Journal of the Egyptian Society of Parasitology, Vol.46, No.3, December 2016
J. Egypt. Soc. Parasitol. (JESP), 46(3), 2016: 563 - 570

ANTIMICROBIAL ACTIVITIES OF CHITOSAN NANOPARTICLES PREPARED FROM *LUCILIA CUPRINA* MAGGOTS (DIPTERA: CALLIPHORIDAE)

By

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Abstract

Chitosan nanoparticles were studied as antimicrobial agent. The antibacterial activity of chitosan nanoparticles were investigated against three Gram-negative bacteria; *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*, and three Gram-positive bacteria; *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus pyogenes*. The antifungal activity were examined against three fungi; *Geotrichum candidum*, *Candida krusei* and *Candida parapsilosis*. The antiviral activities were tested against three viruses; Rift Valley Fever (RVFV), Herpes simplex-1 (HSV-1) and Coxsackie viruses. Chitosan nanoparticles were inhibited all bacteria and fungi except *E. faecalis* seemed to be resistant strain. Infectivity titers of all viruses were reduced by chitosan nanoparticles, which are a natural antimicrobial agent.

Keywords: Antimicrobial activities, Chitosan, Nanoparticles, *Lucilia cuprina*, Maggots

Introduction

Dipterous insects such as mosquitoes and flies are vectors for many pathogens such as protozoa, nematodes, bacteria, fungi and viruses. *Culex pipiens* is a common mosquito species in Egypt which representing a vector for *Wuchereria bancrofti* that causes filariasis or elephantiasis (Khalil *et al*, 1930; Gad *et al*, 1996), West Nile Virus (Taylor *et al*, 1956; Dohm *et al*, 2002), and Rift Valley Fever Virus (Meagan *et al*, 1980; Darwish and Hoogastraal, 1981; Turell *et al*, 1996; Tantely *et al*, 2015).

Flies are very dangerous vector of many diseases such as typhoid, cholera, anthrax, diarrhea, dysentery, African sleeping sickness and etc. Also the flies attracted to the host by the sensilla and may feed on the host tissues or body fluids and may causes myiasis by their maggots. On the other side, the maggots of *Lucilia sericata* and *Lucilia cuprina* (Diptera: Calliphoridae) used for treatment of wounds which called maggot debridement therapy (Gottrup and Jørgensen, 2011; Sherman *et al*, 2013; Hassan *et al*, 2014). Maggots inhibit many bacterial strains such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus* and *S. pyogenes* contaminating wounds (Giacometti *et al*, 2000; Raza *et al*, 2013; Alharbi and Zayed, 2014). The maggots were used for preparation of chitosan the powerful antimicrobial agent.

The present study aimed to evaluate the antibacterial, antifungal and antiviral activities of chitosan nanoparticles prepared from *Lucilia cuprina* maggots.

Materials and methods

Chitosan preparation and characterization: Chitosan and chitosan nanoparticles were carried out (Hassan *et al*, 2016). The degree of deacetylation (DDA) of chitosan was 80.5%.

Antibacterial activity of chitosan nanoparticles against three Gram-nega-tive bacteria strains; *Escherichia coli* (RCMB 010052-6), *Pseudomonas aeruginosa* (RCMB 01002 43-5) and *Salmonella typhi* (RCMB 01002 15-4), and three Gram-positive bacteria strains *Staphylococcus aureus* (RCMB 01001 83-9), *Enterococcus faecalis* (RCMB 01001 54-2) and *Streptococcus pyogenes* (RCMB 01001 74-2) were evaluated by microdilution method (Hassan *et al*, 2016).

Antifungal activity of chitosan nanoparticles against three fungi; *Geotrichum candidum* (RCMB 05097), *Candida krusei* (RCMB 05098) and *C. parapsilosis* (RCMB 05073) were evaluated by microdilution method at Microbiology Unit, The Regional Center of Mycology and Biotechnology, Al-Azhar University.

