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Facile fabrication of abundantly available biopolymer as efficacious vehicles of promising natural therapeutics in breast cancer amelioration

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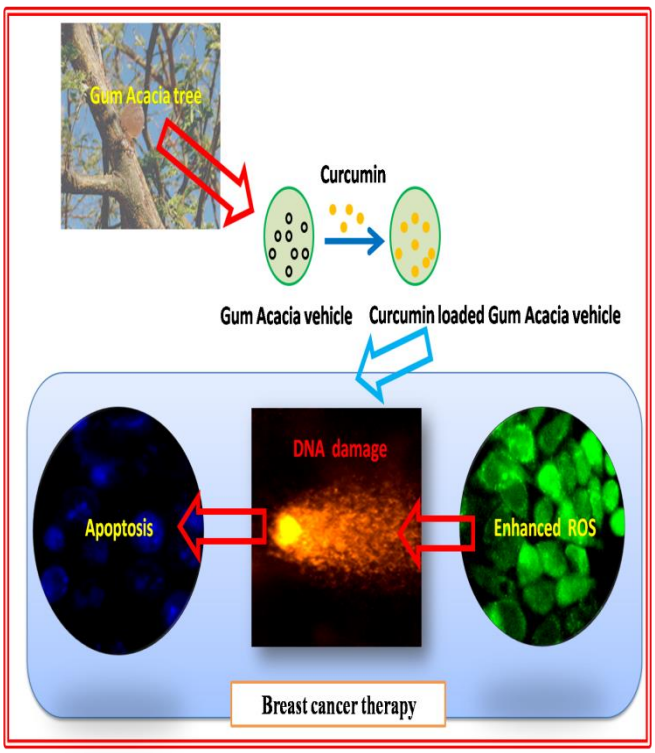
Graphical Abstract



Abstract.

Among the different types of nanoparticles, gum acacia nanoparticles have the potential for controlled drug delivery to cancer cells. We have synthesized gum acacia nanoparticles by nanoprecipitation method. Physical characterization of the synthesized nanoparticles was done by DLS(Dynamic Light Scattering) and Zeta Potential. Hydrodynamic size of Gum Acacia NPs as measured by DLS was 413.3nm and surface charge of the same was found to be -0.348mV.For the possible biomedical application,we have used curcumin, a natural chemotherapeutic agent.Despite its potential anti-cancer activity, solubility of curcumin is very low rendering its limit in application. Several efforts have been made to solubilise curcumin to enhance its bioavailability. We have used gum acacia nanoparticles where curcumin can be loaded comfortably and thereby increases

its bioavailabilty. The cytotoxicity of curcumin-gum acacia NP complex was evaluated on breast cancer cell lines with respect to pre-determined LD50 doses. To investigate the mechanisms, various biochemical assays were also performed. Further, we have explored the fact that the uptake of curcumin by the cells is increased when the curcumin is loaded with gum acacia NPs by utilising the fluorescence property of curcumin. The curcumin-Gum Acacia NP complex was found to induce apoptosis by perturbing the mitochondrial membrane potential of the cancer cells.

Keywords-Biopolymer;GumAcacia;cáncer therapy;breast cáncer;curcumin.

Introduction

Development of biopolymeric nanoparticles for gene and drug delivery has become a emerging and promising sector in the biomedical research field. At present, cancer is one of the most challenging diseases threatening human health and the occurrence of cancer continues to increase day by day. In the past few years, multifunctional nanocarriers have been documented as great potential tool for cancer medicine because of their various biomedical applications like diagnosis,therapy,cell imaging.[1-3]. Over the past decade various nanocarriers such as solids, polymers, micelles and protein-based drug delivery systems have been widely investigated for the detection of cancer, real-time monitoring and targeted anti-cancer drug delivery[4-7]. Nanogels derived from natural polysaccharides (say, Gum Acacia) are of great interest among the nano sized carriers for drug delivery and controlled release due to their biodegradability , biocompatibility and abundance. The properties of nanogels that make them superior drug delivery systems are their small size,high drug loading capacity,controlled and sustained drug release at target site,ability to penetrate small capillary vessels[8]. Apart from being a well known spice, one of the potent medicinal properties of curcumin is its anticancer efficacy which enables it to induce apoptosis in various cancer cells like breast ,lungs, prostate ,colon without causing any cytotoxic effect on other healthy cells[9]. But, curcumin's very low solubility in aqueous medium limits its bioavailability and hence its clinical efficacy. Different types of nanocarriers have been developed to improve the solubility and stability of curcumin in aqueous medium. Encapsulation of hydrophobic curcumin in nanogels makes it readily soluble in aqueous solutions and increases its bioavailabilty in cancer cells. This is because nanogels can easily pass through the cell membrane of solid tumors by

enhanced permeation and retention(EPR) effect owing to their small size[10]. In the present study nanogels are synthesized from Gum Acacia for delivery of curcumin. Gum Acacia (also known as Gum Arabic) is a natural gum obtained from the hardened sap of various species of acacia tree. Gum acacia is a highly branched complex polysaccharide consisting of rhamnose , galactose and glucuronic acid residues in its structure[11]. The present work describes the application of Gum Acacia nanogels for encapsulation and controlled release of curcumin to breast cancer cells.

2.AIMS AND OBJECTIVES

- ***Synthesis of Gum Acacia nanoparticles***
- ***Characterization of the nanoparticles***
- ***Role of Gum Acacia nanoparticles in targeted curcumin delivery to triple negative breast cancer cells.***

3.MATERIALS

- Gum acacia (approx MW 250kDa)
- Curcumin purchased from Sigma-Aldrich(MW .....)
- Cell culture DMEM,RPMI,Fetal Bovine Serum(FBS), JC-1(mitochondrial staining dye) were obtained from Invitrogen-Life Technologies
- Thiazolyl blue formazan(MTT),2'-7'-dichlorofluorescein diacetate(DCF-DA) were purchased from Sigma-Aldrich.
- Deionised water(MilliQ water) was used throughout the experiment with resistivity at least 18MΩ .

All glassware used were washed with aqua regia solution followed by rinsing with ultrapure water.

❖ BREAST CANCER CELL LINES

Triple negative human breast cancer cell lines MCF-7 and MDA-MB-468 were obtained from National Center for Cell Science(NCCS) Pune, India.These were grown in respective DMEM/RPMI 1640 with 10% FBS(Fetal Bovine Serum), penicillin/streptomycin(100units/ml),amphotericin-B(anti-fungal) at 37ºC and 5%C02. All the experiments were done with LD50 dose.All the treatments were done at 37ºC and at a cell density allowing exponential growth.

