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Synthesis and characterization of some acyl thiourea derivatives of chitosan and their biocidal activities

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ABSTRACT

Three acyl derivatives of chitosan (CS) with different side chains were synthesized and their structures were characterized. Their swelling behavior was investigated. The antifungal behavior of these chitosan derivatives was investigated *in vitro* on the mycelial growth, sporulation and germination of conidia or sclerotia of the sugar-beet pathogens, *Rhizoctonia solani* K"uhn (AG2-2) and *Sclerotium rolfsii* Sacc. All the prepared derivatives had a significant inhibiting effect on the different stages of development on the germination of conidia or sclerotia of all the investigated fungi. In the absence of chitosan and its derivative, *R. solani* exhibited the fastest growth of the fungi studied.

1. Introduction

Chitin is a naturally abundant muco-polysaccharide and the supporting material of crustaceans (such as crabs, shrimps and cuttlefish), insects, *etc.* It consists of 2-acetamido-2-deoxy-β-D-glucose through a β-(1–4) linkage. Chitin has proven to be of use in many fields of biomedical and pharmaceutical industry but on top of that it is the source of chitosan the deacetylated form of chitin. Chitosan is much more useful than chitin due to the presence of the amino groups instead of the acetamido groups. The presence of the amino group renders the polymer soluble in dilute acids both organic and inorganic, it also impart a positive charge to the polymer due to the protonation of the NH_2 groups thus enhancing the antimicrobial capacity of the polymer.

Many chitosan derivatives, for example, N, O-acyl and N-alkyl and N-aryl chitosan derivatives were found to have insecticidal and fungicidal activity [1,2]. Novel chitosan derivative with ofloxacin (OFX) has been prepared [3]. The antimicrobial activity of the chitosan/ofloxacin (CH–OFX) complex against various micro-organisms was tested. The prepared chitosan aerogels show important properties such as biocompatibility, non-toxicity, and antibacterial activity, making them suitable for biomedical applications [4]. The antibacterial activity was checked for the synthesis of low-cost iron oxide: chitosan (CH-α-Fe_2O_3) nanocomposites by zone of inhibition method (AATCC 147) [5]. The CH-α-Fe_2O_3 nanocomposite has shown improved antibacterial activity against gram-positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*) when compared to the individual CH and α-Fe_2O_3 nanoparticles [5].

Thiourea chitosan was prepared by the reaction of chitosan with ammonium thiocyanate in ethanol [6,7]. The antimicrobial activity of the Ag^+ complexes of this thiourea compound was evaluated against six species of bacteria and molds. The complex showed a wide spectrum of antimicrobial activities, whose minimum inhibitory concentration (MIC) values against bacteria were 20 times lower than those of chitosan, 100 times lower than those of sodium diacetate and 200 times lower than those of sodium benzoate, respectively; the complex has a better antibacterial activity than antifungal activity [6].

Elsabee et al. [8] modified chitosan by reaction with benzoyl isothiocyanate to give a thiourea derivative. The antifungal behavior of chitosan and its thiourea derivative was investigated. It has been found that this derivative is a much better fungicidal agent (about 60 times more) than pure chitosan against most of the fungal strains tested. The molecular weight and the degree of deacetylation were found to have an important effect on the growth activities of the pathogens. Chitosan derivatives with higher biological activity such as N-sulfonated and N-sulfobenzoyl chitosan [9] have been prepared.

Three different acyl thiourea derivatives of chitosan (CS) were synthesized by Zhong et al. [10] and their antimicrobial behavior against four species of bacteria were investigated. The results

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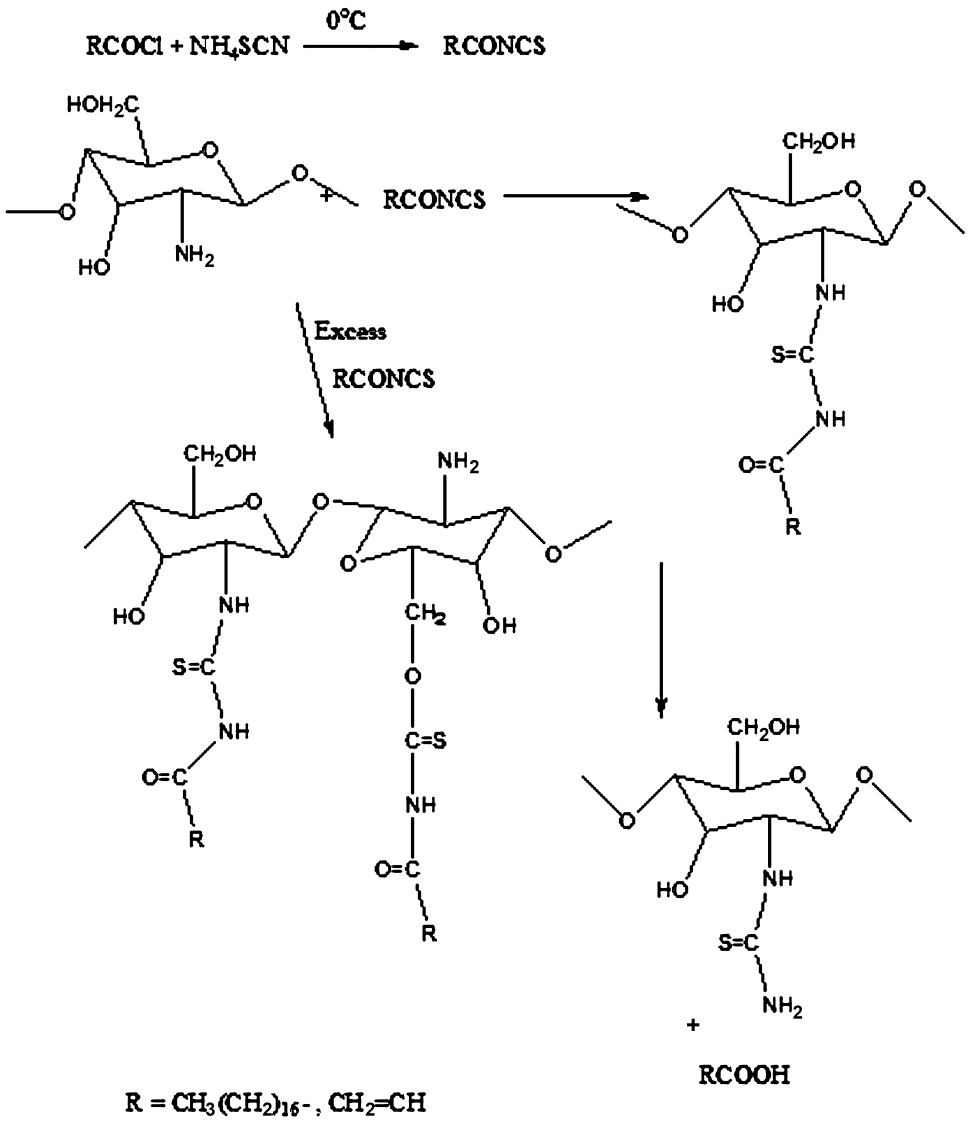

