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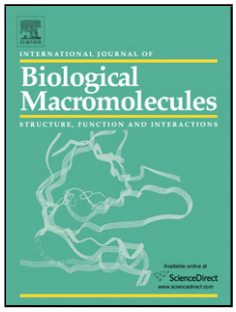

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Author: Vivek Sharma Taranpreet Kaur Helen Bridle Moushumi Ghosh Dr.

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Antimicrobial efficacy and safety of mucoadhesive exopolymer produced by *Acinetobacter haemolyticus*.

Vivek Sharma[1], Taranpreet Kaur[1], Helen Bridle[2] and *Moushumi Ghosh[1]

[1] Department of Biotechnology, Thapar University, Patiala-147004

[2] Institute of Biological Chemistry, Biophysics and Bioengineering, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS, Scotland

Corresponding author: Dr. Moushumi Ghosh

Email: mghosh@thapar.edu; Tel: +91-175-239 3421

Abstract

This study evaluated five extracellular polymers of bacterial origin possessing mucoadhesive properties for their antimicrobial properties and toxicological characteristics. Of the five tested mucoadhesive biopolymers, the extracellular polymer produced by a strain of *Acinetobacter haemolyticus* exhibited broad antimicrobial efficacy towards *Yersinia enterocolitica*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Bacillus subtilis.* Significant ($p<0.05$) inhibition of gram negative bacterial pathogens followed by gram positives were observed with the biopolymer at a dose of 40-60 μg ml-1 at ambient temperature. The cytotoxicity under in vitro conditions and oral toxicity in murine models was also evaluated. The biopolymer did not elicit either haemolytic activity or toxicity in RAW 264.7 cell lines. Haemotological, histopathological and general examinations indicated no adverse effects in Swiss albino mice fed with the biopolymer (120 mg kg-1 body weight-1 day1) over a period of 30 days. These results suggested that the biopolymer was well tolerated without any signs of toxicity and may have several potential biomedical applications where disinfection is desired.

Key words: Antimicrobial, toxicity, polymers, bacterial pathogens, mucoadhesive

1. Introduction:

In the past three decades an increasing number of reports described the bioactivities of polysaccharide glucans and proteoglucans from plant and other sources highlighting the potential use of this class of molecules for immune stimulatory properties. The novel structures and diverse biological activities of microbial exopolysaccharides, lacking in plant polysaccharides have prompted the application of microbial exopolymers in potentially important industrial and biomedical areas. The significantly expanded scope of exopolysaccharide for commercial applications over the current years is based on strong insights on their diverse technological and putative environmental functionalities [1,2]. Additionally, biopolymers represent a low environmental impact technology for applications in a wide sphere of human activities including drug delivery vehicles. Mucoadhesive polymers have recently gained a great deal of attention, in pharmaceuticals, in view of their potential in increasing residence time [3,4,5] and maintaining a high concentration gradient of drug across the epithelium [6]. The usefulness and the ultimate bioavailability of a drug, to an extent, are determined by the length of time it is present at or in the desired site of action. The attribute of mucoadhesiveness can increase the

residence time and hence potentially enhance the bioavailability of a drug. Additionally, mucoadhesives have proven to increase the permeability of the epithelial wall [7]. This has been explained by their tendency to dehydrate the mucous layer causing the cells of the epithelium to 'shrink' and hence open up the tight junctions. Our earlier studies yielded through extensive screening, potential exopolymer producing bacterial isolates from diverse source resulting in an exopolymer library. Characterization of these bacterial exopolymers led to the selection of five biopolymers with reasonable mucoadhesivenesss (unpublished observations).

The emerging interest to create safe, cost-effective biomaterials as non irritant, non toxic biocidals for human applications. While the intrinsic antimicrobial properties have been sought as intriguing feature in biopolymers, few biopolymers with mucoadhesive properties have been characterized with inherent, broad antimicrobial spectrum. Therefore we sought to explore these microbial exopolymers with antimicrobial potential preferably against both gram positive and negative bacterial pathogens. A key aspect for viability of biopolymers relies on their toxicity profile and requires to be addressed through well designed studies. The antimicrobial properties of biopolymers exhibiting mucoadhesive properties and produced by bacterial isolates was evaluated for their antimicrobial activity; the *in vitro* cytotoxicity of the prospective exopolymers determined using the RAW 264.7cell lines and oral toxicity investigated in murine models are reported in this study.