❖ SYNTHESIS OF NANOPARTICLES:-

❖ GUM ACACIA NANOPOLYMER SYNTHESIS

Purchased gum acacia powder was dissolved in de-ionised water with the help of a magnetic stirrer under vigorous stirring conditions for 24 hrs. The solution turned dark brown after complete dissolution of the gum. The solution was then dried in a desiccator at room temperature. The dried sample was collected and mortared for finer grains. The sample was then named as GA and sent for characterization.

❖ CURCUMIN LOADED GUM ACACIA NANOPOLYMER SYNTHESIS

Gum acacia (50 mg/ml) was dissolved in deionised water and vigorously stirred with a magnetic bead for 24 hrs. In another glass tube curcumin (2 mg/ml) was dissolved in acetone and stirred with a magnetic bead for 3hrs. Then the gum acacia solution was added to the curcumin solution drop-wise and was stirred for another 24hrs. After that, centrifugation was done at 10,000 rpm for 10 mins and the precipitate was washed three times with deionised water. Then the solution was dried at room temperature in a vacuum desiccator for 48 hrs. Finally, the dried precipitate was mortared in an agate mortar to achieve finer fractions. The sample was named as GAC and sent for further characterizations

4.METHODS

4.1.CHARACTERIZATION OF NANOPARTICLE:-

4.1.1 XRD:

The XRD patterns of the powdered nanopolymer samples were recorded by X-ray powder diffractometer model-D8, Bruker AXS, Winconsin, USA, using Cu Kα target employing wavelength of 1.5418 Å and operating at 35kV with scan speed of 1sec/step.

4.1.2 UV-Vis:

The nanopolymers were dispersed in MilliQ water to form a diluted suspension of 2 mg/ml using a bath sonicator for 30mins. When particles were completely dispersed in water its absorbance intensity was measured using UV visible spectrophotometer (Bio-Tek).

4.1.3.DLS AND ZETA POTENTIAL MEASUREMENT:-

Average particle diameter and size distribution of biopolymeric NPs were measured by DLS using a Zetasizer(NANO ZS90,Malvern Instruments Ltd,UK). The charge of the NP was also measured by the Zetasizer. The nanoparticles were dispersed in MilliQ water to form diluted suspension of 2 mg/ml using bath sonicator for 30 min. After being completely dispersed in water the particle was analyzed by DLS in Zetasizer.

4.2.CELL CYTOTOXICITY ASSAY

Viability of triple negative human breast cancer cell lines **MCF-7** and **MDA-MB-468** after exposure to various concentration of gum acacia nanoparticles, curcumin loaded gum acacia nanoparticles were determined by MTT assay[12].

The cells were seeded in 96-well plates at 1×10^4 cells per well and exposed to NPs at concentrations of 0 µg/ml, 20 µg/ml,40 µg/ml,60 µg/ml,80 µg/ml,100 µg/ml,120 µg/ ml for 24 hrs. After incubation cells were washed with 1×PBS twice and incubated with MTT solution(450 µg/ml) for 3-4hrs at 37ºC. The resulting formazan crystals were dissolved in an MTT solubilisation buffer and the absorbances were measured at 570 nm by using a spectrophotometer and the value was compared with control cells.

4.3.REACTIVE OXYGEN SPECIES(ROS) ASSAY

Cellular ROS levels can be measured in live cells by a technique that converts 2',7'-dichlorofluorescein diacetate(DCF-DA) which is oxidised to a green fluorescent colored compound 2',7'-dichlorofluorescein(DCF). The fluorescence generated is directly proportional to the amount of oxidised DCF-DA to DCF[13-14].

Stock solution of DCF-DA (10mM) was prepared in methanol and was further diluted with PBS(Phosphate Buffer Saline) to a working concentration of 100 µM. **MDA-MB-468** were treated with LD50 dosage for 24 hrs. Then cells were washed with ice cold PBS and incubated with 100

µM(working solution) of DCF-DA at 37ºC for 30mins in the dark. Then the fluorescence intensity was measured at excitation of 485 nm and emission at 520 nm.

4.4.COMET ASSAY METHOD:-

Comet assay(also known as Single Cell Gel Electrophoresis Assay) is a simple method for measuring DNA strand breaks in eukaryotic cells.The term "comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet[15].

Frosted slide precoated with 200 µl of 0.8% normal melting point agarose in PBS and left briefly to solidify. Cover slips were then removed.90 µl (3×10^5)of **MCF-7** cells were re-suspended in 100 µl of low melting point agarose that was in turn pipetted onto the normal melting point agarose layer spread with cover slips and left briefly to solidify.50 µl of trypsin solution was added onto the slide and left for 30min at 37ºC. Slides were then immersed in ice-cold lysis solution (2.5M NaCl,100mM EDTA,10Mm Tris-HCl,pH-16,1%Triton X-100,10% DMSO) and incubated at 4ºC for 1hr.Then the slides were equilibriated in freshly prepared electrophoresis buffer (1mM EDTA,300Mm NaOH,pH-9.1) for 20mins at 40ºC before performing electrophoresis. Electrophoresed for 20mins at 25V,300 mA at room temperature. The slides were drained and washed three times with freshly prepared neutralizing buffer (0.4M Tris-HCl,pH-7.5) for 20-30mins.The slides were stained with 40 µl of 20 µg/ml of Ethidium Bromide and kept in dark until use. During examination, the slides were washed with PBS three times and mounted with mounting medium and observed directly under fluorescence microscope. A total of about 50 cells per slide were analyzed for comet parameters using an image analysis system.

4.5.MITOCHONDRIAL MEMBRANE ASSAY

The membrane permeant JC-1 dye is widely used in apoptotic studies to monitor mitochondrial health. JC-1 is a novel cationic carbocyanine dye that accumulates in mitochondria. The dye exhibits potential-dependent accumulation in mitochondria indicated by a fluorescence emission shift from green(~529nm) to red (~590nm). Consequently mitochondrial depolarization is indicated by a decrease in the R/G fluorescence intensity ratio. The potential sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates[16-17].

After treatment with JC-1, the **MCF-7** and **MDA-MB-468** cells were washed with PBS and incubated with (10 µg/ml) JC-1 at 37ºC for 30min. Cells were then observed under a fluorescent microscope.

