR: Total RNA was extracted from the control and IC_{50} chitosan treated HEPG-2 and MCF7 cells using the Gene JET RNA Purification kit (Fermantus, UK) according to the manufacturer's protocol.

The concentration and integrity of RNA were assessed spectrophotometrically at 260 /280nm. First-strand cDNA was synthesized with 1μg of total RNA using a Quantitect Reverse Transcription kit (Qiagen, Germany) in accordance with the manufacturer's instructions. These samples were frozen at a temperature of -80◦C until needed for the determination of the expression levels of P53, Bcl-2, HER2 and ER genes using real-time PCR. Quantitative real-time PCR was performed on a Rotor-Gene Q cycler by using Quanti-Tect SYBR Green PCR kits (Qiagen, Germany) and forward and reverse primers for each gene. The nucleic acid sequences of the primers were as follows: P53(F: 5'-TCA GAT CCT AGC GTC GAG CCC-3' & R: 5'-GGG TGT GGA ATC AAC CCA CAG-3') & anti-apoptotic gene Bcl-2 (F: 5'-GTG AAC TGG GGG AGG ATT GT-3' & R: 5'-GGA GAA ATC AAA CAG AGG CC-3') compared to β-actin as a housekeeping gene (F: 5'-CAA GGT CAT CCA TGA CAA CTT TG-3' & R: 5'-GTC CAC CAC CCT GTT GCT GTA G-3'). Real-time PCR mixture consisted of 12.5μL 2x SYBR Green PCR Master Mix, 1μL of each primer (10pmol/μL), 2μL cDNA, and 8.5μL Rnase-free water in a total volume of 25μL. Amplification conditions and cycle counts were a temperature of 95^{o}C for 15min for the initial activation, followed by 40 cycles of denaturation at 94^{o}C for 15s, annealing at 60^{o}C for 30s, and elongation at 72^{o}C for 30s. Melting curves were performed after real time PCR to demonstrate the specific amplification of single products of interest. A standard curve assay was performed to determine the amplification efficiency of the primers used. The relative fold changes in the expression of target genes (P53 & Bcl-2) were accomplished using the comparative $2-\Delta\Delta Ct$ method (Livak and Schmittgen, 2001). β-actin gene was used as an internal control to normalize the level of target gene expression. The $\Delta\Delta CT$ was the difference between the mean ΔCT (treatment group) and mean ΔCT (control group), where ΔCT was the difference between the mean CT gene of interest and the mean CT internal control gene in each sample.

Logarithmic transformation was performed on fold change values before being statistically analyzed, using the fold change values of three replicates for each gene measured.

Statistical analysis: Significance difference between treated and untreated cells was analyzed using one way ANOVA. Difference at P values less than 0.05 was significant.

Result

Cytotoxicity: Chitosan as a natural shrimp derived polysaccharide showed a moderate toxicity relative to concentration, as viability % was increased relative to the lowered concentration (Fig. 1) in the meantime the cytotoxic changes showed a common changes included cell rounding, detaching from TC plates/flasks surface and cellular ruptureing (Fig. 2). The inhibitory concentration (IC_{50}) was cell type dependent and HepG2 cells was significantly sensitive than MCF-7 cell line (Fig. 3).

Cell cycle profiling: The apoptotic profile of chitosan treated cell lines was monitored using annex-V (AV) and Propidium Iodide (PI) processed by Flow-cytometry. Data recorded revealed that there was a significant distributed arrested cell in the G2-M phase.

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sitive than MCF-7 cell line (Fig. 3). Cell cycle profiling: The apoptotic profile of the chitosan treated cell lines was monitored using annex-V (AV) and Propidium Iodide (PI) processed by Flow-cytometry. Data recorded revealed that there was a significant distributed arrest cells in the G2-M phase ($P<0.05$) followed by apoptosis during the pre-G1 compared with the non-treated HepG-2 cell control values. In the meantime, the cell arrest profile was significantly elevated during the G0-G1, S and G2-M phases. While, apoptosis was significantly reported in the PreG1 phase ($P<0.05$) compared with the non-treated MCF-7 cell control. The arrest profile was accompanied with apoptosis, where the total apoptotic profile of chitosan treated cell lines was significantly elevated than control. The total apoptosis profile was insignificantly elevated in case of MCF7 treated cells except the late apoptotic phase than that of HEPG2 cells (Figs. 4-6). The programmed cell death was monitored on the genetic level where the re-corded data showed a significant up regula-tion of pro apoptotic gene of HepG2 cancer cell line namely P53, on the contrary the anti-apoptotic gene BCL-2 was significantly down regulated compared with that of control cells ($P<0.05$). Also, the breast cancer cell specify genes HER-2 and ER were significantly down regulated ($P<0.05$).

