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Isolation and modification of chitin and chitosan from shrimp shells and study as inhibitors of HIV

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ABSTRACT

Chitosan is a nontoxic, biodecomposable and bioconsistent polysaccharide of β (1-4) -linked D-glucosamine and *N*-acetyl-D-glucosamine. It can be derived of raw Chitin (shrimp shelles) which offers outstanding features that have introduced the chitosan in the pharmaceutical and bio-medical fields. This study is concern with extracted and synthesis of some polymers from natural sources from local shrimp shells such as chitin, chitosan and their derivatives: Ethyl amine of chitin, Ethyl amine of chitosan, carboxymethyl of chitin and carboxymethyl of chitosan. Those polymers have been notted as (I-IV) respectively for simplicity. The polymers (I-IV) have been studied on HIV. It has been found that ethyl amine of chitosan has inhibited HIV-1 replication in cell culture at concentration of 100 µM. Ethyl amine of chitosan was much lower than the corresponding lead compounds and more activity than the others polymers; chitin and chitosan were less active than from the others.

Keywords: chitosan,chitin, HIV, shrimp shells

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1. Introduction

Chitosan is a polysaccharide which is simply obtainable in nature from the shells[1] and the other sea shellfish product from deacetylation of chitin. Chitosan is a cationic polysaccharide with linear chain consisting of β-(1, 4)-linked 2-acetamino-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β- D-glucopyranose.[2,3] During the last few years, many of the research directed to the areas of chitin and chitosan materials and can be of benefit greatly in the field of biochemistry. Figure 1 showed the conversion of chitin to chitosan.

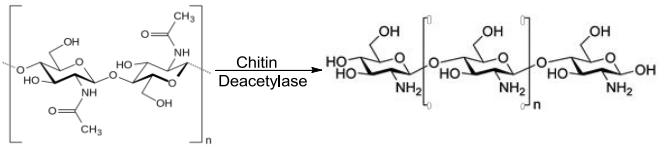


Figure 1. Conversion of chitin to chitosan

1.2 Chitosan based biodegradable hydrogel microspheres for controlled release of an anti HIV drug

Human immunodeficiency virus (HIV) infection reasons acquired immune deficiency syndrome (AIDS) and is a global public health issue.[4]

Anti-HIV therapy, including chemical drugs has improved the life quality of HIV/AIDS patients. However, the emergence of HIV drug impedance, side effects and the necessity for long-term anti-HIV treatment are the main reasons for washout of anti-HIV therapy. Furthermore, it is necessary to isolate novel anti-HIV therapeutics from natural resources. Recently, a great deal of interest has been expressed regarding marine-derived anti-HIV agents such as phlorotannins, sulfated chitin oligosaccharides, sulfated polysaccharides, lectins and bioactive peptides. This contribution presents an overview of anti-HIV therapeutics derived from marine resources and their potential application in HIV therapy so that, there are many ways for treatment at the recent years used Chitosan for treatment as anti- HIV [5] In continuation of our research work on the development of controlled release devices [6,7] utilizing carbohydrate polymers

2. Result and Discussion

2.1 Characterisation of polymers (I-VI)

The extraction and preparation polymers (I-VI) were characterised by using FTIR.

2.1.1 FTIR spectrum of Chitin I

The IR spectrum of chitin showed characteristic bands at absorptions 1660.71, 1624.06, 1554.63 cm^{-1} and assigned as amide I bands.The absorption bands of the hydroxyl and amine groups appeared at 3419.79 cm^{-1} while the C-H stretching band absorbed at 2891.30 cm^{-1}. The bridging oxygen – stretching band showed at 1157.29 cm^{-1} and the C-O stretching bands at 1072.42, 1026.13, 894.97 cm^{-1}.[16,17]

2.1.2 FTIR spectrum of chitosan II

The IR spectrum of chitosan [18] showed characteristic bands absorptions at 3128.54 cm^{-1} and 3442.94 cm^{-1} and assigned as the stretching vibration of OH and NH groups. The band at 896.90 cm^{-1} have been assigned as (C-H) bands of chitosan, the bands at 1035.77, 1155.36 and 1251.80 cm^{-1} corresponds to the asymmetric stretching vibration (C-O-C) and symmetric stretching vibration (C-O-C). The band at 2879.72 cm^{-1} indicates symmetric stretching vibration (C-H)

2.1.3 FTIR spectrum of Ethyl amine of Chitin III

The IR spectrum of ethyl amine of chitin showed that the characteristic band at 3479.58 cm^{-1} is attributed to stretching vibration of NH group. The absorption band at 2877.79 cm^{-1} attributed to aliphatic C-H stretching. The spectrum was characterized by three significant bands at 1653.00, 1624.06, 1558.48 cm^{-1} which correspond to the amide I and amide II. It is important to note that the amide I band of chitin splits at 1653.00 and 1624.06 cm^{-1}, which is attributed to the two types of H-bonds formed by amide groups in the antiparallel alignment present in crystalline region of ethyl amine of chitin. The band at 1379.10 cm^{-1} corresponds to a symmetrical deformation of the CH_3 group and at 1074.35 cm^{-1} for C-O-C vibration inside chitin ring and produced many peaks caused by the presence of hydroxide from chitin which contains a single bond C=O .