4.6.DAPI STAINING AND NUCLEAR MORPHOLOGY ANALYSIS

DAPI staining assay provides a rapid and convenient assay for apoptosis based upon fluorescent detection. Cells undergoing apoptosis display typical features namely cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation, and formation of apoptotic bodies[18-20]. These features can easily be observed under fluorescence microscopy, after appropriate staining of nuclei with DNA-specific fluorochromes like DAPI. DAPI(4',6-diamidino-2-phenylindole) is a popular nuclear stain which produces blue fluorescence. With the process of apoptosis the permeability

of the dye is improved and the apoptotic cells produce high blue fluorescence. DAPI specifically binds to the DNA of the apoptotic cell and stains the condensed chromosome[21].

At first, the **MDA-MB-468** cells were treated with curcumin loaded Gum Acacia NPs for 24hrs at different concentrations (0 µg/ml;20 µg/ml;40 µg/ml). Then the cells were washed three times with 1× PBS and stained with DAPI in Vectashield(0.2g/ml). The morphology of the treated cells with ruptured and de-condensed nuclei were observed under a fluorescent microscope at different time intervals.(excitation and emission wavelengths are 358 and 461nm respectively).

4.7.CELLULAR MORPHOLOGY STUDY

MDA-MB-468 cells were grown on a 35mm plate for 24hrs. After that the cells were treated with different concentrations of curcumin loaded gum acacia (0 µg/ml;20 µg/ml;40 µg/ml) at 37ºC for upto 5days. Pictures of the cells were taken on Day1,Day3,Day 5 respectively and change in their morphology was noted.

4.8.CELLULAR UPTAKE STUDY

The uptake of curcumin loaded gum acacia NPs by **MDA-MB-468cells** is an important factor to assess its nanotoxicity[22].

The **MDA-MB-468cells** were grown in a coverslip for over 24hrs. After that the cells were treated with 0, 20,40 µg/ml of curcumin loaded gum acacia for 24hrs at 37ºC. The cells were washed with 1×PBS. Then they were mounted on a glass slide and observed under fluorescence microscope.

5.RESULTS

5. 1.CHARACTERIZATION OF NANOPARTICLES:-

5.1.1 XRD analysis:

The XRD pattern of Gum Acacia shows a broad peak near 2θ= 14.33º and a small hump at 2θ= 30º. This arises due to the amorphous phase of the gum. No such crystallite nature is seen in the XRD pattern of the un-doped gum.

But, curcumin loaded gum is much more crystalline as it has a sharp peak located at 2θ= 13.41º with a smaller hump at 2θ= 29.51º. The nanocrystallite diameter of the curcumin loaded gum has been calculated using Debye-Schrrer's formula using the relation:

$$< D >_{(020)} = \frac{0.9\lambda}{\beta_{1/2}\cos\theta}$$

Here, D is the average nanocrystallite size, λ is the wavelength of the incident X-ray beam, θ is the corresponding Braggs angle, $\beta_{1/2}$ is the full width at half maximum (FWHM) of the plane.

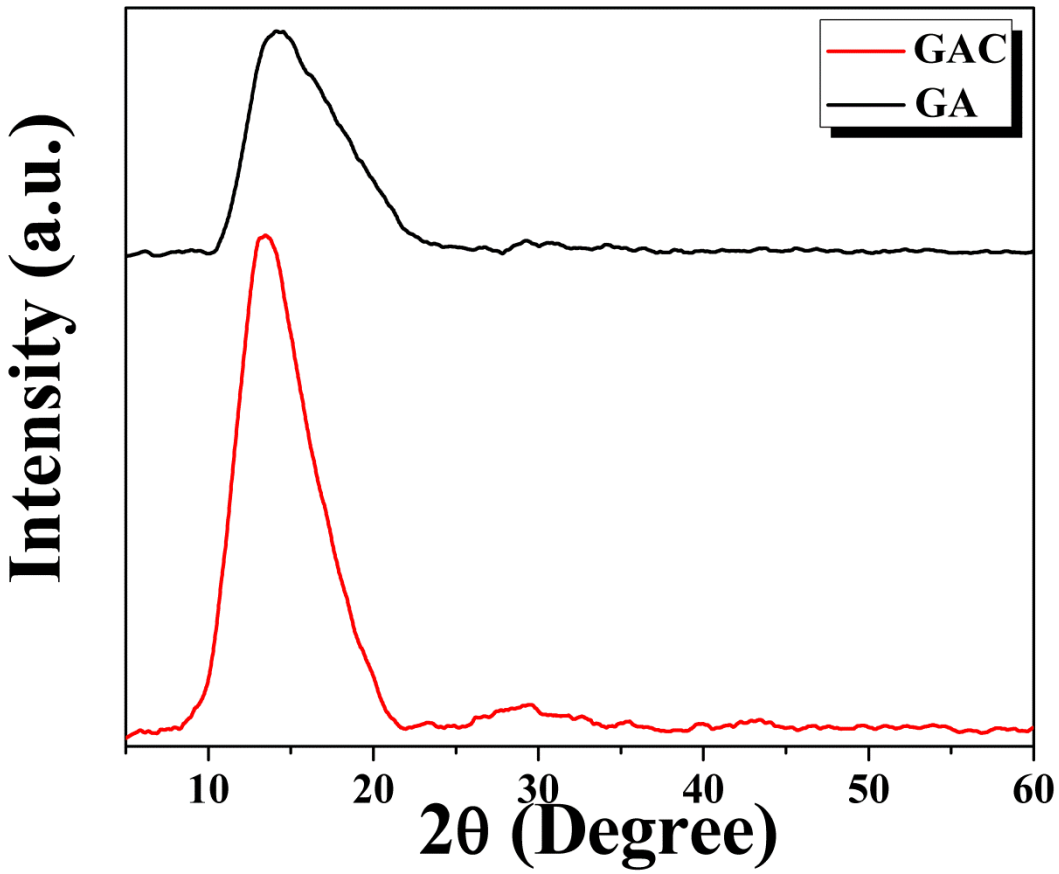


Fig: XRD pattern of GA and GAC

The calculated value for the curcumin loaded gum is 1.65 nm, which supports the formation of curcumin conjugated gum acacia nanopolymer. The XRD pattern of the aforesaid cucumin loaded polymer shows a small peak shift towards the smaller diffraction angles, which is due to the perfect incorporation of the drug inside the polymer matrix.

