RSC Advances

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Unraveling the caspase mediated mechanism for phloroglucinol-encapsulated starch biopolymer against breast cancer cell line (MDA-MB-231)

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

The main objective of the study is to unravel the mechanism underlying the anticancer activity of starch encapsulated polyphenolic (phloroglucinol) compound on breast cancer cell line (MDA-MB-231) in the form of biopolymer. MTT assay confirmed that, MDA-MB-231 cells were highly susceptible during the treatment of biopolymer in a dose-dependent manner. Morphological evidence by fluorescence staining revealed chromatin condensation, nuclear beading and loss of mitochondrial membrane potential. DNA fragmentation and cell cycle analysis confirm the progression of apoptosis in MDA-MB-231 breast cancer cell line. The semi-quantitative RT-PCR showed increased pro-apoptotic genes such as Caspase-3, 8 and 9. Western blotting analysis was done to substantiate the Caspase-3 and 8 expressions in an efficient execution of apoptosis. To conclude, the controlled release of phloroglucinol a polyphenolic compound from starch induces cytotoxicity against MDA-MB-231 breast cancer cell line.

Introduction

The recent advent of science and engineering has produced several new chemical molecules with improved biological activities. However, the use of known conventional drugs leads to immediate release in an unprecedented rate.[1] This can be minimized by controlled release systems, diminishing other side effects with superior therapeutic efficiency.[2] To achieve this, numerous polymeric materials have been employed in manufacturing drugs having controlled release properties. Nevertheless, the use starch has gathered momentum due to its non-toxic, eco-friendly and biocompatibility unlike other synthetic chemicals.[3] The presence of numerous hydroxyl groups in starch can anchor a wide variety of natural and chemical entities.

Polyphenolic compounds are secondary metabolites found in simple prokaryotes to complex eukaryotes.[4] Despite their availability, these compounds are reported to have several aromatic rings bearing one or more hydroxyl groups making this compound to be most studied. During the process of extraction from natural source, these compounds undergo a variety of degradation and require special storage conditions.[5] To circumvent these drawbacks, better encapsulation of polyphenolic could possibly be employed to protect their structural integrity and biological activity. Keeping this in mind, we recently reported the encapsulation of polyphenolic compound (phloroglucinol) onto soluble starch in the form of the biopolymer.[6]

Phloroglucinol are naturally occurring polyphenolic compounds found abundantly in marine brown seaweeds.[7] The enormous biological and pharmaceutical importance of this compound has extended several researchers to synthesis various derivatives of phloroglucinols.[8] On the other hand, targeted release of phloroglucinol into the biological system would be more effective than as bulk. It have been shown that phloroglucinol-encapsulated starch biopolymer possesses improved antioxidant and cytotoxic effect against HepG2 hepatocellular carcinoma cancer cell line.[6] However, the exact mechanism involved in progression of cell death is unclear. Hence, the present study was carried out to unravel the molecular mechanisms involved in the killing effect of biopolymer against breast cancer cell line (MDA-MB-231).

Materials and methods

Chemicals and Reagents

Phloroglucinol, Methylthiazolyldiphenyl-tetrazolium bromide, Acridine Orange, Ethidium Bromide, Hoechst – 33258, agarose (low & high), Propidium iodide, JC-1 were purchased from Sigma Aldrich (St. Louis, MU, USA). Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), antibiotics (streptomycin & gentamycin) was procured from HiMedia laboratories, India. Gene specific primers for β-actin, caspase – 3, 8 & 9 were obtained from Eurofins India. Trizol reagent (Invitrogen, USA), Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA), polyvinyl difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), enhanced chemiluminescence blotting detection kit (Perkin Elmer Life Sciences, Inc., Boston, MA, USA) were also used in this study. Rabbit monoclonal antibody

for Caspase – 3 & 8 were procured from Abcam, USA and β-actin used in the study was obtained from Santa Cruz (CA, USA).

Preparation of phloroglucinol-encapsulated starch biopolymer

Phloroglucinol-encapsulated starch biopolymer (Fig. 1) was prepared as previously described.[6] The loading efficiency of phloroglucinol into the soluble starch was determined spectrophotometrically at 267 nm was 97 %. The H'NMR data exhibited by the biopolymer was comparable with the literature values obtained earlier.

Cell culture and growth

The MDA-MB-231 breast cancer cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's modified eagle minimal medium supplemented with 10 % fetal bovine serum, 100 UmL^{-1} streptomycin, 100 $mgmL^{-1}$ gentamycin. The cells were grown at 37 ºC with 5 % (v/v) CO_2 in a humidified incubator.