Scheme 1. Synthesis of the modified chitosan.

indicated that the antimicrobial activities of the acyl thiourea derivatives are much better than that of the parent CS.

Three novel thiosemicarbazone *O*-carboxymethyl chitosan derivatives were obtained *via* a condensation reaction of thiosemicarbazide *O*-carboxymethyl chitosan with *O*-hydroxybenzaldehyde, *p*-methoxybenzaldehyde, and *p*-chlorobenzaldehyde respectively [11]. The antimicrobial behaviors of the prepared derivatives against three types of bacteria were investigated. The results indicated that the antibacterial and antifungal activities of the investigated derivatives are much higher than those of the parent *O*-carboxymethyl chitosan.

Chitosan sulfate derivatives have been prepared by Huang et al. [12]. It was found that the chitosan sulfate showed no inhibition against *Escherichia coli* (*E. coli*) while they were active against *Staphylococcus aureus* (*S. aureus*). When chitosan sulfate was reacted with caproic anhydride, propanoic anhydride, or 2,3-epoxypropyl trimethyl ammonium N-acyl or N, O-quaternary ammonium derivatives of chitosan were obtained which showed much higher biocidal activity against both strains of bacteria. The activities of N-acyl chitosan sulfates seem to be related to the structures of the covalently bonded acyl moieties, among which the N-hexanoyl moiety was more effective in enhancing the *E. coli* inhibition activities.

In addition to the enhanced antimicrobial property of chitosan–sulfur derivatives they have acquired higher metal uptake capacity. Chitosan sulfur derivatives (thiocarbamoyl chitosan, TC-chitosan) with high substitution degrees has been proposed [13] which provided remarkable increase of its sorption capacity for Au(III), Pt(IV), Pd(II) ions in acidic media. The grafting of thiourea on chitosan backbone allows synthesizing a thiocarbamoyl derivative that was very efficient for mercury sorption in acidic solutions [14,15]. Mercury sorption in acidic solutions is not affected by the presence of competitor metals (such as Zn(II), Pb(II), Cu(II), Cd(II), Ni(II)) or the presence of nitrate anions (even at concentration as high as 0.8 M)). Chitosan-benzoyl thiourea derivative was shown to absorb appreciable amounts of ^{60}Co and $^{152+154}$Eu radionuclide's [16]. Platinum and palladium have been selectively absorbed on chitosan–sulfur derivatives [14]. Diethyl dithiocarbamate chitosan (EtDTCCS) was prepared and the antifungal activity were investigated [17]. Compared with plain chitosan, EtDTCCS shows better inhibitory effect with 93.2% inhibitory index on *G. theaesinensis* at 1.0 mg/mL, even stronger than for polyoxin (82.5%). The

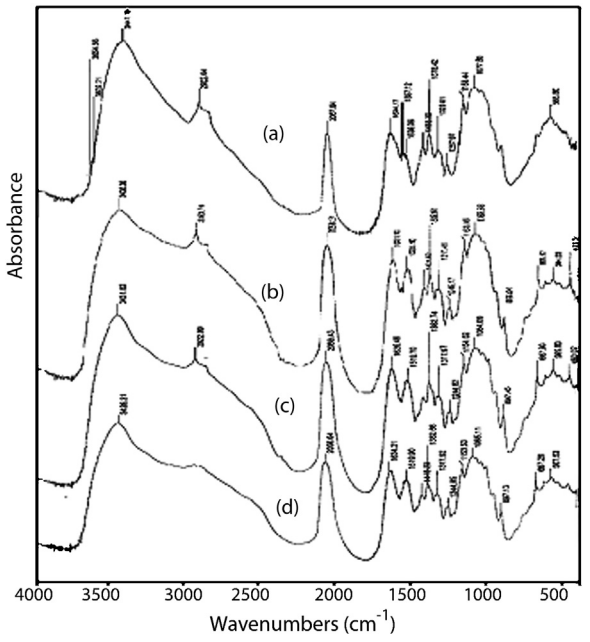

Fig. 1. FTIR spectroscopy of CSTU derivatives (a) 1S (b) 2S (c) 3S (d) 4S.

derivative had enhanced antifungal activity compared with chitosan.

The present work deals with the synthesis of new acyl thiourea derivatives of chitosan with different side chains. The biological activity of the derivatives samples is investigated *in vitro* on the mycelia growth, sporulation, and germination of sclerotia of the sugar beet: *Beta vulgaris* pathogens isolated in Egypt, *Rhizoctonia solani* K"uhn (AG2-2)(*R. solani*) and *Sclerotium rolfsii* Sacc. (*S. rolfsii*). Sugar-beet (*Beta vulgaris* L., Chenopodiaceae) is one of the most important crops grown mainly in areas of temperate climatic conditions for sugar production. It has a great economic importance for Egypt since it is the second crop plant for the sugar production after sugarcane. Sugar-beet is attacked by several root-rot diseases, the most serious of which are those caused by *R. solani* and *S. rolfsii*.

2. Materials and methods

2.1. Materials

Chitosan was isolated from red shrimp that was harvested from the red sea, acetic acid, stearoyl chloride, acryloyl chloride, and ammonium thiocyanate, were of reagent grade chemicals (Aldrich). All solvents, alcohol (methanol and ethanol), acetonitrile, diethyl ether, THF (tetrahydrofuran) and DMF (dimethylformamide), from local market, were purified according to conventional methods.