5.1.2. UV-Vis analysis:

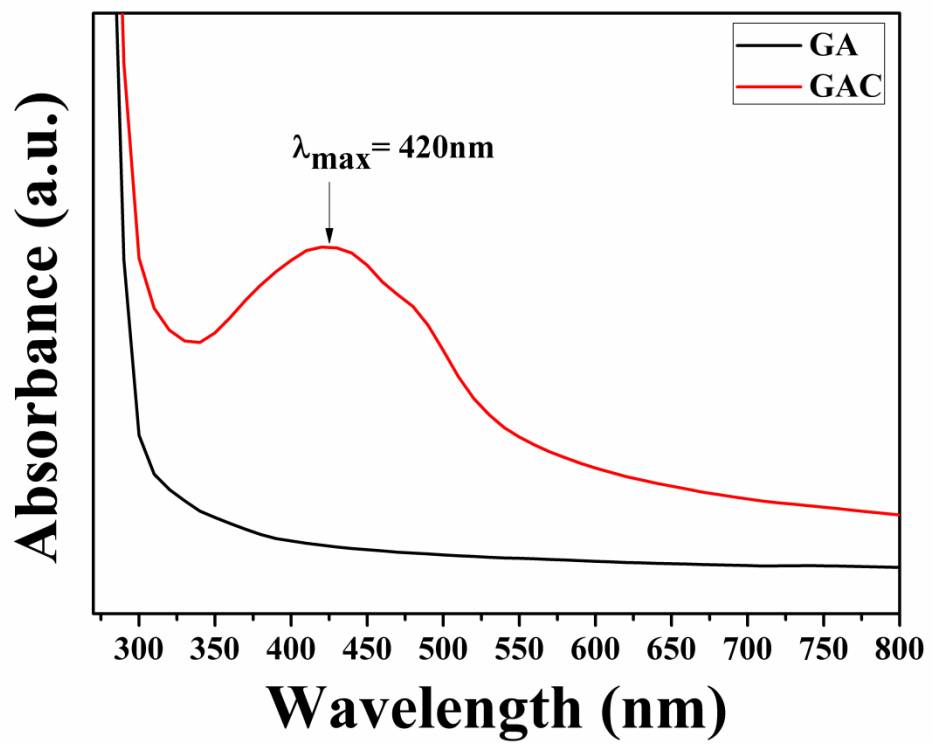


Fig: Absorbance spectrum of GA and GAC

The absorbance spectrum of GA does not show any sharp peak in its spectrum, whereas, GAC shows a broad absorbance peak centred at 420nm and a relatively smaller hump centred at 480nm. The evolution of such peaks in the spectrum of GAC confirms the successful incorporation of curcumin in Gum Acacia's polymeric structure. This also proves that GAC has higher optical activity than un-doped GA.

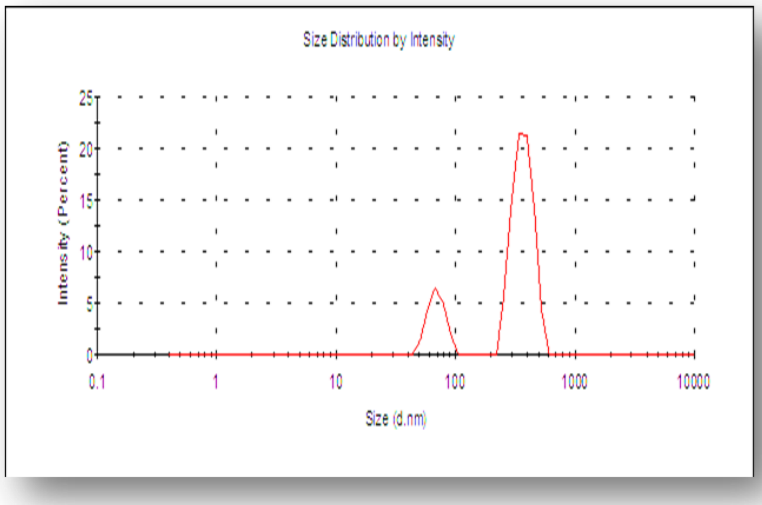
5.1.3.DLS AND ZETA POTENTIAL MEASUREMENT

The Zeta Potential of the nanoparticle as determined by Zetasizer was found to be -0.348mV(for gum acacia nanoparticle) and -0.516mV(for curcumin loaded gum acacia nanoparticle) at a pH of .... The size of the nanoparticle was determined by DLS. Its average hydrodynamic size was found to be 413.3nm(for gum acacia nanoparticle) and 321.3nm(for curcumin loaded gum acacia nanoparticle).

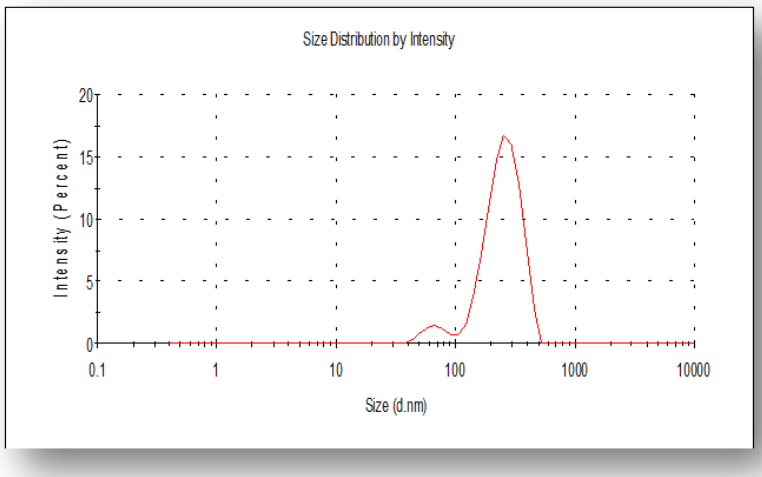
COMPOUNDS	DLS SIZE(dia.nm)	PDI	ZETA POTENTIAL (mv)
Gum acacia nanoparicle	413.3	0.654	-0.348
Curcumin loaded gum acacia nanoparticles	321.3	0.421	-0.516

Table:- Values showing DLS size,PDI and Zeta Potential results

Fig:- Surface charge of nanoparticles



GA



GAC

Fig:-Hydrodynamic size of nanoparticles

5.2.CELL CYTOTOXICITY ASSAY

Cell viability was analyzed with different concentrations of gum acacia nanoparticles and curcumin loaded gum acacia nanoparticles on breast cancer cell lines **MCF-7** and **MDA-MB-468**(0-120 µg/ml). It was found that these biopolymeric NPs had a dose dependent cytotoxicity in these cell lines. The LD50 dose was found to be ....µg/ml and .....µg/ml for MCF-7 and MDA-MB-468 cells respectively.