MTT assay

To evaluate the cytotoxicity of biopolymer, MTT assay was performed against MDA-MB-231 breast cancer cell line.[9] Cells were seeded onto 96 well plates at a density of 1×10^5 cells/well. The MTT assay was performed in triplicates for test compounds such as biopolymer, phloroglucinol and soluble starch at different concentration (0–250 μgmL^{-1}). The half-maximum inhibitory concentration value (IC_{50}) for MTT assay was obtained by plotting a bar graph using OriginPro. 8.0. In addition, the free phloroglucinol present in the encapsulated biopolymer was determined by 2, 4-dimethoybenzaldehyde (DMBA assay) as reported earlier [6].

Morphological staining

MDA-MB-231 cells were seeded in a six-well plate at a density of 1 x 10^5 cells/well. After 24 h, the cells were detached from the surface of the plates and treated with IC_{50} concentration of the biopolymer. The cultured plates were incubated at different time intervals at 24, 36 and 48 h respectively. After the desired incubation, both live and dead cells were collected, incubated and centrifuged at 4000 rpm. The obtained pellet in the form of cell suspension was washed with phosphate buffer saline (PBS) twice, stained with AO/EB (1:1 ratio) and Hoechst 33258 (5 $mgmL^{-1}$). The stained cells were visualized under a fluorescence microscope (Olympus BX-51, Tokyo, Japan) and photographed. The untreated cells were used as control.

JC-1

MDA-MB-231 cells at a confluence of 2 x 10^5 cells/well were trypsinized, centrifuged and collected for counting using hemocytometer. Later in a six-well plate, the cells were incubated (at different time intervals – 24, 36 and 48 h), supplemented with growth media and treated with IC_{50} concentration of biopolymer. At predetermined intervals, the cover slips were stained with a working solution of JC-1 at 37 ºC for 20 minutes [Working solution – 20µl of stain from stock solution in 1 ml of DMSO; stock solution - 5 $mgmL^{-1}$]. The adhered cells in cover slip were kept inversely on a glass slide and visualized under a fluorescence microscope (Olympus BX-51, Tokyo, Japan) using 377-355 nm filter, at 400x magnification and photographed.

DNA laddering

The cells were harvested (after 24 and 48 h treatment with biopolymer), centrifuged (at 10000 rpm for 10 min at 4 ºC) and the pellet was maintained in lysis buffer (10mM Tris Cl, pH. 7.5, 1mM EDTA and 0.2 % Triton x-1000) for 30 min, and the supernatant was collected and incubated with DNA extraction buffer (0.1 $mgmL^{-1}$ proteinase K & 0.2 $mgmL^{-1}$ RNase) for 1 h, at 60 ºC. The resulting mixture was extracted with phenol/chloroform (1 : 1) and allowed to run on agarose gel containing 2% Ethidium bromide. The DNA fragments were visualized under a UV-transilluminator and photographed.

Cell cycle analysis

The cells treated with biopolymer at different time interval (24 & 48 h) were trypsinized, collected, centrifuged (12000 rpm for 10 min) and resuspended in 1 ml of ice cold phosphate buffer solution (free from mg & ca). Then, the cells were fixed with ethanol at 4 ºC for 12 h, and stained with Propidium Iodide before analysis by Flow cytometry (FACS calibur, BD Biosciences).

Caspase activity assay

The caspase activity assay was determined by a chromogenic assay using caspase – 3 (Calbiochem, Merck) and caspase – 8 (Chemicon International Inc.) colorimeter activity assay kit. Briefly, after treatment of biopolymer at different time intervals (24, 36 and 48 h), 1.5 x 10^6 cells were harvested, lysed with cell lysis buffer (50mM HEPES,100mM NaCl, 0.1 % CHAPS, 1mM DTT, 100 mM EDTA) followed by centrifugation at 10,000 rpm for 1 min. About 50 µl of supernatant was incubated with specific substrate (at 37 ºC) for 2 hr in a water bath. The absorbance of the cleaved substrate was measured at 405 nm using a microtitre plate reader (BioRad, UK).

