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Purification, characterization and exploitation of *Azadirachta indica* gum for the production of drug loaded nanocarrier

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Abstract

In this study, gum polysaccharide of *Azadirachta indica* was extracted and purified. The obtained polysaccharide was subjected to TLC chromatography, spectroscopic analysis, thermogravimetric analysis and GC-MS analysis. The polysaccharide was found to have Glucose, Idosan, Allose, Galactose, Ribose and Xylose. The polysaccharide was not having antibacterial activity but possessed good antioxidant and anticancer activity. The extracted polysaccharide was further carboxymethylated and used for the synthesis of nanocarrier to carry anticancer drug, curcumin. Size of the drug unloaded nanocarrier were found to be size below 40 nm, whereas the drug loaded nanocarriers were around 50 to 70 nm. The nanocarriers were studied for cytotoxicity against MCF7 cancer cell line and found to be effective.

1. Introduction

Nature has given tremendous resources with versatile applications. Among all these resources, plant products have been widely used for its recyclability and reliability. Plant gum is one such natural product. These Natural gums exudates are polysaccharides, they are capable of causing an increase in the solution's viscosity, even at small concentrations. They are usually found in the woody elements of plants (bark) or in epidermis of the seeds (Setia 1984). The plant gums are excreted as response to stress created either by abiotic or biotic sources. These plant gums are either hydrophilic or hydrophobic and are widely used for their chemical inertness, biocompatibility, biodegradability and sustainability.

Azadirachta indica also called as Indian Lilac, Margosa, Neem, Nimtree etc, is found in many parts of Asia, Tropical Indian subcontinents like India, Bangladesh and also in Myanmar. Gum production is very often seen with the trees of dry areas. It usually appears in large droplets, nodules, vermiform pieces. Mostly they are found in the wounded areas of the tree. Gum cavities in young stems are responsible for gum production, it commonly secreted out when the cavity is disturbed or broken. These cavities are also found with microbial growth (Setia 1984, Choudhary and Pawar 2014). The gum appears to be clear pale yellow, light brown, amber in colour. With age the colour of the gum becomes darker (Choudhary and Pawar 2014). The gum forms a viscous solution while dissolved with water and reported to consist of L-Arabinose, D-Glucuronic Acid, L-Fucose, and D-galactose (Lakshmi and Pattabiraman 1967, Mukherjee and Srivastava 1955). The other chemical properties have also been studied (Nayak and Pattabiraman 1979).

Polysaccharides can be used as nanocarrier to carry drugs, which is possible when the polysaccharides are carboxymethylated and chelated with an appropriate chelator like STMP (sodium tri metaphosphate), STPP (sodium tripolyphosphate) etc (Samrot *et al* 2017, Samrot *et al* 2018a, Shobana *et al* 2019). These polysaccharide nanocarriers have attracted attention of various researchers in the field of medical field for drug delivery and drug encapsulation (Justin *et al* 2017, Justin *et al* 2018, Sruthi *et al* 2019). This is mainly because particles at smaller size exhibits wide range of properties than the bulk material. Nanocarriers are useful to carry the drug to its site of action without making the drug degraded till it reaches the site of action. In this study, polysaccharides extracted from the *Azadirachta indica* was extracted, purified, characterized and analyzed for its bioactivities. Later, the polysaccharides were carboxymethylated and used for producing curcumin encapsulated nanocarriers and the nanocarrier was characterized by both microscopic and spectroscopic analysis and utilized for invitro bioactivity studies like antibacterial and anticancer activity.

2. Materials and methods

2.1. Materials used

The plant gum exudates of *Azadirachta indica* was collected from Perambur, Chennai. Curcumin was bought from SRL Pvt LTD, India, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), acridine orange and ethidium bromide were bought from Sigma Chemicals, India. STMP, Trichloro acetic acid (TCA), acetone, DPPH (2,2-diphenyl-1-picrylhydrazyl), ethanol and NaOH were bought from Qualigens, India. All the reagents and chemicals of analytical grade was used in this study. Millipore water was used for preparing all the reagents.

2.2. Collection of plant gum

The gum exudates of *Azadirachta indica* was collected from the plant bark of neem tree. The collected gum exudates were completely dried, crushed into powder, sieved and was stored.

2.3. Extraction and purification of polysaccharide

Prior to the extraction of polysaccharide, the plant gum powder was defatted by soaking in ethanol for overnight, followed with drying in hot air oven. 20 g of dried defatted gum was added with 200 ml distilled water, stirred continuously for 4 h and filtered. 100 ml of distilled water was added to the filtrate, stirred under 100 °C for 1 h and filtered. To the obtained filtrate. equal quantity of 10% Trichloroacetic acid was added to precipitate the proteins, which was removed as pellet after centrifugation at 10000 rpm. Supernatant was added with acetone in the ratio 1:0.5 (Supernatant : Acetone). This results in the formation of white mass (Dodi *et al* 2016, Samrot *et al* 2018a, Samrot *et al* 2019a, Samrot *et al* 2019b). The aqueous polysaccharide was then lyophilized and stored in airtight containers.

2.4. Characterization of polysaccharide

The extracted polysaccharide was subjected for UV-Vis Spectroscopic analysis, Fourier Transform Infrared Spectroscopic analysis (Shimadzu, Japan), phenol sulphuric acid assay (Mahendran *et al* 2008, Albalasmeh *et al* 2013), TGA analysis and TLC (having butanol : acetic acid : water (2:1:1) as mobile phase) and GC – MS (JMS-TQ4000GC GC Triple Quadrupole Mass Spectrometer) analysis after derivatization to identify monosaccharides (Gallina *et al* 2004, Raguraman *et al* 2019).

2.5. Bioactivities of polysaccharide

2.5.1. Bioactivity studies

The extracted polysaccharide, at different concentrations, was tested for antibacterial activity against two bacterial strains namely *E.coli* (Gram negative) and *Streptococcus* (Gram positive) by agar well diffusion method (Raji *et al* 2014). Antioxidant assays such as DPPH assay (Radical Scavenging assay) (Maizura *et al* 2011), FRAP (Ferric Reducing Antioxidant Power) assay (Benzie and Strain 1996) and Phosphomolybdenum assay (Saha *et al* 2008) were also performed to identify the antioxidant properties of the extracted polysaccharide. Anticancer activity of polysaccharide against MCF7 (Michigan Cancer Foundation-7) human breast cancer cell line was evaluated by MTT assay (Mosmann (1983)) and apoptosis for IC₅₀ (Inhibition Concentration 50) value obtained by MTT assay was detected by Acridine Orange – Ethidium Bromide staining (AO/EB) (Samrot *et al* 2018a). Pathological changes in cancer cells were observed and recorded using fluorescence microscope (Thermofisher).

2.6. Polysaccharide based nanocarrier synthesis

Prior to nanocarrier preparation, the polysaccharide was carboxymethylated. Two beakers with each containing 100 ml Millipore water was added with 1 g of extracted polysaccharide and stirred at 45 °C for 2 h for complete

dissolution. One of the beakers was added with 20 ml 0.1 M NaOH and another beaker was added with 20 ml of 1 M NaOH and those were designated as CMAI-1 and CMAI-2 respectively. The solutions were heat stirred at 60 °C for overnight and cooled at room temperature. The mixture was then adjusted to neutral pH. Later, the mixture was added equal volume of ethanol and the white precipitates were separated by centrifugation. The pellets were washed with ethanol and were subjected to dialysis against deionized water for 3 days. It was then subjected to lyophilization (Dodi *et al* 2011, Samrot *et al* 2018a).