Chitosan nanoparticles were dissolved in acetic acid 1% (v/v) and diluted to a concentration of 8mg/ml, further 1:2 serial dilutions were per-

formed by addition of culture broth to reach concentrations ranging from 8000 to 0.49µg/ml.

A quantity of 5µl of each dilution was distributed in 96 well plates, as well as a sterility control and a growth control (containing culture broth plus acetic acid 1% (v/v), without antimicrobial substance). Each test and growth control wells were inoculated with 5µl of microbial suspension (10^4 CFU/well). All experiments were performed in triplicate and the microdilution trays were incubated at 37°C for 24h according to the method of Souza, *et al.* (2005).

Ten µl of 3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml) were added to each well and the plates was re-incubated for 3h at 37°C. Fifty µl of Dimethyl sulfoxide (DMSO) solution were added to wells and microbial growth was detected by optical density (ELISA reader, Tecan, Sunrise).

Cell culture: Vero cells line of adult African green monkey kidney (American tissue culture collection, USA) were supplied by Tissue Culture Department, VACSERA. Vero cells were grown in minimum essential medium with Earle's Salts (MEME) supplemented with 10% fetal calf serum (FCS) (GEPCO, USA), 100µg/ml penicillin and 10µg/ml streptomycin.

Cytotoxicity: Cell viability of Vero cells under the effect of chitosan nanoparticles was carried out (Mosmann, 1983) and measured with spectrophotometer (ELISA microplate reader) Bio-Tek, ELx800 at a wavelength 570nm. Cell viability% was calculated by the following equation: Viability% = (mean optical density of tested sample/mean optical density of control) ×100.

The antiviral activities of chitosan nanoparticles against Rift Valley Fever, Herpes simplex (Type 1) and Coxsackie viruses were determined to evaluate the infectivity titer in Vero cells after Hassan, *et al.* (2015) at the laboratory of Virology at the Holding Company for Production of Vaccines and Biological Products (VACSERA).

Results

Complete inhibition percentage (100) of *Escherichia coli* was recorded at concentrations: 8000, 4000, 2000, 1000 & 500µg/ml, and decreased gradually at lowest concentrations until no inhibition recorded at concentrations 3.9, 1.95, 0.98 & 0.49µg/ml. Chitosan nanoparticles showed 100% inhibition against *Salmonella typhi* at concentrations 8000, 4000, 2000, 1000 & 500µg/ml and showed 92.65%, 84.27%, 63.75%, 47.68%, 35.68%, 23.62% & 16.79% inhibitions at concentrations: 250, 125, 62.5, 31.25, 15.63, 7.81 & 3.9µg/ml, respectively.

Pseudomonas aeruginosa was showed resistance to chitosan nanoparticles more than *E. coli* and *S. typhi*, where, the effect of chitosan nanoparticles was detected at 8000, 4000 & 2000µg/ml and showed 50.68%, 36.75% and 15.68% inhibitions, respectively (Tab. 1; Fig. 1).

Chitosan nanoparticles were showed complete inhibition percentage (100) against *Staphylococcus aureus* at concentrations: 8000 & 4000µg/ml and decreased gradually with decreasing concentrations giving 92.65%, 88.33%, 71.55%, 50.64%, 37.24% & 8.68% inhibition at concentrations: 2000, 1000, 500, 250, 125 & 62.5µg/ml, respectively, without inhibition at concentrations: 31.25, 15.63, 7.81, 3.9, 1.95, 0.98 & 0.49µg/ml. Antibacterial activity of chitosan nanoparticles against *Streptococcus pyogenes* was observed at 125µg/ml and increased until 100% inhibition at 8000µg/ml (Tab. 2; Fig. 2). *Enterococcus faecalis* was resistant strain to chitosan nanoparticles.