2.1.4 FTIR spectrum of Ethyl amine of Chitosan IV

The IR spectrum of ethyl amine of chitosan showed characteristic bands at absorptions broad band at 3402.43 cm^{-1} due to the stretching vibration of NH and OH groups, the band at 1558.48 cm^{-1} correspond to NH_2, the band at 894.97 cm^{-1} correspond to the (C-H). The band at 1058.92 1151.50 and 1259.52 cm^{-1} corresponds to the asymmetric stretching vibration (C-O-C) . The band at 1421.54 corresponds to C-H. Meanwhile the band at 2881.65 cm^{-1} indicates symmetric stretching vibration (C-H).

2.1.5 FTIR spectrum of Carboxymethyl of chitin V

The FTIR spectrum of carboxymethyl of chitin [19] showed characteristic bands at absorptions of –COOH group at 1760 cm^{-1}, while the bands at 1720.71 cm^{-1} and 1413.82 cm^{-1} corresponding to the carboxy group (which overlaps with N-H bend) and $–CH_2COOH$ group, respectively. The bands at 1078.21 -1028.06 cm^{-1} (C-O stretch)

2.1.6 FTIR spectrum of Carboxymethyl chitosan IV

The FTIR spectrum of carboxymethyl of chitosan showed abroad absorption band between 3448.72-3150.54 cm^{-1} due to O-H stretching and vibration N-H. The band at 1730 cm^{-1} attributed to the –COOH group. The signals at 2895.15 and 2980.87 cm^{-1} could be assigned to the vibration of methyl and methylene. A band at 1575.84 cm^{-1} was observed, which is attributed to the angular deformation of the N-H bonds of the amino group. A band at 1396.46 cm^{-1} due to the symmetric angular deformation of CH_3 and the amide III band at 1317.38 cm^{-1} were observed. The band corresponding to the polysaccharide skeleton, including vibrations of glycoside bonds, C-O and C-O-C stretching in range 1010.70, 1153.43 cm^{-1} were observed. [20]

2.2 In vitro Anti-HIV Activity

Compounds **I** and **VI**, were tested for their in vitro anti-HIV-1 (strain III_B) and HIV-2 (strain ROD) activity in human T-lymphocyte (MT-4) cells, using MTT method .The results are summarized in Table 1, in which the data for azidothymidine (AZT) and lamivudine (3TC) were included for comparison purposes. Compound **IV** was found to be the only compound from the series inhibiting HIV-1 replication in cell culture at concentration of 100 µM. Compound **IV** showed an IC_{50} value of > 2.17 µM and a CC_{50} value of 6.17 µM, resulting in a selectivity index (SI) of 3, meanwhile exhibited no selectivity can be witnessed (SI < 1) against HIV-2 (IC_{50} > 2.17 µM). Derivatives **I ,II** and **VI** demonstrated no activity since they showed selectivity index (SI) < 1, However, the potency of compound **IV** was much lower than the corresponding lead compounds, AZT, and 3TC.

From the SAR analysis, we found that the free amino group C-2 as well as amino-ethoxy group at C-6 of the sugar moiety *e.g.* compound **IV** would enhanced the anti-HIV activity in comparison to the other analogues including the chitosan itself. These data showed that IV is well tolerated in the hydrophobic region of HIV RT and then showed higher activity than those of the alkylamino substituents at C-2 or ethoxy group having carboxylic group at C-6 of the same ring; *e. g.*: compounds **I , II *and* VI**, and resulted in loss of activity. This means that the amino groups at C-2 and C-4 targeting the hydrophobic binding pocket of HIV-1 RT.

2.2.1 Molecular modeling analysis

Our molecular docking analysis of the new analogs is based on the modeling studies which were performed to understand the binding mode of these analogs with the HIV-1 RT binding pocket (NIBP) (PDB code: 1dtt .The molecular docking was performed using SYBYL-X 1.1, and the results were visualized with PYMOL HIV-1 reverse transcriptase (RT) demonstrates an intrinsic resistance to non-nucleoside RT inhibitors (NNRTIs), one of two classes of anti-AIDS drugs

Table 1 . *In-vitro* anti-HIV-1[a] and HIV-2[b] of new chitosan derivatives.