2. Materials and methods

2.1. Biopolymer source:

The exopolymers were extracted from their respective bacterial cultures (*Acinetobacter haemolyticus*,KP701480; *Bacillus subtilis,* KX610831; *Paenibacillus thiaminolyticus,* KX588229; *Trabulsiella odontotermitis,* KX588299 and *Paenibacillus lentomorbis,* KX608547)

by cultivating them in 2 L of FIB medium on a rotary shaker (120 rpm/min) at 30°C for 48 h as described earlier [8,9]. Bacterial cells were removed by centrifugation at 12,000 g for 30 min at 4°C and polysaccharide was separated from the supernatant by the addition of two volumes of ethanol and precipitation at 4°C for 24 h. The precipitated polysaccharide was collected by filtration (Whatman GF Filter), dissolved in deionized water, dialyzed extensively against deionized water and lyophilized. Crude exopolysaccharide was dissolved in deionized water and re-precipitated by adding a 10% solution of cetylpyridinium chloride (CPC). The precipitated polysaccharide complex was collected by centrifugation at 10,000 g for 20 min at 4°C and re-dissolved in a 10% NaCl solution. The precipitated polysaccharide was recovered by addition of three volumes of ethanol. The extracted polysaccharide was dissolved in deionized water, dialyzed twice against deionized water and lyophilized. The biopolymers were designated as TK15, W2B, X4, P8, Z3 respectively.

2.2. In vitro Antimicrobial Activity and Cytotoxicity of the Molecules

Antimicrobial activity of exopolysaccharides (EPS) was assayed against *Aeromonas hydrophila* ATCC 7966, *Yersinia enterocolitica* MTCC 840, *Salmonella typhimurium* ATCC19585, *Listeria monocytogenes* ATCC 19111and *E. coli* O157:H7 ATCC 43895, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027. The plate well diffusion method was used to visualize the formation of a zone of inhibition in a TSB (tryptic soy broth) solid culture medium.

Approximately 0.25 ml of biopolymer for used for all pathogens, 50 µl suspensions of which were spread onto

plates and the plates incubated for 24 h at 37^{0}C for microorganism growth. Inhibition zones were then measured on bases of the average diameter of the clear area, directly on the dishes.

Antibacterial activity of biopolymer TK15 was assayed by microdilution method, using a sterile 96 well-microtiter plate reader (Bioscreen C, Thermo labsystems, Helsinki, Finland) (Raafat et al. 2009). Briefly, the growth of indicator organisms in broth (300µl) containing biopolymer in different concentrations was studied in micro titre plates (100 wells). Each well was inoculated with 10µl broth culture (grown overnight) of the test organism diluted to about 10^6CFU/ml adjusted as McFarland standard. The optical density at 600nm was measured automatically at 10min interval, using a wideband filter (405-600nm), and the plates were shaken at 3min interval for 20s. Viable counts were performed by withdrawing aliquots in duplicate followed by serial dilution and plating onto TSA, subsequent incubation for 18-24 hours at 37 ^{0}C and recording colony counts. The MIC was defined as the lowest concentration of biopolymer required to completely inhibit microorganisms [10].

EPS cellular toxicity was evaluated by hemolytic assay [11] using sheep blood cells (BioMérieux, France). Control experiments comprised similar combinations but lacked biopolymer. Percentage hemolysis was calculated as under:

$$\text{Haemolysis \%} = \frac{O.Ds - O.Dnc}{O.Dpc - O.Dnc}$$

Where, OD_S, OD_{nc}, OD_{pc} are optical densities of sample, negative control and positive control respectively.

To determine the cytotoxic activity of the microbial exopolymers on macrophages, RAW 264.7 cells (1×10^4 cells/mL) were cultured in Dulbecco's modified Eagle's medium (DMEM; HIMEDIA,Mumbai, India) supplemented with 10% fetal calf serum, 1% penicillin-streptomycin solution,1% L-glutamine and HEPES[4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid] in a 96-well plate at 37°C in an atmosphere of 5% carbon dioxide (CO_2) for 24 hours, followed by treatment of the cells with different concentrations of biopolymers for another 24 hours. To determine the cell viability, MTT (at a concentration of 0.1 mg/mL) was added to the wells and incubated for 4 hours at 37°C in an atmosphere of 5% CO2 in the dark. In metabolically active cells, MTT was reduced to an insoluble, dark purple formazan. The formazan crystals were dissolved in dissolving buffer (sodium dodecyl sulfate[SDS] [11 g] in 0.02 M hydrochloric acid [HCl] [50 mL]and isopropanol [50 mL]). The absorbance was read at 570nm.

2.3 Oral toxicity

Biopolymer solutions were prepared with sterile distilled water and kept under refrigeration until use. The toxicity studies were carried out in accordance with the OECD guidelines 423 (OECD).. Twenty-five pathogen-free male Swiss mice (8 weeks old) were acclimatized to a 12/12 h light/dark cycle for 1 week. They were housed in a polystyrene cage, allowed free access to feed and sterile tap water, divided into five groups of five animals per cage and identified. The biopolymer was administered orally at a dose of 25, 75, 120 and 140 mg kg-^{1} in distilled water for 30 days. The control group received sterile distilled water. The animals were observed for mortality and morbidity such as convulsions, tremors, grip strength and pupil dilation. All animals were weighed before treatment, weekly during treatment and at the end of the study. The feed consumption was recorded weekly.