Two different concentrations of CMAI-1 and CMAI-2 (0.05 and 0.02 % w/v) of carboxymethylated polysaccharides were added separately to 100 ml of 1 M NaOH solution and magnetic stirred for 2 h. The solution was then subjected to probe sonication for 15 min 100 ml of STMP solution (Tri sodium Tri meta phosphate), was prepared as two different concentrations (0.02 and 0.01 % w/v) and were added drop wise to CMAI-1 and of CMAI-2 solutions respectively under continuous stirring and was stirred overnight at 60°C. The stirring was followed by dialysis for 4 days against deionized water to adjust the solution to neutral pH. The nanocarriers were collected through centrifugation. The supernatant was discarded and the pellets were lyophilized (Samrot *et al* 2018a). This results in four different nanocarriers – CMAI 1.1 (0.05 g of CMAI-1 and 0.02 g of STMP), CMAI 1.2 (0.02 g of CMAI-1 and 0.01 g of STMP), CMAI 2.1 (0.05 g of CMAI-2 and 0.02 g of STMP), CMAI 2.2 (0.02 g of CMAI-2 and 0.01 g of STMP). All the synthesized nanocarriers were subjected to characterization under Scanning Electron Microscope.

2.7. Drug loading into nanocarriers

2.7.1. Ultrasonication assisted drug loading

The least sized nanocarrier synthesized was identified using SEM and it was considered for drug loading. The drug (curcumin) solution was prepared by dissolving 15 mg of the curcumin in 10 ml of ethanol. 25 mg nanocarriers dispersed in 10 ml deionized water was added to the drug solution with continuous stirring at room temperature for 15 min, following that it was subjected to high frequency water bath sonicator for about 30 min (Samrot *et al* 2018a). The obtained solution was subjected to centrifugation and washed thrice with Millipore water and lyophilized.

2.8. % Encapsulation efficiency

After sonication, the entire solution was centrifuged as mentioned earlier, the supernatant was checked for absorbance at 425 nm (Senthilkumar *et al* 2017). % drug encapsulation efficiency was determined and calculated as reported earlier (Awotwe-Otoo *et al* 2012, Shobana *et al* 2019).

2.9. Drug release kinetics of curcumin loaded nanocarrier

10 mg of drug loaded nanocarrier was kept submerged in 200 ml PBS at 7.4 pH. At every 15 min time interval, 1 ml of PBS was subjected for spectrophotometric analysis at 425 nm to know the curcumin release (Samrot *et al* 2018a).

2.10. Characterization of nanocarriers

The carboxymethylated polysaccharide was characterized using FTIR. Both the loaded and unloaded nanocarriers were characterized using techniques like FTIR (Shimadzu, Japan), scanning electron microscopy (SEM Jeol JSM-5610LV), Atomic Force Microscopy (Bruker, Dimension icon model) for particle shape and size detection.

2.11. Anticancer activity of loaded and unloaded nanocarriers

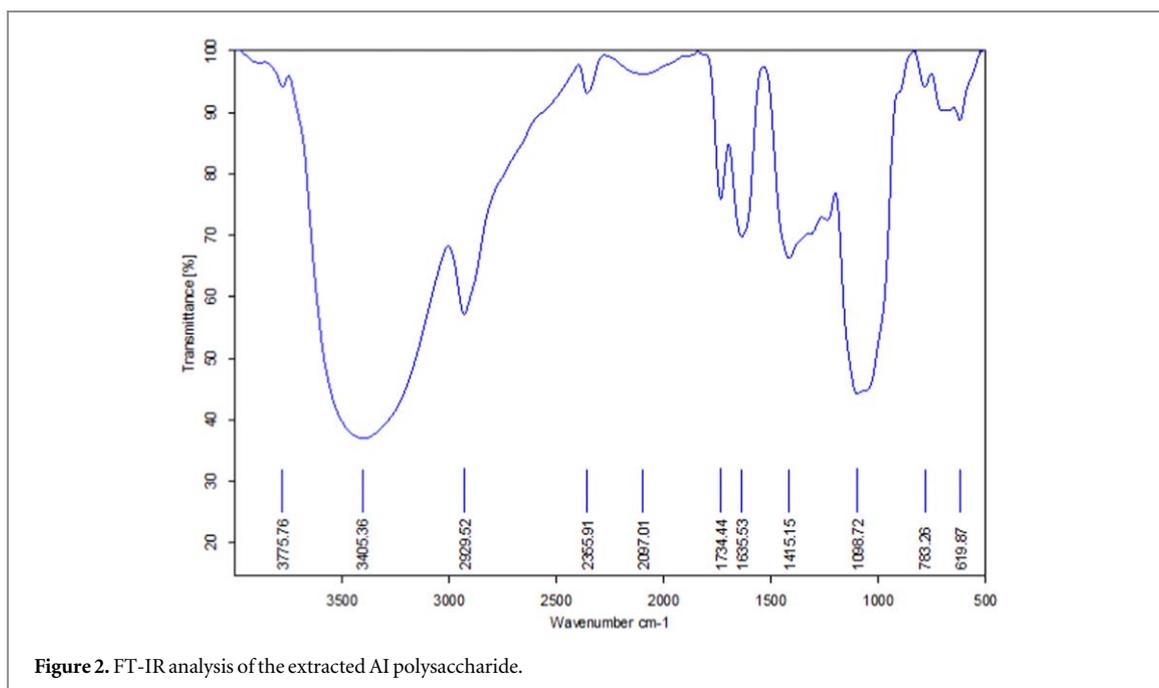
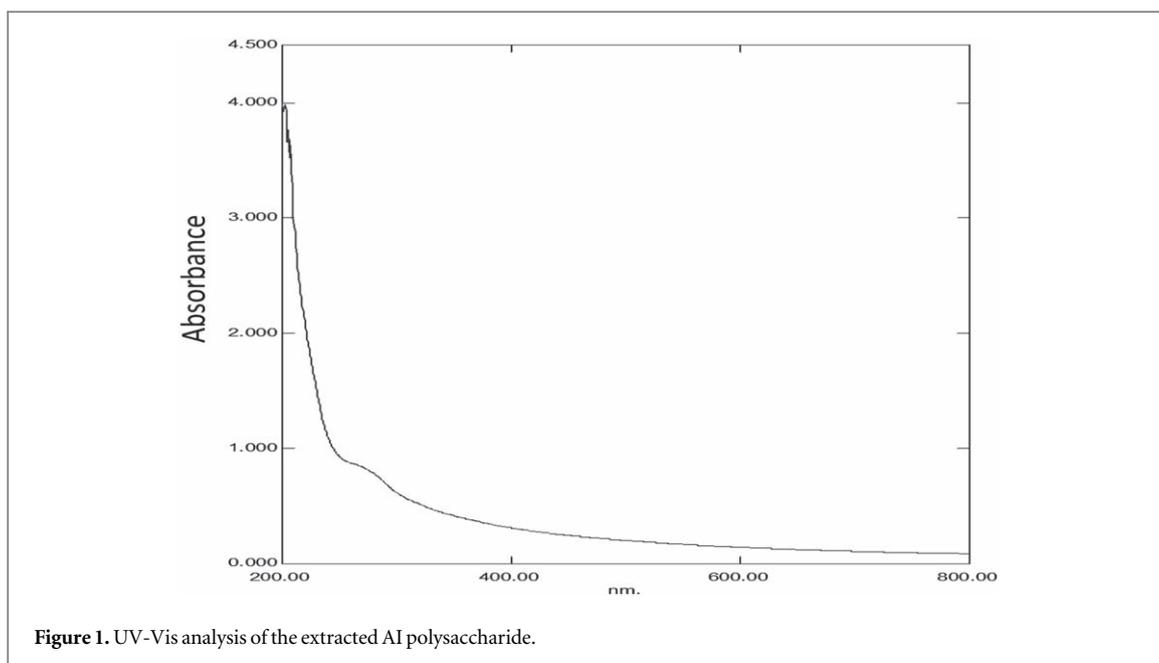
MCF7 breast cancer cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. MTT assay was performed for the various concentration of nanocarriers (Mosmann (1983)).

