

# Antifungal Activities of Chitosan and Nanoparticle Derivatives under Various pH Conditions

Corli de Klerk, Elvis Fosso-Kankeu and Frans Waanders

**Abstract**— The reuse of treated wastewater effluent is receiving considerable attention for possible applications in agriculture and as supplement for drinking water shortages, however acceptable quality must be maintained to ensure that no negative environmental and health effects will transpire. The need to avoid synthetic chemicals is paramount and has led to the development of natural antimicrobial compounds for the removal of harmful microorganisms found in wastewater. This study is aimed at evaluating the effect of chitosan of various molecular weights as possible antifungal agent against fungi likely to occur in wastewater sources, namely *Aspergillus fumigatus*, *Aspergillus ochraceus*, and *Penicillium chrysogenum*. The study will also include the antifungal evaluation of silver nanoparticles that have been immobilized on chitosan films. Chitosan of various concentrations has indicated strong inhibitory activity against *A. fumigatus*, *A. ochraceus*, and *P. chrysogenum* ( $MIC_{HMW} = 37.5 - 75 \mu\text{g/mL}$ ,  $MIC_{MMW} = 75 - 150 \mu\text{g/mL}$ ,  $MIC_{LMW} = 37.5 - 150 \mu\text{g/mL}$ ). The combination of chitosan with silver nanoparticles has exhibited the strongest antifungal effects with MICs reduced to a range of  $18.75 - 37.5 \mu\text{g/mL}$  against the *Aspergillus* species while pristine silver nanoparticles having higher overall MIC values between  $50 \mu\text{g/mL}$  and  $100 \mu\text{g/mL}$  with maximum inhibitory zones of  $12.79 \pm 1.52 \text{ mm}$  for *A. fumigatus*,  $14.25 \pm 0.98 \text{ mm}$  for *A. ochraceus*, and  $14.71 \pm 0.51 \text{ mm}$  for *P. chrysogenum*. Lower pH values also influenced the complex efficacy with significant increase of inhibition at pH 5.

**Index Terms**— Antifungal activity, chitosan, disc diffusion, nanoparticles, molecular weight.

## I. INTRODUCTION

With the ever-growing global population, extensive industrialization, and urbanization of societies it is almost certain there will be a severe shortage of water in the near future [1]. Even though 70% of the earth is covered with water, only 1% is available for human consumption, therefore wastewater treatment is the only recycling process that is able to overcome this problem [2,3]. Furthermore, the water supply is not evenly distributed which leads to serious health hazards in developing countries where water sources are scarce and commonly contaminated with various pathogens and contaminants. Fungi present in wastewater are an imminent threat to the health of immunocompromised individuals relying on contaminated waters for domestic

needs including drinking [4-6]. There are numerous processes that are being used for wastewater clean-up, however, the lack of adequate sanitation requires robust processes and techniques for remediation of wastewater for reclamation. Furthermore, the use of common water treatment processes can have a negative environmental impact, since overdosing occurs regularly and can influence ecology [7]. Nanotechnology has been recognised as a potential technology that could greatly influence water purification systems and over the past few decades growing interest in the modification and application of chitosan and chitosan derivatives in wastewater purification has arisen [8,9].

Chitosan is a biopolymer derived from chitin and is a choice material for the preparation of nanoparticles as it is biodegradable, nontoxic, and abundantly available compound that provides a wider range of applications and that has tremendous antimicrobial activity [10,11]. Chitin is traditionally derived from crustaceans' skeleton and shells, but it can also be found in insects and the cell walls of some bacteria and fungi [12,13]. The molecular weight is one of the main parameters that defines the physical and chemical properties of the polymer. Additionally, metal nanoparticles have a long history as antimicrobial agents. Silver nanoparticles (Ag NPs) have also been proven to have a strong effect on harmful bacteria and fungi and have stimulated interest in wastewater purification applications since it has a large surface area, is chemically stable, and has good mechanical strength capable of hard-wearing applications [14,15]. Comparative studies have shown that chitosan-silver complexes have strong antibacterial effects and are more effective than pure chitosan, however, there are not significant information available for the effect of chitosan complexes against fungi sources [16]. Chitosan is also used for the composite formation as it acts as a protective agent for the Ag NPs [17]. For these reasons, these materials were selected and used to evaluate the antifungal effect of commonly found wastewater fungi. Commercial chitosan of various molecular weights was used as to evaluate the antifungal capabilities of the polymer. Ag NPs has been synthesized by a chemical reduction process.