2.2. Methods

2.2.1. Chitosan extraction

Chitosan was extract according to the procedure in Ref. [18]. The degree of deacetylation DDA of all chitosan samples was determined by acid–base potentiometric titration [19], and by elemental analysis [20]. The degree of deacetylation was found to be equal 90.7%.

2.2.2. Preparation of the chitosan thiourea derivatives

Dry ammonium thiocyanate (NH_4SCN) was dissolved in acetonitrile at 0 °C and then an equivalent amount of acid chlorides was added drop wise. The reaction medium was stirred at 0 °C for 1 h, giving a yellowish white product which was filtered, and the

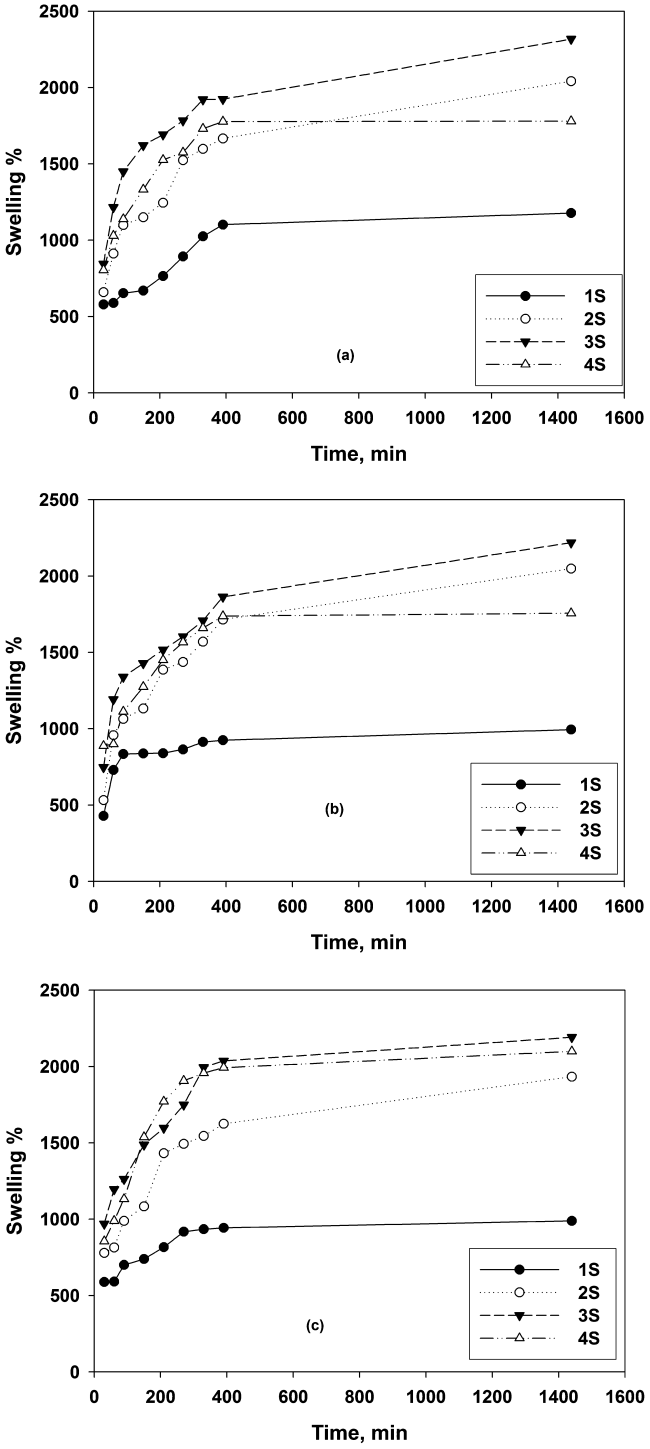

Fig. 2. Swelling behavior of CSTU derivatives at (a) pH 3, (b) pH 7, and (c) pH 10.

filtrate was added to chitosan with stirring for 24 h. The product was filtered and washed with acetonitrile and diethyl ether and dried at 60 °C. But in case of acryloyl chloride, another method was used where in samples 1AC and 2AC acryloyl chloride was added at room temperature to a chitosan dissolved in acetic acid (5%) to give a homogeneous product then the product was poured over ethyl alcohol and after precipitation was filtered and dried at 60 °C. The obtained material was analyzed by elemental analysis and FTIR spectroscopy. The synthesis of the modified chitosan is illustrated in Scheme [1].

