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Antimicrobial Screening Of Carboxymethylated *Caesalpinia Pulcherrima* Galactomannan

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ABSTRACT :

Caesalpinia pulcherrima galactomannan (CPG) is a natural polysaccharide consisting of a (1→4)-linked α-D-mannopyranosyl backbone, substituted at O-6 by single units of β-D-galactopyranose. Literature reveals that the carboxymethyl group is the key factor responsible for increasing antibacterial activity. Current study focussed on screeniong of *in vitro* antimicrobial properties of carboxymethylated galactomannan (CMCPG) against gram-positive and gram-negative bacteria (*S. aureus* and *E. coli)*. Derivatization of galactomannan was performed by using a carboxymethylation reaction with variation of reaction conditions such as the concentration of monochloroacetic acid and temperature. Optimized derivative were evaluated

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for degree of substitution(DS), FTIR, percentage yield and solubility. Minimum Inhibitory Concentration (MIC) was tested by using the liquid dilution method whereas zone of inhibition (ZOI) was evaluated by the well diffusion method. Synthesised carboxymethylated derivative detected with degree of substitution of 0.56 ± 0.05, percentage yield of 144.02 ± 1.5 % and solubility of 80.96 ± 1.3 %. FTIR spectrum of CMCPG detected with presence of the characteristic peak at 1589 cm^{-1} and 1319 cm^{-1} which confirmed introduction of carboxymethyl group into the molecular structure of CPG. Absentism of *in vitro* antibacterial activity detected in range of concentration 50 mg/mL to 0.78 mg/mL for CMCPG alongwith lacuna of MIC in range of concentration 13.75 mg/mL to 1.25 mg/mL). As per the screening of Carboxymethylated *Caesalpinia pulcherrima* Galactomannan, zone of inhibition, minimum inhibitory concentration and antibacterial activity against gram negavtive *E. coli and gram-positive S. aureus*. Thus, screened carboxymethylated *Caesalpinia pulcherrima* derivative may not be a promising antimicrobial agent.

KEYWORDS: *Caesalpinia pulcherrima*, Modification, Carboxymethylation, Solubility, MIC

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INTRODUCTION:

Biodegradable polymers are suitable biomaterials for the design of polymeric drug delivery devices for many classes of bioactive agents. These polymers have been used in various macromolecular architectures: linear, cross-linked and branched. Galactomannans (often called "Pharaoh's Polysaccharides") are water-soluble hydrocolloids, high molar mass, water-soluble, non-ionic polysaccharides forming highly viscous, stable aqueous solutions They have special properties such as high molar mass, water solubility, non-ionic character, galactomannans extracted from seeds of four different species of *Leguminosae, Adenanthera pavonina, Caesalpinia pulcherrima, Gleditsia triacanthos and Sophora japonica*, showed adequate characteristics for food and biomedical industry applications[1,2]. In simple aqueous systems, they are effective viscosifiers and thickeners and are also excellent stiffeners and stabilizers of emulsions. The absence of toxicity also favors their use in the textile, pharmaceutical, biomedical, cosmetics, and food industries[3] which direct their potential use as paper, films/coatings, as gel agent, and as a part of mixed systems such as hydrogels[4]. They are also used in formulations such as gels, mouth-dissolving film, scaffolds, tablet, emulsions. Galactomannans are neutral, water-soluble polysaccharides that due to their economical, biocompatible, non-toxic, biodegradable features, have been used as functional biopolymers for applications in papermaking, food and pharmaceutical industry. Modification of natural polysaccharides is a new strategy for preparing environmentally friendly biomaterials and biopolymers with special properties which can extend their applications[5,6]. CPG has the unique property of increasing viscosity when dissolved in water; it swells instantly in the presence of moisture as soon as it reaches the stomach. As a result, the absorption of glucose is delayed, which leads to a decrease in blood sugar level[7].

Srivastava and Kapoor[8], have reported more than 40 different leguminous species, such as the already commercialized sources of seed gums and many other are constantly described in the literature[9]. The general structure of those neutral polysaccharides which are comprised of a main chain of (1→4)-(α)-D-mannan linked to single α-D-galactopyranosyl groups attached within the O-6 position of the D-mannopyranosyl residues makes the galactomannans really specie-variable materials. These gums, although sharing similar basic structures, may have very different fine structures regarding the degree and distribution of branching, the ratio mannose: galactose and their molecular weight being potentially useful for a wide variety of applications[10].