The overall objective of this study was to investigate and evaluate the antifungal efficacy of chitosan in combination with silver nanoparticles on various pathogenic fungi potentially found in wastewater sources. A scanning electron microscopy (SEM) technique was used for the characterization of the structure of the chitosan-Ag complex and the antifungal effectiveness was determined by measuring the fungal growth radius as well as the Minimum Inhibitory

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Concentration (MIC) that inhibit the selected fungi as it is used as an indicative measure to assess the antifungal activity of the compound.

## II. METHODOLOGY

### A. Materials

Chitosan from various molecular weights (low molecular weight (LMW), medium molecular weight (MMW), high molecular weight (HMW)), and Sabouraud Dextrose (SD) nutrient broth was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) all with a degree of deacetylation of 75-85%. Silver nitrate ( $\text{AgNO}_3$ ), sodium citrate, dimethyl sulfoxide (DMSO), and glacial acetic acid were purchased from Associated Chemicals Enterprises (ACE, Johannesburg, South Africa). All chemicals were of analytical grade and used without further purification. *Aspergillus fumigatus*, *Aspergillus ochraceus*, and *Penicillium chrysogenum* were supplied by the Department of Biotechnology at the University of Johannesburg (South Africa).

### B. Methods

#### B.1 Preparation of chitosan solution.

The chitosan solution was prepared by the acid degradation method where a mass of 0.1 g chitosan of each molecular weight was dissolved in a 1 % v/v acetic acid solution. The pH was adjusted by the addition of sodium hydroxide (NaOH) to reduce the acidity, but still provide natural growth conditions for the fungi. Due to the poor solubility of chitosan, the solution mixed vigorously and left overnight to ensure complete dissolution and a clear solution was obtained.

#### B.2 Particle synthesis and impregnation.

Chitosan-based silver nanoparticles were prepared from the addition of 8 mL of 52 mM  $\text{AgNO}_3$  in a 20 mL chitosan-acetic acid solution. The solution was mixed until a homogenous solution was obtained and the solution was left for 12 h at 95°C. The colour progressed from colourless to brown and the samples were directly used for the antifungal activity. To determine performance of all the chitosan derivatives, three solutions were prepared: (1) LMW chitosan-silver, (2) MMW chitosan-silver, and (3) HMW chitosan-silver. The synthesis of silver nanoparticles was achieved by pouring the solution of 0.17g/L  $\text{AgNO}_3$  into water, heating to 60°C and adding 10 mL of 36 mM sodium citrate. The solution was stirred vigorously at 60°C until the nucleation and growth of the silver nanoparticles were observed.

#### B.3 Particle characterization.

To verify the formation of the metal nanoparticles, the particles were analysed by means of a scanning electron microscope (SEM, FEI Quanta 250 FEG ESEM with an integrated Oxford Inca X-Max EDS system, Czech Republic) to determine the morphology of the samples. The samples were coated with a layer of gold/platinum prior to SEM analysis.

### B.4 Preparation of fungi samples.

Fungi were harvested and grown in sterilized SD nutrient broth (40 g/L dextrose and 10 g/L peptone) for 24 hours. The fungi were grown on sterilized SD agar at a pH of 5.6 by placing a drop of each suspension on the centre of each culture medium. The dishes were sealed with parafilm and incubated in an inverted position at 36°C for 48 hours. For each fungus, five petri dishes of SD agar were used as the control. The radial growth rates were measured in four directions and recorded for 48 hours at a constant interval. Growth inhibition was documented as the fungal growth reduction in the presence of chitosan and chitosan-complexes and compared to the control.

### C. Antifungal screening

The antifungal activity of chitosan (LMW, MMW, and HMW) and Ag NPs was tested on *A. fumigatus*, *A. ochraceus*, and *P. chrysogenum*. The MIC of the sample was regarded as the amount of antifungal agent that is required to kill or inhibit the visible growth of the fungus colonies. The samples that exhibited lower MIC values were considered to have stronger antifungal effects. The activity of the compounds was compared to reference drugs.

