

Bio-Medical Applications of Chitosan Linked *Rosmarinus Officinalis* Leaf Extract Nanopolymer

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Research Article

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Abstract

Nanotechnology has numerous applications in science and technology. The nanomaterial which are plant based have drawn more attention due to its immense application in various fields because of their physico-chemical properties. Physical, chemical, and biological processes are used to create nanomaterials. Physiochemical processes are costly and hazardous to the environment, whereas biological processes involving nanoparticles are thought to be environmentally friendly. In recent times considerable research interest has evolved using chitosan nanoparticles as they have emerged as one of the most exciting tools due to their increased surface-volume ratio and are of great interest for nanomedicine and development of new therapeutic drug release systems with improved bioavailability, increased specificity and sensitivity, and reduced pharmacological toxicity. In the present investigation, we have synthesized chitosan nanoparticles using *Rosmarinus officinalis* leaf extract by the ionic gelation technique and was characterized using UV spectrophotometer and FTIR. The optical density of the broad peak of Chitosan-Rosemary Leaf nanopolymer was found to be at 0.587. The FTIR results recorded the absorption peaks at 3381 cm^{-1} , and 1666 cm^{-1} which represented the O-H stretch and C=C bond stretching respectively. Thus, there was a stretching from higher wavelength region to lower wavelength region demonstrating the interaction of tripolyphosphate with chitosan and biomolecules by reduced hydrogen bonding. Further, the study was designed to determine the cytoprotective and DNA protection activity. The results of our study lend pharmacological credence to the bio-medical applications, ethno medical use of this plant in traditional system of medicine.

Introduction

Nanobiotechnology is the application of nanotechnology in biological fields. Nanotechnology is a multidisciplinary field that utilizes both conventional and advanced engineering, physics, chemistry, and biology technologies and facilities [1]. Nanobiotechnology has great potential in a wide range of life science fields. It also has potential applications in nanotechnology fields, such as nanomedicine and nano-biopharmaceuticals [2].

Chitosan is a polycation, soluble in acidic medium (pKa 6.5) in which the negatively charged TPP interacts with the positively charged groups of amino acids of Chitosan forming inter or intramolecular structure [3]. Chitosan is a partial N-deacetylated chitin β (1 \rightarrow 4)-2-amino-2-deoxy-D-glucan. The nanoparticulate forms of chitosan demonstrated improved solubility and bioavailability, resulting in more efficient drug delivery [4]. Chitosan is famous for anti-properties like antimicrobial, antioxidant, and chelating effects, as well as its nontoxic and biocompatible existence [5].

Green synthesis is an emerging area of bio-nanotechnology where plants are employed to produce environmentally friendly nanoparticles rather than the chemical technologies. This is supported by the fact that plant-derived products have lesser negative effects than many synthetics. Plants can be used to make nanoparticles instead of other biological processes because they reduce the time-consuming process of maintaining cell cultures and can be ramped up for large-scale synthesis of nanoparticles in a

non-aseptic environment. In addition, it has quality role in the field of high sensitivity bio-molecular detection, catalysis, biosensors, and medicine [6,7].

Rosmarinus officinalis, more commonly known as rosemary, is a Mediterranean herb that belongs to the Lamiaceae family of mints. It's an annual aromatic plant with shrub-like branches brimming with leaves. *R. officinalis* is used in cooking as a spice, as a natural preservative in the food industry, and as an ornamental and medicinal plant. *Rosmarinus officinalis* shows adequate antioxidant activity due to the presence of phenolic acids like caffeic, rosmarinic acids and phenolic diterpenes like carnosol, carnosic acid and rosmanol. It also possesses antimicrobial, anti-inflammatory, antitumor, antifungal, antiviral activities. [8,9,10,11,12]

The present investigation aims at the synthesis and characterization of Chitosan linked nanopolymer using *Rosmarinus officinalis* leaf extract to identify DNA protection activity, DNA inhibition activity and cytotoxicity of chitosan Rosemary leaf nanopolymer (CNP(L)).