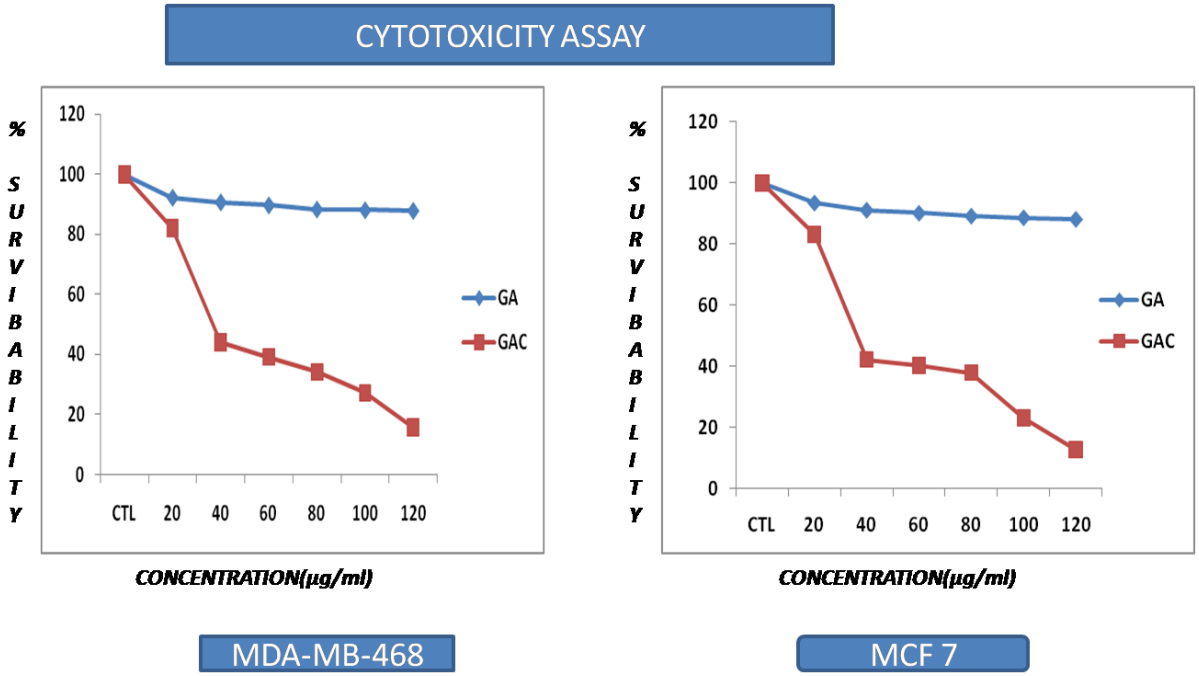
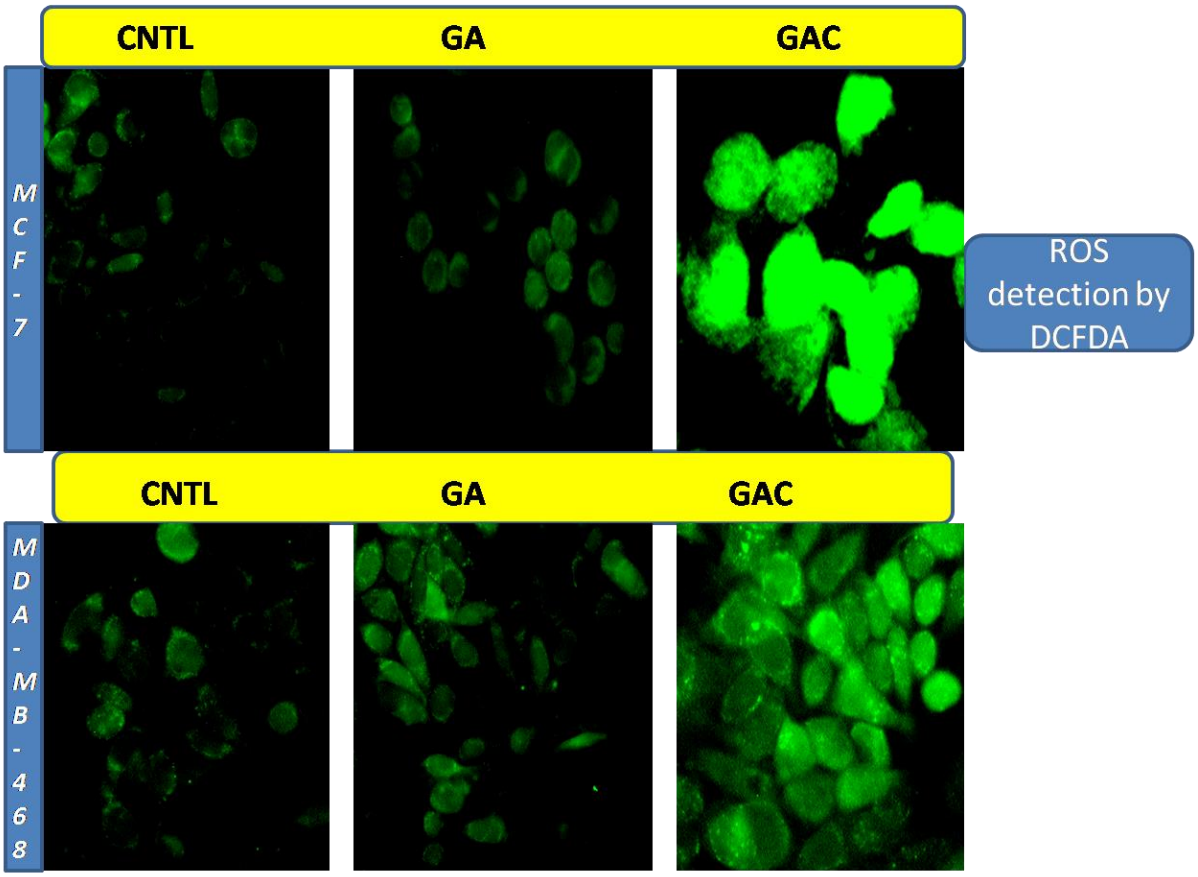


