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***In vitro* evaluation of anti-schistosomal properties
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In vitro evaluation of anti-schistosomal properties of diethyldithiocarbamate sodium salt loaded with chitosan-nanoparticles

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ABSTRACT

Biologically active agents show several biomedical applications. Non-chitosan loaded diethyldithiocarbamate (DDC) was previously found to have anti-schistosomal activity. The present work aimed to prepare DDC-Chitosan-Nanoparticles (DDC-Chitosan-Na-NPs), and to investigate its *in vitro* anti-schistosomal activity with slow-release approach and as well as its mortality % against both concentrations and exposure time. NPs were prepared by suspension cross linking method, in which 0.16 g chitosan were dissolved in 16 ml 2% acetic acid solution (ratio 1% wt/v.). Then after, 0.1g DDC-Na salt was added to the chitosan solution and 3.2 ml of glutaraldehyde as a cross linking agent was added into the mixture dropwise. NPs were washed several times with ethanol 70%, dried under vacuum, prepared for chemical characterization and biological assessment. Our data showed that the slow release of DDC-chitosan-NPs resulted in mortality effects on Schistosomal worms *in vitro* when applied with small dose for longer time. These data compared to those of praziquantil (PZQ) as a reference drug showed that administration of DDC loaded with chitosan-NPs was more effective on the mortality of Schistosoma worms. In conclusion, DDC-Chitosan-NPs have lethal effects on *S. mansoni* worms *in vitro* by a unique mode of action in a way of concentration and exposure time dependent. Also, we recommend that the development of DDC as a local, cheaper and more effective agent against *S. mansoni* worms.

Keywords: Metformin; Hamsters; Pancreatic cancer; N-Nitrosobis (2-oxopropyl) amine

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INTRODUCTION

The controlled-release technology is used to improve the biological activity and safety of many active agents such as pharmaceuticals and agrochemicals. This technology of biologically active agents has certain advantages over conventional methods of application of such agents (Eftaiha et al., 2010).

Diethyldithiocarbamate (DDC) has a functional group of sulfhydryle (SH) where in this compound, both oxygen atoms are substituted with sulfur ones. Most primary and secondary amines react with carbon disulfide and sodium hydroxide to form dithiocarbamates which are used as ligands for chelating metals. DDC has many

medical uses such as pharmacological agents (Nagendra et al., 1994). It is used as anti-leishmanial (Meshnick and Eaton 1981), anti-trypanosomal (Meshnick et al., 1986), anti-fungal (Cid et al., 1990), as anti-schistosomal substance *in vitro* (Rizk and El-Bolkiny, 1997). DDC served as adjuncts for chemotherapy (Arbuckle and Sever, 1998 and Bach et al., 2000), anti-inflammatory (Walters et al., 1999 and Voll et al., 1999), immune-suppressive agent (Ishiyama et al., 2000), alcohol aversion and anti-cataract therapies (Ito et al., 2001; Wang et al., 2004 and Li et al., 2005), and a therapy for AIDS (Pande and Ramos, 2003). Other medical uses of DDC as an immuno-modulator and therapeutic agents for cancer (Wang et al., 2003), potential

uses in treating cocaine addiction (Sofuoglu and Kosten, 2005), against inflammation (Yang et al., 2005) and against viral infections (Krenn et al., 2005). Also, DDC is used extensively in economy and industry such as manufacturing of pesticides (Bergendorff and Hansson, 2002; Zucconi et al., 2002 and Laurenzi et al., 2003).