#### C.1 Disc diffusion method

A disc diffusion technique was used *in vitro* to determine the antifungal effect of the complexes. The relevant fungi were harvested and suspended in nutrient broth. 1 mL conidial suspension of each isolate was spread on a 90 mm Petri dish containing 20 mL SD agar and the excess isolate decanted and left to dry. The prepared complexes were dissolved in DMSO and 4 sterile 6 mm discs (Davies Diagnostics (Pty) Ltd, South Africa) were impregnated with 15  $\mu\text{L}$  antimicrobial solution (containing 12.5, 25, 50, 100, 200, 400 and 800  $\mu\text{g}/\text{disc}$ ) and air-dried before being carefully placed on the surface of each agar plate with a pair of sterile forceps. After 10 minutes at room temperature, the plates were incubated in an inverted position for 48 h at a temperature of 38°C and the diameter of the inhibition zone was measured. The control was an agar plate with the commercial antimicrobial compounds. The experiments were conducted in quadruplicate (four discs with identical concentrations of the same compound) and the diameter zone reported was the average value of replicate measurements. Amphotericin B (AmB) was used as the positive control at a concentration of 20  $\mu\text{g}/\text{disc}$ , while DMSO was used as the negative control. The radius of the inhibition zone of the fungal growth was measured on two axes that are at a right angle to one another and expressed as the percentage Minimum Inhibitory Zone (MIZ) that is expressed by the following equation:

$$\% \text{ MIZ} = \frac{\pi(r_2 - r_1)^2}{\pi R^2} \times 100 = \frac{\pi r^2}{\pi R^2} \quad [1]$$

Where  $r_2$  represents the radius of the zone of inhibition of AmB (control compound) and  $r_1$  the radius of the inhibition

zone of the tested compound, and R is the radius of the petri dish.

### C.2 Radial growth inhibition

The antifungal activity of the complexes was obtained by a radial growth inhibition assay. Fungi grown in an SD broth suspension were incubated for 24 h at 37°C in the presence of varying concentrations antimicrobial compounds. A drop of the suspension of each fungus was inoculated on the centre of the SD culture medium. The plates were incubated in an upright position at 37°C. Each experiment was performed in 5 replicates. The fungal growth was measured after 48 h.

The reverse side of the colonies was measured with a ruler and a non-inoculated sample was used as the control. The percentage radial growth was determined by equation [2]:

$$\% \text{ Growth inhibition} = 100 - \left( \frac{\text{Growth in chitosan medium}}{\text{Growth in control medium}} \right) \times 100 \quad [2]$$

### C.3 Effect of pH on fungi

The pH tests were conducted in three sets: one control test with no antimicrobial compounds, one test with acetic acid at specific pH values, and one test with the chitosan solution at specific pH values. Since there is no significant literature data available regarding the influences of pH on the antifungal activity of chitosan of various molecular weights, the experiments were conducted over a pH range of 5 and 6.5 and varied with the addition of NaOH.

## III. RESULTS AND DISCUSSION

### A. Characterization of chitosan and nanoparticles

Transparent and flexible chitosan films were obtained from various amounts of chitosan and metal nanoparticles. The SEM micrographs presented in Figure 1 indicates that the surface is smooth with the Ag NPs forming crystals structures within the film. Figure 1(A and C) indicates a more branched Ag crystal structure whereas Figure 1(B and D) is dominated by denser NPs forming granular-like structures as the NPs has agglomerated and formed closer to the chitosan surface.

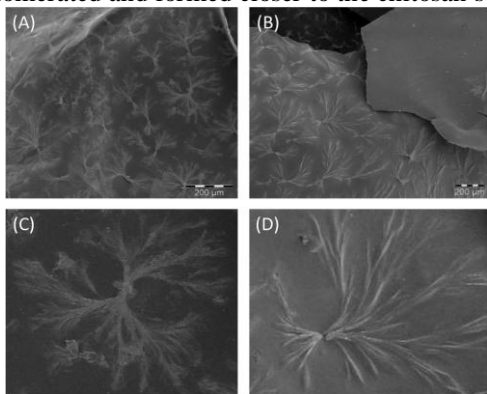


Fig. 1. Micrograph images: (A) Low molecular weight chitosan impregnated with silver nanoparticles. (B) High molecular weight chitosan impregnated with silver nanoparticles. (C) Magnified micrograph of branched silver crystals in low molecular weight chitosan. (D) Magnified micrograph of silver crystals in high molecular weight chitosan.