Material And Methods

Chemicals : Ethanol, Ferric chloride, Sodium hydroxide and Hydrochloric acid, glacial acetic acid, Chloroform, Sulphuric acid, Dragendroff's reagent, n-butanol, Ethyl acetate, L- Ascorbic acid, Ascorbic acid, Ferrous sulphate, Aluminum chloride, Potassium Acetate obtained from Fischer Scientific, Quercetin, Chitosan, Sodium Tripolyphosphate (TPP), Salmon Milt DNA were analytical grade chemicals from HiMedia, Karnataka, MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Thin layer chromatography Silica gel plate was of GF $\times\times\times$ Merck.

Plant source: The Fresh Rose Mary (*Rosmarinus officinalis*) plant was collected from Lal Bagh Botanical garden and Kaval Bysandra, Bangalore through random selection.

Preparation of plant extract: The plant extract was prepared from leaves of Rose Mary (*Rosmarinus officinalis*). The leaves were washed properly under tap water to remove the dust particles and other impurities from the plant. These leaves were then oven dried overnight at 60°C and was made into a coarse powder. A 5% aqueous and ethanolic (50% ethanol) extracts of the leaves of the Rosemary plant was prepared. The weighed amount of the coarse powder was mixed with the solvent system and placed on a magnetic stirrer for 30 minutes. After which the solution was centrifuged at 8,000 rpm for 18 mins at 25°C. The supernatant was then separately collected and used for further experimental analysis.

Synthesis of chitosan nanopolymer. The ionic gelation method was employed for the synthesis of chitosan nanoparticle using sodium tripolyphosphate (TPP) as a cross linking agent . About 1% (w/v) of the plant extract was mixed with 0.5% (w/v) of TPP and the solution was added drop wise into the chitosan solution containing 0.5% (w/v) chitosan and 1% (v/v) acetic acid under gentle magnetic stirring of REMI 2-MLH. The solution was incubated for 20 mins and used for characterization [13]

Characterization of Chitosan nanopolymer:

a. **Ultra-Visible spectrophotometer:** The chitosan and the target compound interaction were monitored by measuring the UV-Vis spectrum of the nano-polymer suspension. The absorbance spectrum of the nano-polymer suspension was recorded immediately after the synthesis and a reference of de-ionized water was recorded before the actual sample analysis. To check the stability of nano-polymer, the absorption spectrum was recorded for pure chitosan, Rosemary extract, TPP, TPP – Rosemary extract, Chitosan – TPP, Chitosan-Rosemary extract and Chitosan-TPP-Rosemary extract. The spectrophotometer used was UV Scan 2600 (Thermo Fisher) and the software was spectrum TM version 6.87. Absorbance spectra were recorded over the range of 220-400 nm. Wavelength of peak absorbance and λ max was calculated.

b. **Fourier Transform Infrared Spectroscopy analysis:** The biomolecules stacked chitosan nanoparticles were freeze dried and the powdered test was used for FTIR spectroscopy studies. The FTIR examination of chitosan nanoparticles sample was performed with a2 technologies portable attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (ATR-FTIR). Sample spectra were recorded within the range of 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} in the absorbance mode for 10 scans at room temperature. FTIR spectra of chitosan nanoparticles were obtained by placing 1mg of test on the sensor of the instrument and spectrum was then compared with the spectrum of Chitosan standard.

Biomedical applications of chitosan nanopolymer: The characterized nanopolymer was used to carry out the following assays:

Detection of DNA protection by chitosan nanopolymer using Ultra Visible spectrophotometer: UV spectroscopy was used to compare the level of DNA protection provided by synthesized chitosan Rosemary leaf nanopolymer to that provided by the standard antioxidant (ascorbic acid). Standard antioxidant Ascorbic acid (10 mM) and standard oxidant - ferrous sulphate (10 mM) were used in the reaction mixture with Salmon milt DNA (200g/mL). TAE buffer was used as a reference standard and the absorbance was estimated at 220-400 nm. Different reaction mixtures were prepared as follows and the absorbance of each was recorded:- DNA, DNA + buffer, DNA +ascorbic acid+ buffer, DNA+ ferrous sulphate + buffer, DNA + ferrous sulphate + ascorbic acid, DNA + ascorbic acid + ferrous sulphate, DNA + nanopolymer + buffer, DNA + ferrous sulphate + nanopolymer and DNA + nanopolymer + ferrous sulphate with 5 min intervals. [14]