Fig:- Cytotoxicity Assay of MDA-MB-468 and MCF7 cells

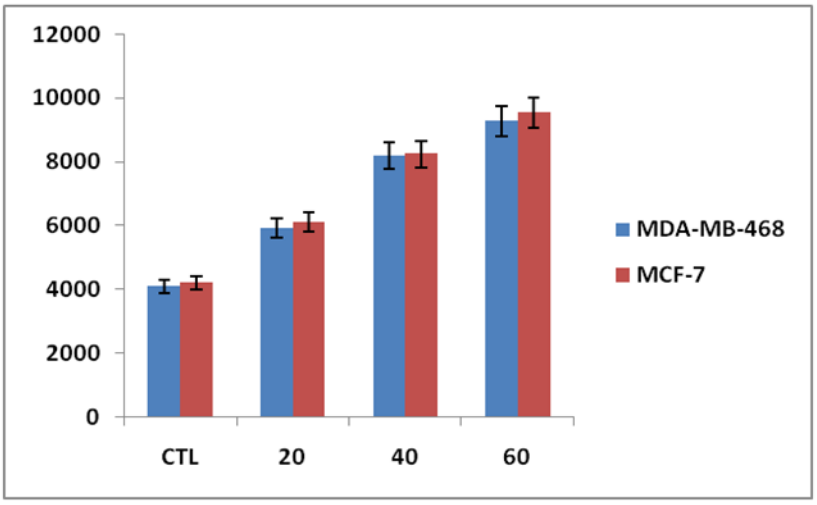
5.3.ROS ASSAY:-

MDA-MB-468 cells were used for determination of reactive oxygen species for 12hrs. It was observed that ROS intensity increased to more than 2fold at 40µg/ml concentration and 3fold at 60µg/ml concentration for the cell line. Fluorescence microscopy also suggested the same with considerably increased intensity being observed at 40µg/ml concentration in comparison to control.



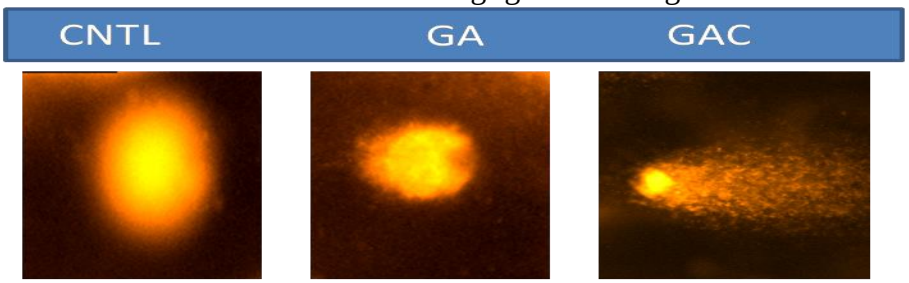
Ros detection formation in control,20µg/ml,40µg/ml treated cells respectively.

Fig:- ROS Detection in Control,20µg/ml,40µg/ml treated cells



5.4.COMET ASSAY

ROS induced DNA damage was also measured in **MCF-7** cell line. DNA damage was analyzed by Comet assay, which is based on damage DNA migration upon electrophoresis. From Comet assay it was observed that tail length upon treatment with a LD50 dose for 24hrs were 28±3nm for MCF-7 cells. This increase of tail length is an indication of DNA damage in cells resulting from the treatment of untreated cells which had no or negligible tail length.



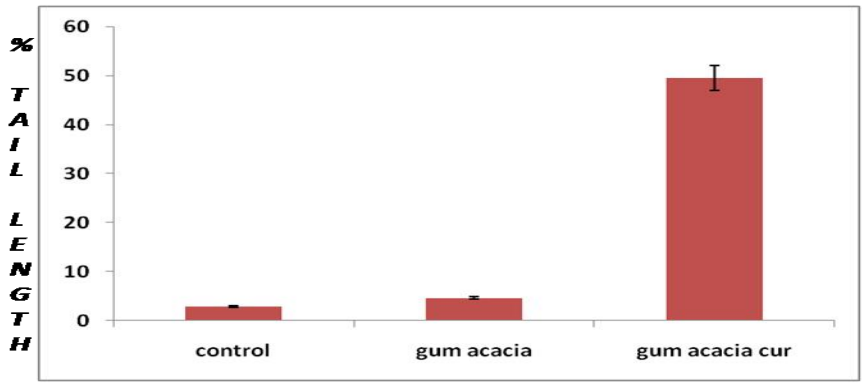


Fig:- DNA Damage (Comet) assay of MCF-7 cell line

5.5 MITOCHONDRIAL MEMBRANE ASSAY

Mitochondria serve as the powerhouse of cells. ROS production is also linked with mitochondrial depolarization. Mitochondrial membrane potential can be measured by JC-1 fluorescent dye. Inside cells it can exist in aggregate form during healthy mitochondria giving red fluorescence and in monomer form during depolarised mitochondrial membrane potential emitting green fluorescence. In our study, treated as well as control cells were stained with 10 µg/ml JC-1 dye. After 24hrs treatment, both the cells were found to have higher intensity of green fluorescence compared to untreated cells which shows strong red fluorescence. This shift of fluorescence intensity from red to green indicates the depolarization or loss of mitochondrial membrane potential in the treated cells.