2.4 Haematology

At the end of the experiment, animals were anaesthetized in an ether chamber, and blood was collected by cardiac puncture. Analysis of red blood cell count (RBC), haematocrit (Hct), haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and differential cell count were conducted.

2.5 Histology

Necropsies were performed on all study animals; the liver and kidney were analyzed macroscopically. In order to microscopically examine the tissues, the latter were fixed

in aqueous Bouin, processed, embedded in paraplast, sectioned to a thickness of 7 μm, and stained with haematoxylin and eosin. Histological analysis of organs was done as described by [12]

2.6 Statistical analysis

Data was presented as mean± SD of three independent experiments. During the 30-day oral toxicity studies, treated and control groups were compared by ANOVA with significance level of 5 % using Statistica 11.01, (2012).

3. RESULTS

In a prior study, the mucoadhesive properties of five biopolymers were evaluated by texture analysis and rheology and compared these with three established biopolymers: chitosan, pectin and sodium alginate, using rheological methods.

Tensile test offers a better method of assessment on the strength of mucoadhesion as shown by the tested mucoadhesive polymer with mucin layer. The higher peak force indicated the higher interaction capability of mucoadhesive polymer. Additionally the total of work or area under curve suggested the ability of the mucoadhesive polymer to withstand the continuous forces (Table1a).

The comparative rheological profile of the biopolymer TK15 depicting apparent, observed viscosity with 10% mucin is presented in Table 1(b) Based on the value of viscosity synergism and normalized parameter, the rank order of mucoadhesion for the biopolymers tested was found to be sodium alginate > Chitosan > TK15. We observed that several factors such as concentration, contact time and ionic strength could affect the mucoadhesive property. These findings were similar to those reported earlier [13]. Since the mucoadhesiveness of the 4 other biopolymers were lower in rank than TK15, the latter was selected for further studies.

Antimicrobial activity:

TK15 demonstrated notable inhibition and its potency against both gram positive and gram negative bacterial pathogens tested as shown in Fig 1 (a). The antimicrobial activity of the biopolymer was evident over an average concentration range of 40-80 μg/mL. Further increase in concentration failed to alter the antimicrobial efficacy significantly ($p>0.05$).In liquid media, inhibition could be clearly observed (Table 1 a, sup) after 10 minutes. Over 4 log reduction was evident after 30 minutes in all cases. The inability of re-growth of each pathogen, if any, following injury was confirmed using TAL (Thin agar layer plates, results not shown). Gram positive pathogens required higher doses (80μg/mL) of the biopolymer.

Molecular characterization of the producer bacterial isolate was performed by 16S rRNA sequencing using universal primers. The TK15 DNA was extracted, amplified and 16S rRNA PCR products were sequenced and aligned by multiple sequence alignment. Based on the phylogenetic tree constructed with sequences of representative bacteria, the strain which was isolated from the effluents of a textile dyeing unit, was ascribed to genus *Acinetobacter* and species *haemolyticus* (Fig.1); the sequence of this strain has been deposited in GenBank database (Accession No KP701480).

Biochemical characterization of biopolymer TK15:

Pentoses were identified as the second major components in addition to amino sugars, uronic acid and pyruvic acid which constituted approximately 15% weight fraction of the biopolymer, while DNA and RNA were not detected. FTIR analysis (Fig.2) of TK15 revealed presence of a broad peak characteristic of stretching vibration of hydroxyl (O-H) and amino (N-H) groups at 3408 cm^{-1}. The peak at 1650 cm^{-1} represents N-H bending of primary amines while the peak at 1421 cm^{-1}represents C-N stretching of amines. The peak at 1032 cm^{-1} was attributed to CO ether linkage of sugars while the peak at 875 cm^{-1}indicated presence of β glycosidic linkage in the biopolymer TK15.

Cytotoxicity: ASTM F 756-00 (2000) classifies materials into three categories based on their hemolytic index. Those exhibiting>5% hemolysis are considered hemolytic, between 2-5% are considered slightly hemolytic while those having<2% hemolysis are non hemolytic materials [16].Haemolysis observed by TK 15 was less than 2% indicating a strong possibility for being safe. The risk of hemolytic character is evaluated against the clinical benefits. To further substantiate the finding, RAW 264.7 cells were challenged with various concentrations of this biopolymer. The cell viability was not significantly affected at 24 hours of incubation in the presence of the biopolymer (Table 3). Microscopic observations of the treated macrophages were also carried out. The macrophage cell morphology in a monolayer culture after the treatment with varying concentrations of TK15 exhibited no distinct morphological changes in the cells treated with the bactericidal doses (results not shown) even upon incubation for 48-72 hours.