DNA inhibition assay using agarose gel electrophoresis: The ability of Chitosan nanopolymer to protect Salmon milt double stranded DNA from devastating effects of free radicals generated was assessed by DNA damage inhibition assay. The reaction mixture contained 2mL DNA (200 μ g/ mL), 1mL 10mM ferrous sulphate and 1 mL 10mM ascorbic acid or 1mL of 1% nanopolymer rosemary (leaf) extract. The DNA was processed and checked in two different treatments. In the first treatment, (treatment 1) reaction mixture was prepared with DNA and FeSO $_4$ incubated for 5 minutes at 37 $^{\circ}$ C, and then addition of the chitosan rosemary leaf nanopolymer (CNP(L)) extract which was again incubated for 5 minutes at 37 $^{\circ}$ C. In the second treatment (treatment 2), reaction mixture constituted DNA and (CNP(L)) was incubated for 5 minutes at 37 $^{\circ}$ C followed by addition of FeSO $_4$. This mixture was incubated for 5 minutes at 37 $^{\circ}$ C. The

samples were then read under UV spectrophotometer from 320-520. 20µL of each reaction mixture were loaded on 0.8% agarose gel. Electrophoresis was carried out at 100V and 120A for 1 hour. The gel was then visualized under Gel Documentation for the appearance of bands [15, 16].

Cytotoxicity studies by MTT assay: MTT (3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide) assay was performed to check cell viability and cell toxicity. MTT is a yellow colour solution which gets reduced by the action of dehydrogenases and other reducing agents produced by metabolically active cell. This reduction of MTT finally yields a violet-coloured water insoluble product called formazan which indicated the non-viability of the cells. The dead or non-viable cells will not cleave MTT, so this assay is very sensitive to the presence of living cells [17].

0.25 g of liver tissues were weighed and placed in cell culture plate. 1 ml of DMEM (Dulbecco's Modified Eagle Medium) containing 1% penicillin was added to each of the wells containing the liver tissues. A comparative study was performed. 50µl, 100µl and 150µl of the Chitosan linked Rosemary leaf extract nanopolymer were added into a cell culture plate containing the weighed liver tissues. The same aliquots of the 5% ethanolic Rosemary leaf extract was added into another cell culture plate containing the liver tissues. The plates were then incubated at 37°C for 24 hours in an incubator. After incubation, 20µl of MTT (0.5 mg/ml) was added to each well and again incubated for 4 hours at 37°C in an incubator. To stop this reaction, 1 ml of 0.04 N HCl in isopropanol was added and the reaction mixture was centrifuged at 3000 rpm for 10 mins at room temperature. The wells 4 and 5 of both the cell culture plates had none of the contents added to it. The absorbance of the supernatant was read at 570nm in UV-Vis spectrophotometer [18]

Results And Discussion

Rosemary plant has various pharmacological, ethnobotanical and other miscellaneous uses. Rosemary plant has enormous benefits in medical field and chitosan linked nanopolymer will be more effective than its original form which is proved in the present study.

CHARACTERISATION OF NANOPOLYMER-

UV Spectrophotometer analysis : A UV-Vis spectrophotometer was used for the preliminary characterisation of the prepared Chitosan-Rosemary nanopolymer. The maximum optical density of the Chitosan-Rosemary nanopolymer was found to be at 0.587 while the pure chitosan showed an optical density of 0.352. The bonding of pure chitosan with Rosemary leaf extract resulted in an increase in the optical density. This revealed the formation of chitosan plant nanopolymer. Our current study correlated with the revelation of the broad peak for chitosan nanoparticle at the range of 220-322 nm [19].

FTIR Analysis : The capability of the ionic gelation protocol to form the plant-chitosan nanoparticles loaded with biomolecules was assessed by FTIR with transmission values on the Y-axis and wavelength on the X-axis. The spectral analysis of pure chitosan is depicted in fig 2(a) and fig 2(b) shows the spectral analysis for the chitosan rosemary leaf nanopolymer loaded with bioactive molecules. The IR spectra of

pure chitosan nanoparticles (CNPs) showed 2 major peaks at 3333 cm^{-1} and 1637 cm^{-1} , while the FTIR spectra of the Chitosan-Rosemary leaf nanoparticle revealed two major absorption peaks at 3381 cm^{-1} and 1666 cm^{-1} . The presence of O-H bonds was confirmed by the spectra observed at 3381 cm^{-1} for the synthesised nanoparticle. For the biomolecules loaded chitosan nanoparticles, the isolated alkene groups of the C=C stretch were observed at 1666 cm^{-1} [20].