For a long time, chitosan as a cationic natural polymer, has been widely used as a topical dressing in wound management owing to its hemostatic, stimulation of healing, antimicrobial, non-toxic, biocompatible and biodegradable properties. Previous studies showed the importance of chitosan in the management of wounds and burns by virtue of its ability to deliver extrinsic antimicrobial agents to wounds and burns (Ref). Also, it used as a slow-release drug delivery vehicle for growth factors to improve wound healing (Dai et al., 2011). Utilization of chitosan in the development of oral sustained release preparations was suggested by experience with intragastric floating tablets of chitosan (Hou et al., 1985; Inouye et al., 1988 and Kim et al., 1992). Chitosan due to its antacid and antiulcer characteristics prevents or weakens drug irritation in the stomach (Hou et al., 1985). Therefore, chitosan has a great potential for use as a suitable carrier in sustained drug delivery systems. Several approaches have been taken in the past by many workers to prolong the retention of the dosage form in the stomach (Miyazaki et al., 1981; Chandy and Sharma 1993; Yao et al., 1994 and Yuji et al., 1996). The oral sustained release formulation is subjected to sequentially changing environment during transit through the gastro-intestinal tract as it passes from the strongly acidic to the weakly alkaline medium in the small intestine. The variable absorbing surfaces over the length of the gastrointestinal tract add further constraint to the design of oral dosage forms (Gupta and Ravi Kumar, 2000). Therefore, there is an emergency to prepare DDC- chitosan granules and to investigate the in vitro DDC release from chitosan beads by using Shimadzu 1601 UV-VIS spectrophotometer and its antishistosomal effects on *S. mansoni* adult worms.

MATERIALS AND METHODS

Sodium diethyldithiocarbamate trihydrate (DDC-Na)

Salt of DDC-Na is a product of sigma Chem. Co. USA, MSDS available, for research and diagnosis use only, not for drug. It has CAS 20624-25 3, and chemical formula of $C_5H_{10}NNaS_2 \cdot 3H_2O$, molecular weight of 225.31, and melting point 95-98.5 °C, density 1.1 g/cm³. DDC-Na salt is produced under the batch number 084k2608, with the molecular structure shown in Figure 1. However, this salt can be prepared in the laboratory by treating carbon disulfide with diethylamine in the presence of sodium hydroxide according to the following equation: $CS_2 + HN(C_2H_5)_2 + NaOH \rightarrow NaS_2CN(C_2H_5)_2 + H_2O$.

Chitosan

Chitosan series: PM100, with degree of deacetylation above 85% was obtained from Biro Rundingan & Inovasi, University of Kebangsaan, Malaysia. Chitosan specifications were obtained from the producer. chitosan (Chito-Chem Chitosan) series: PM100, Physical form: powder, 100 mesh, Colour: white / off white, Moisture content (%): 10 – 13, Ash content (%): 0.3, pH of water extract (1% w/w): 7.63, viscosity (0.5% solution) (in CPS): 30 – 50 (using Brookfield Digital Viscometer Model DV-2, Spindle NO: 1), it's molecular structure showed in Figure 2. Glutaraldehyde, buffer and other reagents were of high analytical purity and purchased from El Gomhoria for Chemicals, Tanta, Egypt, and they were used as received.

Instruments

The UV absorbance was measured using UV-spectrophotometer (UV-1601PC, Shimadzu, Polymer Research group, Chemistry Department, Faculty of Science, Tanta, Egypt). Infrared Spectra: were recorded on a PERKIN- ELMER 1430. Elemental analysis of DDC was determined on Heraeus (Microanalysis Centre, Faculty of Science, Cairo University, Giza, Egypt). Scanning electron microscopic images were taken using scanning electron microscope (JEOL, JSM 5610LV) at Microanalysis Centre, Faculty of Science, Cairo University, Giza, Egypt.

Preparation of chitosan-NPs

Chitosan NPs containing diethyldithiocarbamate sodium salt (DDC- chitosan-NPs) was prepared according to the principles of Nayak et al. (2009), based upon cross linking approach, in which 0.16 g chitosan were dissolved in 16 ml 2% acetic acid solution (ratio 1% wt/v). Then after, 0.1g DDC-Na salt was added to the chitosan solution and 3.2 ml of glutaraldehyde as a cross linking agent was added into the mixture dropwise. The final obtained NPs were washed several times with ethanol 70 %, dried under vacuum, prepared for chemical characterization and biological assessment. *In vitro* anti-schistosomal efficacy of the prepared upgraded concentrations DDC-chitosan-NPs was examined. The mortality % against both concentration and exposure time of DDC-Chitosan-Na was investigated.

Determination of maximum wave length (λ_{max}) of DDC-Na

The λ_{max} was determined using UV spectrophotometer by dissolving 10 mg of DDC-Na in distilled water then λ_{max} was measured using computerized UV spectrophotometer.