3. Results and discussion

3.1. Characterization of gum polysaccharide

The aqueous solution of the extracted polysaccharide of *Azadirachta indica* was analyzed with UV-Visible Spectroscopy (Shimadzu UV-1800, Japan). Absorbance maximum was found between the range of 265 – 285 nm (figure 1) and further no absorbance was found. Presence of peak at 210 nm and around 265 nm might be for xylose and glucose respectively.

FT-IR analysis showed peak at 3405 cm^{-1} , indicated H-O-H stretching, (figure 2) 2929 cm^{-1} indicated -CH Stretching in Alkyl group. 1418 cm^{-1} corresponded to CH_3 and CH_2 bending, peak 1098 cm^{-1} corresponded to C-O-C esters (Samrot *et al* 2018, Samrot *et al* 2019a).



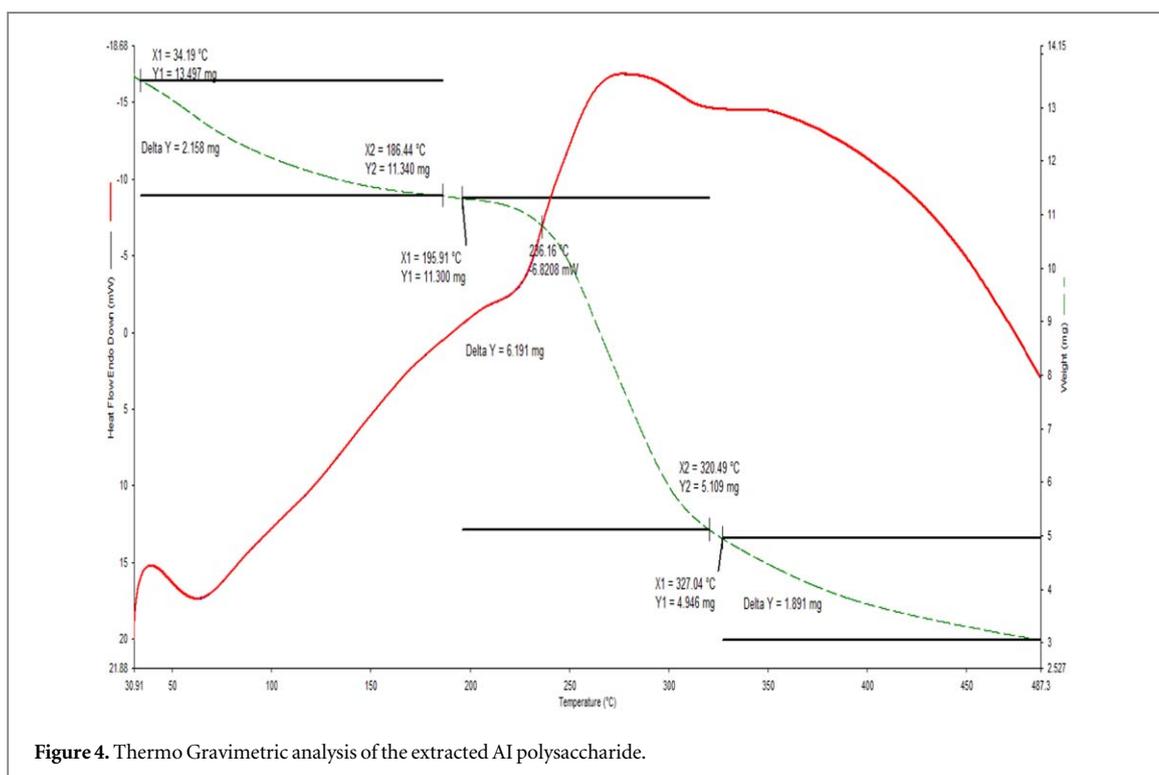
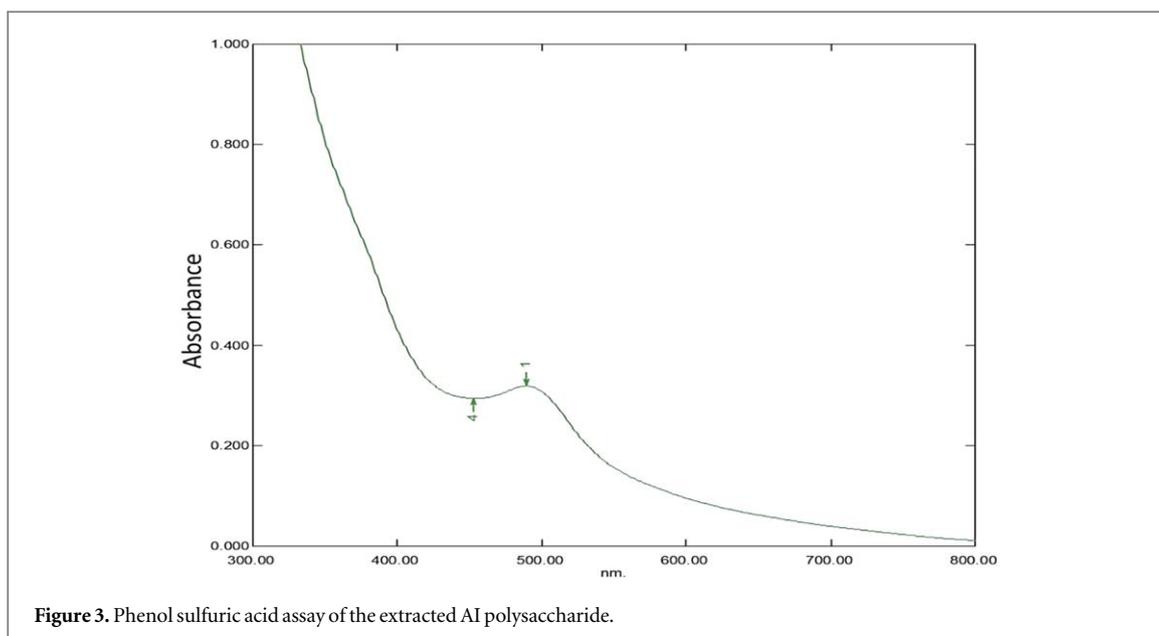
Phenol sulphuric acid assay clearly indicated the presence of various sugars present as absorbance was found between 420 and 530 nm, which corresponded to glucose, galactose, mannose, xylose (figure 3) (Dodi *et al* 2016). The thermo gravimetric analysis was done to detect the thermal stability of the polysaccharide (Jamaludin *et al* 2017). The polysaccharide showed a loss of weight from 100 % to 63.3 % between the temperature range of 50 and 485 °C (figure 4).

TLC for the derivatized polysaccharide was performed having Butanol : Acetic acid : Water (2:1:1) as mobile phase. Galactose, glucose, fructose, arabinose, xylose with the respective corresponding R_f value of 0.37, 0.46, 0.52, 0.48, R_f –0.55 was found (figure 5) (Boual *et al* 2012).