Detection of DNA protection assay: Reactive oxygen species are thought to cause severe oxidative damage to various macromolecules, including DNA, RNA, and protein [21]. In our study Salmon milt DNA which is double stranded has a molecular mass of 1.3×10^6 Da (~2,000 bp). The ability of the CNP(L) to protect the DNA strands from the oxidative damage caused by the oxidant was by two treatments. The absorbance value higher than the standard DNA indicating strand breakage. Treatment 1- was the pre-treatment of DNA with the CNP(L) followed by treatment with the oxidant to analyze the DNA protection activity and Treatment 2- was the pre-treatment of the DNA with oxidant- FeSO_4 , followed by the treatment with the synthesized chitosan plant nanopolymer to assess the DNA repair activity. In fig (3), the addition of FeSO_4 to the pure DNA resulted in an increase in the absorbance at 260 nm. The addition of the CNP(L) showed a relatively less increase in absorbance, indicating that the nanopolymer does not cause DNA damage.

The results indicated that chitosan-rosemary leaf nanopolymer contemplated higher DNA protection activity than DNA repair activity. The oxidative damage caused by the oxidant, FeSO_4 , lead to denaturation of the double stranded DNA which gave a high optical density value than the standard DNA. The reduction in the optical density value on treatment with the low concentrations of the Chitosan-rosemary nanopolymer indicated the significant DNA protection potential of the nanopolymer.

DNA INHIBITION ASSAY USING AGAROSE GEL ELECTROPHORESIS: DNA damage can also be caused by alkylating agents. Reactive carbonyl species are some of the chemical agents that results the DNA damage. These highly reactive oxygen species attack the bases or the deoxyribosyl backbone of DNA leading to an interruption in the normal structure of the DNA causing single or double stranded breaks. [22]

In the fig 4 a), DNA revealed the least absorbance serving as the control for the assay. A slight increase was observed in the absorbance when DNA was incubated with the oxidant, FeSO_4 . This increase in absorbance is due to the formation of free radicals by FeSO_4 that led to the DNA damage. There was a small decrease in optical density when DNA was treated with an antioxidant (ascorbic acid), indicating the release of oxidative stress on DNA. In contrast, when the DNA was treated with the antioxidant followed by the oxidant (FeSO_4) an optical density was noted in between the antioxidant and oxidant treated. This indicated that the stress was caused by the addition of FeSO_4 to DNA but at the same, the antioxidant ascorbic acid suppressed the additive effects of FeSO_4 and protected the DNA from any further damage. Furthermore, when the pre-treated DNA and CNP(L) mixture was treated with the oxidant, the optical density was found to be similar to that of DNA+ Ascorbic acid+ FeSO_4 optical density values.

This indicated that plant nanopolymer was found to have antioxidant activity and DNA damage inhibition properties alike the standard antioxidant (ascorbic acid).

The DNA inhibition activity was confirmed on an agarose gel. The mixtures used for the UV spectral analysis of DNA inhibition assay was mixed with small aliquots of EtBr and loaded on to an 0.8% agarose gel. Ethidium bromide aids in the visualization of DNA bands as well as the size determination of separated DNA fragments [23].

In figure 4 b), the Lane 1 which consisted of DNA (control) showed an orange-fluorescent band as the DNA existed in its supercoiled form itself. Lane 2 showed a much lighter fluorescent band due the oxidative stress caused by the free radicals on addition of FeSO₄. Lane 3 indicated the DNA protection by the addition of ascorbic acid with orange-fluorescent bands which was in same lines with the control. Lane 4 indicated DNA protection by ascorbic from the oxidative stress caused by FeSO₄. In Lane 5, the DNA was pre-treated with the Chitosan- Rosemary nanopolymer followed by addition of FeSO₄. A single orange bright fluorescent band was observed which indicated a potent inhibition effect towards DNA damage which could be caused by FeSO₄. This proved that CNP(L) has significant DNA damage inhibition activity.