As per literature survey polymer modifications are intended to attribute different, typically desired properties to the newly modified material properties such as enhanced thermal stability; multiphase physical responses; biological resistance, compatibility or degradability; impact response; flexibility; rigidity; etc. Polymers can be modified by various methods like thiolation,

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carboxymethylation, hydroxylation, sulphation, and PEGylation[11]. Many articles reported about carboxymethylation of polymers and an increased in antimicrobial activity.[12] Sulphation of polymers causes enhance in the antioxidant activity of polymers[13]. Natural gum mucilage is used for their multiple activities and beneficial properties[14,15]. The current research focused on the screeing of the antimicrobial property of carboxymethylated *Caesalpinia pulcherrima galactomannan.*

MATERIAL AND METHODS :

Materials :

The seeds of *Caesalpinia pulcherrima* plant were obtained from Nasik, Maharashtra, India. All other chemicals were of analytical grade and are used as received.

Extraction of CPG:

Pods of *Caesalpinia pulcherrima* were collected from specific shrubs, growing in Nasik, Maharashtra, India. The seeds were separated from the pods, cleaned and suspended in 99% ethanol in ratio 1:3 (seeds: ethanol by volume) at 60–80 ^{0}C for 30 min. to inactivate the enzymes and separate low-molecular-weight compounds. Then ethanol was poured out. The treated seeds were mechanically crushed. The endosperms were manually isolated from the germ and the husk of the seeds. The distilled water was added in a proportion of 1:5 (endosperm: water in terms of weight) and reserved or approximately 24 h. Further, distilled water was added in a double proportion followed by mixing in a blender for 5 min. The suspension was filtered through nylon net and further centrifugation was continued at 25 000 rpm for 30 min at 25 ± 0.5 ^{0}C to eliminate insoluble matter. The galactomannan was precipitated by the addition of 99% ethanol. The ethanol was decanted and the precipitated galactomannan was lyophilized and reserved in a dry place (desiccators) till further use.

Synthesis of Carboxymethylated galactomannan:

Various methods are available for the carboxymethylation of galactomanans which are tried for the modification of CPG as follows

Method 1:

Galactomannan gum was derivatized to carboxymethyled galactomannan by mixing with 4 mL water heated to 80 ^{0}C for 15 min. Further 56% w/v of ice-cold sodium hydroxide solution was added dropwise over a period of 45 minutes. Monochloroacetic acid solution was added slowly for a period of 1 hour to the above mixture and maintained at 15 ^{0}C. The temperature of the mixture was raised slowly to 65 ^{0}C and stirred for another hour. The wet mass was washed with methanol for 15 minutes[16]. The pH of the suspension was adjusted to neutral with glacial acetic acid, followed by drying at 50 ^{0}C to 60 ^{0}C.

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Method 2:

Briefly, an accurately weighed 2 g quantity of CPG was dispersed in 6.72 ml ice-cold solution of 45% w/v NaOH. The 3.33 mL of 45.05 % w/v monochloroacetic acid solution(MCA) was then slowly introduced with continuous stirring at 15-18 ^{0}C. The temperature of the reaction mixture was gradually raised to 60-65 ^{0}C and agitated for another 2 h. The resulting gum was washed thrice with 80% v/v methanol. During the last wash, the pH of the methanolic suspension of gum was adjusted to neutral with glacial acetic acid. Finally, the product was washed with methanol, dried at 50-60 ^{0}C until two successive weights were constant, followed by drying at 50 ^{0}C to 60 ^{0}C[17].

Method 3:

2 g CPG was soaked in 30 ml of 50 % w/vNaOH solution at (-18 ^{0}C) for 48 h. After two days after thawing, 10 mL of isopropyl alcohol (IPA) was added to it. Galactomannan solution was then allowed to react with the 6.25 g MCA in 25 mL of IPA. The mixture was allowed to stir at 25 °C for 8 h followed by addition of 200 mL of distilled water with continuous stirring. The undissolved matter was then filtered off. The pH of the filtrate was then adjusted to 7 using concentrated hydrochloric acid to give a clear solution. The derivative was then precipitated by adding an excess ethanol. Further it was filtered and dried under a vacuum.

Method 4:

1.65 g of CPG was mixed with Sodium hydroxide into glass beakers. The mole ratio of MCA:NaOH used was 1:0.55. Further, 1 mL ethanol 96% was added to it. It was heated at 30 ^{0}C with constant stirring using a magnetic stirrer for 20 min. 1.05 g of MCA was further added to it with constant stirring using a magnetic stirrer for another 20 minutes. It was heated in the oven for 10 h at 60 ^{0}C. The carboxymethyl polysaccharide produced was dried at room temperature, crushed, and stored in a desiccator[18].