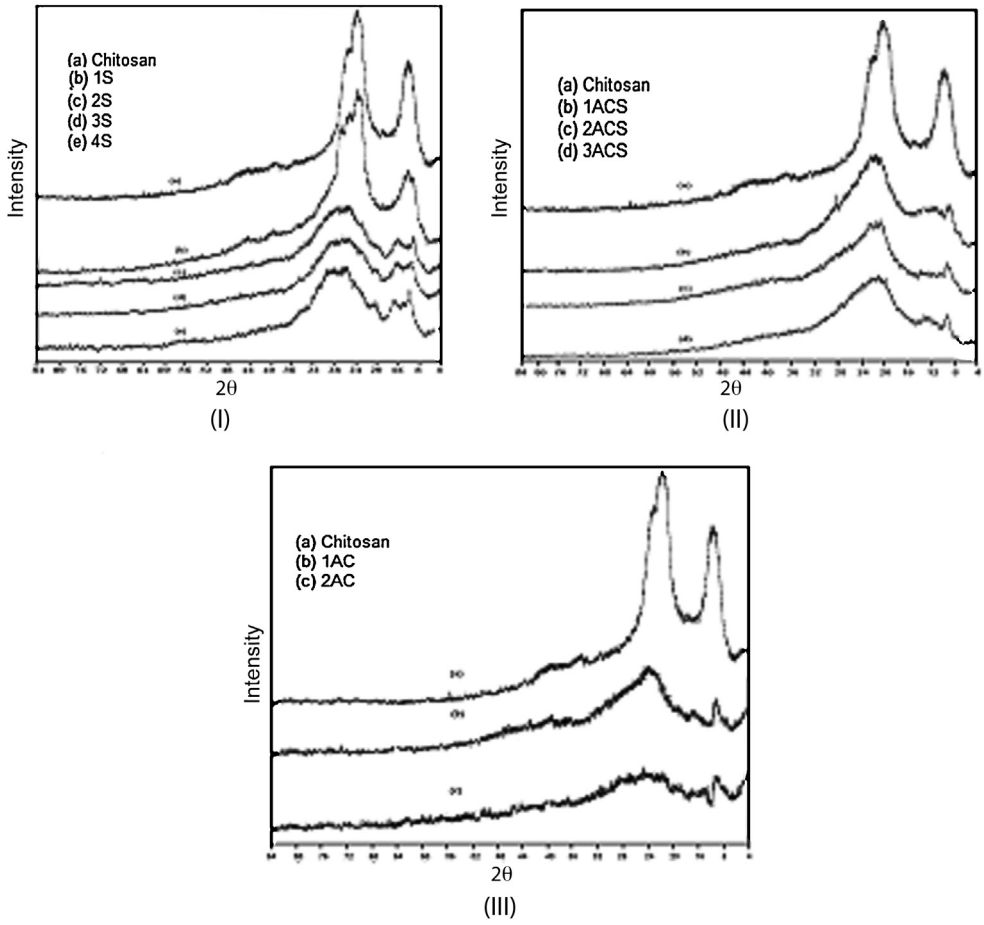

Fig. 3. X-ray diffraction patterns of chitosan, S, ACS and AC derivatives.

Four chitosan stearoyl thiourea derivatives were prepared by the same procedure by changing the stearoyl isothiocyanate: chitosan ratio: 1:3, 1:1, 5:3, and 2:1 and are designated as: 1S, 2S, 3S and 4S respectively. Three chitosan acryloyl thiourea derivatives were prepared with the ratio of acryloyl isothiocyanate: chitosan ratio of 1:1, 3:1, and 5:1 and are designated as 1ACS, 2ACS, and 3ACS respectively.

Chitosan acryloyl derivatives were prepared with the ratios of acryloyl:chitosan ratio of 2:1 and 5:1 and designated as 1AC and 2AC respectively.

2.3. Measurements

2.3.1. Swelling measurement of chitosan derivatives

The swelling behavior of the prepared samples was studied at 25 °C as a function of time in distilled water and in buffered solutions at different pH-values using the well known filter paper bag method.

An exact amount of pre-dried sample was placed into a 12.5 cm filter paper bags; this was then immersed in buffer solution at 25 °C. After certain time, the filter paper bag containing swollen sample was taken out and hung up for 5 min in order to eliminate excess unabsorbed liquid and then weighed. The degree of swelling at time t was calculated using the following relation:

$$\text{Swelling degree} = \frac{W_s - W_o}{W_o}$$

where W_s and W_o, weights of swollen and dry sample respectively.

2.3.2. Characterization of extracted chitosan and prepared chitosan derivatives

The FTIR spectra were measured in KBr pellets in the range 400–4000 cm^{-1} using Perkin-Elmer 2000 spectrophotometer.

X-ray diffraction analysis (XRD) was carried out by a Scintag powder diffractometer between 2θ angles of 5° and 40°. Ni-filtered CuKα-radiation was used as the X-ray source. The relative crystallinity of the polymers was calculated by dividing the area of the crystalline peaks by the total area under the curve.

Thermogravimetric analysis TGA was carried out using Shimadzu TGA-50H instrument at a heating rate of 10 °C/min under nitrogen atmosphere.

2.3.3. Biological properties of chitosan thiourea derivatives

Sources and culture of fungi, germination of sclerotia, dry mass determination and production of sclerotia has been given in details in previous works [8,21].

2.3.4. Statistics

The experiments were conducted in three to five replicates and the results obtained were treated statistically with an analysis of variance and the significance was expressed at L.S.D. 5% and 1%.

3. Results and discussion

3.1. Chitosan stearoyl thiourea CSTU derivative

Preparation of chitosan derivatives with long side chain could be favorable in terms of enhancing the antibacterial properties. It