The thinning of bands were observed in all the wells indicating the complete degradation of DNA. DNA molecules are divided in a pattern based on their size, with the distance travelled being inversely proportional to the log of their molecular weight. These findings were in accordance with the UV spectrophotometric analysis.

Cytotoxicity studies by MTT assay: Cytotoxicity of the CNP(L) was studied using liver cells by MTT assay. The MTT assay is a colorimetric assay in which NAD(P)H-dependent cellular oxido-reductase enzymes, under defined conditions, reflect the number of viable cells present. These enzymes can reduce the tetrazolium dye MTT, a yellow-coloured solution, to its insoluble purple coloured formazan.. According to the study, the liver cells were viable even after 24 hours of incubation with DMEM containing 1% penicillin with different concentrations of the Chitosan plant nanopolymer. When MTT was treated with CNP(L), no change in colour from yellow to purple was detected. This revealed that the plant nanopolymer is non-toxic and keeps the liver cells viable.

A negative result was observed when the liver cells were treated with different aliquots of the plant extracts under the same conditions. The liver cells lost their viability after the incubation period and all the wells in the cell culture plate turned purple indicating toxicity.

The picture above shows the results when liver tissues were being treated with the chitosan nanopolymer and the negative control. The picture below shows the results when the liver tissues were treated with just plant extract.

Conclusions

The necessity of looking for alternative plant products to combat a variety of ailments linked to Reactive Oxygen Species has remained popular due to the lower toxicity of natural goods compared to synthetic solutions.

In the present research, the Chitosan Rosemary leaf nanopolymer was synthesized using the ionic gelation technique. The synthesized chitosan Rosemary nanopolymer was characterized using UV spectrophotometer and FTIR analysis. The characterized nanoparticles were then investigated for biomedical applications such as DNA protection, DNA inhibition and Cytotoxicity studies by MTT assay. From the results of UV spectrophotometric analysis for the DNA protection and repair assay it was observed that the synthesized Chitosan Rosemary nanopolymer showed significant DNA protection activity and moderate DNA repair activity. The invitro DNA inhibition activity of CNP(L) was evaluated by UV spectrophotometric analysis. It revealed that the CNP(L) prevented the degradation of DNA into small fragments and had convincing DNA damage inhibition properties. This was confirmed on an agarose gel which should a brighter orange fluorescent band. In the MTT assay, cell viability and cytotoxicity was studied in response to the chitosan-plant nanopolymer. No colour change was observed when the cells were treated with MTT and CNP(L) indicating that they were still viable. This assay proved that the chitosan-plant nanopolymer synthesized does not affect the viability of the cells and is also non-toxic. Since the biomolecules loaded chitosan nanopolymer conferred potential abilities, the finding of this study opens future applications in the field of drug and gene delivery, tissue engineering and various other biomedical applications.

Declarations

CONFLICT OF INTEREST :

The authors declare that they have no conflict of interest.

FUNDING :