Using the GC-MS chromatogram (figure 6), the sugars like Glucose, Idosan, Allose, Galactose, Ribose and Xylose were identified (Gallina *et al* 2004).

3.2. Bioactivities of polysaccharide

The *Azadirachta indica* gum polysaccharide showed inhibition against *E.coli* strain at the maximum concentration (table 1), but no zone of inhibition was recorded against the gram positive stain. Neem extracts and neem exudates are known to exhibit various biological activity (Bijauliya *et al* 2018).



Activities like free radical scavenging and antioxidant activity and reducing property of gum derived polysaccharide showed an increasing activity with increase in concentration (figure 7). Polysaccharide of *Terminalia catappa* has shown increased antioxidant activity as the concentration increased (Samrot et al 2018a).

Gum polysaccharide was found with anticancer activity with IC_{50} around $220 \mu\text{g}$ against the cell line used (figure 8(a)). The obtained IC_{50} value of polysaccharide was utilised for apoptosis detection against MCF7 cell line. The control cells were with green fluorescing nuclei (figure 8(b1)), where the treated cell lines were with red fluorescence of apoptotic cells indicating the anticancer activity of polysaccharide (figure 8(b2)). Similarly, the polysaccharides of *Terminalia catappa* was also found to show anticancer activity when treated against HepG2 cell line (Samrot et al 2018a).

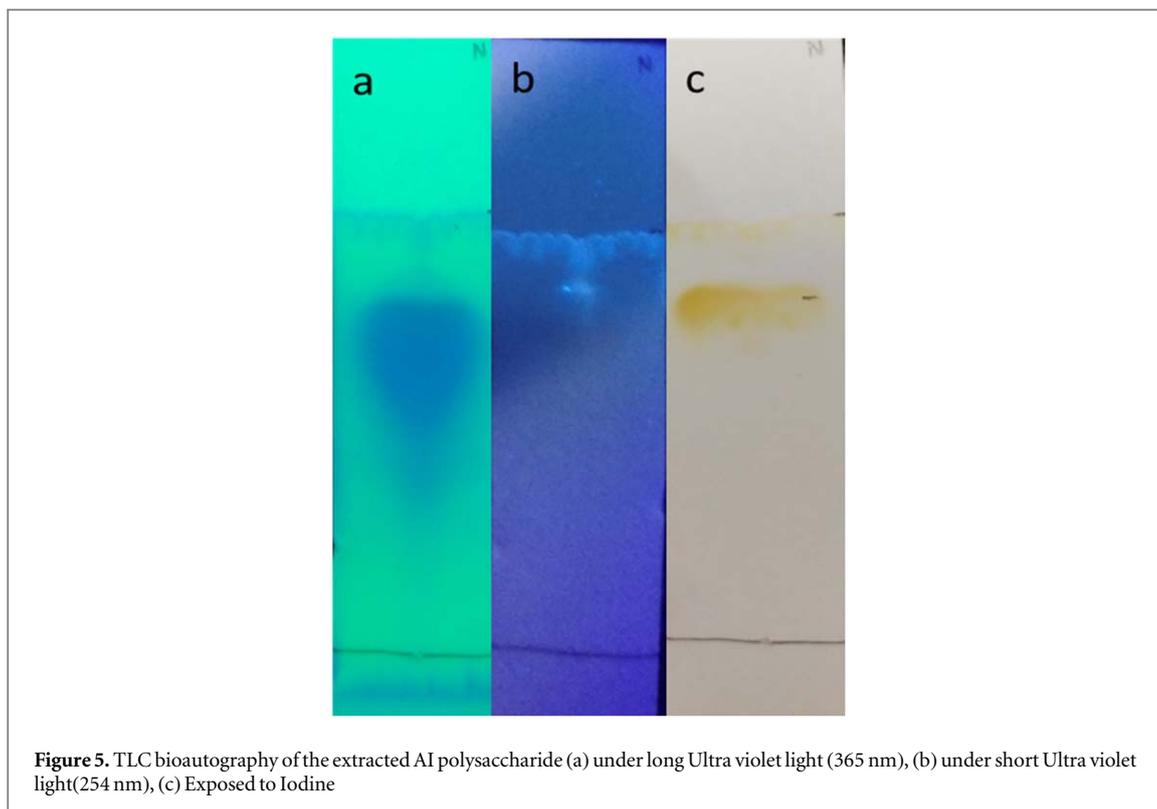


Figure 5. TLC bioautography of the extracted AI polysaccharide (a) under long Ultra violet light (365 nm), (b) under short Ultra violet light(254 nm), (c) Exposed to Iodine