Entry	HIV-1 (IIIB) Av IC_{50} (μM)[c]	HIV-2(ROD) Av IC_{50} (μM)[c]	av. CC_{50} (μM)[d]	SI[e]
I	>32.21	>32.21	32.21	<1
II	>30.90	>30.90	30.90	<1
III	>11.95	>11.95	11.95	<1
IV	>2.17	>12.30	6.17	3
V	>25.29	>25.29	25.29	<1
VI	9.90	>9.90	9.90	<1
AZT	0.0019	0.0018	>25	>13144 (>14245)
3TC	0.51	2.02	>20	>39(>10)

[a] Anti-HIV-1 activity measured with strain III_B; [b] Anti-HIV-2 activity measured with strain ROD; [c] Compound concentration required to achieve 50 % protection of MT-4 cells from the HIV-1 and HIV-2 induced cytopathogenic effect; [d] Average CC_{50}: compound concentration that reduces the viability of mock-infected MT-4 cells by 50 %; [e] SI: selectivity index (CC_{50}/IC_{50}).

that target the viral RT, however, HIV-1 RT has a similar overall fold to HIV-2 RT but has structural differences within the "NNRTI pocket" at both conserved and nonconserved residues. Compound **IV** has been selected for the docking modeling study, since showed a lowest binding energy score (-8.2 kcal/mol) (Figure 2). The aminosugar backbone is located in the middle of the binding pocket, anchoring the two amino groups: one at C-2 whereas the other one of the ethoxyamino residue at C-6 in a favorable position for hydrogen bonding with the Tyr316 and Lys101 of the reverse transcriptase (RT) enzyme, respectively. Further, the oxygen atom of the ethoxyamino group and Lys103 of RT enzyme. Overall, there are several amino acids like Lys100, Asp318 and Glys99 surronded the binding pocket of the compound **IV** molecules together with the amino acids Tyr 316, Lys101 and Lys103 which finally govern the binding of compound IV with HIV-1 RT. terminal NH_2 group of Lys101 with NH_2 group of ethoxyamino residue at C-6, and oxygen atom of ethoxygroup with terminal NH2 group of Lys103 of reverse transcriptase (RT) enzyme residues of HIV-1.

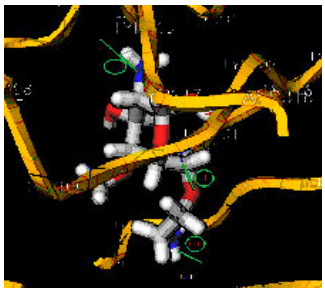
3. Experimental details

3.1 Extraction of chitin (I)[8]

In this study the shells of local marine shrimps has been Used. The marine shrimps brought from the local market, in the city of Basra. First the collected shrimp shells were washed with Tap water and then the samples were sun light dried for 24 hours, and further dried in oven at 80° C, after that shell grinded by electric grinder to get the fine powder.

3.1.1 Demineralization

50g from the fine shells powder were suspended in 500 ml 5% HCl temperature 25° C with continues stirring. After 1 hour the mixture were quite squashy, then the solution were filtered and the precipitate washed well with distilled water to remove the residue of the acid , then dried in oven at 50° C



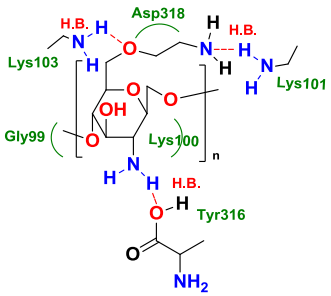


Figure 2. Docked conformation of compound IV showing three hydrogen bonds: oxygen atom of the carboxylic group of Tyr 316 with NH_2 group at C-2 of aminosugar, Lys101

3.1.2 Deproteninization of Shells

Deproteinization of shrimp shell was done with 500 ml of 1.2 N of NaOH of the product demineralized precipitate, was heated to 111° C for 3 h with continues stirring. Then the residue product was washed by distilled water to remove the excess of sodium hydroxide.

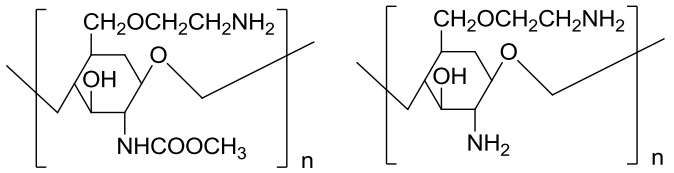
3.2 Preparation chitosan from chitin (II) [9]

To the round bottom flask with two neck fitted and reflex condenser , added 25 g of chitin and 250 ml of 50% sodium hydroxide , then the mixture was heated with stirring for 2 hours at 130° C. The resulting chitosan was washed with distilled water, ethanol and acetone. The product filtered and then dried overnight at 60° C. The final weight of chitosan was 15 g.