Semi-quantitative RT-PCR

The total RNA from the IC_{50} treated biopolymer at predetermined intervals (24, 36 & 48 h) was prepared by using Trizol reagent (Invitrogen, USA). 1 µg of RNA was reverse transcribed and amplified by one step semi-quantitative RT-PCR using specific primers (Supplementary Table. 1). The conditions for PCR are as follows: 1 x (94 ºC for 5 min); 35 x (94 ºC for 1 min; 55 ºC for 1 min & 72 ºC for 1 min) & 1 x (72 ºC for 10 min). The amplified products were verified by 1 % agarose gel electrophoresis under a UV-transilluminator and then photographed. β-actin used in the study served as an internal control.

Western blotting

After the treatment with IC_{50} concentration of biopolymer (24, 36 & 48 hr), the adherent and floating cells were harvested and lysed in lysis buffer (50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 0.02 % sodium azide, phenyl methane sulfonyl fluoride (PMSF), aprotinin, and 1% Triton-X 100) for 30 min. The lysed cells were centrifuged at 12,000 rpm for 10 min at 4 ºC. The total protein content present in the sample was determined by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The obtained protein (30 µg) was subjected to 12.5 % sodium dodecyl sulfate-polyacrlamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinyl difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was then blocked and probed with primary antibodies (Caspase – 3 & 8) overnight at 4 ºC. The membrane was again incubated with horseradish peroxidase conjugated-secondary antibody for 2 h at room temperature. Finally, the blots were visualized using enhanced chemiluminescence blotting detection kit (Perkin Elmer Life Sciences, Inc., Boston, MA, USA). β-actin was used as an internal control.

Result and discussion

In vitro cytotoxic assay

The *in vitro* cytotoxicity of soluble starch, phloroglucinol and phloroglucinol-encapsulated starch biopolymer was investigated against MDA-MB-231 cell line using MTT assay. The cytotoxicity of biopolymer is pronounced to have a high inhibitory effect on MDA-MB-231 breast cancer cell line in a dose-dependent manner with an IC_{50} value of 187.5 μgmL^{-1} (Fig. 2). However, the phloroglucinol (255.54 μgmL^{-1}) and soluble starch (854.67 μgmL^{-1}) show reduced cytotoxic effect as compared to the biopolymer. The increased cytotoxic effect of biopolymer may well be compromised by the loss of cell membrane integrity. Altogether, the controlled release phloroglucinol within the cancer cell at physiological pH (7.4) induces cell death leading to apoptosis.[6] Meanwhile, the actual phloroglucinol present in the biopolymer was found to be 45.49 μgmL^{-1} by DMBA assay.

AO/EB staining

The morphological changes within the cells were examined by using Acridine orange/Ethidium bromide (AO/EB) by treating the cancer cell line with biopolymer (IC_{50} - 187.5 μgmL^{-1}) at different time intervals (12, 24 & 36 h) (Fig. 3A). It is well known that, Acridine orange stains both live and dead cells and emit green fluorescence whereas Ethidium bromide stains the cells whose membrane integrity is lost emitting red fluorescence.[10] Hence in the present study, distinct cytological changes within the cells have been characterized according to the fluorescence emission and morphological features are shown in the Fig. 3A) a) well organized, uniform live cells stained green; b) early apoptotic cells with condensed chromatin at the perinuclear region in red; c) preliminary late apoptotic cells emitting bright red fluorescence; d) occurrence of prominent cell death by apoptosis and swollen necrotic bodies.

Hoechst staining

Hoechst staining was performed to differentiate the dead from live cells by examining their nuclear morphology under fluorescence microscope.[11] Hoechst 33258 is cationic, lipophyllic dye that permeates the cell membrane and effectively binds AT-rich region of double stranded DNA.[12] A time-dependent study was carried out with IC_{50} concentration of a biopolymer (IC_{50} - 187.5 μgmL^{-1}) showed typical apoptotic nuclear morphology namely nuclear shrinkage, DNA condensation and fragmentation (Fig. 3B). However, the untreated cells remain with intact nuclear morphology. The drastic change occurs within the cells could be due to the controlled release of phloroglucinol. It is also observed that, change in nuclear morphology of the cancer cells is directly proportional to controlled release of phloroglucinol.[6] Altogether, the loss of cell volume upon time is an attributed to cytoskeleton breakdown and plasma membrane blebbing.[13]

Mitochondria membrane potential (Δψm)