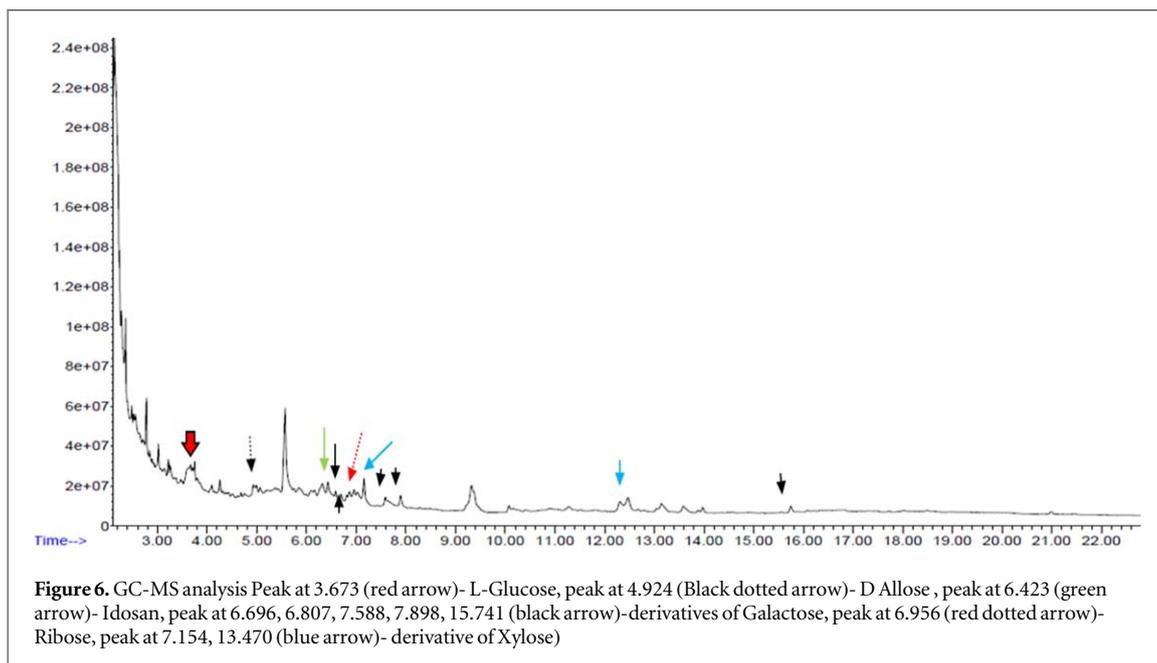
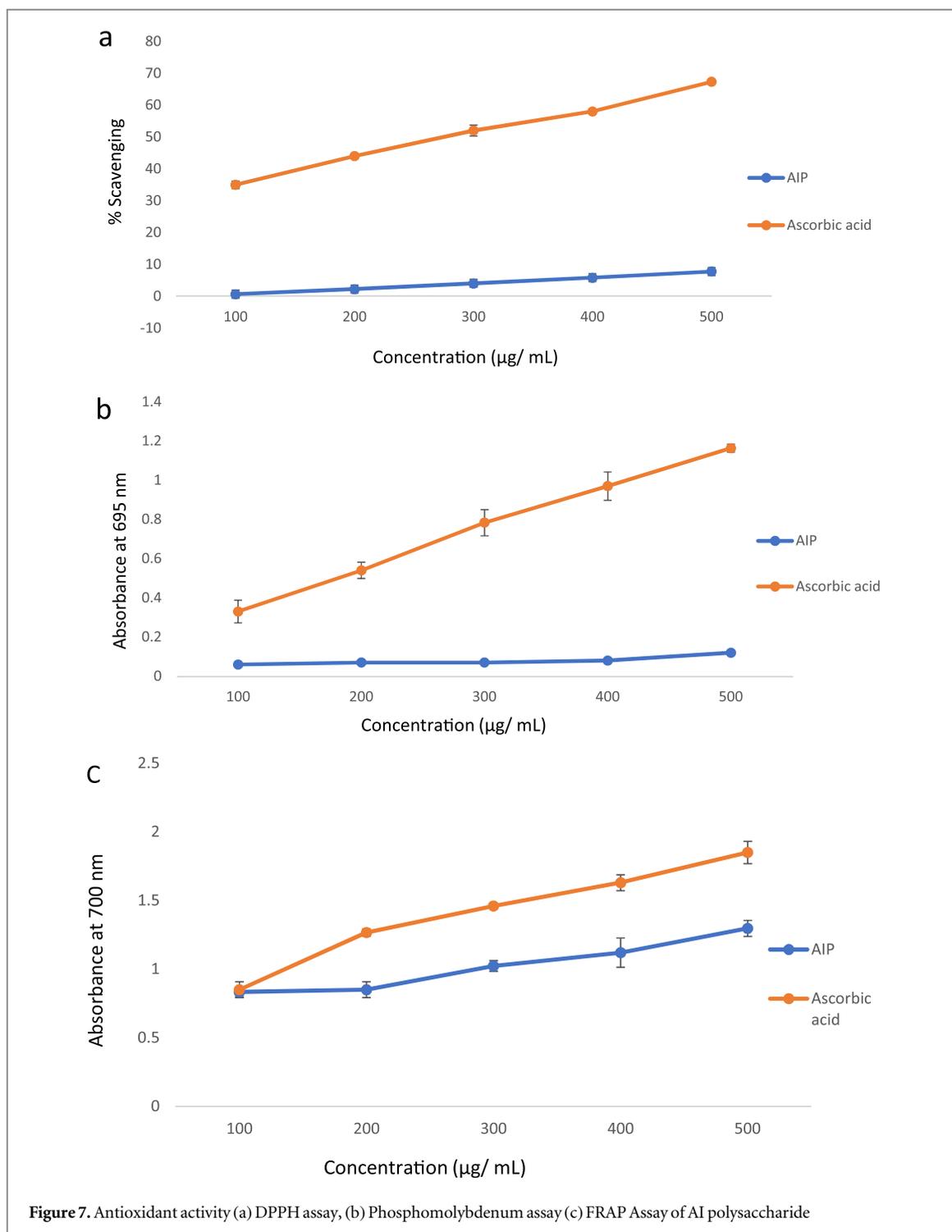


Figure 6. GC-MS analysis Peak at 3.673 (red arrow)- L-Glucose, peak at 4.924 (Black dotted arrow)- D Allose , peak at 6.423 (green arrow)- Idosan, peak at 6.696, 6.807, 7.588, 7.898, 15.741 (black arrow)-derivatives of Galactose, peak at 6.956 (red dotted arrow)- Ribose, peak at 7.154, 13.470 (blue arrow)- derivative of Xylose)