Determination of degree of carboxymethyl substitution:

Five hundred milligrams of CMCPG were dispersed in 5 ml 80% (v/v) methanol/water mixture. Concentrated hydrochloric acid was added in excess amount and stirred for 2–3 h. The mixture was filtered through Whatman filter paper with pore diameter 11 µm. Residue was washed with successive 5 ml of methanol til neutral pH. The residue was dried to constant weight. Accurately weighed, 200 mg of dried CMCPG was mixed with 1.5 ml of 70 % (v/v) methanol/water mixture. Further 20 mL of water and 5 mL of 0.5 N NaOH were added. The mixture was shaken for 3–4 h. To form complete miscibility. The solution was then back titrated with 0.4 N HCl to a phenolphthalein end-point. The degree of substitution (DS) of carboxymethyl group was calculated from the equation:

$DS = 0.162A/(1 - 0.058A)$,

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where 'A' is milliequivalents of NaOH required per gram of sample.[19]

Fourier Transform Infrared Spectroscopy (FT-IR):

Major structural groups of CPG and CMCPG were detected using FTIR. For FTIR spectrum of galactomannan was obtained using the KBr method. Biopolymer samples were pressed into KBr pellets at the sample: KBr ratio 1:100. Fourier transform-infrared spectra were recorded on an FTIR (Shimadzu FTIR–8400S spectrometer Kyoto, Japan) in a region of 4000–400 cm−1, at a resolution of 4 cm−1.[20]

***Water Solubility* (WS):**

CPG is soluble in cold water, forming a high-viscosity solution at low concentrations which increases in viscosity at high concentration and hence has wide application for its gelling and thickening properties. CMCPG (500 mg) was suspended with 20 mL of distilled water in 50 mL centrifuge tubes, which was agitated for 5 min using a vortex mixer.[21] The samples stood at room temperature for 20 min. Followed by centrifugation at 6000 rpm for 10 min. The supernatant was dried at 108 ^{0}C to obtain constant weight. WS was calculated by Equation:

$$WS\ (\%) = \frac{m_1}{m_2} \times 100$$

where m_1 was the mass of the original sample (mg), and m_2 was the mass of supernatant after drying (mg).

Minimum inhibitory concentration (MIC):

MIC is the lowest concentration of an antibacterial agent that under strictly controlled in vitro conditions, completely prevents visible growth of the test strain of an organism. By using the liquid dilution method, CMCPG was tested with 13.75 mg/mL, 11.25 mg/mL, 8.75 mg/mL, 6.25mg/mL, and 3.75mg/mL and 1.25 mg/mL concentrations. A pure culture of a single microorganism was grown in Nutrient Broth. The culture was standardized using standard microbiological techniques to have a concentration of very near 1 million cells per milliliter. After the antimicrobial agent has been diluted, a volume of the standardized inoculum equal to the volume of the diluted antimicrobial agent was added to each dilution tube, bringing the microbial concentration to approximately 500,000 cells per milliliter. The inoculated, serially diluted antimicrobial agent was incubated at an appropriate temperature for the test organism for 18 hours. After incubation, the series of dilution tubes was observed for microbial growth, usually indicated by turbidity and/or a pellet of microorganisms in the bottom of the tube. The last tube in the dilution series that does not demonstrate growth corresponds with the minimum inhibitory concentration (MIC) of the antimicrobial agent. Sterilized physiological saline was used as the blank control, and all the media were incubated for 24 hours at 37^{0}C.[22]

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Antimicrobial Activity of CMCPG :

The test organisms i.e., gram-negative bacteria *E. coli and gram-positive S. aureus*, were obtained from the Microbiology Department, MET Institute of Pharmacy, Adgaon, Nashik, Maharashtra, India.

The cultures of bacteria were maintained on a nutrient agar slant at 4°C and subcultured onto nutrient broth for 24 hours prior to testing. The antibacterial activity was assessed using the well diffusion method. In nutrient agar medium, 100 microliters of diluted inoculums of 1×10^5 CFU/mL of 24-hour-old cultures of test organisms were mixed and shaken. After that, media (20-25 mL) was poured into sterilized Petri dishes (20 x 90 mm). Wells with a diameter of 5 mm were punched into the agar medium and filled with the test samples (50 mg/mL to 0.78 mg/mL). As a positive control, an antibiotic (cefalexin) was used. The plates were then incubated for 24 hours at 37 °C. The antibacterial activity was interpreted based on the diameter of the inhibition zone measured in millimetres.[11]

RESULT AND DISCUSSION

Carboxymethylation of CPG is achieved with monochloroacetic acid and sodium hydroxide. In the present investigation, a natural polysaccharide, CPG was chemically modified to carboxymethyl CPG [CMCPG] and characterized with respect to its percentage yield, degree of carboxymethyl substitution, FTIR analysis, aqueous solubility, MIC and Antibacterial activity (ZOI). The p*ercentage yield for* CMCPG synthesised by four methds are as mentioned in *Table 1*.