3.1 Oral toxicity studies

Mortality of animals was not observed over the period of experiment. Body weight gains (Table 4 a), rectal temperature profile (results not shown) and feed consumption in all concentrations of the biopolymer tested was comparable to control group values. Behavioral changes or changes in body weight of TK15 exposed mice were not observed. Gross and microscopic examination revealed no changes attributable to the administration of either of the biopolymers. Haematology indicated no significant ($p > 0.05$) treatment related changes that were evident during the experimental period (Tables 4b and 5) upon comparison to the control group. Liver sections from mice fed with biopolymers exhibited a similar profile as the blank groups in liver tissues, suggesting the absence of any untoward response.

4. DISCUSSION

Rheological technique which studies the flow and deformation of materials (fluid) under applied stress is useful in determining the mucoadhesion ability of biopolymers. In this technique, mucin is used as the adherent substrate surface onto which the biopolymer would adhere. The technique indirectly assesses the interactions between the biopolymers and mucin through the measurements of viscosity and viscoelasticity. The parameter ηenh (viscosity enhancement or viscosity synergism) characterizes the strength of interaction between the biopolymers with mucin[14].The mucoadhesiveness of the biopolymers were evaluated using this rheological technique as well as for their tensile strength in a prior study. Tensile test results indicating high peak force and total area under curve were in agreement to our observations from rheological experiments and indicated the intrinsic capability of biopolymer TK15.

In view of this, we extended our investigations further to evaluate the antimicrobial potential and safety of this biopolymer. Amongst the few biopolymers investigated for antimicrobial applications as well safety, chitosan has been extensively reported in a number of biomedical applications, including drug-delivery systems, tissue engineering and healing; Pullulan, a neutral linear polysaccharide composed of (1→6)-linked α-D-maltotriose residues synthesized from starch or sugar by the microorganism *Aureobasidium pullulan*, has adhesive properties and safety but lacks potential antimicrobial properties.

The TK 15 bipopolymer was characterized to be a polysaccharide with molecular weight of 48.9 kDa, composed of hexoses and pentoses in equal proportions. Although pentose sugars have been reported in small amounts in exobiopolymer produced by *A. baumannii* strains [16], the significant contribution of pentoses (approximately 50%) to polysaccharide composition suggests that *A. haemolyticus* TK 15 is unique as compared to other members of *Acinetobacter* genus.

Bonding to the mucin layer or mucoadhesion of polymers has been explained by several theories [26]. Amongst the key factors responsible for binding by an ideal polymer, the molecular weight, spatial arrangement and hydrogen bonding (presence of hydroxyl, carboxyl and amino groups) may be primarily responsible for binding of TK 15 biopolymer to mucin layer. Moreover, scanning electron micrographs have indicated the biopolymer to be a highly porous structure and ability to form hydrogels which may have implications in drug targeting and bioavailability of poorly water soluble drugs and for designing sustained release drugs[27, 28].

The agar well diffusion assay offers a visual method for checking the local inhibition; therefore this was used initially to confirm the antimicrobial activity of the biopolymer for each pathogen. With liquid culture, we observed little or no increase of antimicrobial property at concentrations higher than 80µg biopolymer. It may be hypothesized that the formation of hydrogen and covalent bonds amongst the functional groups of biopolymer is facilitated at higher concentrations. The spatial restrictions borne upon the functional groups [22] in such cases, afford fewer charged sites available for interaction with the bacterial cell wall. In fact similar results have been shown with chitosan as well as chemically modified chitosan recently [23]. The complete inactivation of the pathogens after a period of 30 minutes was interesting, however the dose requirements of

gram positives lay in a higher range of 80µg/mL; possibly the inherent architectural features assumed importance in this case.

The observed antimicrobial properties of the biopolymer TK15 may be attributed to phenolic and or carboxylic groups or to the presence of short peptides strongly associated with the polymer.

The antimicrobial mechanism may be explained by considering that the Gram-negative cell wall is thinner and more electronegative which facilitates effective binding. This results in blocking of nutrient flow and consequently enhanced susceptibility to lysis. On the other hand, cell walls of Gram bacteria positive bacteria are made up of peptidoglycans which contain N-acetyl glucosamine, N-acetyl muramic acid, D and L- aminoacids, glutamate and teichoic acid. These positively charged amino acids can bind with the biopolymer resulting in the cell wall distortion, exposure of cell membrane to osmotic shock and exudation of cytoplasmic contents [19,21].

Recently, gum tragacanth a high arabinose, protein containing acidic heteropolysaccharide was shown to possess antimicrobial activity against *K.pneumoniae* and *C.albicans*; the active index (AI) in comparison to standard antibiotics of this biopolymer was notably higher than when compared to guar gum for bacterial pathogens [15].