Table 1: Antibacterial activity of chitosan nanoparticles against *Escherichia coli*, *Pseudomonas aeruginosa* & *Salmonella typhi.*

Concentration (µg/ml)	Inhibition (%)		
	Escherichia coli	*Pseudomonas aeruginosa*	*Salmonella typhi*
8000	100	50.68	100
4000	100	36.75	100
2000	100	15.68	100
1000	100	-	100
500	100	-	100
250	89.64	-	92.65
125	73.65	-	84.27
62.5	61.57	-	63.75
31.25	43.68	-	47.68
15.63	36.76	-	35.68
7.81	17.49	-	23.62
3.9	-	-	16.79
1.95	-	-	-
0.98	-	-	-
0.49	-	-	-

Table 2: Antibacterial activity of chitosan nanoparticles against *Staphylococcus aureus, Enterococcus faecalis* and *Streptococcus pyogenes.*

Concentration (µg/ml)	Inhibition (%)		
	Staphylococcus aureus	*Enterococcus faecalis*	*Streptococcus pyogenes*
8000	100	-	100
4000	100	-	90.14
2000	92.65	-	80.33
1000	88.33	-	62.76
500	71.55	-	43.68
250	50.64	-	23.69
125	37.24	-	8.76
62.5	8.68	-	-
31.25	-	-	-
15.63	-	-	-
7.81	-	-	-
3.9	-	-	-
1.95	-	-	-
0.98	-	-	-
0.49	-	-	-

Antifungal activity: *Geotrichum candidum* was a sensitive strain to chitosan nanoparticles which produced 87.66%, 71.65%, 59.37%, 51.24%, 36.76% & 7.49% inhibition at concentrations of 1000, 500, 250, 125, 62.5 & 31.25µg/ml, respectively. Complete inhibition (100 %) was produced at the higher concentrations: 2000, 4000 & 8000µg/ml, while the concentrations lowest than 31.25µg/ml didn't produce inhibition. Chitosan nanoparticles showed 100% inhibition against *Candida krusei* at concentration 8000µg /ml, while they showed 82.64%, 71.68%, 53.69%, 43.62%, 32.69% & 15.87% inhibitions at concentrations: 4000, 2000, 1000, 500, 250 & 125µg/ml, respectively.

The effect of chitosan nanoparticles on *Candida parapsilosis* was began at concentration 125µg/ml and showed 31.03% inhibition, while, the concentrations 250, 500, 1000 & 2000µg/ml recorded 50.68%, 62.79%, 69.39% & 85.69% inhibition, respectively. The completely inhibition of *Candida parapsilosis* was recorded at concentrations: 4000 and 8000µg/ml (Tab. 3; Fig. 3).

Table3: Antifungal activity of chitosan nanoparticles against *Geotrichum candidum, Candida krusei* and *Candida parapsilosis.*

Concentration (µg/ml)	Inhibition (%)		
	Geotrichum candidum	*Candida krusei*	*Candida parapsilosis*
8000	100	100	100
4000	100	82.64	100
2000	100	71.68	85.69
1000	87.66	53.69	69.39
500	71.65	43.62	62.79
250	59.37	32.69	50.68
125	51.24	15.87	31.03
62.5	36.76	-	-
31.25	7.49	-	-
15.63	-	-	-
7.81	-	-	-
3.9	-	-	-
1.95	-	-	-
0.98	-	-	-
0.49	-	-	-

Antiviral activities: Regarding to the evaluation of chitosan nanoparticles cytotoxicity on Vero cell line, the present data showed that, the viability of Vero cells was inversely proportional with the concentration of chitosan nanoparticles solution. The safe concentration of chitosan nanoparticles solution which used in the antiviral investigations was observed to be 60µg/ml. The virus infectivity titer of Rift Valley fever virus on Vero cell (pre-treatment as control) was 6.66 log (10)/0.1ml, while, the virus infectivity titer of RVFV on Vero cell treated with chitosan nanoparticles (post-treatment) was 5 log (10)/0.1ml. The log difference was 1.6 with reduction of 24.9%. The chitosan nanoparticles reduced the infectivity titer of Herpes simplex-1 (HSV-1) on Vero cell from 5.32 log (10)/0.1ml to 4.32 log (10)/0.1ml with one log difference and the

reduction percent was 18.8%. Reduction of infectivity titer of Coxsackie virus on Vero cell was observed with chitosan nanoparticles, where, the infectivity titer of Coxsackie virus pre-treatment was 3.83 log (10)/0.1ml, and infectivity titer of Coxsackie virus post-treatment was 2.83 log (10)/0.1ml (Tab. 4; Fig. 4). The reduction percent was 26.1 %.