Mitochondria membrane potential (Δψm) is a valuable indicator to assess the induction of cellular apoptosis.[14] As mitochondria are considered to be the "power house" several notable anticancer drugs target them to induce apoptosis without affecting normal cells. Keeping this in mind, to study the effect of biopolymer on the mitochondrial Δψm, JC-1 staining was performed. JC-1 is a cationic, lipophyllic dye that selectively enters the mitochondria emitting red (untreated cells) and green (treated cells) fluorescence depending upon the mitochondrial Δψm.[15] As expected, in the present study time dependent treatment confirms the loss of mitochondrial Δψm against breast cancer cell line as shown in Fig. 3C. In normal/untreated cells, JC-1 enters the mitochondria forming J-aggregates enabling red fluorescence. However, in the case of apoptotic (or) unhealthy cells with low mitochondria Δψm, JC-1 remains in monomeric form emitting green fluorescence.[16] Further, this indication confirms the release of apoptogenic proteins such as cytochrome *c* and apoptosis inducing factor (AIF) into the cytoplasm leading to the activation of the caspase cascade.[17]

DNA fragmentation

DNA fragmentation is considered to be a hallmark event during the progression of apoptosis. However, apoptosis can also occur without DNA fragmentation. In the present study, the biopolymer treated cells showed apoptotic DNA fragmentation whereas the untreated cells show no such event (Fig. 4). Several *in vitro* studies pertain to explain the participation of caspase-9 dependent activation of caspase-3 in the development of DNA fragmentation.[18] The caspase-3 readily cleaves the inhibitor of caspase activated DNase (ICAD) and activates caspase activated DNase (CAD) enzyme. On the other hand CAD cleaves the internucleosomal DNA, leading to apoptotic DNA fragmentation.

Cell cycle arrest

The effect of biopolymer on cell cycle progression was also examined on treatment with IC_{50} concentration after 24 & 48 h incubation (Supplementary Table. 2). After 24 h, the biopolymer caused an increase of cells in S phase as compared to G_2 phase

and untreated cells. This could possibly due to controlled release of phloroglucinol that affect the formation of replication fork formation by inhibiting topoisomerase I leading to the occurrence of double stranded DNA break.[19] However, there is a steady increase in G_2 phase as compared to S phase after 48 h indicates the DNA damage from S phase has prevented G_2 phase in entering the process of mitosis.[20]

The role of caspases in apoptosis induced by biopolymer

Caspases are aspartate-specific cysteine proteases enzyme that play a vital role in the induction, transduction and amplification of intracellular apoptotic signals in eukaryotes.[21] Based on the amino acid composition, caspases are divided into three sub-families, namely activator, executioner and mediator caspases. Most importantly the initiator and executioner caspases get involved in the apoptotic signalling cascade. For the first time, the molecular mechanism involved during the effect of phloroglucinol-encapsulated starch biopolymer was studied on MDA-MB-231 breast cancer cell line. The results from the study suggest that, the involvement of both extrinsic and intrinsic pathways towards the induction of apoptotic cell death. To ensure this, caspases were quantified based upon a colorimetric assay kit on treatment with IC_{50} concentration of biopolymer at different time intervals (24, 36 and 48 h). It is observed from the study that, caspase-3 and 8 showed a time-dependent increase in activity as compared to untreated cells (Fig. 5). Altogether, it is also confirmed that the involvement of caspase – 3 and 9 in apoptotic event. This has made us to study the expression pattern of caspases at gene and protein level during the induction of apoptosis. During the internalization of biopolymer, the modified form of a starch matrix will interact with Fas (or) TNF receptor in the plasma membrane towards the recruitment of procaspase-8. Meanwhile, the autoactivation of procaspase-8 into caspase-8 lead to the activation of downstream caspases.[22] This has been confirmed by the upregulation of caspase-8 through differential gene expression (Fig. 6a) and western blotting analysis (Fig. 7c). It is also observed that, there was an increase in the expression pattern of pro-caspase-8 to active caspase-8 at IC_{50} concentration within 24 h. This suggests that a minimum time interval is sufficient for the modified form of starch to interact with receptors in the plasma membrane. In addition, the cleavage of pro-caspase-8 to active caspase-8 was time-dependent. Hence, the modified form of starch matrix plays an important role towards the diffusion and internalization. After the internalization, the weak hydrogen bondings between the soluble starch and phloroglucinol disrupts towards controlled release.[6] This paves the way for phloroglucinol to attack mitochondria and increase its mitochondrial membrane potential towards the activation of caspase-9 as the evidence from JC-1 staining and DNA fragmentation.[23] The expression pattern of caspase-9 was found to be in a time-dependent manner as validated by semi-quantitative RT-PCR (Fig. 6b). This results in increase mitochondrial membrane potential to release cytochrome c into the cytosol that binds pro-caspase-9 leading to formation of apoptosome.[24] Meanwhile, these confirmation changes will activate caspase-3 to play a crucial role in apoptosis via intrinsic pathway of cell death (Fig. 6c & 7b). It is also reported that exceutionary caspases (caspase–3), may cleave cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and so on by activating cytoplasmic endonuclease.[25] In our study, it is also found that the expression pattern of gene and protein for β-actin (internal control) was equal (Fig. 6d & 7a). Meanwhile, statistical data from expression fold ratio analysis of western blot are significantly higher as compared to control (Fig. 8). The overall findings suggest that, caspase mediated apoptosis are provoked by phloroglucinol-encapsulated starch biopolymer in MDA-MB-231 cancer cells (Fig. 9). Hence, the phloroglucinol-encapsulated starch biopolymer is worth enough to serve as an anticancer or chemotherapeutic agent for treating breast cancer in near future.