When the  $\text{AgNO}_3$  was mixed with the chitosan solution, the  $\text{Ag}^+$  ions bound to the chitosan macromolecules by an electrostatic interaction between the ether groups and electron-rich oxygen atoms of the polar hydroxyl of the chitosan and the silver cations.

### B. Antifungal activity of Ag-NPs, chitosan and derivatives

All three of the fungi tested showed sensitivity when exposed to the chitosan-rich environment. Pristine Ag NPs are the most popular inorganic NPs used for antimicrobial treatments [16] and the effect can be clearly observed at low concentrations. Inhibition zones of  $12.79 \pm 1.52$  mm and  $14.25 \pm 0.98$  mm was observed for the *Aspergillus* species at 100  $\mu\text{g}/\text{disc}$  and  $14.71 \pm 0.51$  mm for *P. chrysogenum* at a lower 50  $\mu\text{g}/\text{disc}$ .

As shown in Table 1, the Minimum Inhibitory Zone (MIZ) determined from the disc diffusion assay presented a significant improvement in the presence of the Ag NP-CS complex with an MIZ of  $2.02 \pm 0.35\%$  for *A. ochraceus* compared to  $1.21 \pm 0.71\%$  and  $1.10 \pm 1.20\%$  for pristine Ag NPs and HMW CS, respectively. A similar trend is observed from *A. fumigatus* and *P. chrysogenum*.

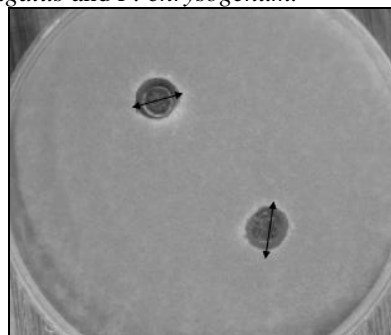


Fig. 2 Photograph of the disc diffusion measurement procedure.

The clear zone surrounding the silver impregnated diffusion disc is the area where no fungal growth has been observed (Figure 2). Surrounding the clear zone is a white precipitation zone where the cells have been injured. Regrowth of these cells is accompanied by a longer lag phase and slower overall growth. Similar trends were observed in the presence of the chitosan-complex, however the precipitation zone increased with a decrease in molecular weight.

TABLE I  
MIZ VALUES OF THE FUNGI SOURCES IN THE PRESENCE OF VARIOUS ANTIMICROBIAL COMPLEXES. RESULTS ARE PRESENTED AS A MEAN VALUE AND STANDARD DEVIATION, N=3.

Complex	Conc.	% MIZ		
		<i>A. fumigatus</i>	<i>A. ochraceus</i>	<i>P. chrysogenum</i>
Ag	100 $\mu\text{g}/\text{disc}$	$0.89 \pm 0.53$	$1.21 \pm 0.71$	$0.78 \pm 0.37$
CS LMW	75 $\mu\text{g}/\text{disc}$	$0.14 \pm 0.12$	$0.87 \pm 0.48$	$0.71 \pm 0.34$
CS MMW	150 $\mu\text{g}/\text{disc}$	$0.25 \pm 0.14$	$0.44 \pm 0.32$	$1.24 \pm 0.46$
CS HMW	75 $\mu\text{g}/\text{disc}$	$0.63 \pm 0.47$	$1.10 \pm 1.20$	$0.52 \pm 0.47$
CS-Ag	75 $\mu\text{g}/\text{disc}$	$1.59 \pm 0.14$	$2.02 \pm 0.35$	$1.61 \pm 0.54$

Table 2 presents the MIC values of the tested antimicrobial complexes. The MIC varies with fungal species, with *P. chrysogenum* being the most susceptible to pristine Ag NPs, and the *Aspergillus* species exhibiting a stronger resistance to higher concentrations of the complex. However, the Ag CS complex has proved to reduce the MIC considerably. It is also observed that the antifungal effect is increased with increasing concentration of Ag CS until it reaches a maximum dosage and no significant effect can be observed.