Table 4: Antiviral activity of chitosan nanoparticles against Rift Valley Fever (RVFV), Herpes simplex-1 (HSV-1) and Coxsackie viruses.

virus	Titer pre-treatment log (10)/0.1ml	Titer post-treatment log (10)/0.1ml	Log difference
Rift Valley fever virus	6.66	5	1.66
Herpes simplex-1 virus	5.32	4.32	1
Coxsackie virus	3.83	2.83	1

Discussion

Antibacterial activity: The antibacterial assay of chitosan nanoparticles against Gram-negative and Gram-positive bacteria was applied with different concentrations. Chitosan nanoparticles were showed antibacterial activities against Gram-negative and Gram-positive bacteria. The inhibition percentages were directly proportional with the chitosan nanoparticles concentrations. Generally, the Gram-negative bacteria were more sensitive to chitosan nanoparticles than Gram-positive bacteria.

The results agreed with Liu *et al.* (2001); Qi *et al*. (2004); Balicka-Ramisz *et al.* (2005); Fujimoto *et al.* (2006); Liu *et al.* (2006); Andres *et al.* (2007); Jing *et al.* (2007); Chung and Chen (2008); Mohy eldin *et al.* (2008); Li *et al.* (2010); Tang *et al.* (2010); Tayel *et al.* (2010a); Islam *et al.* (2011b); Benhabiles *et al.* (2012); Younes *et al.* (2014) and Ma (2015), the chitosan was showed antibacterial activity against *Escherichia coli.*

In the present study, the chitosan showed antibacterial activity against *Pseudomonas aeruginosa*, this result was similar with that of Balicka-Ramisz *et al.* (2005); Andres *et al.* (2007); Jing *et al.* (2007); Chung and Chen (2008); Mohy eldin *et al.* (2008); Tayel *et al.* (2010a); Tao *et al.* (2011) and Benhabiles *et al.* (2012).

Qi *et al.* (2004); Balicka-Ramisz *et al.* (2005); Tayel *et al.* (2010a); Islam *et al.* (2011a); Benhabiles *et al.* (2012); Rodrigues-Nunes *et al.* (2012) and Younes *et al.* (2014) recorded the antibacterial activities of chitosan against *Salmonella sp.*, these records were hassling with the antibacterial activity of chitosan nanoparticles against *Salmonella typhi* obtained in the present study.

Corresponding with the findings of Liu *et al.* (2001); Qi *et al*. (2004); Balicka-Ramisz *et al.* (2005); Fujimoto *et al.* (2006); Jing *et al.* (2007); Tayel *et al.* (2010a); Islam *et al.* (2011a; 2011b); Tao *et al.* (2011); Benhabiles *et al.* (2012); Rodrigus-Nunez *et al.* (2012); Salmabi and Seema (2013); Van Toan *et al.* (2013) and Younes *et al.* (2014), the *Staphylococcus aureus* was inhibited with chitosan. In addition, *Streptococcus pyogenes* were inhibited also by chitosan nanoparticles as observed in the present study. In agreement with Younes *et al.* (2014), *Enterococcus faecalis* was resistant strain to chitosan.

Antifungal activity agreed with Yien *et al.* (2012), the chitosan nanoparticles were observed to be natural antifungal agents. The antifungal activity of chitosan against *Geotrichum candidum, Candida krusei* and *Candida parapsilosis* in the present study were similar with *Candida albicans* (Balicka-Ramisz *et al*, 2005; Seyfarth *et al*, 2008; Ballal *et al*, 2009; Tayel *et al*, 2010b), *Candida tropicalis* (Allan and Hadwiger, 1979), *Candida krusei* and *Candida glabrata* (Seyfarth *et al*, 2008).