Table 1. Antibacterial Activity of AI polysaccharide.

SAMPLE	ORGANISM	ZONE OF INHIBITION (cm)					
		CONTROL	CONCENTRATION (μ l)				
		+	—	5	10	15	20
<i>Azadirachta indica</i>	<i>E. coli</i>	1.6	—	—	—	—	0.6
	<i>Streptococcus</i>	1.7	—	—	—	—	—

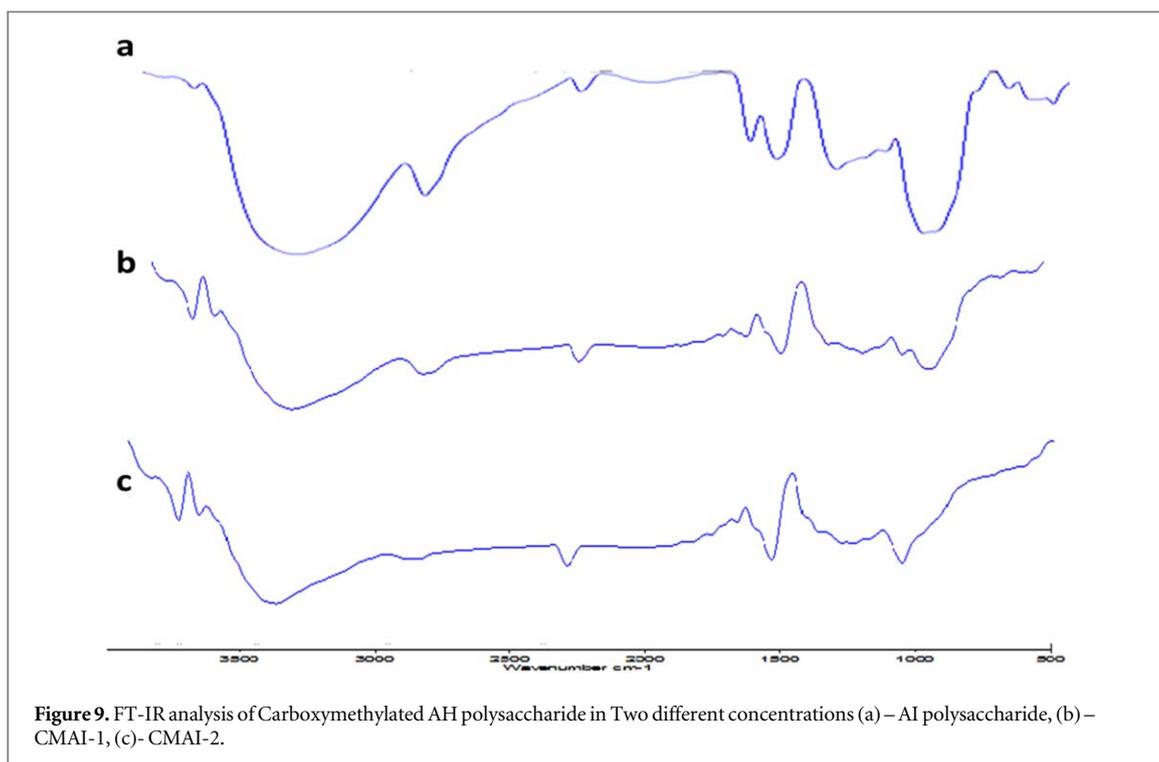
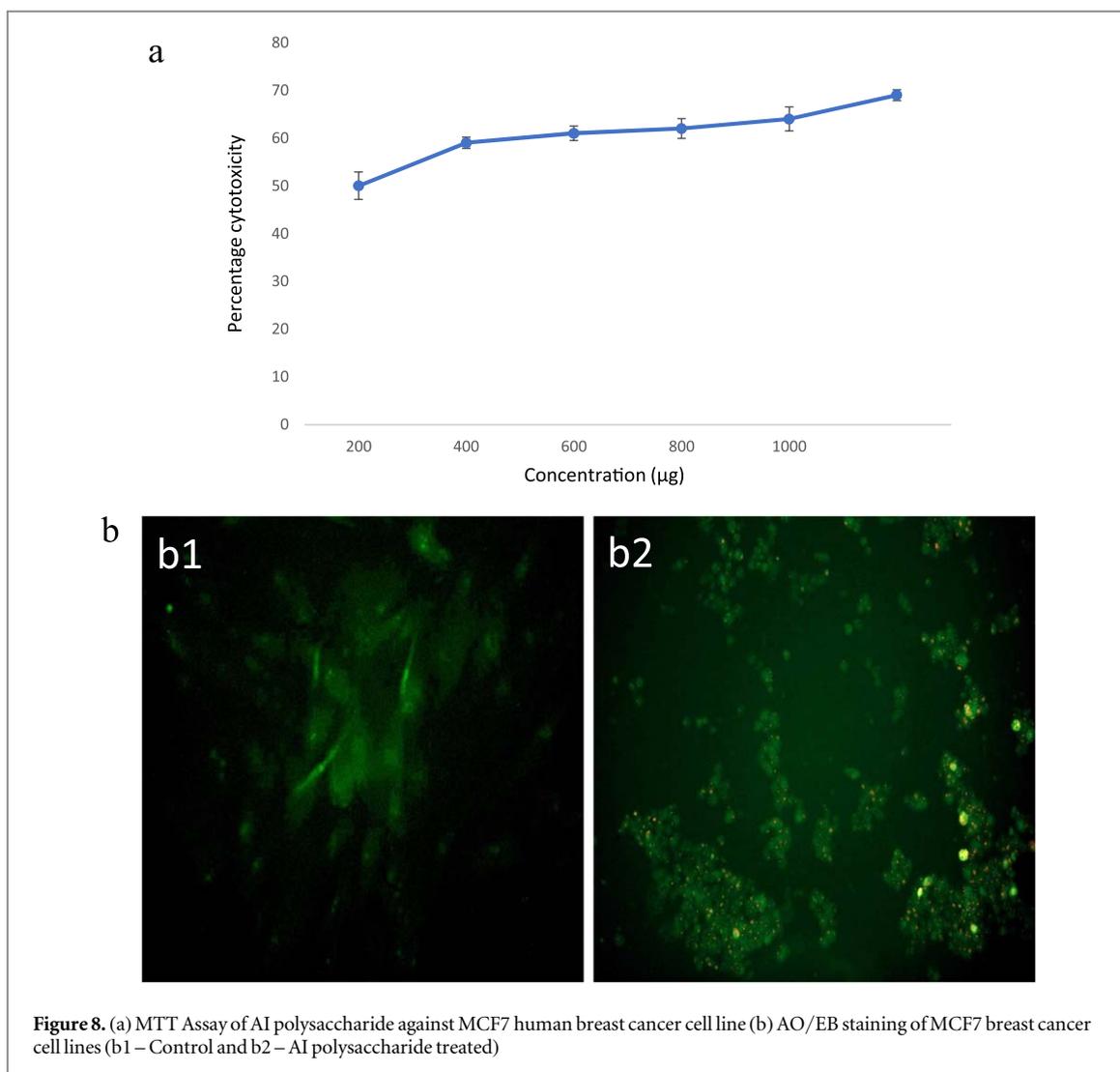