TABLE II  
ANTIMICROBIAL COMPLEX MIC SUMMARY

Complex	<i>A. fumigatus</i>	<i>A. ochraceus</i>	<i>P. chrysogenum</i>
Ag	100 µg/mL	100 µg/mL	50 µg/mL
CS LMW	75 µg/mL	75 µg/mL	37.5 µg/mL
CS MMW	150 µg/mL	150 µg/mL	75 µg/mL
CS HMW	75 µg/mL	75 µg/mL	37.5 µg/mL
Ag-CS	18.75 µg/mL	18.75 µg/mL	37.5 µg/mL

Figure 2 represents the reduction in radial growth at concentrations of 0, 0.25, 0.5, 1, 3, and 6 mg/mL. The results showed that the antifungal effectiveness is greatly dependant on the strain of fungi. At low concentrations (0.25 mg/mL), the fungi showed decreased growth rates, however only radii reductions between 10-15% were observed for LMW, MMW, and HMW CS. As the CS concentration increased, the growth reduction indicated significant effects, especially at the HMW CS of 6 mg/mL where *A. fumigatus* had a final growth reduction of 80.26%. LMW CS resulted in a growth reduction of 32.01% for *A. ochraceus* where the HMW CS produced growth inhibition of 76.88%. *P. chrysogenum* had the strongest inhibition of 87.32% in the presence of the HMW CS, 1.7 times stronger than MMW CS and 1.8 times stronger than LMW CS. It is also observed that the *Aspergillus* species was more resistant to the increased concentrations. Generally, chitosan with a higher molecular weight was more effective than lower molecular weight chitosan. Similar trends were observed by a previous study [18]. It is observed that Ag NPs have a strong antifungal effect against all three fungi sources, but dominates the *Aspergillus* species at concentrations of 100 µg/disc. MMW CS has the lowest toxicity, with the highest MMW CS result occurring at a higher concentration of 150 µg/disc. HMW CS also has a strong toxic effect at lower concentrations of 75 µg/disc. The combination of Ag-NPs and CS leads to an increased antifungal result at a concentration of 75 µg/disc, a lower concentration than that of both CS and Ag-NPs.

The effect is, however, only fungistatic and not fungicidal with effective inhibition of spore germination and radial growth. The Ag CS films showed a minimum required concentration, thus suggesting that low concentrations of the complex is sufficient for cell death. These effects are as a result of the complex morphology: a larger surface area per unit volume provides more accessible reaction capabilities that occur on a nanoscale.

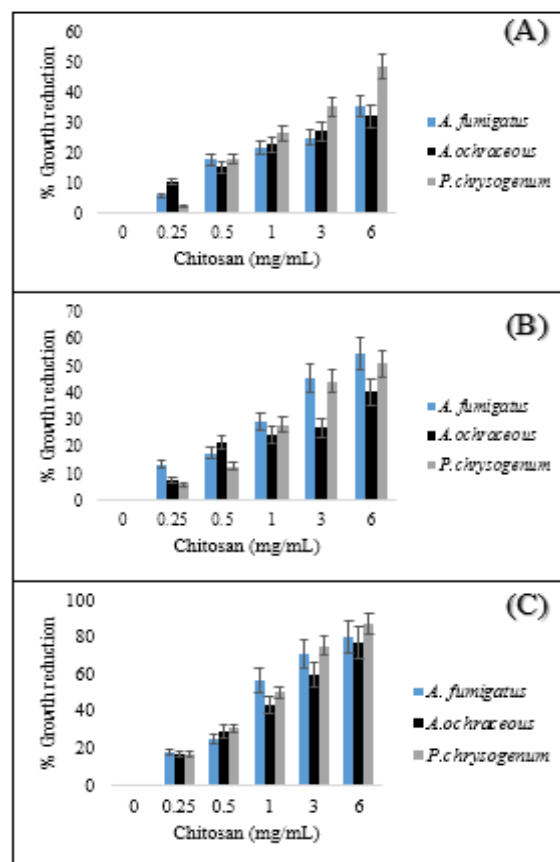


Fig. 1 Effect of increasing concentration chitosan on the radial growth of the fungi. Increasing concentration is accompanied by increased inhibition and HMW-CS producing almost complete inhibition for *P. chrysogenum*. Results are presented as a mean value and standard deviation, n=3. (A) LMW, (B) MMW, and (C) HMW chitosan.