Nil

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Figures

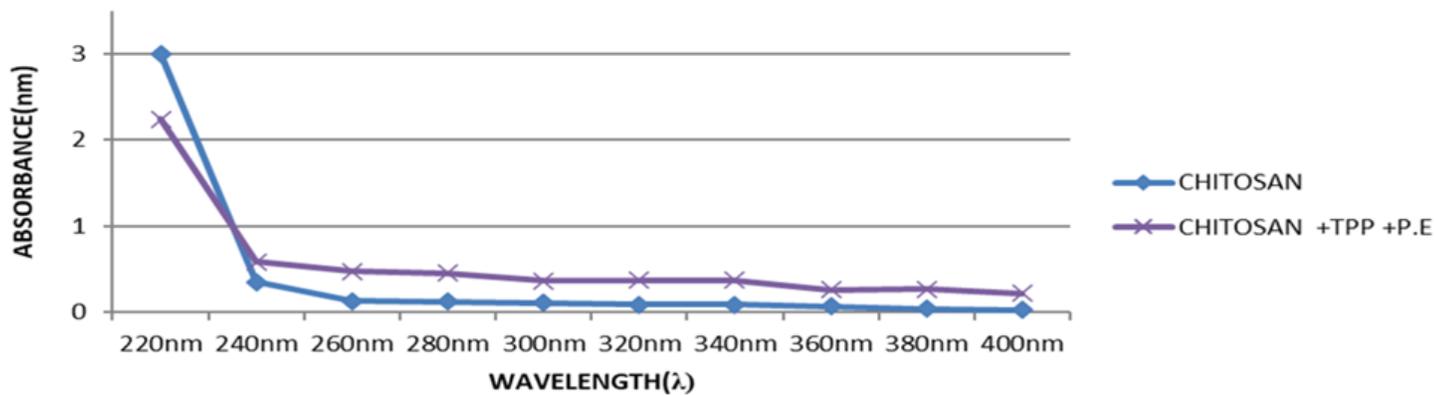


Figure 1

UV-Vis spectrum of pure chitosan solution; Solution of 0.5% chitosan+ TPP+ P.E.

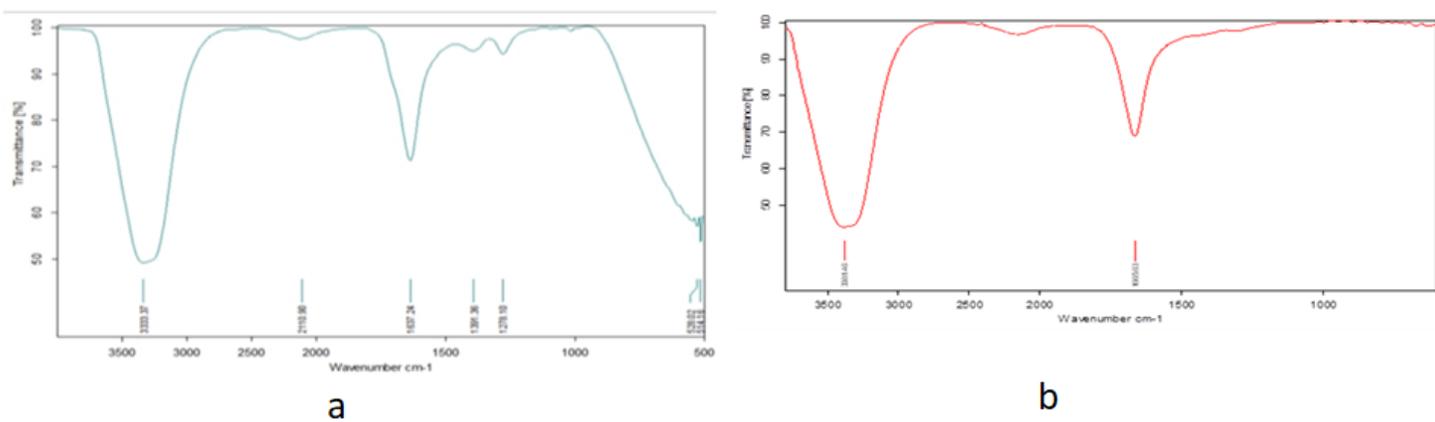


Figure 2

FTIR spectrum of a) chitosan nanoparticles b) Rosemary leaf extract cross linked with chitosan nanopolymer