Discussion

Cancer is the disease of uncontrolled cell proliferation, occasionally it might be considered a genetic disorder, also considered to be the multifactorial heterogeneous disease, and the main induction potential for morbidity and mortality worldwide (Jemal *et al*, 2011). The breast cancer (BC) is the most commonly diagnosed malignancy among the women, globally contributing to the high mortality rates (Siegel *et al*, 2018). Moreover, Cancer Stem Cells (CSCs) played an important role in drug resistance (Ashour et al, 2018). Carcinogenesis is the development of tumor that processed in the multistep process including initiation, promotion, and progression steps (Oliveira *et al*, 2007). The cellular evolution during carcinogenesis drives cells to acquire six phenotypic hallmarks of cancer, which were maintenance of the proliferative signaling, evading growth suppressor, invasion and metastasis enhanceement, inducing the angiogenesis, and the resisting apoptotic signals; initiating the transformation to malignant cells (Hanahan and Weinberg, 2011). The targeting multiple molecular pathways that the in-capability of deregulation during carcinogenesis; was the major focus on the cancer prevention and treatment. Over the last decades research was established beyond doubt that the diverse of epigenetics and environmental factors played an important role in development and progression of cancer (Minamoto *et al*, 2000; Pogribny and Beland, 2013). Despite the significant advancements in the treatment options, the incidence and mortality rates from cancer continued to increase (Jemal *et al*, 2011; Siegel *et al*, 2015). It is predictable that by the year of 2025, there will be virtually 20 million cancer patients (Siegel *et al*, 2016). Consequently, attention was focused on cancer prevention as an ultimate strategy for management of the cancer epidemic (Key *et al*, 2004; DiMarcoCrook and Xiao, 2015). In addition to that two the thirds of cancer related deaths might be prevented through lifestyle variation, mostly through the dietary means (Barnard, 2004). Cancer chemoprevention refers to the usage of synthetic or natural chemical agents that were able to prevent, or suppress the carcinogenesis processes or the tumor progression (Priyadarsini and Nagini, 2012). The dietary plants such as fruits, vegetables, spices, and nuts are important source of bioactive natural compounds with anticarcinogenic activity (Huang, 2010). So, far more than 10000 different dietary phytochemicals were described; as being widely present in the plant derived foods and beverages (Russo *et al*, 2010; Tasinov *et al*, 2012; Uzhachenko *et al*, 2015; Goda *et al*, 2018). Cancer prevention by dietary phyto-chemicals was an attractive

approach due to the natural biological activity, affordability, easy availability, lack of toxicity and ability to modulate a variety of signaling pathways and cell processes (Priyadarsini and Nagini, 2012).

In the present study, morphological changes of chitosan treated cells agreed with Yuniardini *et al.* (2014) reported that Ca922 cells exposed to LMWC showed morphological characteristics of apoptotic cell death including the membrane blebbing and cell shrinkage were observed. SEM showed that the 125 degree of membrane blebbing of LMWC-exposed cells was less than cells exposed to the cisplatin as the reference anticancer drug (Coleman *et al*, 2001). Also, the anticancer activity/cellular toxicity was attributed to the positive surface charges adsorb with the high affinity onto the negatively charged tumor cell membrane (Qi *et al*, 2005).