Cationic polymers like chitosan have been shown to interact with the anionic membrane of the bacterial cells thereby initiating cell disruption [17]. Moringa seed polymer a cationic polypeptide acts directly and non-specifically upon bacterial membranes causing leakage of cytoplasmic content whereas, polymers from *Gum acacia* contains cyanogenic glycosides that exhibit antibacterial properties [18]. It is possible that the presence of amino and carboxyl groups in TK15 play an important role in their antimicrobial property, however more detailed studies are necessary for understanding the exact mechanism of the bactericidal activity of this biopolymer.

An important aspect for any molecule to be used as an antimicrobial agent is that the molecule should be capable of eliminating the target pathogen without affecting the viability of mammalian cells. The RAW 264.7 has been extensively used for screening the cytotoxicity of plant and microbial biopolymers apart from bioactives, with reliable interpretations [24].The MTT assay was used to test the cytotoxicity of TK15 biopolymer treated macrophages. The MTT assay relies on the fact that metabolically active cells reduce MTT to purple formazan; hence, the intensity of dye read at 570 nm is directly proportional to the number of viable cells. However, the IC50 values could not be determined even after exposure of the macrophages to the biopolymer at the maximum concentration of 80µg/mL and after 72 hours incubation.Plant bioactives/biopolymers are considered to be cytotoxic is less than 20 mg/mL [25].Additionally, in agreement with the viability results, microscopic observations of the treated macrophages displayed a distinct morphology as that of control cells indicating the non toxicity of TK15. Therefore the non cytotoxic nature of the biopolymer was concluded from these observations.

Elaborate documentation for the safety of polysaccharide biopolymers intended for human applications have been suggested; for instance chitins from crustacean shells [19] β-glucan from barley and yeast [20, 21] have been extensively evaluated. A general interpretation from these studies highlight that differences in the molecular structures of polysaccharide chains (e.g., chain length, type of linkage) in biopolymers influence

physico-chemical properties and, consequently, biological activity as well as a differential response in mice under in vivo conditions (intestinal behavior); however, these biopolymers were destined as dietary supplements. Such detailed examination, including caecum, urine and faeces, was not deemed important since the present study was designed to judge the toxicity of the antimicrobial polymers. The absence of lipids and the presence of a primarily polysaccharide composition of TK15, eliminated the possibility of a cytotoxic effect as indicated in toxicity studies with cell lines. A critical comparison of the results obtained from our studies was not possible due to the lack of similar studies, where microbially derived biopolymers have been screened for antimicrobial property. The absence of negative effects of the biopolymers on behavior, body weight, haematology and organ histology at higher concentrations through oral route representing 1,000-fold more than the anticipated application suggest the safety, and therefore, a potential applicability of the biopolymer. To the best of our knowledge, extracellular polymers of microbial origin as well as antimicrobial properties with mucodahesive attributes have not been reported. Given its safety and intrinsic characteristics we presume potential applications of TK15; further studies regarding its other functions are currently underway.

5. Conclusion

TK15, an extracellular polymer with mucoadhesive properties, produced by *Acinetobacter haemolyticus* demonstrated potential antimicrobial characteristics against both gram positive and gram negative bacterial pathogens under *in vitro* conditions. The biopolymer was non haemolytic and non cytotoxic as evident from results of in vitro toxicity tests. Oral toxicity of the biopolymers in murine models suggests its safety for up to a concentration of 40-80 µg/mL. The promising functionalities of this biopolymer and its safety obtained from the results of this study imply an interesting commercial prospect for its further exploitation.

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Conflict of interest: The authors confirm that there are no conflicts of interest pertaining to the material in the manuscript.

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Figure(s)