Conclusion

In conclusion, phloroglucinol-encapsulated starch biopolymer induces apoptosis in MDA-MB-231 breast cancer cells via extrinsic and intrinsic pathway. These findings suggest, the encapsulation of phenolic compound will pave new avenue in prevention and treatment of cancer.

Acknowledgements

The authors are grateful to Natural Resource Data Management System (DST-NRDMS), Government of India, New Delhi for their financial assistance through major research project. The authors acknowledge Dr. Hairul Islam, Pondicherry Centre for Biological Sciences, Puducherry for his help in cell culture facilities.

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Figure caption

Figure. 1. Phloroglucinol-encapsulated starch biopolymer

Figure. 2. Cytotoxic effect of biopolymer, phloroglucinol and soluble starch against breast cancer cell line (MDA-MB-231)

Figure. 3. Morphological assessment of MDA-MB-231 cancer cell line using fluorescence staining A) AO/EB staining; B) Hoechst 33259 staining; C) JC-1 at different time intervals with IC_{50} concentration of biopolymer - a) untreated as control; b) 24 h treatment; c) 36 h treatment; d) 48 h treatment.

Figure. 4 DNA fragmentation of MDA-MB-231 breast cancer cell line – 1) Ladder; 2) Untreated cells as control; 3) IC_{50} treated cells at 24 h; 4) IC_{50} treated cells at 48 h.

Figure. 5 Phloroglucinol-encapsulated starch biopolymer induced caspase – 3 and 8 activity assays at different time intervals

Figure. 6 Semi-quantitative RT-PCR – Effect of biopolymer on expression of a) Caspase-8; b) Caspase-9; c) Caspase-3; d) β-actin (control) at different time intervals

Figure. 7 A time dependent treatment with IC_{50} concentration of phloroglucinol-encapsulated starch biopolymer against MDA-MB-231 breast cancer cell line. SDS-PAGE was carried out & proteins were detected by Western blotting – a) β-actin; b) Caspase-3; c) Caspase-8

Figure. 8 Changes in pro-apoptotic protein (Caspase-3 & Caspase-8) induced by phloroglucinol-encapsulated starch biopolymer at different time intervals assessed by Western blot analysis in MDA-MB-231 breast cancer cell line. Significant differences from the control (untreated) are indicated by *** $p = 0.001$ and $^{\#\#\#}$ $p = 0.002$.

Figure. 9 Overall possible mechanism involved in Caspase mediated apoptosis induced by phloroglucinol-encapsulated starch biopolymer against breast cancer cell line (MDA-MB-231).