In the present study, BV loaded CSNPs was bind to the tumor cell membrane via the electron interactions, entered the cell via endocytosis and release the encapsulated BV into the cytosol (endosomal burst). Due to increased bio-availability, BV may exhibits its antitumor effect through up regulation of P53 and Bax and down regulation of Bcl2 genes which would eventually lead to cell death the cytotoxicity of chitosan also could be explained via its potential to preferentially delivered into the nucleus inside the cells for eliciting a better therapeutic, where chitosan is able to escape the endosomes offering high potential for nuclear delivery. Molecular entry into the nucleus occurs through the nuclear pore complexes; the efficiency of which was dependent on NP size and the presence of nuclear localization sequence (Salama and Tammam, 2015). The increased caspase activity in Ca9-22 cells post chitosan exposure. Normally, numerous microvilli would be present on the surface of the cells (Dou *et al*, 2004). No apoptotic bodies were detected by SEM in Ca9-22 cells exposed to LMWC. Paul *et al*. (2007) detected protein kinase activity in the CVPE. Heating CVPE at 60^{o}C for 30min destroyed all three activities, and suggested that one or more polypeptides in CIV induced the apoptosis. Domnina *et al*. (2004) reported that marginal blebbing as the early microfilament-dependent apoptotic event, and that it was initiated by minimal activation of the caspase-3 and the following local Rhokinase-dependent stimulation of actinmyosin cortex contractility.

In the present study, blebs resulted from changes in the contractility of the actmyosin through only partial activation of caspase-3. Also, the present SEM showed only a marginal membrane blebbing; while the TEM showed cytoplasmic alteration of LMWC treated Ca9-22 cells. Besides, positive control, cisplatin treated cells showed nuclear fragmentation and numerous mitochondria, whereas post LMWC exposure cells showed only nuclear margination with the prominent mitochondria. At the same-time, the Ca9-22 cells exposure to LMWC induced G1/S cell cycle arrest, This might have involved changes in the expression of proteins regulating this phase, especially those that prevent cells from entering S phase, and might take place in a P53 independent setting (Vermeulen *et al*, 2003). The presence of DNA damage sometimes required a rapid response that did not involve transcription or translation, and therefore protein needed to be synthesized faster (Bartek and Lukas, 2001). When the checkpoint was presented in the early G1/S phase, there was possibility that Cdk-4/Cdk-6-Cyclin complex would be inhibited by the CKI P15 and P27 induced by TGF-β. This would prevent RB phosphorylation independently of P53 (Reynisdóttir *et al*, 1995).

Salehi *et al*. (2017) reported that the initial microscopic examination of cells after 24hr showed vivid morphological alterations in cancer cells such as the cell rounding, detachment, and cytoplasmic vacuolation indicating the CS cytotoxicity. The physical and biologic properties of chitosan are dependent on its molecular weight (MW) and degree of de-acetylation (DD). The cytotoxicity of chi-

tosan as a soluble molecule was reported as a function of MW, DD, and concentration (Panel *et al*, 2015). Also, anti-proliferative activity was measured by MTT assay and IC50s was calculated. As exposure of three breast cancer cell lines to low Mw CS with 85% DD clearly decreased cellular viability in a dose and time dependent manner while inducing a much less toxic effect on normal L929 cells, indicating a favorable selectivity towards cancer cells. Among the breast cancer cell lines tested, MDAMB-231 was a highly invasive, basal-like phenotype31 and often considered as an apoptosis-resistant cell line (Salehi *et al*, 2017). Thus, to study the mechanism by which CS decreases cell viability in breast cancer cells, the study focused on MDA-MB-231 cells. CS induces apoptosis in MDA-MB-231 cells, and in order to verify apoptosis as the underlying mechanism of CS growth inhibitory effect, AO/EB staining, Annexin V-FITC/PI MDAMB-231, T47D, and MCF-7 cells after 24hr treatment and staining with AO/EB showed clear apoptotic morphological alterations, while, L929 normal cells did not give significant changes (scale bars=50mm). Annexin V-FITC/PI Flow cytometry analysis of MDAMB-231 cells treated with CS IC50 for 4hr induced (21.91%) shift of cell population to right compared to control (4.56%) indicated a significant apoptotic cell population in CS treated cells. Cell nuclei and cell membrane integrity of the control samples did not change significantly, while treated ones showed different extents of chromatin condensation, nuclear fragmentation, and destruction of cell membrane integrity. To further confirm apoptosis, MDA-MB-231 cells were treated with IC50 concentration (290mg ml) of CS for 4hr and apoptosis induction was evaluated by Annexin V-FITC/PI double staining. Annexin V-FITC was capable of binding tightly to phosphatidylserines externalized to outer plasma membrane during early stages of the apoptosis, and simultaneously, the propidium iodide was included to differentiate between apoptotic and necrotic cells. This approach allowed for the further distinction of early apoptotic (Annexin V+/PI) and late apoptotic/necrotic (Annexin V+/PI+) cells.