3.3. FT-IR of carboxymethylated polysaccharide

Substitution of -OH groups was observed in carboxymethylated polysaccharide as observed by reduction of peaks at 3400 cm^{-1} (figure 9). Carboxymethylation was reported to reduce peak at 3417 cm^{-1} in almond gum polysaccharide (Samrot *et al* 2018).

3.4. Characterization of nanocarrier (Loaded and Unloaded)

The synthesized nanocarriers were analysed using SEM for particle size and morphology identification. The nanocarriers were synthesized using four different concentrations (CMAI-1.1, CMAI-1.2, CMAI-2.1, and CMAI-2.2) by varying the amount of carboxymethylated polysaccharide and the cross-linking agent (STMP). All the four concentration nanocarriers (figure 10 a-d) were analyzed. The nanocarriers CMAI-1.1, CMAI-1.2, CMAI-2.1 were highly aggregated and had no regular shape, whereas the CMAI-2.2 had a proper spherical shape and the particles were around 40 nm and this particle was used for curcumin loading (figure 10(d)).



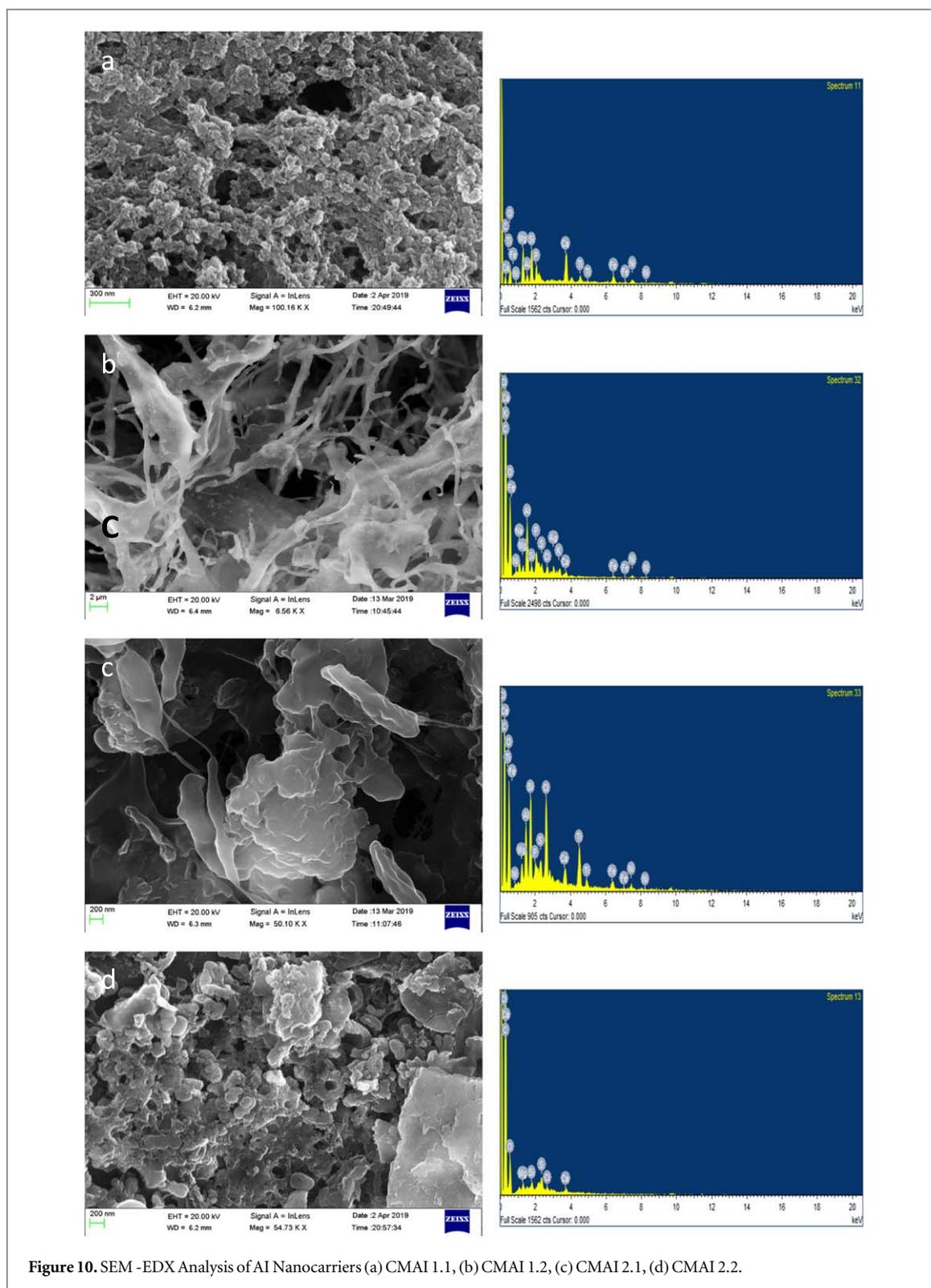
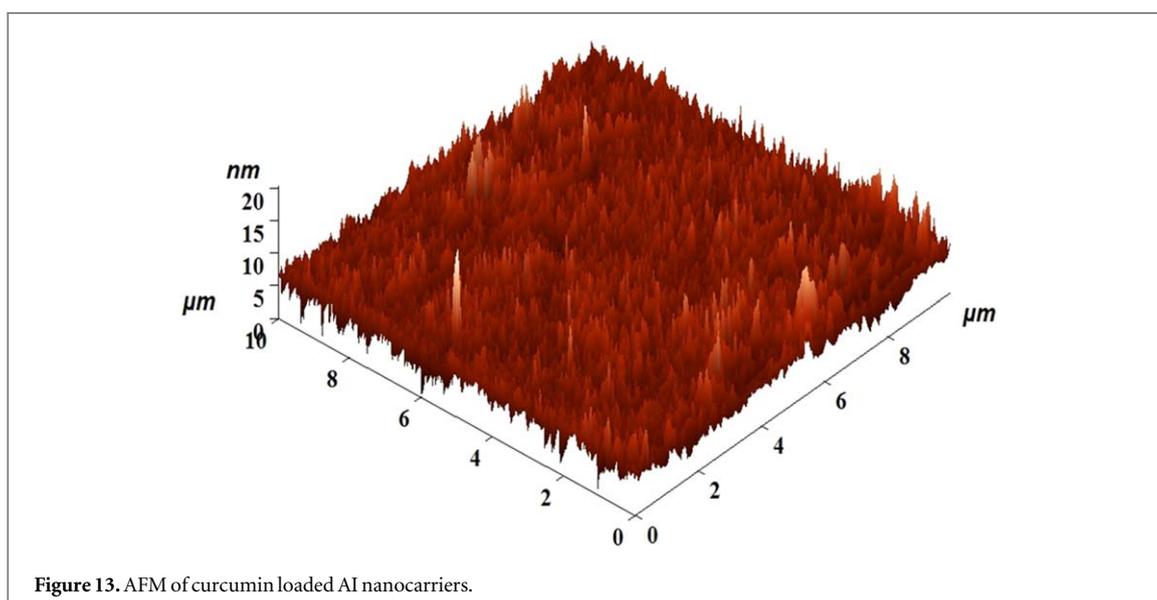
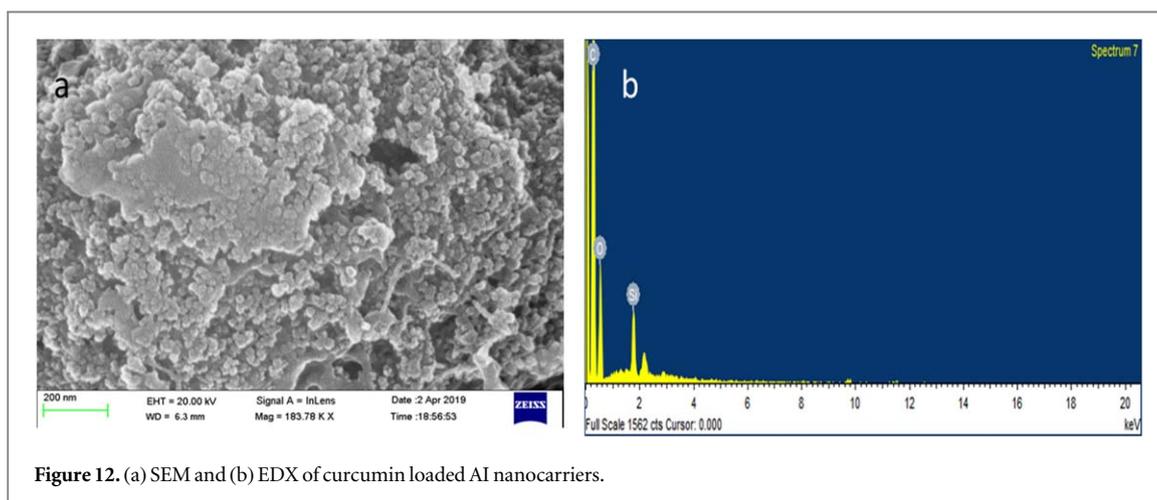
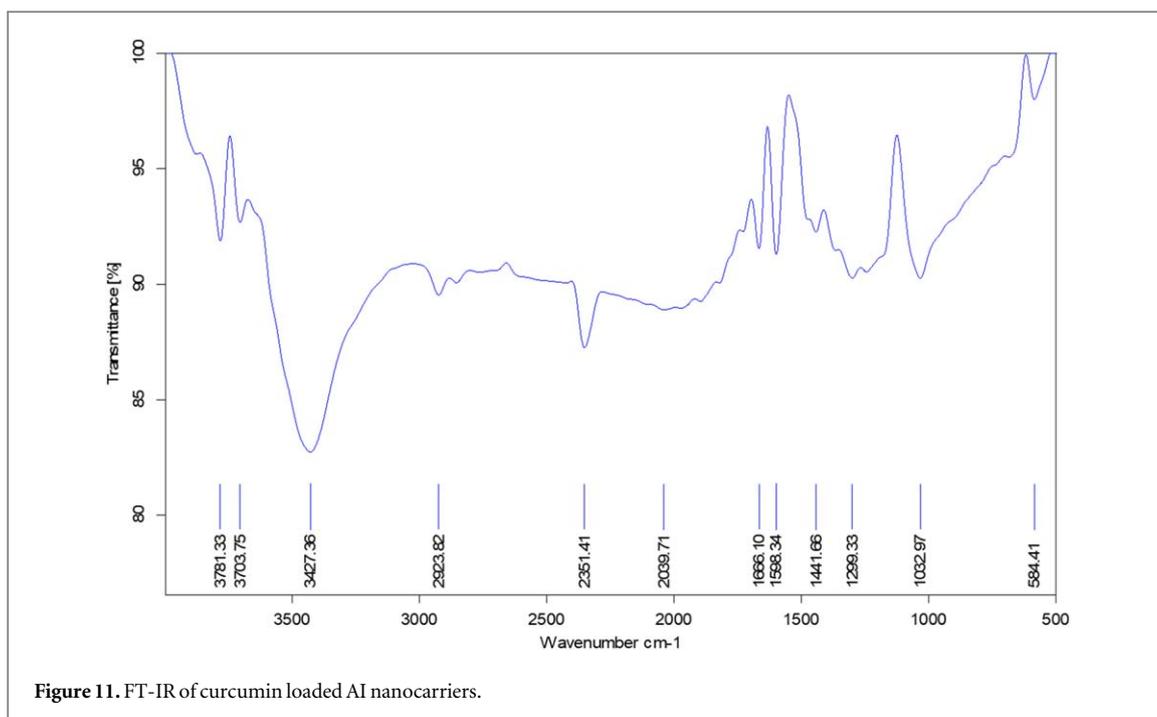


Figure 10. SEM -EDX Analysis of AI Nanocarriers (a) CMAI 1.1, (b) CMAI 1.2, (c) CMAI 2.1, (d) CMAI 2.2.