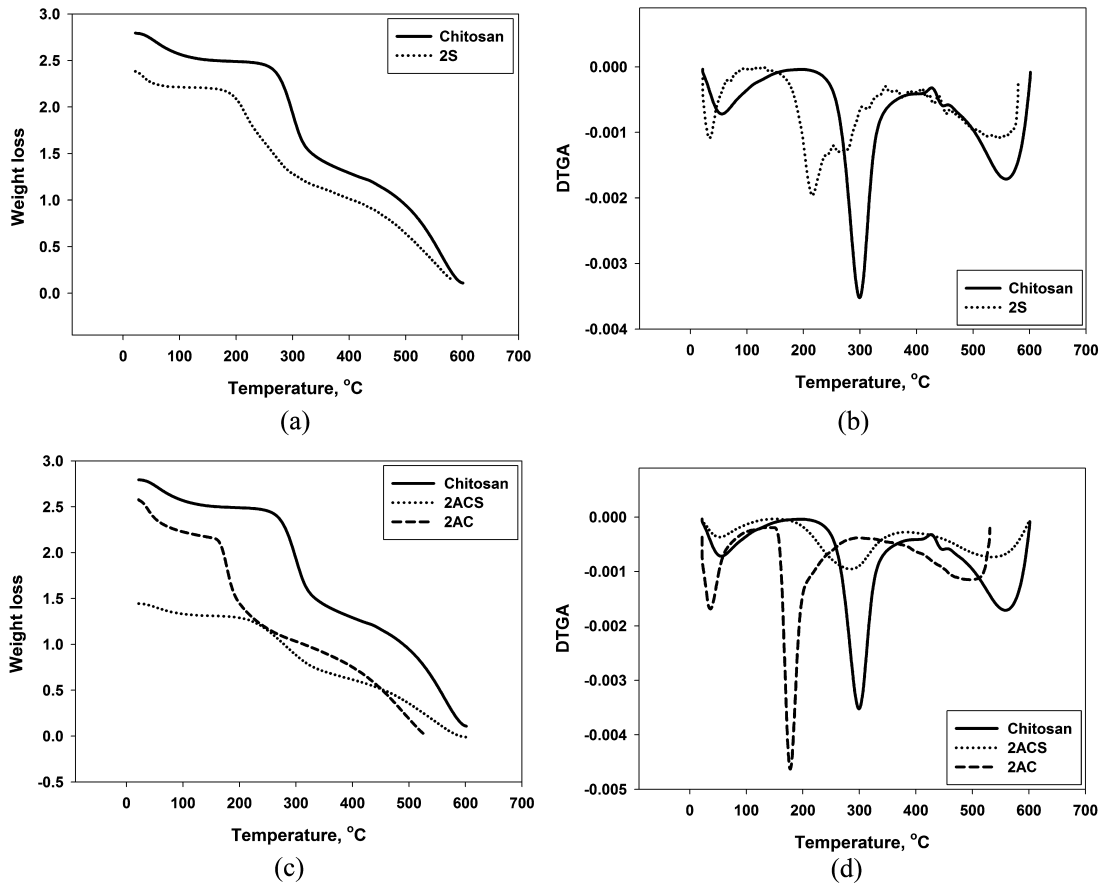

Fig. 4. The thermogravimetric analysis and deferential thermogravimetric analysis of: (a) TGA of chitosan and 2S, (b) DrTGA of chitosan and 2S, (c) TGA of chitosan, 2AC and 2ACS and (d) DrTGA of chitosan, 2AC and 2ACS.

is quite reasonable that the antimicrobial activity is dependent on the spacer length due to the change in both conformation and charge density of the polymer, which consequently affect the mode of interaction with the cytoplasmic membrane [22]. The hydrophobic characteristic of N-acylated chitosan can be favorable for the interaction of polymer molecule and bacterial cell, where the hydrophobicity is likely to be a contributing factor for its enhanced inhibitory effect [23]. Together with the long chain the presence of thiourea moiety is an additional factor enhancing the antimicrobial properties of the prepared derivatives.

3.1.1. Elemental analysis of the CSTU series

The elemental analysis data of the four stearoyl thiourea derivatives of chitosan were as follows (%): 1S C (43.26), H (7.26), N (8.76), and S (3.7). 2S, C (36.5), H (6.10), N (10.8), and S (10.3). 3S C (35.6), H (5.92), N (11.4), and S (11.2). 4S C (34.9), H (5.93), N (11.8), and S (11.6). The N and S contents increase systematically with increasing the stearoyl thiocyanate ratio. The most plausible explanation to this increase in the N and S contents could be based on the assumption that the stearoyl chloride has lost the oxy alkyl radical to form stearic acid and the isothiocyanate group has reacted with chitosan forming thiourea derivatives (Scheme 1). In this way the contents of N and S will increase otherwise if not then increasing the substitution should have decreased their content due to the long aliphatic carbon chain in the stearoyl chloride molecule. Further support comes from the low carbon content which indicates a big loss of carbon content.

Accordingly the final product of the reaction should be a mixture of chitosan thiourea and chitosan with long side chain *via* a thiourea connection. An example of the approximate ratios of these two components in the 2S sample (1:1 ratio), using the obtained elemental analysis, show that we have about 31% chitosan with long side group and 69% a thiourea component.

3.1.2. FTIR spectroscopy of CSTU derivatives

Fig. 1 shows FTIR spectra of 1S, 2S, 3S, and 4S. The stearoyl derivatives show similar pattern as the benzoyl derivatives [16] with only slight variation in the peak (N—H and O—H stretching) around 3441 cm^{-1} as it shifts toward shorter wavelength 2428 cm^{-1} as the degree of substitution (DS) increases. The band at 2058 cm^{-1} is very pronounced in all the samples and appears almost at the same position augmenting the tautomerism idea [12,16], a band at 1634 cm^{-1} due to the stretching vibration of the C=O group and another weaker around 1538 cm^{-1} due to the C=S group.

3.2. Chitosan acryloylthiourea CATU derivatives

Chitosan acryloyl thiourea derivatives were prepared with acryloyl isothiocyanate: chitosan ratios of 1:1, 3:1 and 5:1 and are designated as 1ACS, 2ACS, and 3ACS respectively.

3.2.1. Elemental analysis of the CATU series

The elemental analysis of the ACS series is: 2ACS C (38.6), H (6.10), N (9.16), (7.24). 3ACS C (38.2), H (6.15), N (9.05), S (7.22)

Elemental analysis and FTIR spectroscopy indicate that the two samples (2ACS and 3ACS) are almost identical in structure and that the DS is almost the same.