Fig 1: Phylogenetic tree of the mucoadhesive biopolymer TK 15

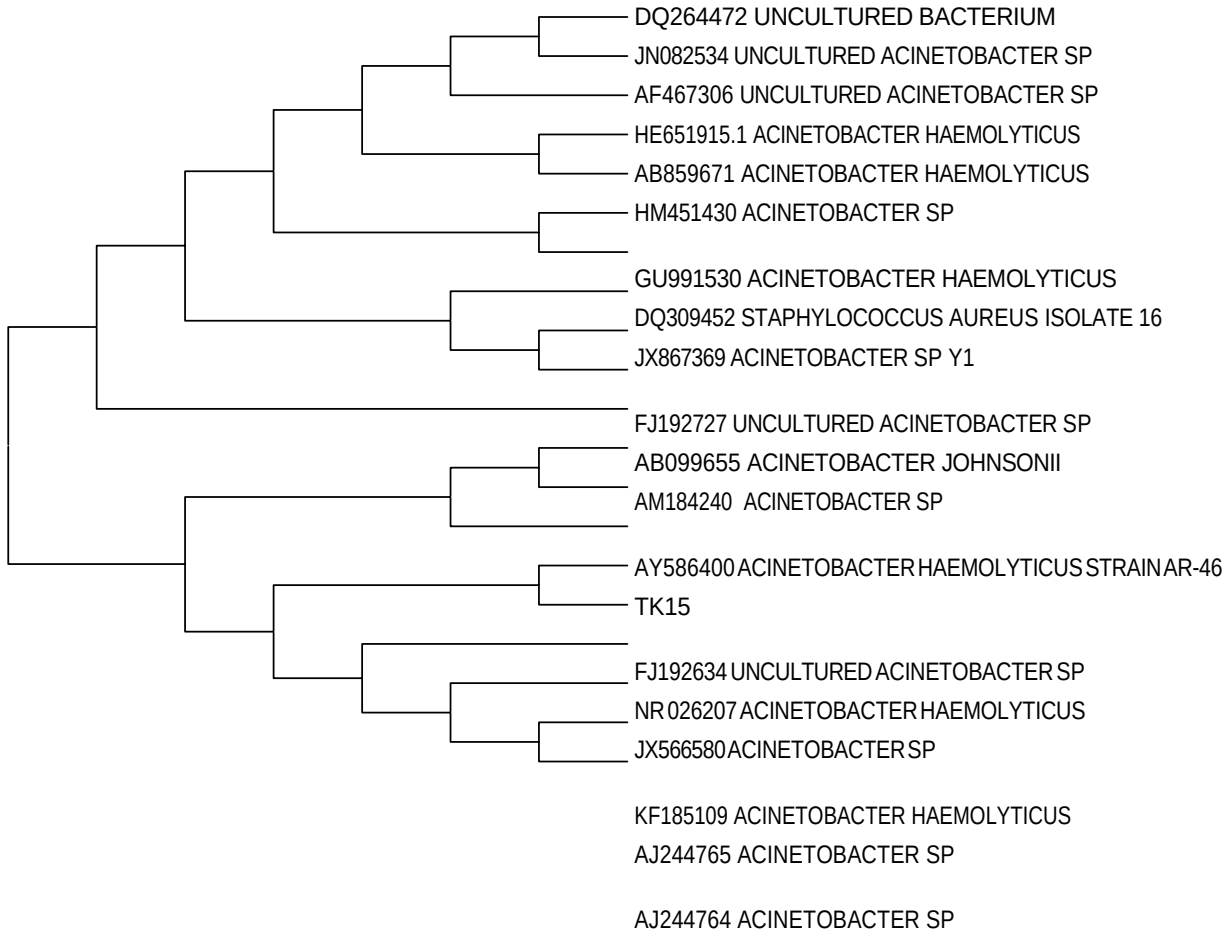
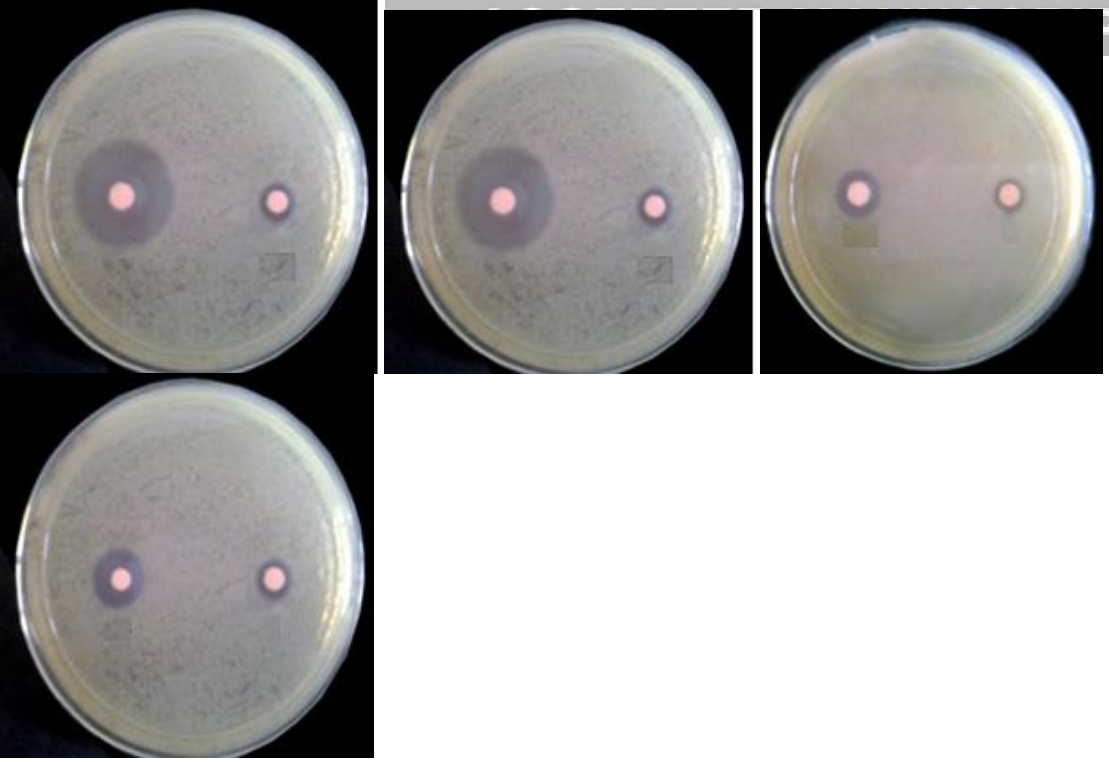