Figure 1

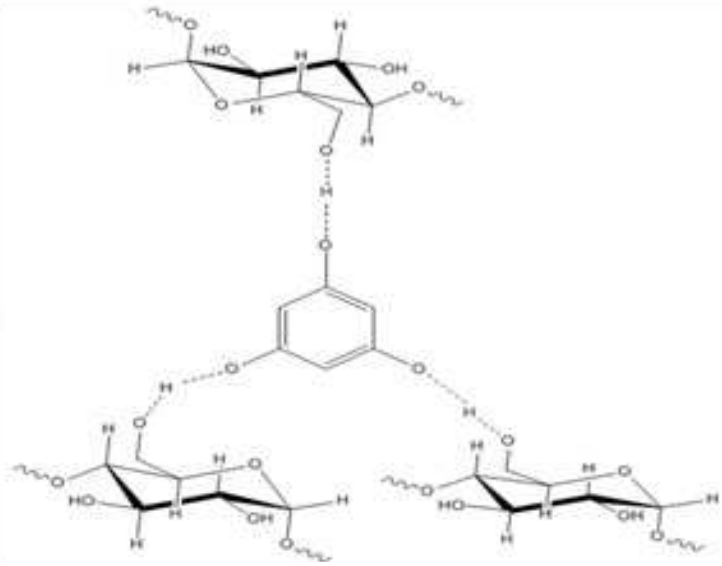

Figure 2

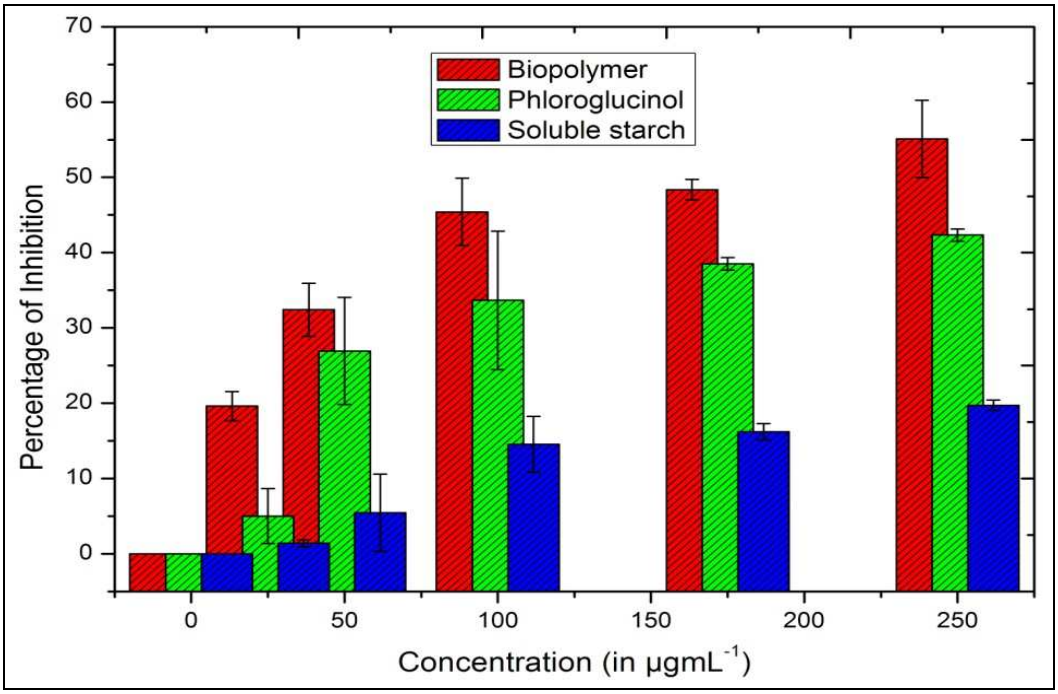

Figure 3

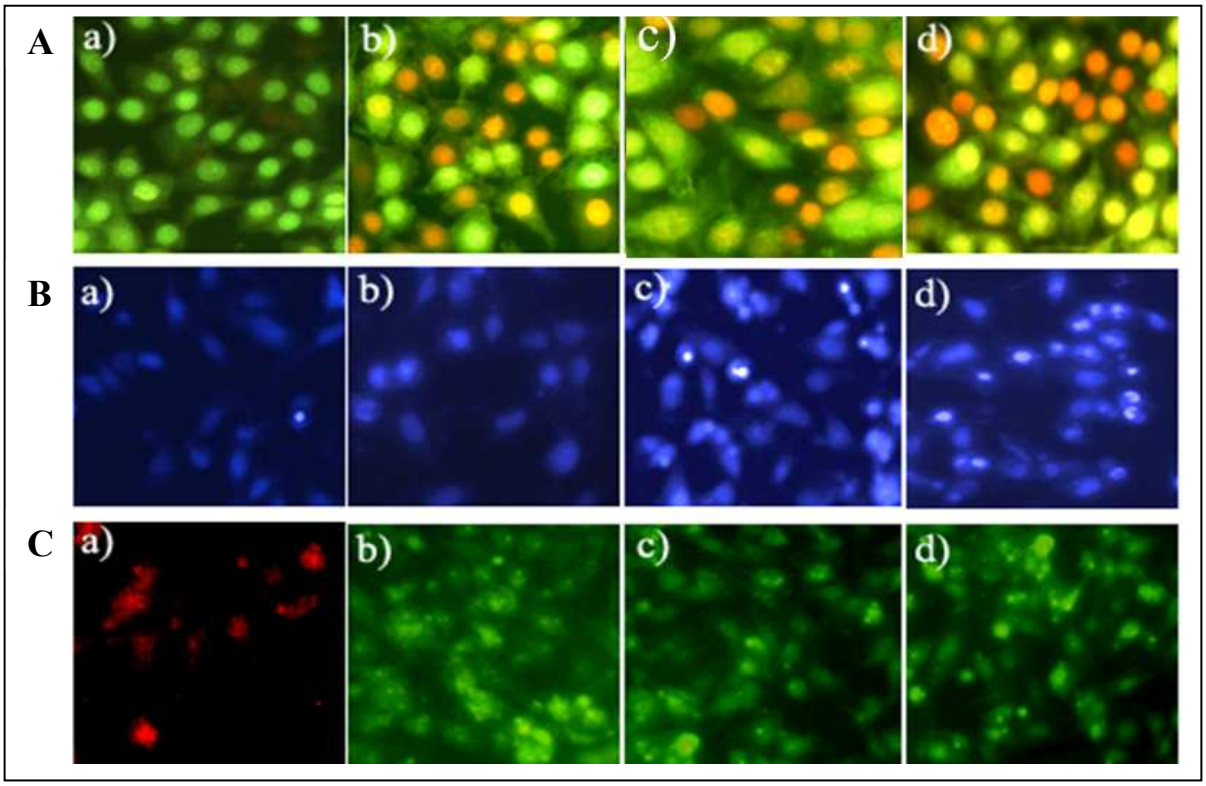