The present results showed that the CS markedly induced apoptosis, and population of early apoptotic cells was 34%, higher than untreated ones. DNA fragmentation was a major hallmark of apoptosis restricted endonuclease activity and were evaluated quantitatively by TUNEL assay. DNA fragmentation in MDA-MB-231 cells treated with IC50 concentration of CS for 4hr, quantitated by Flow cytometry as compared to non-treated control cells 21.9% apoptosis was observed. CS induced apoptosis was attributed to generated ROS. So, to examine changes in intracellular ROS level and ROS involvement in apoptosis induced by CS, DCFH-DA assay was conducted on the MDAMB-231 cells treated with 70, 145 & 290mg/ml CS for 12hr and 9.29, 13.40 & 20.50% increase in ROS generation respectively, but, untreated cells showed 3.69% DCF positive cells. Consequently, the CS triggered ROS generation in a dose-dependent manner in treated cells.

Conclusion

The shrimp (Crustacean) derive chitosan proved to have anticancer activity that assured via induced cell toxicity that was cell type and concentrations dependent. Also, the cell cycle, cell arrest profile was accompanied with the up and down regulation of the pro-apoptotic and the anti-apoptotic genes.

Recommendation

Evaluation of anticancer activity of free and natural and synthetic anticancer material loaded chitosan to maximize the anticancer potential finally evaluation of anticancer activity against deferent cancer cell lines and arrange for in vivo studies. Also, evaluation of antioxidant level that may the anticancer may attributed.

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Explanation of figures

Fig. 1: Evaluation of HepG2 and MCF-7 cell lines using MT assay relative to concentration

Fig. 2: Evaluation of inhibitory (IC50) of chitosan treated HepG2 & MCF-7 cell lines using MA sterplex.

Fig. 3: Evaluation of cytoxic effect of chitosan of both liver (HepG2) and breast (MCF-7) post exposure to dissolved chitosan

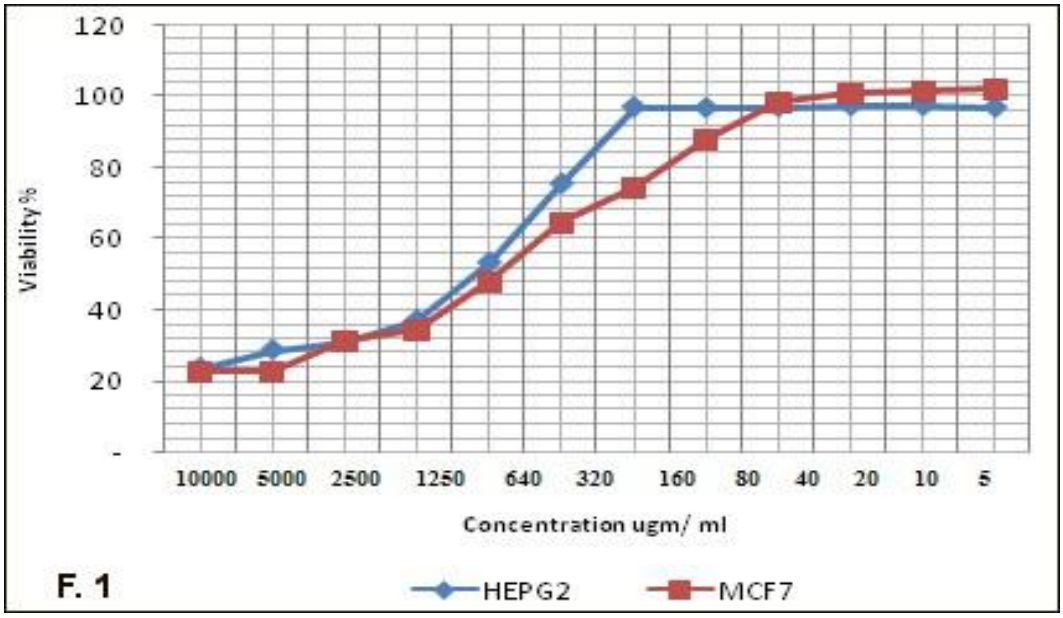
Fig. 4: Evaluation of cell cycle and apoptotic profile of chitosan MCF-7 treated cells: A: cell cycle & B- apoptosis; Non-treated cell control: C- cell cycle & D- apoptosis.