Table 1 : Percentage yield obtained from four different methods of carboxymethylation:

Sr. No.	*Method*	*Percentage yield (%)*	*Appearance of CMCPG*
1	Method I	52.0 ± 0.8	Non-Sticky Flakes
2	Method II	47.6 ± 0.75	Solid White Crystals
3	Method III	144.02 ± 1.5	White fine powder
4	Method IV	192.0 ± 1.9	Reddish White Fine Powder

Synthesised CMCPG by method I was obtaioned as nonsticky flakes whereas synthesised CMCPG by method II was crystalline in nature. Method III synthesised CMCPG was detected as white fine powder and synthesised CMCPG by method IV was reddish fine powder in nature (Figure 1).

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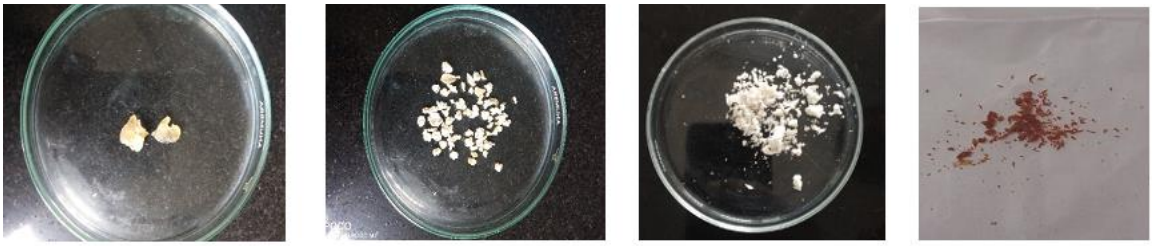

Figure 1 : Synthesized Carboxymethylated galactomannan by four different methods

Determination of degree of carboxymethyl substitution:

The substitution degree was determined using titrimetric method. The substitution degree (DS) is the average value of the hydroxyl group exchanging with the carboxymethyl group. A substitution degree test was conducted to determine the number of carboxyl compounds present in each polysaccharide monomer. It describes the quality of the carboxymethyl polysaccharide produced. In carbohydrate chemistry, the level of modification is expressed in terms of the degree of substitution, which is the average number of newly substituted carboxymethyl groups. The average degree of carboxymethyl substitution in CPG was found to be 0.56 ± 0.05. Further the substitution of carboxymethyl groups in the native CPG aws confirmed by FTIR analysis.[19]

Fourier Transform Infrared Spectroscopy (FT-IR):

FTIR analysis has been used as a significant tool to get important knowledge and primary identification of biopolymers from diversified sources[20]. Galactomannan; as a biopolymer has been characterized using FTIR previously in the literature[23]. *Figure 2* represents the FTIR spectrum of CPG and CMCPG. FTIR spectra depicted various band stretching ranges from 4000 cm^{-1} to 400 cm^{-1}. Peak at 2924 cm^{-1} is an indication of the C–H stretch; whereas the peak at 1041 cm^{-1} is an indication of the C-O bond from the alcohol group. Combine stretch in the region of 2924 cm^{-1} and region of 956.69-1041.56 cm^{-1} showed the presence of C-O-C and C-O functional groups that are characteristic of carbohydrates polymers. The characteristic absorption peaks appeared at 1589 cm^{-1} and 1319 cm^{-1} which were related to the stretching vibration of –COO and symmetric stretching of C-H.[24] The stretching in the region 1651.07 cm^{-1} is indication of amides which confirmed our proximate analysis result that protein is present in moieties in galactomannan sample. The IR spectra of galactomannan depicts peaks at 810 cm^{-1} and 879 cm^{-1} that are associated with the occurrence of anomeric configurations (CH oscillations of α and β conformers) and glycosidic linkages.