### C. Effect of pH on fungal inhibition

At pH values of 5.6 and higher, the dissolved chitosan formed fibres that precipitated and lower antifungal effects was recorded. A pH of 6.5 was taken as the control experiments and the samples were compared to similar pH values without antimicrobial compounds. The effect of acetic acid on the fungi resulted in small growth variations. As the pH decreased, the inhibition increased, however not as significant as in the presence of chitosan, since fungi generally grow well in acidic conditions [19]. *A. fumigatus* showed to be the most susceptible to low pH values in chitosan-rich environments (90.52%, pH 5), compared to acetic acid that only inhibited a maximum of 41.11%. *A. ochraceus* was also strongly inhibited with growth reductions of up to 80.15% for HMW chitosan. It was also found that the MIC values were up to 2 times lower at low pH values and fungal regrowth was delayed with a longer lag phase. The mechanism of inhibition suggest that the decrease in pH can influence the cell growth by acidifying the cell, consuming a large amount of energy to maintain the intracellular pH for homeostasis.



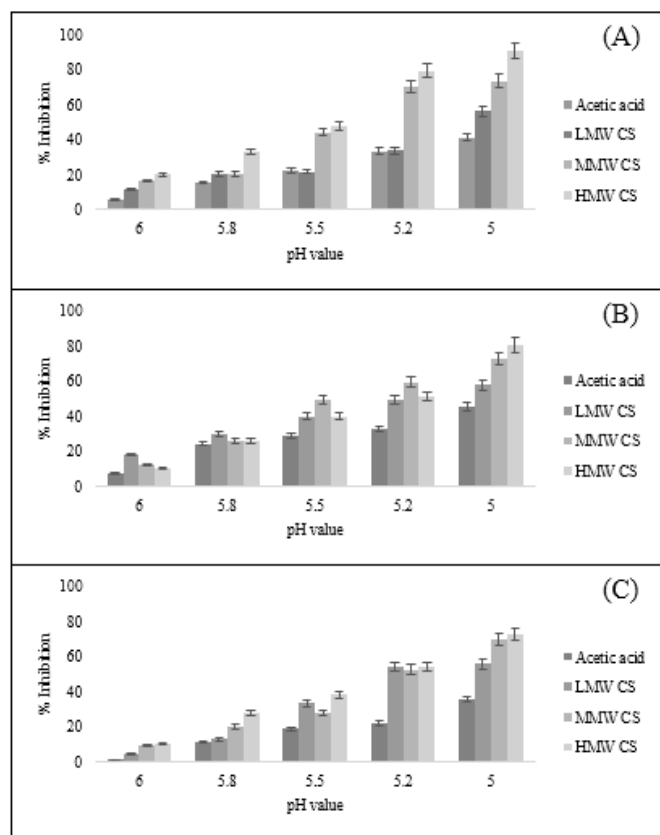


Fig. 4 Effect of pH decreasing pH values in the presence of different molecular weight grades chitosan. The control is observed as the same pH in the presence of acetic acid as inhibitor. (A) *A. fumigatus*, (B) *A. ochraceus*, and (C) *P. chrysogenum*. The data is presented as an average value of 4 dishes with the standard deviation of the measured value.

#### IV. CONCLUSION

In summary, chitosan is a versatile compound that has successfully proved to have antifungal properties, especially when applied in combination with silver nanoparticles. Results indicated that the degree of fungal inhibition is dependent on the molecular weight of chitosan as well as the concentration of silver nanoparticles. Silver NPs had satisfactory inhibitory effects and all chitosans also inhibited the growth of each of the fungi tested and correlates to research done by Akmaz *et al.* in 2013 [17]. It is also suggested that the presence of only a small concentration of Ag in the chitosan complex was satisfactory to enhance the antifungal activity. The efficacy differed with the chitosan molecular weight as well as the particular fungi source. The antifungal effectiveness was collectively more pronounced in the *Aspergillus* species with lower growth capabilities observed over a fixed period of time. Low molecular weight chitosan also has inferior inhibition capabilities that improved with increasing molecular weight. The proposed mechanism of inhibition for the synthesised complex is the ionic cell surface interaction, resulting in membrane damage and cell leakage. The higher antifungal activity at the lower pH values suggest that the addition of chitosan can be effectively applied at lower pH values. This correlates to a similar study done by Jumaa and co-workers in 2002 [20].

Furthermore, chitosan is an abundantly available natural biopolymer with inherent positive properties, making it ideal for polymer fabrication with enhanced effects by adding already established successful antimicrobial compounds.

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**Prof Elvis Fosso-Kankeu** has been the recipient of several merit awards.