Calibration curve for DDC-Na

Stock solution of 5000 ppm was prepared by dissolving 50 mg DDC-Na in 10 ml saline. Gradually descending concentrations were prepared from the stock solution. Absorbance was determined at 350.5 nm using UV spectrophotometer. Concentrations against absorbance were plotted and the linear diagram associated with equation was resulted.

Characterization of DDC-Chitosan-NPs Surface structure of the DDC-Chitosan-NPs

The surface structure of the NPs was characterized from the photomicrographs taken with the scanning electron microscope (JEOL, JSM 5610LV, microanalysis Centre, Cairo University, Giza, Egypt).

Infrared spectroscopy

Infrared spectra of DDC-Na, chitosan and DDC-chitosan-NPs loaded NPs were recorded using a PERKIN-ELMER 1430 Spectrophotometer (Microanalysis Centre, Cairo University, Giza, Egypt).

Elemental analysis

Elemental analysis for both nitrogen and Sulphur atoms was determined on Heraeus (Microanalysis Centre, Cairo University, Giza, Egypt). Release of DDC-Na from NPs at pH 8 and pH 2 To prepare phosphate buffer (PB) of pH 2 and pH 8, a mixture of di-sodium hydrogen phosphate and potassium dihydrogen phosphate with different concentrations (W/V) were used. The release of DDC-Na from NPs (pH 2 and 8) was done spectrophotometrically. Absorbance of each was measured at zero time and every 15 minutes for consecutive 6 hours and after 24 hours. At the end of the experiment, the two concentrations were boiled to determine the maximum content of DDC-Na salt in the NPs. Concentrations of test samples were derived from the calibration curve and expressed as ppm to determine the rate of DDC-Na release.

Animals

Schistosoma mansoni cercariae required for the infection of albino mice, *Mus musculus*, were obtained from Theodore Bilharz Research Institute, Cairo, Egypt. The collection of cercariae and infection of mice were based on the method described by Christensen et al. (1984). Mice were individually infected with 80-100 freshly shed cercariae and mice were of 6-weeks old. Seven weeks later, adult worms of *S. mansoni* were obtained by perfusion through hepatic portal veins of the anaesthetized infected mice. The medium of perfusion was PB solution. The obtained adult worms were recovered on a mesh screen and finally maintained in the same buffer until experimentation.

Anti-schistosomal efficacy of DDC-chitosan-NPs A

Anti-schistosomicidal activities of DDC- chitosan-NPs *in vitro*, we used *S. mansoni* worms in various ascending of DDC-Chitosan- Na concentrations. Exposure time and concentration that induce 10, 50 and 90% mortality of worms of were calculated. In this case, a standardized stock solution of DDC-chitosan-NPs was prepared by dissolving 200 mg of DDC-chitosan-NPs in 100 ml PB to get a final concentration of 2000 ppm. Working concentrations (200–2000 ppm) were freshly prepared from the stock solution. In small Petri dishes, 10 adult worms of *S. mansoni* were

exposed to different concentrations of DDC-chitosan-NPs solution in triplicate trails. The number of dead or immobilized worms was counted using dark-field dissecting microscope and the mean of mortems was recorded at 15 minutes' intervals and after the 1st and 2nd hours. The average of the exposure time, inducing 10, 50 and 90% mortality were calculated from linear equations produced. Also, the mortality percent of worms exposed to a definite concentration (200 – 2000 ppm) were calculated and after 1st and 2nd hours of exposure.

Statistical analysis

Obtained data were analyzed using IBM SPSS software package version 20.0. Quantitative data were represented as means + SD. For normally distributed data, comparison between more than two populations were analyzed by F-test (ANOVA) followed by post hoc t-test by Duncan's method. Duncan's method was used to find the significant between each two groups.