In the present work, the chitosan nanoparticles showed antiviral activity and protect the Vero cells from cytopathic effect of viruses compared with the control, this finding corresponded to Chirkov (2002) who reported that, chitosan have antiviral activity and suppress the viral infection. The antiviral activities of chitosan nanoparticles against Rift Valley fever, Coxsackie viruses as RNA virus and Herpes simplex-1virus as DNA virus were hassling with the result of Artan *et al.* (2008) on *Lentivirus* human immunodeficiency virus 1 (HIV-1).

In this study, the chitosan prepared from maggots of *Lucilia cuprina* was showed antiviral activities on tested viruses and this similar with the result of chitosan prepared from *Musca domestica* maggots on the *Bombyx mori* nuclear polyhydrosis virus (Ai *et al*, 2012).

Conclusion

The antibacterial, antifungal and antiviral activities of chitosan nanoparticles evaluated in this study. The chitosan nanoparticles were inhibited the growth of all tested Gram-negative

bacteria strains; *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* and two Gram-positive bacteria strains *Staphylococcus aureus* and *Streptococcus pyogenes*, while the *Enterococcus faecalis* was seemed to be resistant strain. Antifungal activity of chitosan nanoparticles were observed against *Geotrichum candidum*, *Candida krusei* and *Candida parapsilosis*. Infectivity titers of tested viruses; Rift Valley fever, Coxsackie viruses as RNA virus and Herpes simplex-1virus as DNA virus were reduced by chitosan nanoparticles.

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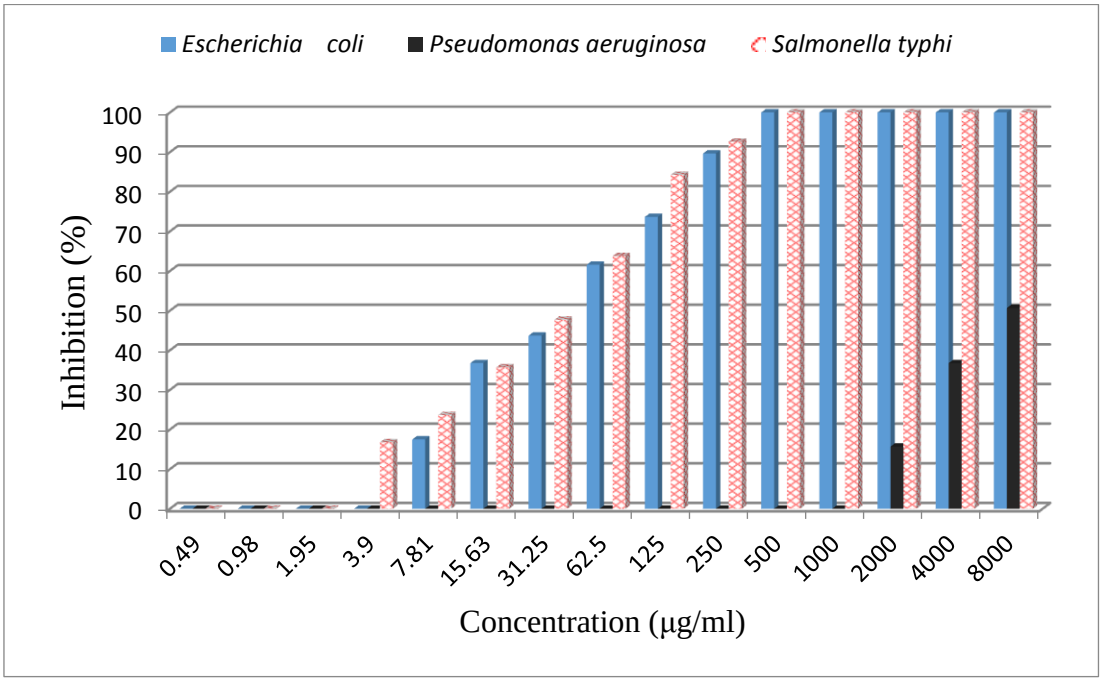