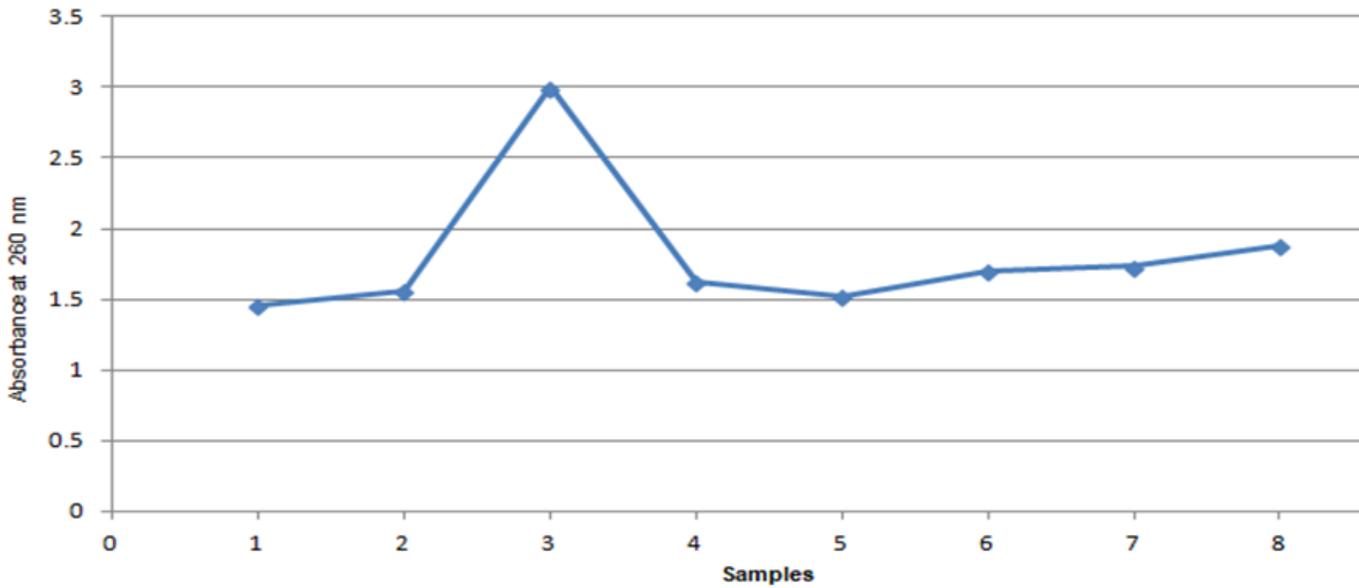
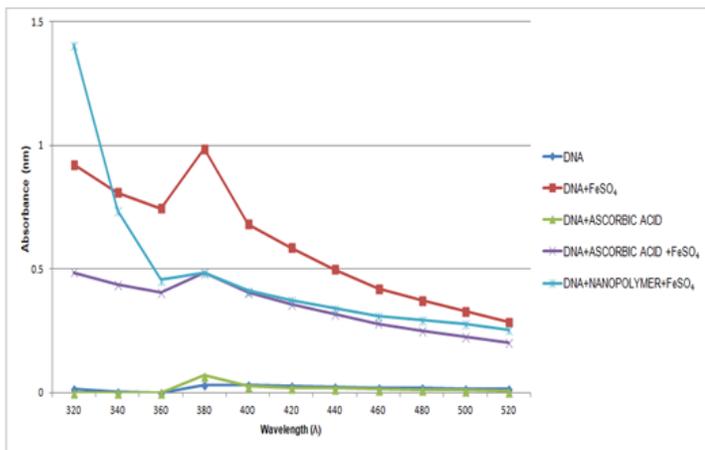


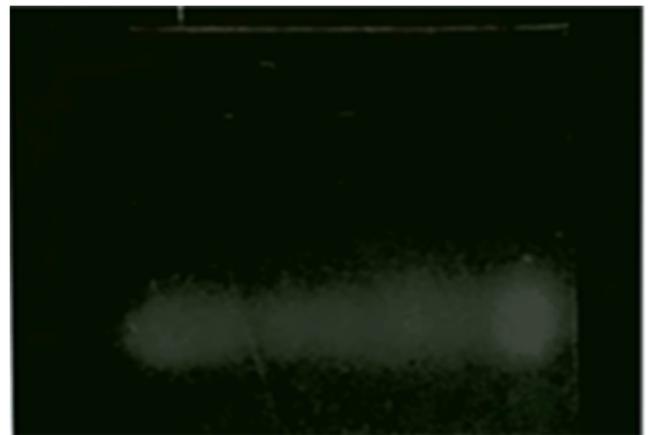
Figure 3

1)DNA + Buffer, 2)DNA +Ascorbic acid +Buffer,)DNA +FeSO₄+ Buffer, 4)DNA+ FeSO₄+ Ascorbic acid, 5)DNA+ Ascorbic acid +FeSO₄, 6) DNA +Chitosan Rosemary nanopolymer +Buffer, 7) DNA + Chitosan Rosemary nanopolymer +FeSO₄ 8) DNA+ FeSO₄+ Chitosan Rosemary nanopolymer



a

1 2 3 4 5



b

Figure 4

a) Spectrophotometric analysis of DNA inhibition by Chitosan- Rosemary nanopolymer:-(1) DNA, (2) DNA+ FeSO₄, (3) DNA+ Ascorbic acid, (4) DNA+ Ascorbic acid+ FeSO₄, (5) DNA+ Chitosan-Rosemary leaf Nanopolymer b) DNA Inhibition by nanopolymer observed by agarose gel electrophoresis: - LANE 1: DNA+ EtBr; LANE 2: DNA+ FeSO₄+EtBr; LANE 3: DNA+ Ascorbic acid+ EtBr; LANE 4: DNA+ Ascorbic acid+ FeSO₄+EtBr; LANE 5: DNA+NP(L)+ FeSO₄+ EtBr