RESULTS

In vitro anti-schistosomal efficacy of DDC-chitosan-NPs

The schistosomicidal activity of DDC-chitosan- NPs was extensively studied in buffered solutions. Data obtained by using upgraded concentrations of DDC-chitosan-NPs (200–2000 ppm) were depicted in Figures 2A-J. At a particular concentration, the mortality percentage of worms was recorded at 15 minutes' intervals up to 120 minutes. In this case, linear relationship between the mortality percentage and the exposure time was resulted, presenting linear equations. That is to say, the mortality percentage of the worms exposed to a definite concentration is proportional to the exposure time. From these equations, time values of the sub-lethal concentrations (LC10, LC50 and LC90) (Table 1). Observed that the mortality % of worms following the first and second hour of exposure was increased by increasing DDC concentrations from 200-2000 ppm, and the most potent effect was recognized after 2 hours of exposure. Hence, the in vitro lethal effect of DDC at a particular time period on *S. mansoni* worms was also found to be concentration dependent.

The data in Table 2 give an evidence of decreasing time periods of lethality of Schistosome worms by increasing concentration used. Based on data obtained in Tables 1 and 2, the lethal effect of DDC-Chitosan-Na-NPs was found to be concentration and time dependent.

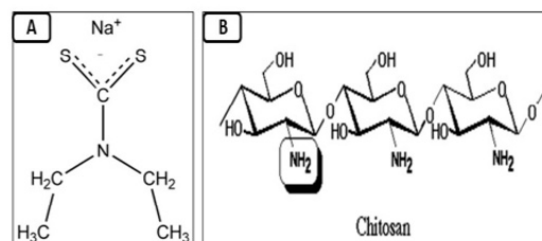


Figure 1. Structure of sodium diethyldithiocarbamate trihydrate (DDC-chitosan- Na) (A); Structure of completely deacetylated chitosan (B).

Table 1. The exposure time (minutes) required for mortality percentages at sub-lethal concentrations of DDC-CS-Na-NPs (LC10, LC50 and LC90) of adult *S. mansoni* worms at laboratory conditions.

DDC Conc. (ppm)	Exposure time required for		
	10 % mortality	50 % mortality	90 % mortality
200	64.83	103.3	141.8
400	54.23	97.31	140.4
600	60.41	94.93	129.5
800	53.89	92.56	131.2
1000	50.23	87.1	124
1200	41.89	83.54	125.2
1400	40.46	81.66	122.9
1600	29.93	77.72	125.5
1800	26.83	75.92	125
2000	23.72	71.51	119.3

The number of worms was 10 / concentration in triplicates. * Significant at $P < 0.05$ (t-test)

Table 2. Effect of various DDC-CS-Na-NAPs concentrations (ppm) on the mortality % of *S. mansoni* adult worms after one and two hours, respectively at laboratory conditions.

DDC Conc (ppm)	Worms' lethality after			
	1 st hour		2 nd hour	
	X ± SD	%	X ± SD	%
200	0.33 ± 0.58	3.3	6.67 ± 0.58*	66.7
400	0.67 ± 0.58	6.6	7.3 ± 1.15*	73.3
600	0.67 ± 0.58	6.6	8.0 ± 1.0	80
800	0.67 ± 0.58	6.6	8.67 ± 0.58*	86.7
1000	1.33 ± 0.58	13.3	9.67 ± 0.58*	96.7
1200	1.67 ± 0.58	16.7	10.0 ± 0.0*	100
1400	2.00 ± 0.00	20	10 ± 0*	100
1600	2.33 ± 0.58	23.3	10 ± 0*	100
1800	2.67 ± 0.58	26.7	10 ± 0*	100
2000	3.33 ± 0.58	33.3	10 ± 0*	100

The number of worms was 10 / concentration in triplicates. * Significant at $P < 0.05$ (t-test).