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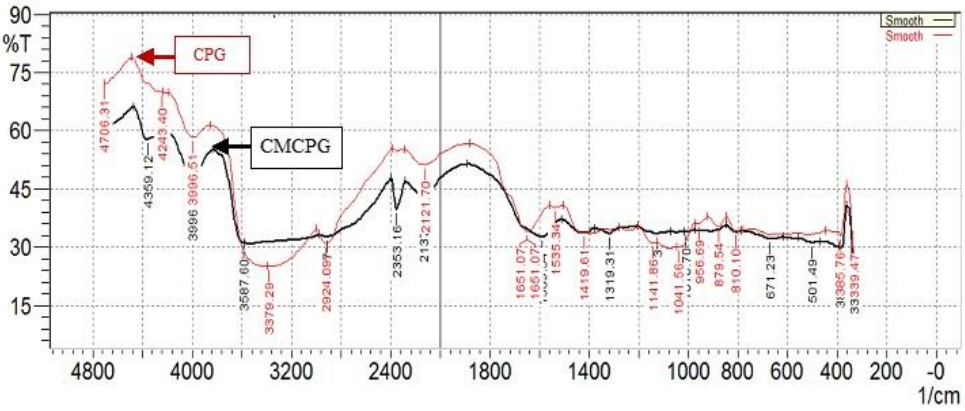

Figure 2 : Superimposed FTIR Spectra of A) CPG B) CMCPG

In the FTIR spectrum, the appearance of the characteristic peak 1589 cm^{-1} and 1319 cm^{-1} indicated that the carboxymethyl group was successfully introduced into the molecular structure of the CMCPG. Thus current spectra confirmed CPG is modified as CMCPG.

Water Solubility (WS):

It is well known that CPG is soluble as well as swells and creates a viscous solution in water at room temperature. However, CMCPG became soluble in distilled water at room temperature and a clear solution was achieved. The improvement in aqueous solubility could be attributed to the hydrophilic nature of carboxylate functional groups of CMCPG.[19] In addition, it could be seen that its water solubility was increased, which was attributed to the excellent hydrophilicity of the carboxymethyl group. The water solubility of the sample was found to be 80.96 ± 1.3 % and it increased with the DS. The hydrogen bonds between the carboxymethyl groups, and water increase with the increase of the number of carboxymethyl groups, which reduced the formation of intramolecular hydrogen bonds and improved the water solubility[25]. Carboxymethylation contributed to improved water solubility. The insertion of carboxymethyl groups decreased the intra-molecular and intermolecular interactions making the molecule more soluble.

Minimum Inhibitory Concentration of CMCPG :

The lowest concentration of an antimicrobial agent required to prevent a microorganism's ability to grow visibly is determined using Minimum Inhibitory Concentration (MIC) experiments. There are three main reagents necessary to run this assay: the media, an antimicrobial agent, and the microbe being tested. The most commonly used media is cation-adjusted Mueller Hinton Broth, due to its ability to support the growth of most pathogens and its lack of inhibitors towards common antibiotics. The adjusted antimicrobial was serially diluted into multiple tubes (or wells) to obtain a gradient. For verification, the positive control was plated in a hundred-fold dilution to count colony-forming units. The microbes inoculated the tubes (or plate) and were incubated for 16–20 hours. The MIC was generally determined by turbidity using liquid dilution method. CMCPG was tested for 13.75 mg/mL, 11.25 mg/mL, 8.75 mg/mL, 6.25 mg/mL, 3.75

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mg/mL and 1.25 mg/mL concentration. The minimum inhibitory concentration for CMCPG was not found.

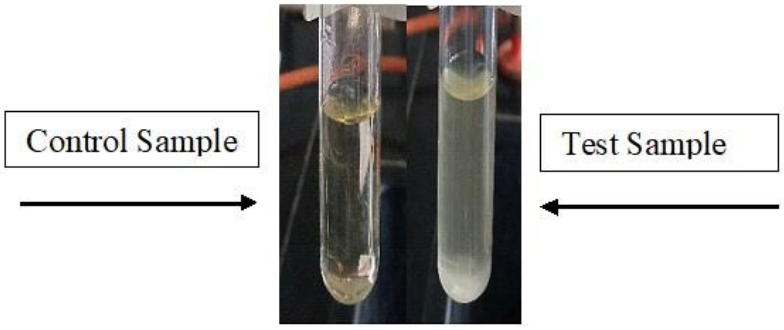

Figure 3 : MIC Outcome of Synthesized CMCGP

According to the MIC, concentration of CMCPG in the range of 13.75 mg/mL to 1.25 mg/mL needed for the inhibition of microbial growth which was not detected nealy for the modified galactomannan i.e.CMCPG. Thus MIC is valuable parameter for screening of antibacterial activity (Figure 3).