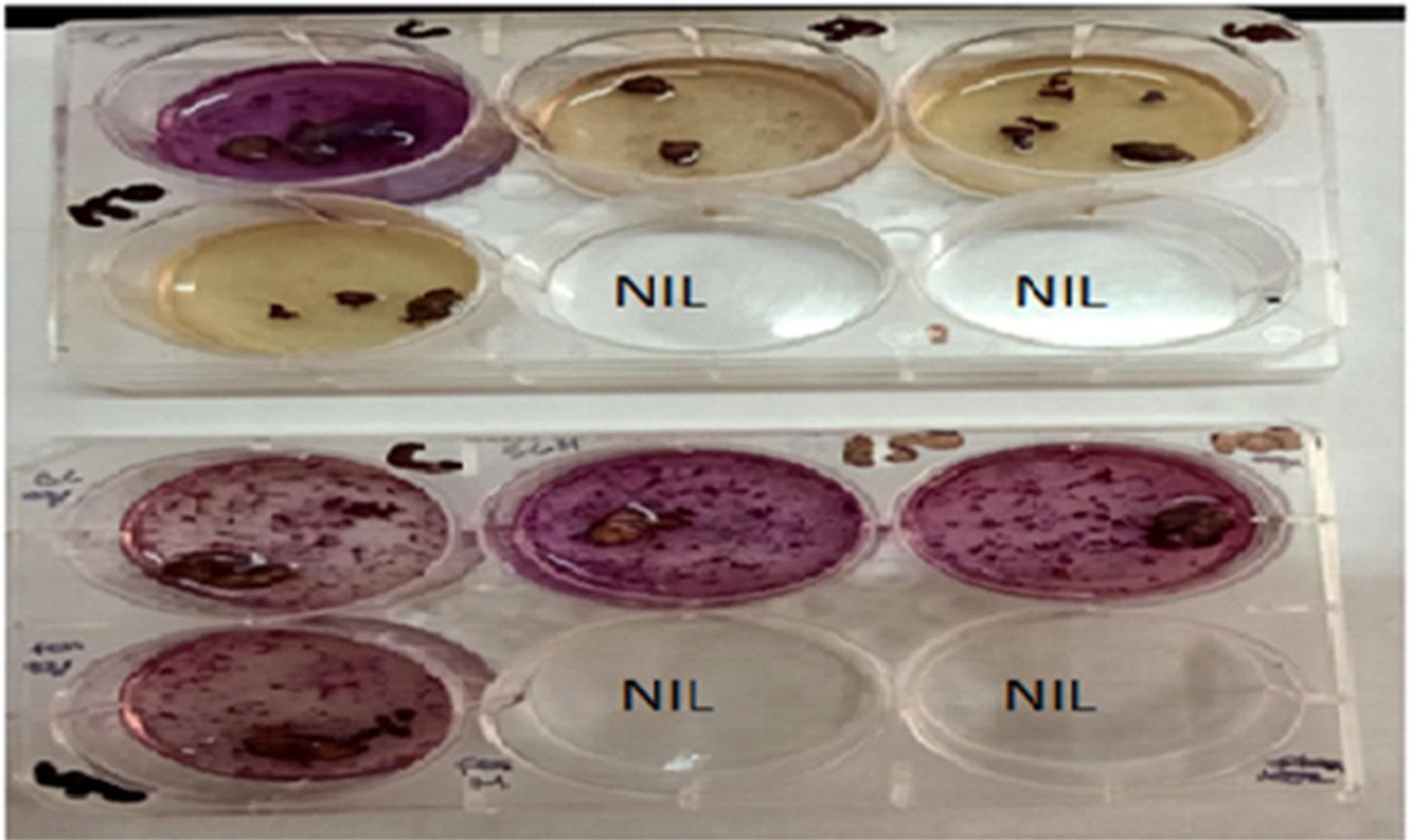


Figure 5

Cytotoxicity by MTT Assay