Fig. 1: Antibacterial activity of chitosan nanoparticles against *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi.*

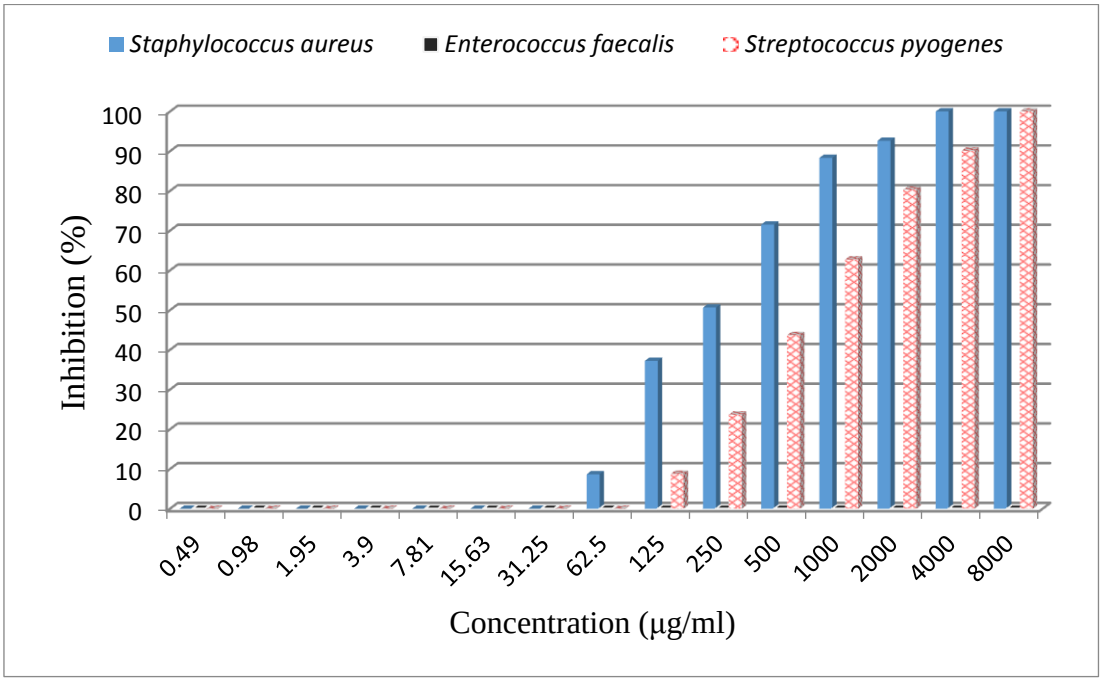

Fig. 2: Antibacterial activity of chitosan nanoparticles against *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus pyogenes.*