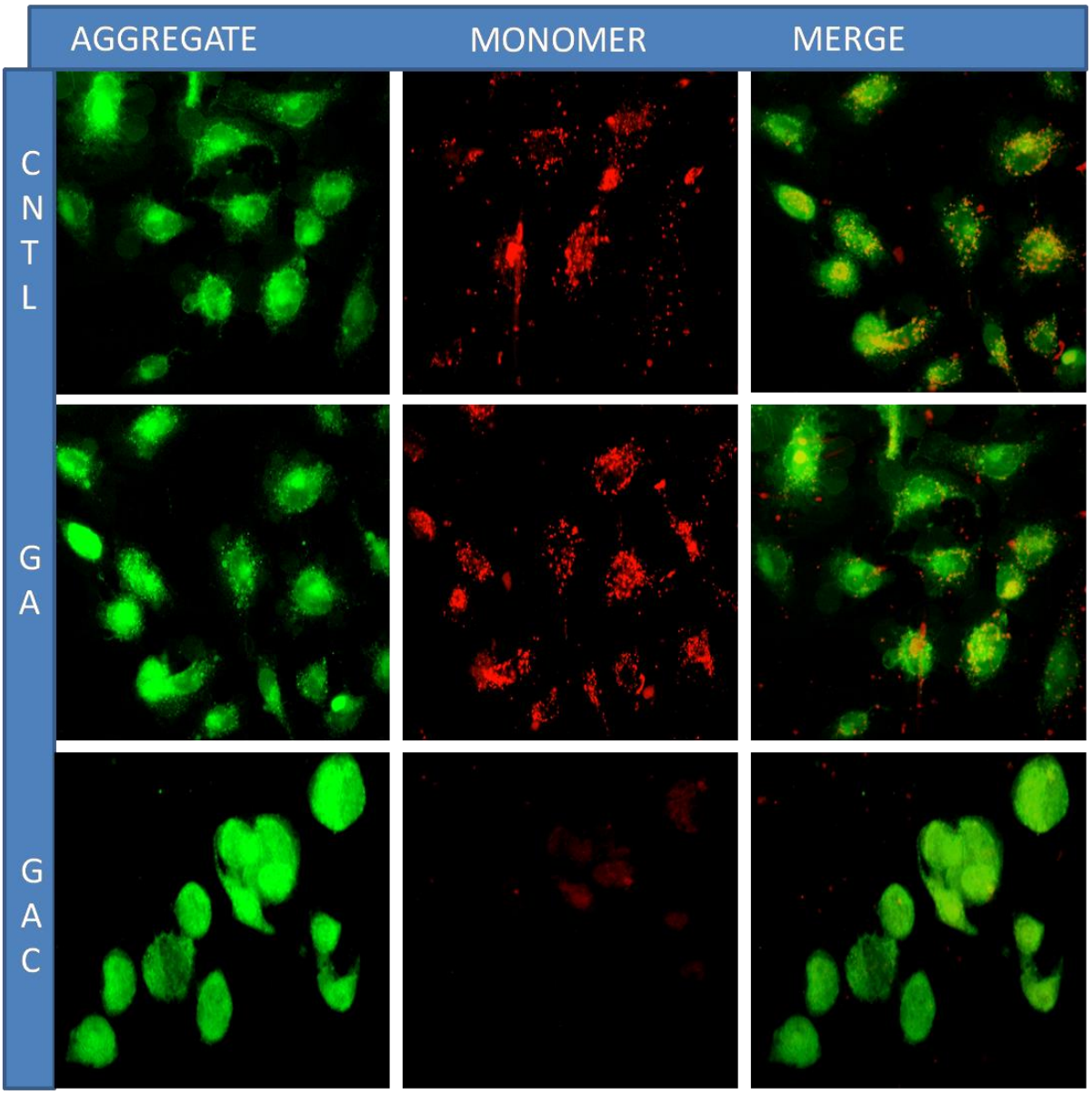


Fig:- Mitochondrial Membrane Assay of MDA-MB-468 and MCF-7 cells

5.6. DAPI STAINING AND NUCLEAR MORPHOLOGY ANALYSIS

To confirm if curcumin loaded gum acacia NPs could induce cell death an apoptosis study was performed **on MDA-MB-468** cells. We have studied the nuclear morphology of treated and control cells by DAPI staining and visualized by fluorescence microscopy. In the control cells an intact nucleus was found but in the treated cells the morphology of the nucleus changed. Apoptotic morphology with disjointed nuclei was detected which confirms that the nanoparticle induced apoptotic cell death in a concentration dependent manner.Large numbers of apoptotic nuclei were observed when **MDA-MB-468** cells were treated with 20 and 40 µg/ml of curcumin loaded gum acacia NPs. This result suggests that curcumin loaded gum acacia nanoparticle could competently deliver the drug to the cancer cells, thus preventing cell growth and inducing apoptosis.

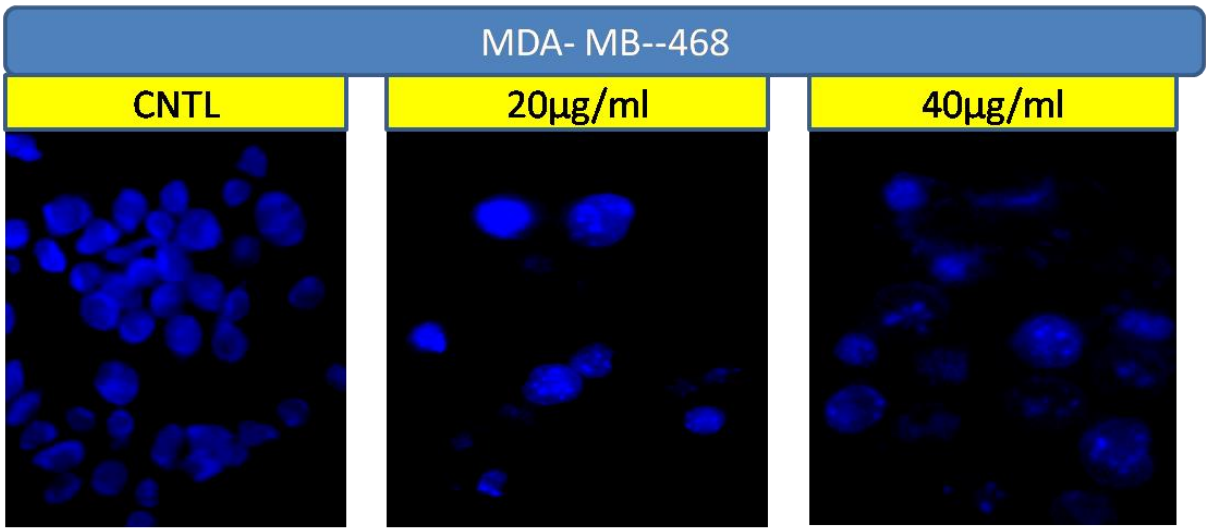


Fig:- Nuclear morphology study by DAPI staining

5.7. CELLULAR MORPHOLOGY STUDY

To verify whether curcumin loaded gum acacia nanoparticles could exert any effect on the cellular morphology of **MDA-MB-468cells** were treated with different concentrations(0,20,40 µg/ml)of the nanoparticles at 37ºC for about 5days.It was observed that in the control cells there was no change in morphology and the nucleus was intact. But in the treated cells the nucleus was disintegrated and various morphological distortions were noticed. This indicates that curcumin loaded gum acacia NPs are toxic towards **MDA-MB-468cells**.