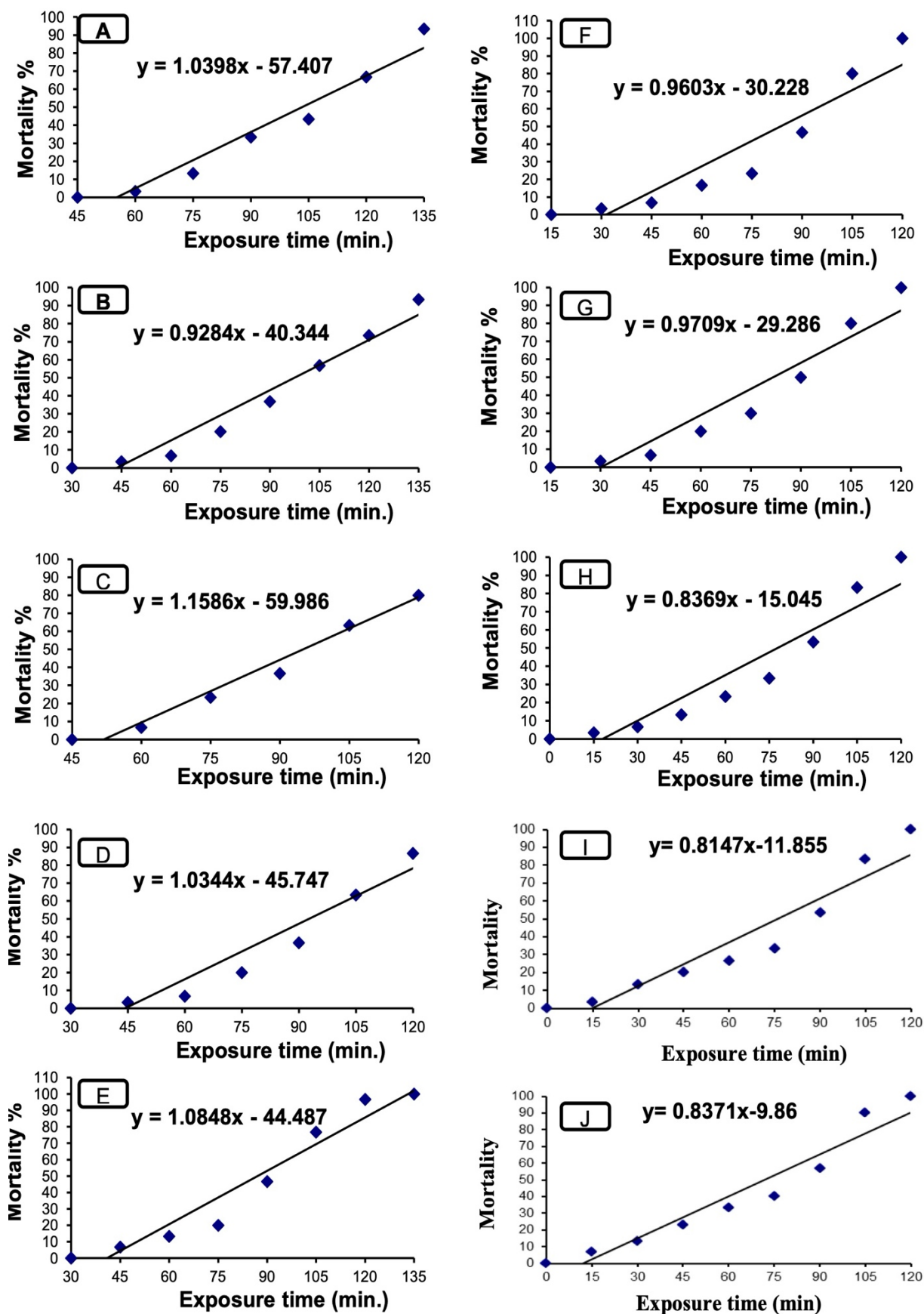


Figure 2. Correlation between exposure time and mortality % of adult worms of *S. mansoni* exposed to 200 (A), 400 (B), 600 (C), 800 (D), 1000 (E), 1200 (F), 1400 (G), 1600 (H), 1800 (I) and 2000 (J) ppm of DDC-CS-NAPs in phosphate buffer solution at the laboratory conditions.

To attain LC10, LC50 and LC90 after the exposure of adult worms to 200 ppm of DDC, it consumes a time period of 64.83, 103.3 and 141.8 minutes, respectively. The increasing DDC-concentration, the decreasing values of the exposure time. Also, after the exposure of worms to 2000 ppm, the time values of sub-lethal concentrations decreased to 23.72, 71.51 and 119.3 respectively. These results indicate that the time required for the sub-lethal concentrations (LC10, LC50 and LC90) reduced from 64.83 to 23.72, 103.3 to 71.51 and 141.8 to 119.3 respectively, after the exposure of worms from 200–2000 ppm.