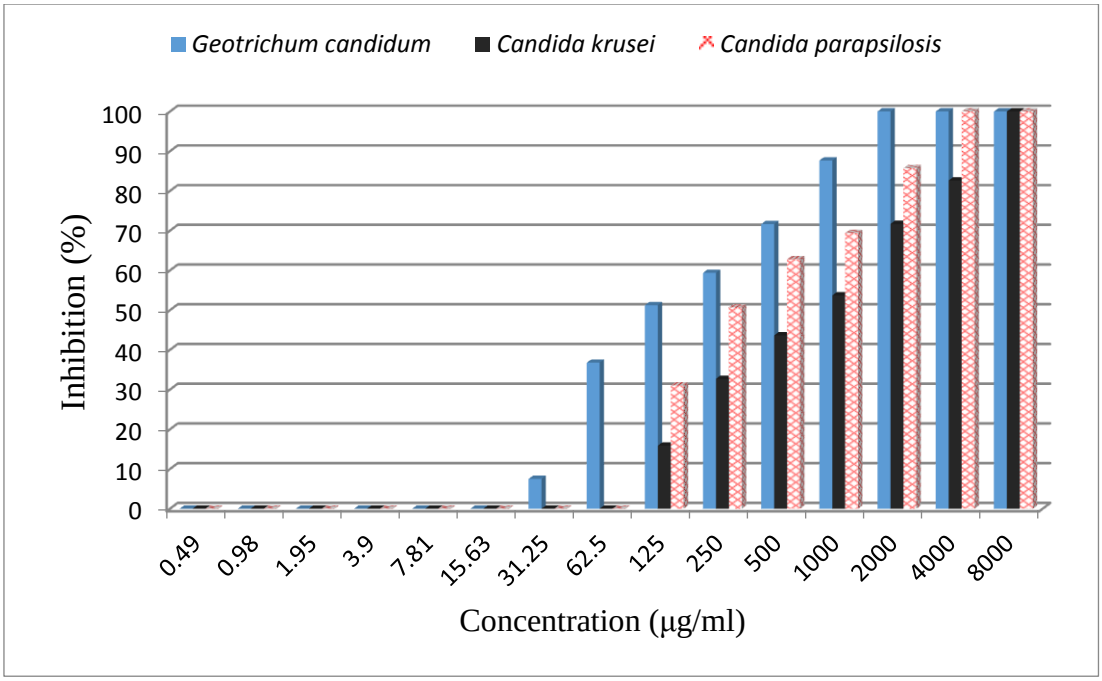

Fig.3: Antifungal activity of chitosan nanoparticles against *Geotrichum candidum*, *Candida krusei* and *Candida parapsilosis.*

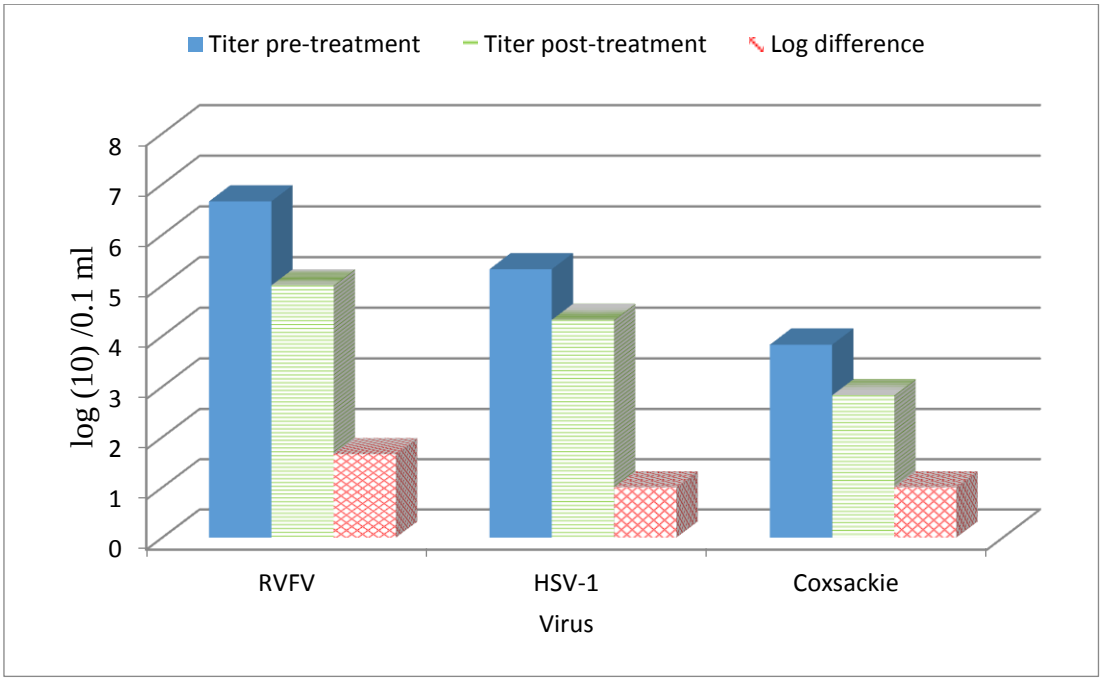

Fig. 4: Antiviral activity of chitosan nanoparticles against Rift Valley Fever (RVFV), Herpes simplex-1 (HSV-1) and Coxsackie viruses.