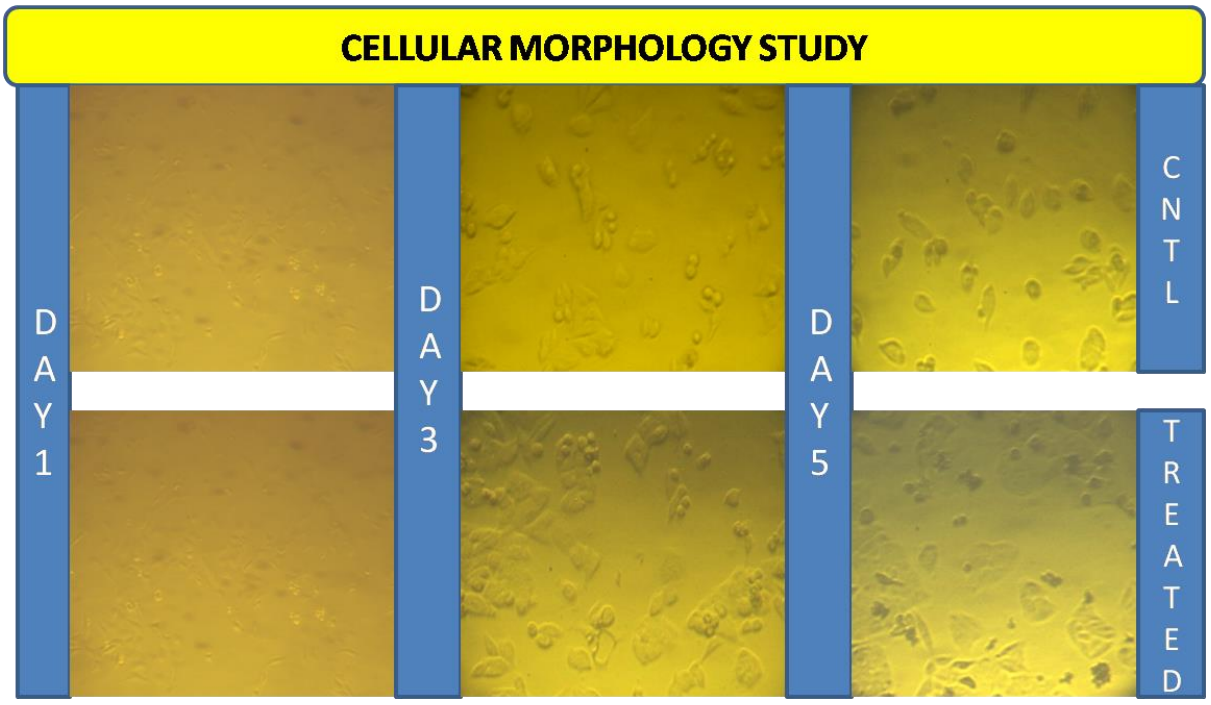


Fig:-Study of change in morphology of treated MDA-MB-468 cells

5.8 .CELLULAR UPTAKE STUDY

This fluorescence microscopy study is further performed to ensure the cellular uptake of curcumin loaded gum acacia by **MDA-MB-468cells**. From the fluorescence image it is observed that with increasing time, more nanoparticles are internalised.

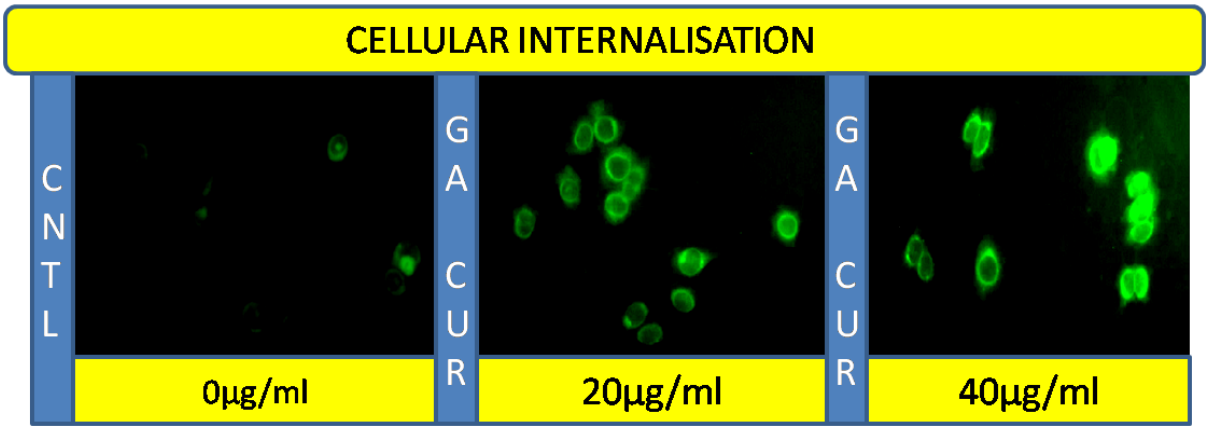


Fig:- Cellular Internalisation

6.CONCLUSION:-

Curcumin loaded Gum Acacia nanoparticles are created by nanoprecipitation technique. Encapsulation of curcumin in nanospheres increases its water solubility and bioavailability.Dynamic light scattering studies revealed the nanosize and negative zeta potential of the nanoparticles. Curcumin loaded nanospheres showed controlled curcumin release profile. MTT assay in **MCF-7** and **MDA-MB-468** cells showed that the curcumin loaded nanospheres were toxic towards the cells.ROS assay showed that the treated cells had increased ROS intensity. In mitochondrial membrane assay, the shift of fluorescence intensity from red to green indicated depolarisation or loss of mitochondrial membrane potential in the treated cells. In DAPI staining presence of apoptotic morphology with distorted nucleus in the treated cells proved that curcumin loaded gum acacia nanoparticles induced apoptosis in them. Changes in the cell morphology upon treatment with the nanoparticles was also studied.The cell uptake studies also revealed that nanospheres could enter into the cells and making use of the green fluorescence of curcumin it could illuminate the cells. All these results prove that curcumin loaded Gum Acacia nanospheres are promising as carriers for curcumin drug delivery to breast cancer cells.

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