Fig. 5: Evaluation of cell cycle and apoptotic profile of chitosan HepG2 treated cells: A- cell-cycle & B- apoptosis; Chitosan non-treated: C- cell cycle & D- apoptosis

Fig. 6: Evaluation of cell cycle arrest of MCF-7 & HepG2 cell lines by Flow-cytometry

Fig. 7: Evaluation of propedium iodide staining apoptotic profile by Flow-cytometry

Fig. 8: Evaluation of pro- and anti-apoptotic genes using real time PCR post HepG2 and MCF-7 treated with chitosan


120
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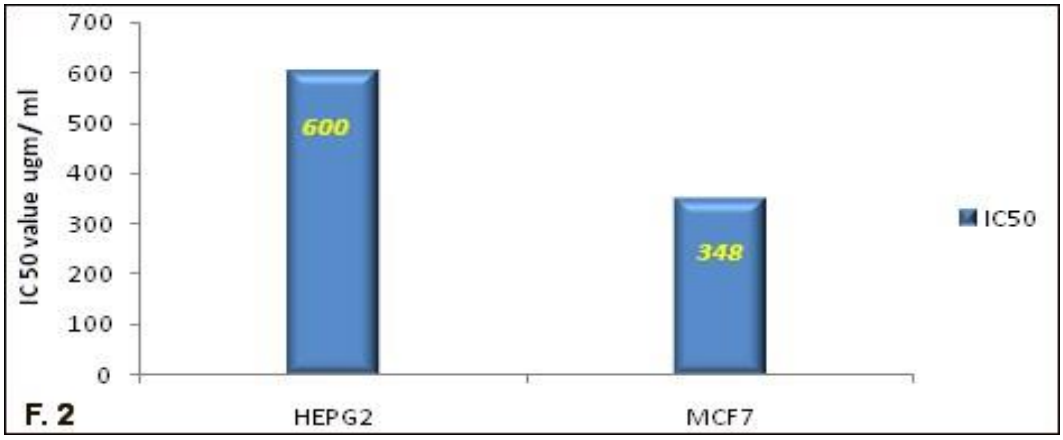
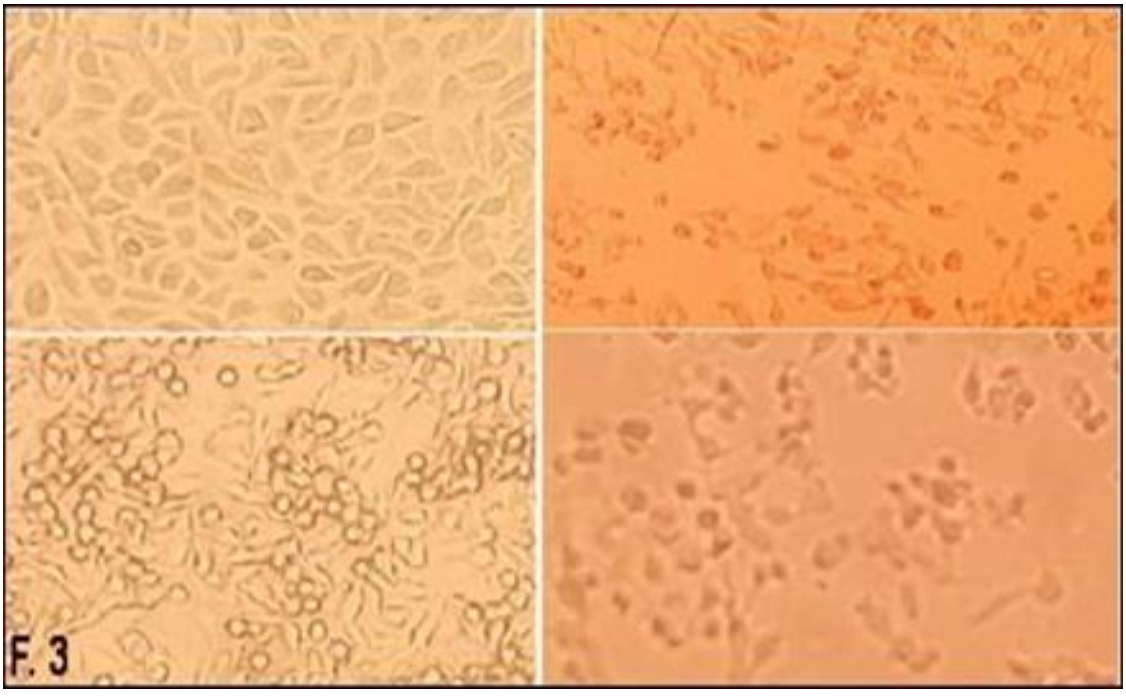
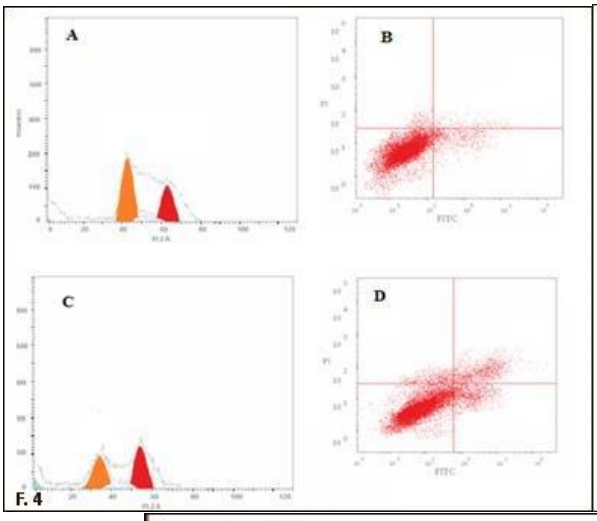
-
Viability %
10000 5000 2500 1250 640 320 160 80 40 20 10 5
Concentration ugm/ ml
HEPG2
MCF7