Fig 1(a) : Antimicrobial activity of biopolymer TK15 against selected pathogens(A) *Aeromonas hydrophila* (B) *Salmonella typhimurium* (C) *Listeria monocytogenes* and (D) *Staphylococcus aureus*.Indicator pathogens are on the left and control on right.

(A) (B) (C)



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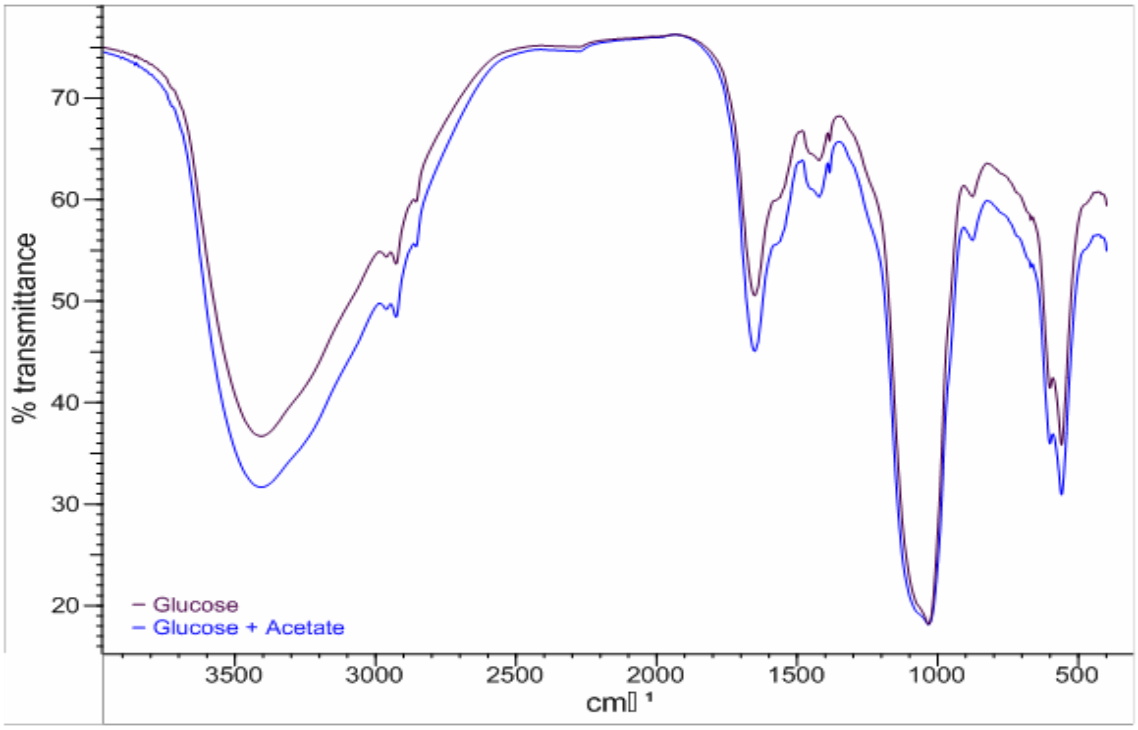

Fig 2: FTIR spectra of the mucoadhesive biopolymer TK 15

Table(s)

Table 1(a): Apparent viscosity and observed viscosity of the different polymers and their mixture with 10% (w/v) mucin (1.5 ml of polymer solution used for the mixing process) at 50 s-1, 5 minutes holding time at ambient temperature.

Biopolymer	**Biopolymer concentration (w/v)**	Hp	η obvs	η exp	η enh
Sodium Alginate	1%	0.023 ± 0.02	1.30 ± 0.3	0.43 ±0.05	0.871± 0.04
High DE Pectin	1%	0.017±0.01	1.323± 0.51	0.432±0.07	0.891±0.09
TK15	1%	0.024±0.001	1.283 ±0.33	0.438 ±0.06	0.845±0.08
Mucin	0	0.00079±0.002	0.414+0.08	0.414 ±0.04	0

Table 1(a): Total work (area under curve) of tensile test for different of concentrations and biopolymers. Tensile strength was determined by a texture analyzer. Figures in parentheses indicate standard deviation of mean.