3.3 Preparation of Ethyl amine of Chitin (III) and of Chitosan (IV)

To 250 mL round-bottom flask was added 20 mL of mono ethanolamine and two drops of phosphoric acid. 1 gm of chitin or chitosan dissolved in 15 mL of mono ethanolamine was added drop wise to the mixture for 30 min. The mixture heated to 50 ° C, and then the heat was increased gradually to 80 ° C (reflexes for 6 hours).

Following the same previous method, the ethyl amine of chitosan was prepared. The chemical structure of ethyl amine of chitin and ethyl amine of chitosan showed in Figure 3 .



a **b**

Figure 3: (**a**) The chemical structure of ethyl amine of chitin (**b**) the chemical structure of ethyl amine of chitosan.

3.4 preparation of Carboxymethyl of Chitin [10] (v)

10 g of chitin was mixed well with 40 ml of 60% (w/w) NaOH . The alkali mixture was kept in ice for 1 hour. Then, kept overnight at a freezer of refrigerator.

200 mL of isopropyl alcohol have been poured to the prepared alkali chitin, and monochloro acetic acid then added at periods with continuous stirring. The addition of monochloro acetic acid was stopped as soon as the whole mixture became neutral. When the monochloro acetic acid was added to mixture the reaction temperature was increased, therefore, for each concentration of alkali, it is has been used one set of carboxymethylation which allowed to take place at room temperature, and reation temperature maintained at 35-40° C by using ice bath, the resulting viscous solution was dissolved in 1 litter of distilled water, and then precipitated using acetone. The precipitated mass of carboxymethylation was filtered and then washed for 24 hours in running water using filter papers to remove the byproduct salts and then carboxymethylation dried at 55° C overnight at oven. The weight of the product was 13 g, the chemical structure of Carboxymethyl chitin showed in Figure 4.

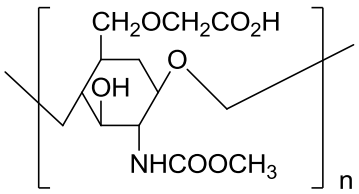


Figure 4. The chemical structure of Carboxymethyl chitin

3.5 Preparation of Carboxymethyl Chitosan (VI) [11]

Carboxymethyl chitosan was prepared according to the method reported by Chen and Park. Briefly 10 g of chitosan were dispersed in 100 ml of aqueous isopropyl alcohol (50 %) with stirring at room temperature for 30 min. Aliquot portions of 15 ml sodium hydroxide (10 M) were added at an interval of 5 min under agitation at room temperature. The reaction mixture was stirred for an additional 45 min. Then, 30 g of chloroacetic acid was dissolved in this mixture portion wise at interval of 5 min under warming at 55-60 °C with stirring for 1 h. The resultant solution was filtered. The filtrate was washed with aqueous solution of ethanol (80 % v/v), then dried in a vacuum oven at 50 °C for 24 hrs. The chemical structure of carboxymethyl chitosan showed in Figure 5.

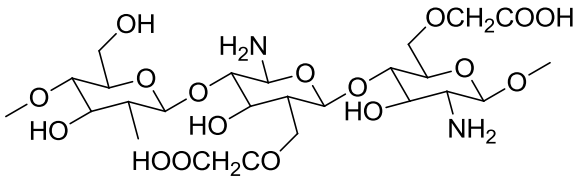


Figure 5. The chemical structure of Carboxymethyl chitosan

3.6 HIV

In vitro HIV assay

Evaluation of the antiviral activity of compounds **I** and **IV** against the HIV-1 strain (III_B) and the HIV-2 strain (ROD) in MT-4 cells was performed using an MTT assay as described previously[12]. In brief, stock solutions (10 times final concentration) of test compounds were added in 25-µl volumes to two series of triplicate wells to allow simultaneous evaluation of their effects on mock and HIV infected cells at the beginning of each experiment. Serial 5-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments). Untreated control, HIV and mock-infected cell samples, were included for each sample. HIV-1 (III_B) [13] or HIV-2 (ROD) [14] stock (50 µL) at 100-300 CCID50 (50 % cell culture infectious dose) or culture medium was added to either of the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compound on uninfected cells in order to assess the cytotoxicity of the test compounds. Exponentially growing MT-4 cells [15] were centrifuged for 5 min at 1000 rpm, and the supernatant was discarded. The MT-4 cells were resuspended at 6×105 cells per ml, and 50-µL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of the mock- and HIV-infected cells was examined spectrophotometricall

4. Conclusion

According to the results of this study the following conclusions can be summarized:

1. The polymers (I-VI) are non-toxic, prevents the incidence of side effects and Cheap, chitosan is biodegradable and biocompatible

2. The new polymers (III, IV) were found more active from the others because the medications containing amine and carboxyl groups be more effective in the drug

3. The compound (IV) has effectively worked in treatment of HIV. It has been found that ethyl amine of chitosan has inhibited HIV-1 replication in cell culture at concentration of 100 µM.

5. References

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