The relationship between the mortality percent of *S. mansoni* worms and DDC-concentration at a particular exposure time was also shown in table (2) where, mortality % of worms increased gradually with increasing DDC concentration. Therefore, the lethality % of worms increased from 3.33% to maximal 33.33% by increasing DDC concentration from 200- 2000 ppm, respectively. In spite of the maximal mortality % of Schistosome worms reached 33.33% following 1 hour of exposure to 2000 ppm, after 2 hours of the exposure, lethality % of worms increased gradually to reach 100% at 1200 ppm of DDC buffered solution. Therefore, it was

DISCUSSION

Controlled release technology is used to improve the biological activity and safety of many active agents such as pharmaceuticals and agrochemicals. This technology has certain advantages over conventional methods of application of such agents. As chitosan is a natural polysaccharide, shows no inhibitory effect on cell growth with blood compatibility (Zhao et al., 2010), its NPs derivatives have been potentially applied in the field of drug delivery. Various methods have been developed to prepare chitosan microparticles such as emulsion crosslinking (Thanoo et al., 1992 and Genta et al., 1998) ionotropic gelation (Berger et al., 2004 and Ko et al., 2002), emulsification/solvent evaporation (Genta et al., 1997) spray drying (He et al., 1999) and conservation/precipitation (Bayomi et al., 1998 and Vandenberg et al., 2001). Thanoo et al., (1992) prepared chitosan microspheres (425–600 μm) by glutaraldehyde crosslinking of an aqueous acetic acid dispersion

of chitosan in paraffin oil. The highly crosslinked microspheres of chitosan showed a slower release rate whereas the less crosslinked ones showed a faster rate observed in simulated gastric and intestinal fluids.

The positively charged microspheres of chitosan are beneficial of enhancing the mucoadhesion, enabling them suitable for delivery of drugs via the gastrointestinal or nasal routes of delivery. The mucoadhesive properties of the microspheres can be mediated by the preparation parameters. At a lower cross-linking degree, both the mucoadhesive characters and the particle size were improved. However, chitosan-NPs have brought much attention in virtue of their large drug loading capacity, good adsorption performance and long half-life. Several techniques have been developed to prepare chitosan-NPs because of the morphology of the chitosan-NPs have a highly sticky property, the chitosan particles, in particular in a nanometer range, are rather easy to combine with each other. Chitosan-NPs resulted after crosslinking by glutaraldehyde, the shape and size of the chitosan droplets are fixed. Therefore, by this process the chitosan-NPs with good diversity are successfully obtained by Hui et al., (2007).

The present data showed that DDC released from DDC-chitosan-NPs occurred at pH 8 even at zero time, where small concentration was observed. Release of DDC continues with time and the concentration increased tells 1558 ppm after 24 hours. While no release observed for DDC from DDC-chitosan-NPs at pH 2. The data was reliable with those of Ubaidulla et al. (2007) who found that in simulated gastric fluid (pH 2.0), insulin release from the chitosan phthalate microspheres was very low. They added that as pH of the medium was changed to simulated intestinal fluid (pH 7.4), a rapid release of insulin occurred. The relative pharmacological efficacy for chitosan phthalate microspheres administration was almost four-fold higher than the efficacy of non-microspheres chitosan phthalate-insulin solution. These phthalate microspheres of chitosan sustained the plasma glucose at pre-diabetic level for at least 16 h.

Present results are also in parallel with Wang et al. (2009 & 2010) who generated a new type of NPs

made of quaternized chitosan and poly (aspartic acid), and showed that fast release occurred in 0.1M phosphate buffer solution (PBS, pH 7.4), while the release was slow in 0.1M HCl (pH 1.2). Onishi et al. (2011) found that simple Eudragit microparticles loaded with prednisolone and chitosan-succinyl- prednisolone conjugate microparticles coated with Eudragit exhibited suppressed release at pH 1.2, gradual release at pH 6.8 and rapid release at pH 7.4. Conjugate microparticles (chitosan-M1) showed no release at pH 1.2, and very slow release at pH 6.8 and 7.4. chitosan-M1 regenerated poorly from EuS- coated conjugate microparticles (chitosan- M1/S) at pH 6.8.