Biopolymer	MP(g.s)	WP(g.s)	MW(g.s)	Net(g.s)	Normalized parameter
1% Sodium alginate	2149.64±57.12	46.87±11	26.75±13	256±20	3.5
2%Sodium alginate	3227.34 ± 61	98±12.02	26±11.5	491±31	3.93
1% Pectin	1039.23± 97	7.25±3.3	421±20.7	612±33	1.81
2% Pectin	2446.14 ± 107	730.77±21	421.38±24	1297.69±113	1.13
1% TK 15	2045.23 ±112	44.7±13	22.4±13	251±43	3.1
2% TK15	3132.21± 139	99±14	23±11	483±37	3.7

Table 2: Zones of Inhibition of selected bacterial pathogens observed with different concentrations of the biopolymer TK15

Indicator pathogen	**10µg**	**20µg**	**40µg**	**60µg**	**80µg**
Yersinia enterocolitica	9.6mm	12.5mm	17mm	16.5mm	16.8mm
Salmonella typhimurium	10mm	9.6mm	16mm	15.3mm	14mm
Listeria monocytogenes	10mm	8.8mm	14.3mm	15.0mm	14.7mm
Escherichia coli O157:H7	11.4mm	11mm	14mm	13.7mm	13.5mm
Bacillus subtilis	11mm	10.7mm	11.6mm	12mm	11.3mm
Pseudomonas aeruginosa	8.7mm	10mm	13mm	12.2mm	12mm

Table 3: Cytotoxic effect of the biopolymer TK 15 in murine macrophages.

Biopolymer dose	**Cell Viability**
10	98.2% ± 5.3
20	98% ± 4.8
40	98.3% ± 5.2
60	99% ± 8.8
80	98.6% ± 7.5
Control	100% ± 9.6

Table 4(a): Mean body wt profile after every 5th day of administration of TK15

Group	**Dose**	**Days**						
	(mg/Kg bw	0	5	10	15	20	25	30
G1	60	23.5 ±	23 ±	24 ±	24 ±	23.5 ±	23.4 ±	23.3 ±
G2	180	23.0 ±	22 ±	22.5 ±	23.5 ±	23.0 ±	22.8 ±	22.8 ±
G3	220	23.0 ±	22.5 ±	23.4 ±	22.2 ±	22.6 ±	23.5 ±	23.2 ±

Table 4(b): Means of red blood cell parameters in mice administered biopolymer by gavage for 30 days, TK15

Parameter	Dose 0	25	80	120	140
Red blood cells (millions/mm3)	9.0± 0.2	7.4 ± 0.2	6.5 ± 0.9	7.0 ± 1.5	8.3 ± 0.2
Haemoglobin (g dL 1)	13.1± 2.0	13.3 ± 1.2	46± 1.2	15.4 ± 2.4	18.5 ± 3.5
Haematocrit(%)	49.3± 5.3	53 ± 3.8	51 ± 3.4	50 ± 5.1	48.7 ± 2.0
Mean corpuscular volume (µm3)	50.8± 2.2	60 ± 10.5	46 4± 7.3	63.2 ± 10.5	50 ± 2.3
Mean corpuscular haemoglobin (pg)	17.2± 0.7	18.2 ± 1.0	21 ± 3.0	20.5 ± 2.5	21.9 ± 4.0
Mean corpuscular haemoglobin concentration (%)	28.2± 4.3	25 ± 4.8	26 ± 4.2	30 ± 2.5	39 ± 9.3

Table 5: Means of white blood cell parameters of mice administered TMB by gavage for 30 days, TK 15

Parameter (%) 0		5	15	25	35
Eosinophils	0.8 ± 0.9	1.3 ± 0.7	1.6 ± 0.6	1.4 ± 1.0	1.34± 0.6
Monocytes	4.4± 1.0	4.8 ± 1.5	4.8 ± 1.2	4.2 ± 1.5	4.2± 1.6
Lymphocytes	.07± 2.5	83 ± 6.4	8.8 ± 3.0	80.3 ± 7.2	80.1± 8.5
Neutrophils	1.2 ± 2.0	13 ± 4.2	1.4 ± 4.1	12.3 ± 8.2	13.02± 4.2

Table 6: Chemical Composition of TK15

Constituent	
Sugars	74.2
Proteins	8
Amino sugars	2.5
Pyruvic acid	0.98
Uronic acids	2.1
DNA	ND
RNA	ND
Carbon	18.7
Hydrogen	4.35
Nitrogen	6.14
Sulphur`	0.2