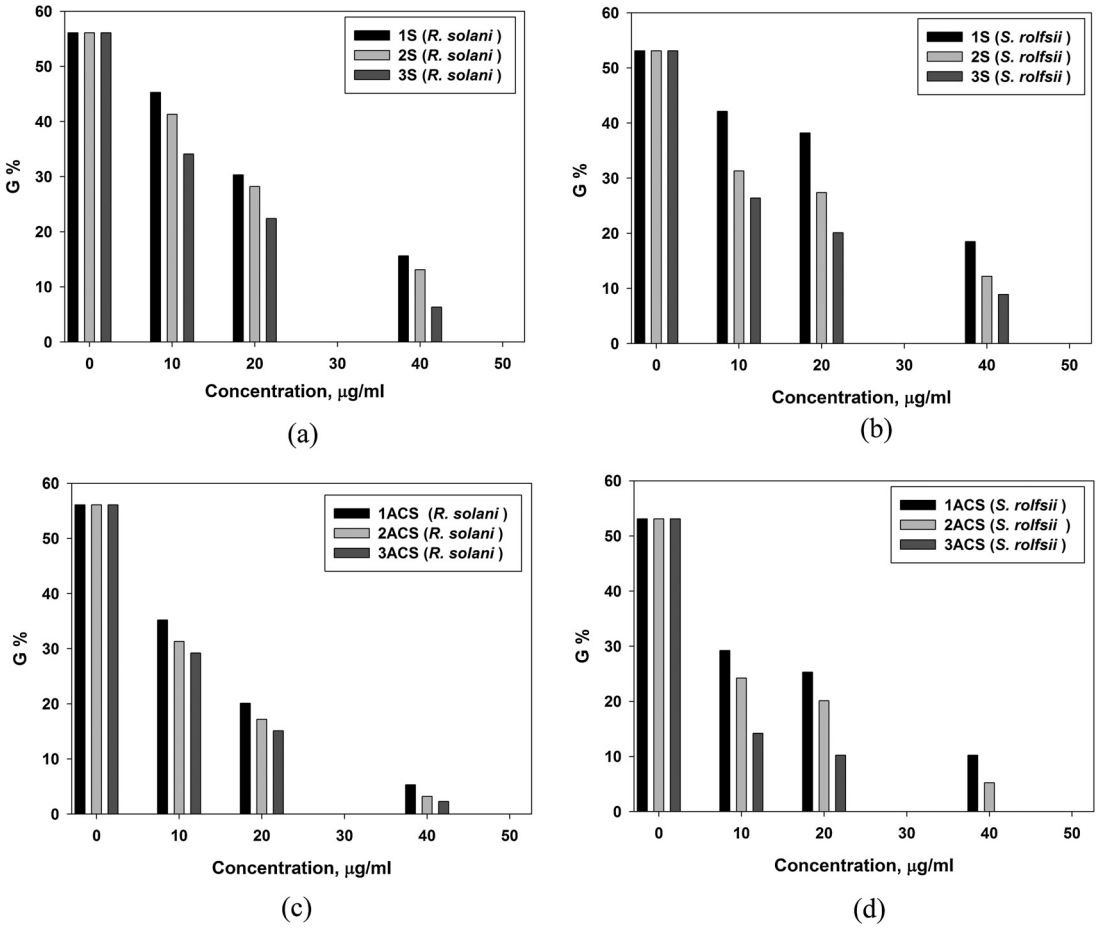

Fig. 5. Effect of chitosan derivatives concentration, µg ml^{-1} (S and ACS) on the germination % (G%) of (a) (*R. solani*), (b) (*S. rolfsii*), (c) (*R solani*) and (d) (*S rolfsii*).

3.2.2. FTIR Spectroscopy of CATU derivatives

The FTIR spectra of two derivatives 1ACS and 3ACS show high similarity with the spectra of the stearoyl derivatives. Strong peak at 3439–3435 cm^{-1} due to OH and NH stretching a pronounced peak at 2058 cm^{-1} due to the tautomeric structure (HN—C═S— ↔ N═C—SH—) band at 1635–1639 cm^{-1} due to carbonyl stretching. The spectra for the 1ACS and 3ACS are quite similar indicating similar DS in agreement with the elemental analysis data.

3.3. Chitosan acryloyl CA derivatives

Chitosan acryloyl derivatives were prepared with the acryloyl:chitosan ratios of 2:1 and 5:1 and designated as 1AC and 2AC respectively. Elemental analysis of the prepared sample: 1AC C (39.8), H (7.0), N (6.55) and 2AC C (40.63), H (6.7), N (6.31).

3.3.1. FTIR spectroscopy of chitosan acryloyl 1AC

The FTIR spectrum for the AC derivatives shows a band at 3449 cm^{-1} due to N—H and O—H stretching, a band at 1629 cm^{-1} characteristic to the carbonyl band and the absence of the 2058 cm^{-1} band due to the absence of tautomerism between (HN—C═S— ↔ N═C—SH—)

3.4. Swelling measurement of chitosan derivatives

From Fig. 2 N-acylation of chitosan with longer chain acid (C_{16}) chlorides increased its hydrophobic character (hydrophobic self-assembly) and made important changes in its structural features derivatives. The swelling characteristics of the chitosan derivatives depend on both the acyl chain length and the degree of acylation. The swelling behavior follows to a great extent the behavior of the benzoyl derivatives [24].

The swelling behavior of the ACS series showed the same trend as the S series. The swelling extent changed slightly with pH in the following order: pH 7 > pH 10 > pH 3 reaching a maximum of 1900%, 1800%, 1700% respectively. The swelling is highest at pH 7 similar to the swelling of the S series.

3.5. X-ray diffraction of derivatives

Fig. 3 shows the X-ray diffraction pattern of the acyl derivatives of chitosan. Crystallinity % of the derivatives was calculated and was found to be: 1ACS (52.1%), 2ACS (42.1%) and 3ACS (41.0%). Fig. 3 illustrates clearly the decrease in % crystallinity with increasing the DS for the stearoyl derivative, as follows: 1S 46.5%, 2S 23.5%, 3S 21.0% and 4S 24.0%. A drastic reduction of crystallinity has occurred after the reaction with acryloyl chloride (1AC 20.2%, 2AC 10.8%), which could be due to the strong acidic property of the acid chloride leading to partial hydrolysis and degradation of chains simultaneously with the reaction of the amino groups. The diffraction patterns for the prepared chitosan derivatives indicate that substitution in every case lowered the degree of crystallinity and the decrease is proportional to the extent of substitution. This is a reasonable finding since the chemical reaction leads to disruption of the polymeric chains and attacking the crystalline parts as well.