***Evaluation of antibacterial activity* :**

An important task of the clinical microbiology laboratory is the performance of antimicrobial susceptibility testing of significant bacterial isolates. The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. The main focus of antimicrobial therapy is selective targeted therapy in order to avoid toxicity and the emergence of resistance.[26] The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. CMCPG samples were investigated to screen its antibacterial activity against strains of Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*) using the well diffusion method. Screening of the antibacterial activity of CMCPG samples against *S. aureus* and *E. coli* was as follows in *figure 4.*

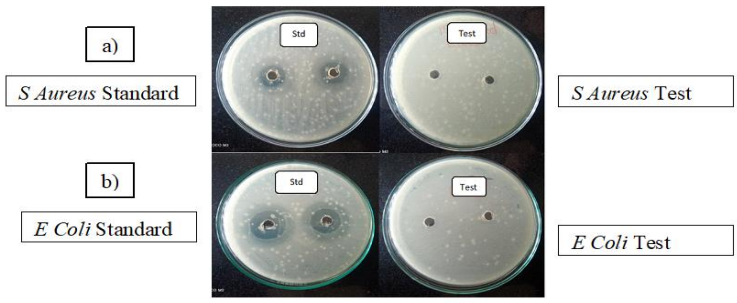

***Figure 4 :* CMCPG antimicrobial activity against (a) *Staphylococcus aureus* and (b)**

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Escherichia coli

The results revealed that the growth of microorganisms was detceted after incubation of CMCPG, while the standard drug showed a zone of inhibition against the microorganisms at a concentration from 50 mg/mL to 0.78 mg/mL. Thus as per the outcome of *in vitro* screening, it was found that CMCPG is inactive against gram-positive and gram-negative bacteria whereas as per literature survey carboxymethylated galactomannan shows antimicrobial activity which was ineffective with absence of zone of inhibition. Meanwhile, in recent years, many pieces of research have also shown that carboxymethylation can promote the antibacterial effect of polysaccharides which was not effective in CMCPG.[27]

Discussion:

The biological activity of many plant preparations is attributed to galactomannan and can be enhanced depending on the substituent groups present in a structure. The derivatization protocol used on galactomannan was effective to produce a carboxymethylated polysaccharide with a DS of 0.56 ± 0.05, percentage yield of 144.02 ± 1.5 %, aqueous solubility 80.96 ± 1.3 %. Carboxymethyl polysaccharide of CPG was synthesized with two stages, namely alkalization and carboxymethylation. FT-IR analyses confirmed that galactomannan was carboxymethylated derivative. Carboxymethylation contributed to improved water solubility. The insertion of carboxymethyl groups decreased the intra-molecular and intermolecular interactions with more soluble, which can strengthen its application as a healthcare product. The results of the work *suggested* that carboxymethylation of galactomannan may be a viable mechanism to enhance the solubility of the parent polysaccharide (CPG), resulting in interesting biomedical and industrial applications of the derivatized biopolymer. The higher solubility of the carboxymethylated biopolymer (CMCPG) can possibly contribute to an improvement of its biological properties. However, this requires further studies for evidence of such activities. Through *in vitro* results, it was demonstrated that CMCPG is ineffective against gram-positive and gram-negative bacteria through carboxymethylation. CMCPG at a concentration range of 13.75 mg/mL to 1.25 mg/mL was disappointed for minimum inhibitory concentration against the microorganisms (*Figure 3)* and free from zone of inhibition from the concentration of 50 mg to 0.78 mg (*figure 4)*. In the traditional system of medicine, CPG and its derivatives are used for treating a number of ailments, but the CMCPG of seed shows a lack of antimicrobial activity. Furthermore, synthesised CMCPG detected free from MIC and ZOI results.

CONCLUSION :

From the present study it was concluded that, *Caesalpinia pulcherrima* galactomannan was successfully carboxymethylated as CMCPG. As per screening of CMCPG for antimicriobial activity, it has insignificant action against the tested microorganisms. The growth of microorganisms was detected after incubation of CMCPG, while the standard drug showed a

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zone of inhibition against the microorganisms. As per outcome of *in vitro* study, CMCPG did not showed zone of inhibition or MIC against the tested microorganisms. Thus as per results obtained in current research work, it can be suggested that the carboxylated derivative of CPG may not be promising antimicrobial agent. The present study will be helpful to avoid any study repeated in such direction in the future.

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