Unloaded nanocarriers was showing an intense peak at 3452 cm^{-1} for OH stretching present in the gum polysaccharide. 2352 cm^{-1} peak was indicating the P–O bending and the peak at 886 cm^{-1} was because of CH bending (Samrot *et al* 2018). In loaded nanocarriers, the curcumin loading was confirmed from the stretching peak formed at 2117 cm^{-1} for C=C aromatic ring. 1280 cm^{-1} peak corresponded to CO which were not found in the unloaded nanocarriers (figure 11).

The SEM analysis of drug loaded nanocarriers showed that the loaded particles were spherical in shape and the size was found to be around 50–60 nm (figure 12(a)). Polysaccharide derived nanoparticles were reported to be used for loading drugs and drug delivery (Bhaskar *et al* 2010, Samrot *et al* 2018). In EDX analysis, the curcumin loaded nanocarriers predominantly had a strong carbon and oxygen peaks and also had sodium peaks



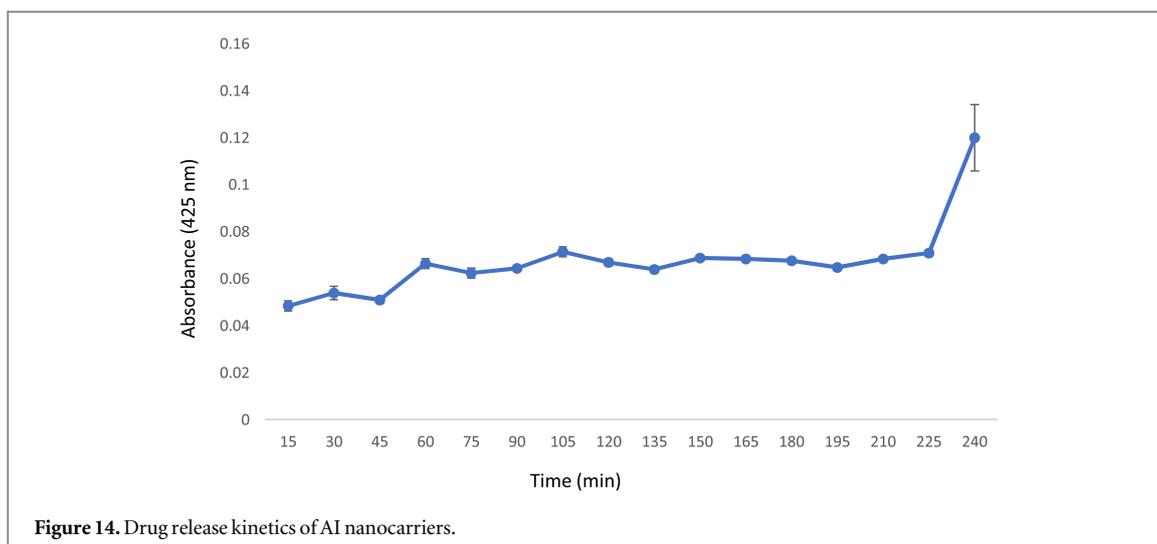


Figure 14. Drug release kinetics of AI nanocarriers.

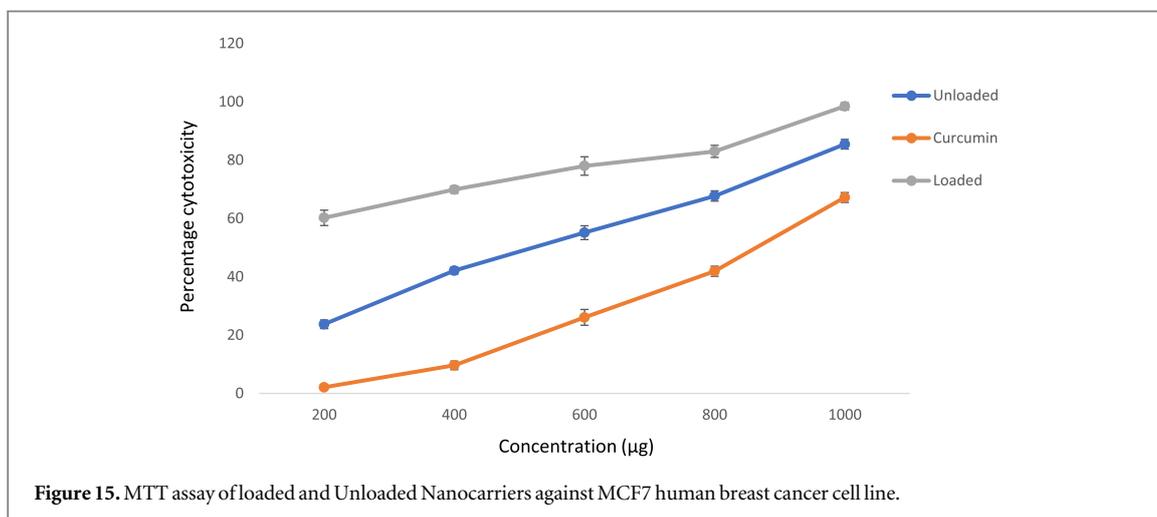


Figure 15. MTT assay of loaded and Unloaded Nanocarriers against MCF7 human breast cancer cell line.

(figure 12(b)). The AFM analysis shows that the loaded nanocarriers were found around 60 nm and the particles were spherical in shape (figure 13) and those particles were also found to be spherical.

3.5. Drug loading efficiency and drug release kinetics

The nanocarriers were found to encapsulate 81 % of curcumin used in this study after 30 min of sonication (result not shown here) and when it was subjected for drug release, it was steadily releasing till 240 min and it was continuing (figure 14). The polysaccharide nanocarriers are having tendency to swell and slowly dissolve in solvent and release the encapsulated drug (Samrot *et al* 2018a).

3.6. Anticancer activity of nanocarriers

The IC_{50} value of curcumin loaded nanocarrier was observed to be higher i.e. 200 μg . (figure 15) polysaccharide itself was showing anticancer activity earlier too. Likewise, Nanocarrier assisted drug delivery of curcumin using polymeric nanoparticles produced from chitosan and gum arabic was found to successfully express anti-colorectal cancer activity due to increased cellular uptake (Udompornmongkol and Chiang 2015).

4. Conclusion

In this study, extraction and purification of plant gum of *Azadirachta indica* was done. Purified polysaccharide was analysed spectroscopically and found to have sugars like glucose, allose, xylose etc. Polysaccharide was recorded to have antibacterial activity against the gram negative organism and have a good antioxidant and anticancer activity. Purified polysaccharide was carboxymethylated and chelated to form nanocarrier using

STMP and characterized using SEM/EDX. The nanocarrier formed at the concentration CMAI-2.2 was reported with regular shape and also the particle was at the least size (40 nm). This nanocarrier was loaded with curcumin using ultrasonication and the loaded nanocarriers were subjected to SEM, EDX, FTIR and AFM. The size of loaded nanocarrier was around 60 nm. The nanocarriers (both loaded and unloaded) were studied for cytotoxicity study against MCF7 human breast cancer cell line and were found to have significant anticancer activity. The curcumin loaded nanocarrier was found with enhanced activity.

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Conflicts of interest

The authors of this paper have no conflict of interest

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