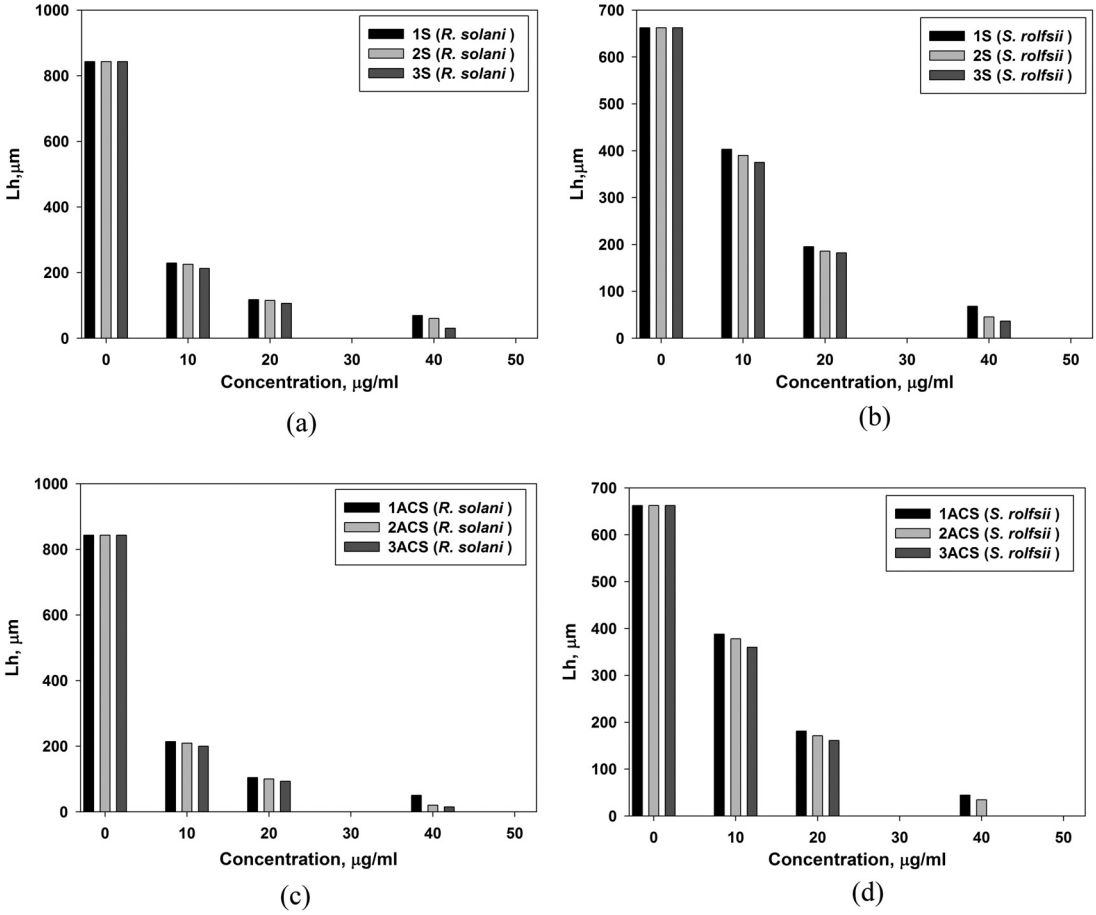

Fig. 6. Effect of S concentrations, on the Lh of (a) (*R. solani*), (b) (*S. rolfsii*) and ACS concentration on (c) (*R. solani*) and (d) (*S. rolfsii*).

3.6. Thermal analysis of CSTU derivatives

The thermal behavior of the chitosan and prepared chitosan derivatives is shown in Fig. 4. The unmodified chitosan shows a small weight loss before 100 °C due to the loss of water followed by one major decomposition band with a maximum temperature at 300 °C, while the derivatives 2S, 2ACS, and 2AC show major decomposition at 216.63 °C, 286.66 °C, and 173.33 °C respectively. Fig. 4 shows also a declining in thermal characterization with substitution in which the side groups seem to accelerate the thermal decomposition of the polymers. Even low cross linking extent did not affect much the thermal behavior of chitosan.

3.7. Antifungal activity for chitosan thiourea derivatives

The fungicidal activity of different chitosan thiourea derivatives toward two pathogens was investigated *in vitro* and the results are depicted in Figs. 5–7. From these figures (germination) one can see that the percent germination (G %) of sclerotia of *R. solani* and *S. rolfsi* decreased with increasing the chitosan derivatives concentration for both species (Fig. 5). The average length of hyphal (Lh) extension (Fig. 6) and dry mass (DM) yield was affected similarly, decreasing proportionally to the chitosan thiourea derivatives concentration. The pH of the growth medium shifted toward alkalinity for the *R. solani*. The pH increase in the culture medium during fungal growth may have been caused by differential uptake of cations and anions. Transport of anions such as phosphates may act as the hydroxide exchange system with the medium becoming more basic. The number of sclerotia produced by *R. solani* and *S. rolfsii* at chitosan thiourea derivatives concentrations ranging from 20 to 45 µg ml^{-1} were reduced proportionally to the chitosan thiourea derivatives concentration (Fig. 7). Approximately no sclerotia were produced by either species at concentration of 40 µg ml^{-1}.

Dry weight estimations showed that mycelial tolerance to chitosan thiourea derivatives concentration was low for *R. solani* and *S. rolfsii*.

From Figs. 5–7 it can easily be seen that chitosan thiourea derivatives has a profound effect on the growth activities of the studied pathogens. A similar report by Benhamou et al. [25] indicated that chitosan derived from crab-shell at concentration of 0.5 and 1 mg/ml was effective in reducing disease incidence caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Chitosan at pH 5.8 (when most of the amino groups are protonated) was found to induce massive leakage of UV absorbing materials in *Pythiumparoecandrum* [26]. At the same time El-Ghaouth et al. [27,28] revealed that chitosan was effective in inhibiting mycelial growth of *Pythiumaphanidermatum* completely at a concentration of 400 µg ml^{-1}. While at a concentration of 100 µg ml^{-1} it causes a 75% reduction of the mycelial dry weight. In addition, chitosan appeared to affect the development of *P. aphanidermatum* in submerged culture. At a concentration of 400 µg ml^{-1}, *P. aphanidermatum* grew in the form of cell clusters, indicating that chitosan may have affected the process of hyphal extension [25]. In comparison with the El Ghaouth work the thiourea derivatives prepared in this work can affect dramatic reduction in germination sclerotia production at much lower concentration (40 µg ml^{-1}) which almost 25 times less than chitosan itself.