Figure 4

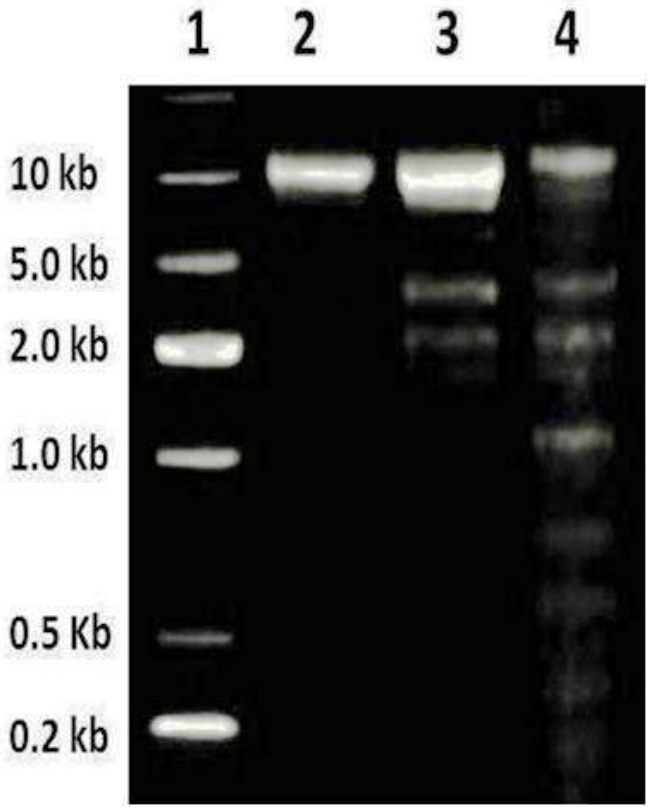

Figure 5

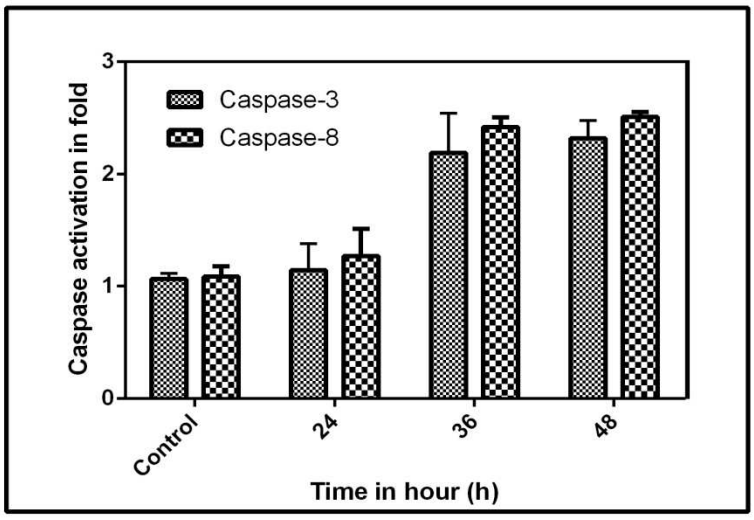

Figure 6