Our result was in contrary with that of Chandy and Sharma (1992 and 1993) who found that higher release rates of drugs from all studied chitosan delivery systems at pH 1–2 than at pH 7.2–7.4. The medium effect was explained by the necessity of chitosan ionization, which could be attained by the acidic simulated gastric fluid (SGF) or microacidic environment created by the used acidic salt of a basic drug in water, but not in simulated intestinal fluid (SIF) (Bani-Jaber and Al-Ghazawi, 2005).

The carbamates, stabilized derivatives of carbamic acid, are potent biological agents used extensively in applications ranging from agriculture to medicine and industry. The two major classes of carbamates are cholinesterase-inhibiting and non-cholinesterase inhibiting carbamates, including sulfur containing carbamates (DDC) from which DDC is derived and characterized with reversible neurotoxicity. The neurotoxic actions of the DDC may be related to their metal-chelating and enzyme-inhibiting properties (Miller, 1982). Moreover, the toxicity of almost carbamates is due to their ability to disrupt the impulses transmission along the nervous system of animals. Simply, carbamate toxicity is due to inhibition of acetylcholinesterase (AChE) at certain synaptic junctions in the nervous system and that AChE inhibition is the prime cause of death. Inhibition of AChE may explain the activity of some carbamate chemicals as selective rodenticides (Peardon et al., 1972), molluscicides (Judge and Kuhr, 1972; Triebskorn and Kohler, 1992; Rizk and El- Bolkiny, 1997) and schistosomicide (El-Bolkiny et al., 2000).

Schistosomiasis remains a major public health concern affecting billions of people around the world. Currently, praziquantel is the only drug of choice for treatment of human schistosomiasis. The emergence of drug resistance to praziquantel in schistosomes makes the development of novel drugs an urgent task. Thioredoxin glutathione reductase in *Schistosoma mansoni* and some other platyhelminths have been identified as alternative targets (Song et al., 2012). Parasitic infection such as helminthiasis and protozoosis causes major problems of public health, especially in underdeveloped countries, affecting mainly youth populations. Generally, treatment of intestinal helminthes is carried out with carbamates derivatives such as albendazole and mebendazole. The antihelmintic activity of this class of compounds appears to be related to their selective antimitotic action, due to the preferential binding of these agents to helminthes tubulin; the biological activities of some carbamates as inhibitors of mammalian tubulin have been reported. In spite of these compounds having a wide spectrum of action, they have polarity and hence they are poorly absorbed orally (Angeles et al., 2000).

Some carbamates have anti-parasitic activity such as anti-trypanosomal, anti-malarial, anti-flarial and anti-schistosomal activity. Parasitic flatworms of the genus *Schistosoma* are the causative agents of schistosomiasis, a highly prevalent, neglected tropical disease that causes significant morbidity in hundreds of millions of people worldwide. The current treatment of choice against schistosomiasis is praziquantel (PZQ), which is known to affect Ca²⁺ homeostasis in schistosomes, but which has an undefined molecular target and mode of action. PZQ is the only available antischistosomal drug in most parts of the world, making reports of PZQ resistance particularly troubling (Salvador-Recatalà and Greenberg, 2012).

The current antischistosomal activity of DDC after incorporation with chitosan polymer several criteria for the assessment of drug activity are being used to cause mortality. The present results showed that upgraded DDC-Chitosan-Na-NPs concentration increasing mortality % even in very low concentration and in

addition, increasing exposure time, that was in agreement with Khalil (2000) who recorded that all worms died due to treatment with PZQ. The activity of the DDC-chitosan was judged by marked reduction in the number of the deaths as compared with the control.

CONCLUSION

In conclusion, DDC-Chitosan-NPs of slow delivery system resulted in a low concentration of DDC for prolonged time of release could improve the antischistosomal activity of DDC against *S. mansoni* that appeared in reduce of worm survival.

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