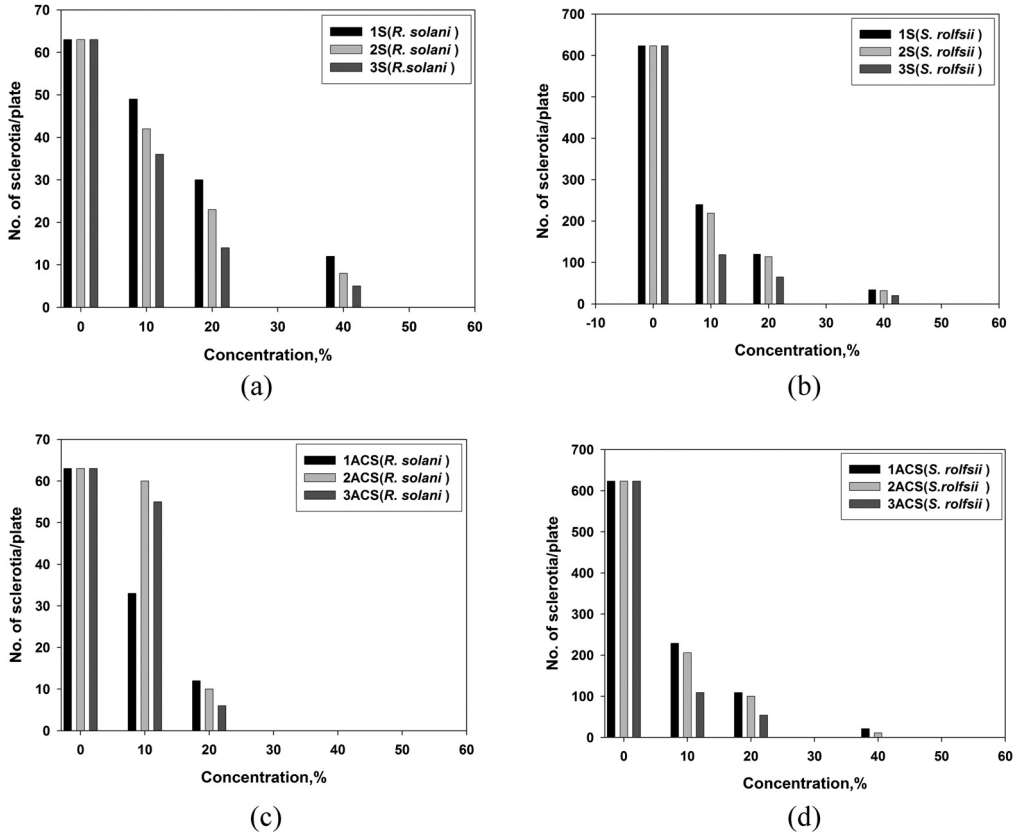

Fig. 7. Relation between S concentration, μg ml^{-1} and no. of sclerotia/plat for (a) (*R. solani*) (b) (*S. rolfsii*) and ACS concentration, μg ml^{-1} and no. of sclerotia/plat for(c) (*R. solani*) and (d) (*S. rolfsii*).

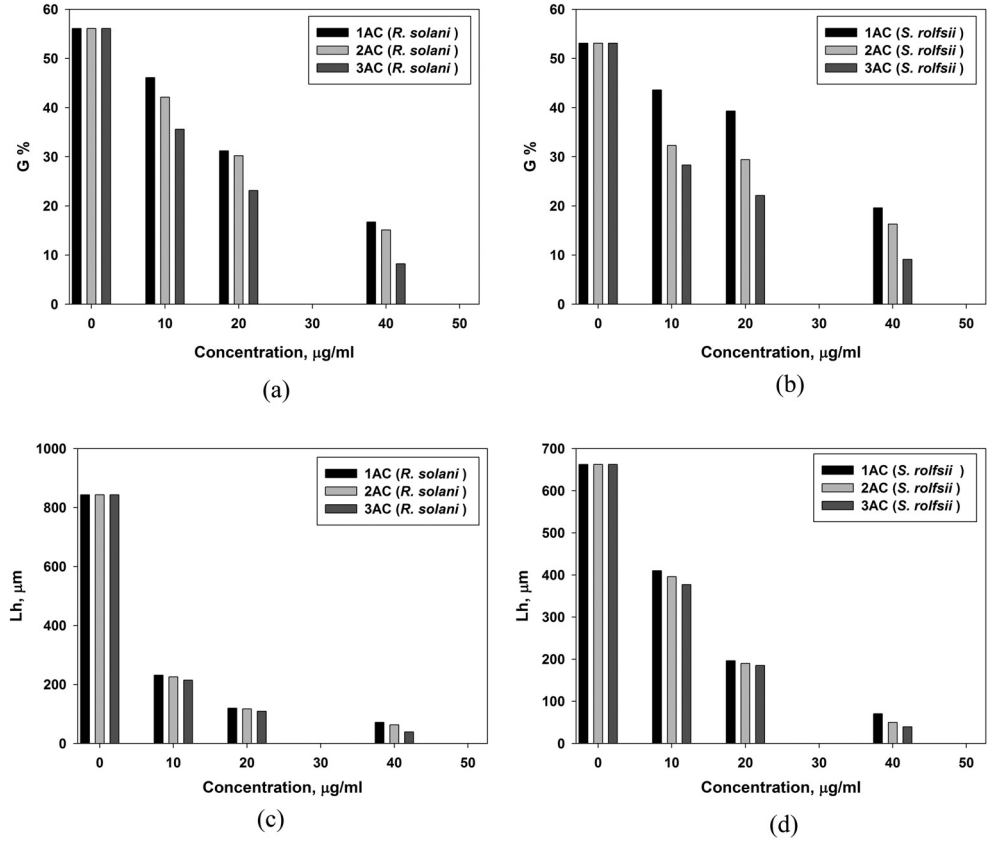

Fig. 8. Relation between CA concentration, μg ml^{-1} and G% of (a) (*R. solani*), (b) (*S. rolfsii*) and Lh, μm for (c) (*R. solani*) and (d) (*S. rolfsii*).

3.8. Antifungal activity for CA derivatives

Fig. 8 shows the effect of the acryloyl derivative of chitosan (AC) on the G% and the Lh (hyphal length). The reduction of both G% and Lh is lower than the corresponding sulfur derivatives which illustrates the effect of the presence of the sulfur in the form of thiourea derivative on the antifungal efficacy of chitosan.

4. Conclusion

Three acyl chitosan derivatives were successfully prepared by different methods. The prepared derivatives have been characterized by several techniques, since these are biologically active polymers swelling was conducted for any anticipated potential biomedical applications of these polymers. It was found that substitution of chitosan increases its swelling capacity. The TGA analysis revealed that the thermal stability of chitosan decreases with substitution. The crystallinity of chitosan was also found to declines as was shown by X-ray diffraction measurements.

The antifungal behavior of chitosan and its thiourea derivative was investigated *in vitro* on the mycelial growth, sporulation and germination of conidia or sclerotia of the following sugarbeet: *Beta vulgaris* pathogens isolated in Egypt, *Rhizoctonia solani* K"uhn (AG2-2) *and Sclerotium rolfsii* Sacc. All the prepared thiourea derivatives had a significant inhibiting effect on the different stages of development on the germination of conidia or sclerotia of all the investigated fungi in the polymer concentration range of 5–40 $\mu g\,ml^{-1}$. In the absence of chitosan and its derivative, *R. solani* exhibited the fastest growth of the fungi studied.

The most sensitive to the modified chitosan stress with regard to their germination and number produced were the sclerotia of *S. rolfsii*. Ithas been found that the chitosan thiourea derivatives are a much better fungicidal agent than the pure chitosan against most of the fungal strains tested.

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