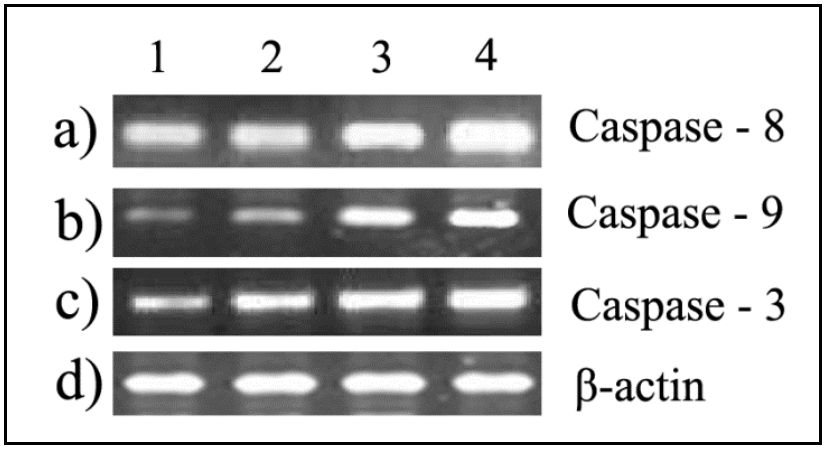

Figure 7

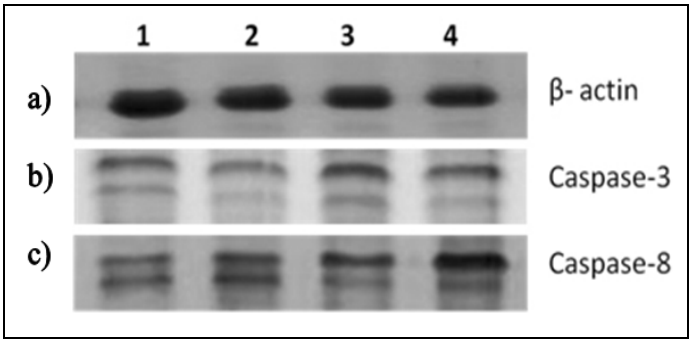

Figure 8

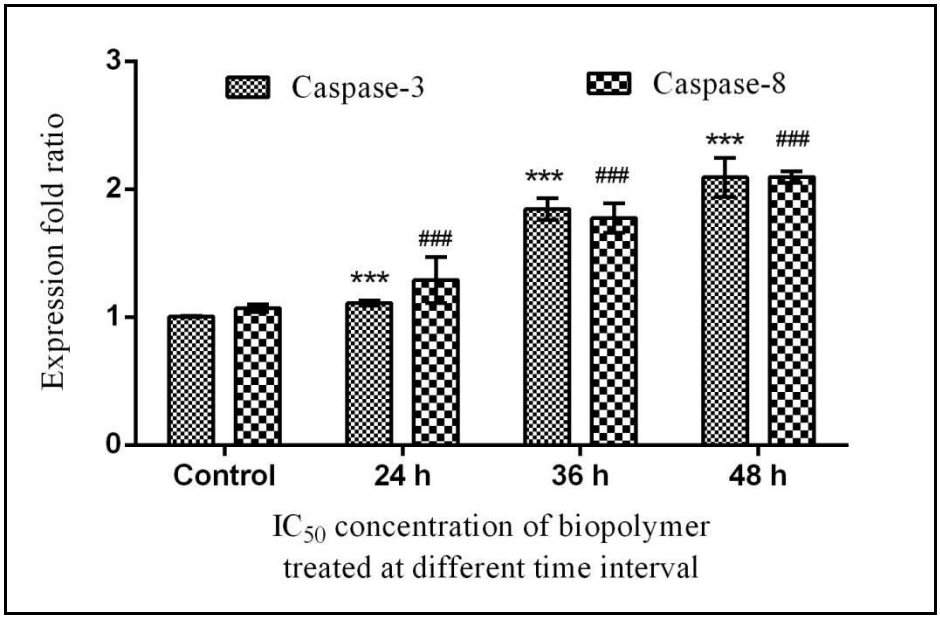

Figure 9