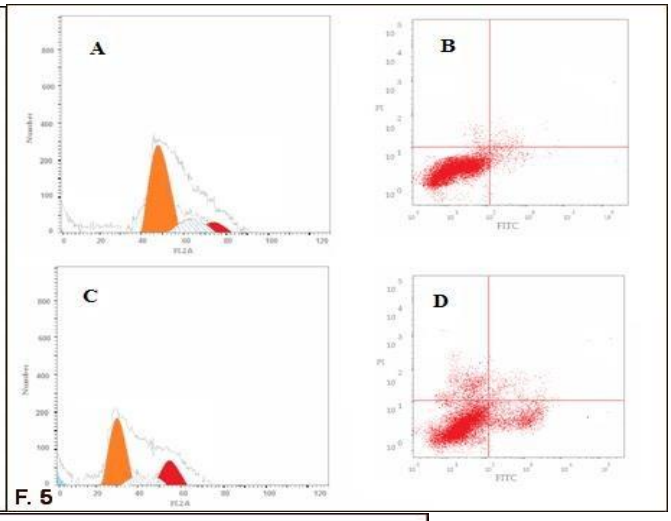
F. 1

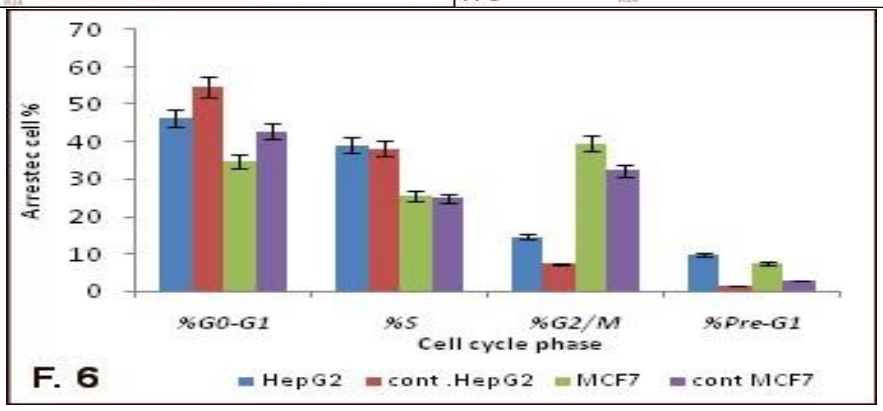

700
600
500
400
300
200
100
0
IC 50 value ugm/ ml
600
348
IC50
HEPG2
MCF7

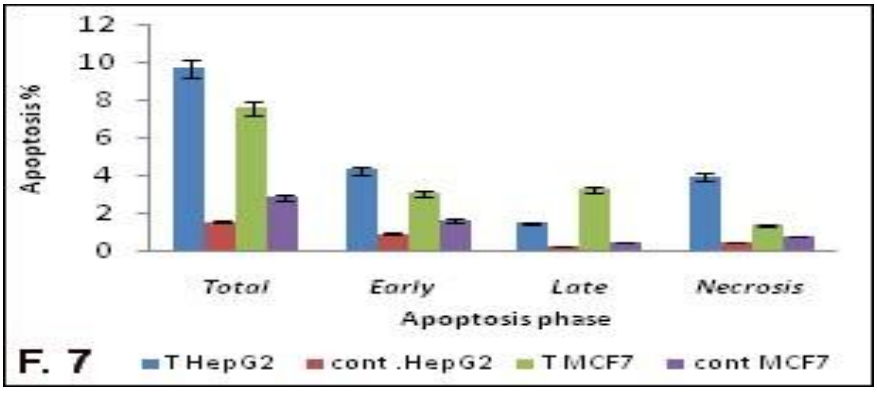
F. 2

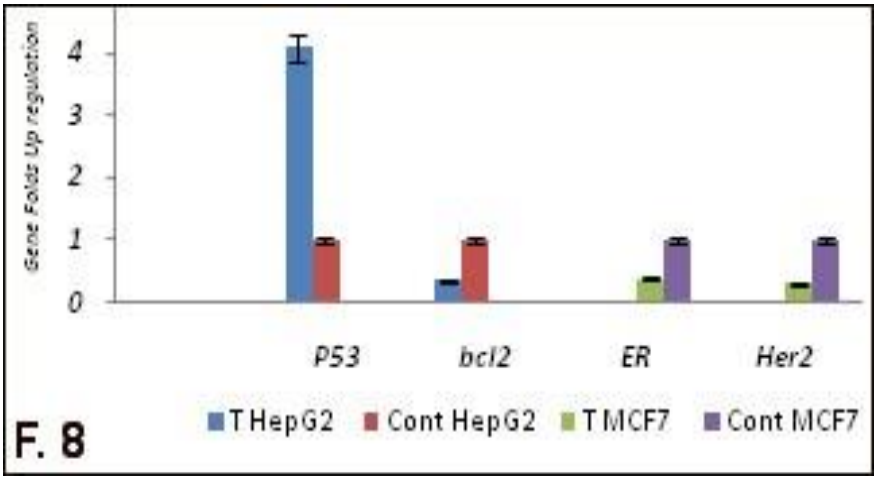

F. 3


A
B
C
D
F. 4


A
B
C
D
F. 5


Arrestec cell %
70
60
50
40
30
20
10
0
%G0-G1
%S
%G2/M
%Pre-G1
Cell cycle phase
HepG2
cont .HepG2
MCF7
cont MCF7
F. 6


Apoptosis %
12
10
8
6
4
2
0
Total
Early
Late
Necrosis
Apoptosis phase
T HepG2
cont .HepG2
T MCF7
cont MCF7
F. 7


Gene Folds Up regulation
4
3
2
1
0
P53
bcl2
ER
Her2
T HepG2
Cont HepG2
T MCF7
Cont MCF7
F. 8