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Der Pharmacia Lettre, 2015, 7 (10):107-117  
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## Chitosan-benzofuran adduct for potential biomedical applications: Improved antibacterial and antifungal properties

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### ABSTRACT

Chitosan-benzofuran adduct showed better antifungal and antibacterial properties compared to chitosan or benzofuran alone. The new polymer hydrogel has been characterized using FTIR, SEM, DSC, TGA and XRD. FTIR proved the formation of Schiff's base reaction at  $1632\text{ cm}^{-1}$ , between the chitosan's amino group and the khellinone's ketonic group. The X-ray diffraction patterns of chitosan-benzofuran showed the formation of a porous xerogel network. The TGA and DSC results were in accordance with the results obtained from the SEM. The TGA and DSC revealed slight loss in the chitosan-benzofuran' thermal stability compared to chitosan hydrogel and that could be regarded to the increase in porosity as shown in the SEM. The novel chitosan-benzofuran hydrogel has shown an outstanding antifungal and antibacterial effect. Moreover, the cellular cytotoxicity of baby hamster kidney cells culture by adding different concentrations of chitosan-benzofuran hydrogel were greater than those obtained from benzofuran or chitosan alone as they were  $4.81\pm 2\%$ ,  $5.27\pm 2\%$  and  $9.43\pm 5\%$ , respectively.

**Keywords:** chitosan, chitosan-benzofuran hydrogel, characterization, Schiff's base, antimicrobial activities.

### INTRODUCTION

Polysaccharides are widely distributed in nature. These materials are important in different fields since they possess unique structures and characteristics that are different from those of typical synthetic polymers. Chitin, poly(b-(1→4)-N-acetyl-D-glucosamine), is synthesized by an enormous number of living organisms and depending on its source, occurs as two allomorphs, namely the  $\alpha$  and  $\beta$  forms. Chitosan is an abundant heteropolysaccharide obtained from deacetylation of chitin, which is the most abundant polysaccharide after cellulose. Considerable interest to researchers lies in the biocompatible, biodegradable, cytocompatible, hemocompatible and adsorption properties of chitosan, which can be exploited to create unique building blocks with novel function via Schiff's base [1&2]. Schiff's base techniques has been successfully used with other biopolymers [3-6] These properties are attracting interest for use in pharmaceutical and biomedical fields, including antimicrobials, gene delivery, carriers of immobilized enzymes and cells, biosensors, artificial organs, and biodegradable packaging, as well as wound

healing and as scaffolds for tissue regeneration [7-9]. The organic biopolymer is containing chromophoric groups, whose optical property make the material much more important from a biotechnological and medicinal application point of view [10&11].

Although chitosan is widely known for its antibacterial and antifungal properties, there were some studies that chitosan activity varies depending on chitosan molecular weight [12]. Other studies demonstrated that low molecular weight chitosans are more antibacterial than high molecular weight chitosan [13&14].

On the other hand, benzofuran have drawn considerable attention over the last few years due to their physiological and chemotherapeutic properties as well as their widespread occurrence in nature [15]. Benzofuran derivatives display potent biological properties including antihyperglycemic [16], analgesic [17], antiparasitic [18], antimicrobial [19], antitumor and kinase inhibitor activities [20]. In addition, substituted benzofurans find application such as of fluorescent sensor [21], oxidant [22], antioxidants, brightening agents, a variety of drugs and in other field of chemistry and agriculture [23].

To that end, we are proposing incorporation of benzofuran to low molecular weight chitosan to form chitosan-benzofuran hydrogel for the sake of improving chitosan's antibacterial properties. According to our knowledge, there is no previous report that studied low molecular weight chitosan doped with benzofuran from this point of view. In this work, the structure and morphology of the new compound, chitosan-benzofuran, were characterized by FTIR, SEM and TGA. We also reported the physicochemical, optical and biological activity of chitosan-benzofuran hydrogel for potential biomedical applications.

## MATERIALS AND METHODS

### 2.1. Materials and equipment

Chitosan (low molecular weight, 20-300 cP, 1 wt. % in 1% acetic acid (25 °C, Brookfield), 75-85% deacetylated), khellin and other chemicals and solvents were purchased from Aldrich products Co. As a general rule, the percentage of cell viability was presented as mean  $\pm$  SD for at least three replicates with  $P < 0.05$ .

### 2.2. Preparation of chitosan gel (1.5% w/v chitosan) (1)

Dissolve 3g of low molecular weight chitosan in 100 mL distilled water containing 2 mL concentrated acetic acid + 100 mL ethanol absolute. Mix well using magnetic stirrer at 40°C.

### 2.3. Preparation of 4,7-dimethoxy-5-acetyl-6-hydroxy benzofuran (2)

Khellin (10 gm) was dissolved in 100 mL 3% potassium hydroxide. The reaction mixture was refluxed for 2 hours, after cooling it was acidified by 10% (v/v) HCl. The formed product was filtered off and washed with water.

### 2.4. Preparation of chitosan-benzofuran hydrogel (3)

Dissolve 10g of benzofuran derivative (2) in 400 mL ethanol absolute. In a separate container, dissolve 12 g of low molecular weight chitosan in 400 mL distilled water containing 8 mL concentrated acetic acid and stir at 40°C till complete dissolution. Mix the two beakers to each other gradually and under stirring.

### 2.5. Characterization Methods

#### 2.5.1. Cell culture

The cells were obtained from Egyptian Holding Company for Biological Products & Vaccines (VACSERA), Giza, Egypt and then maintained in the tissue culture unit. The cells were grown in RPMI-1640 medium (Roswell Park Memorial Institute), supplemented with 10% heat inactivated FBS (Fetal Bovine Serum), 50 units/mL of penicillin and 50 mg/mL of streptomycin and maintained at 37 in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were maintained as monolayer culture by serial sub-culturing. Cell culture reagents were obtained from Lonza (Basel, Switzerland)

### 2.6. Antimicrobial activity assay

Antimicrobial activity of the tested compounds was determined using the disc diffusion method with slight modifications [24]. Briefly, 100  $\mu$ l of the tested bacteria/fungi were grown in 10 mL of fresh media until they reached a count of approximately 10<sup>8</sup> cells/mL for bacteria or 10<sup>5</sup> cells/mL for fungi [25]. 100  $\mu$ l of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Isolated colonies

of each organism that have pathogenic role should be selected from primary agar plates and tested for susceptibility by disc diffusion method [26].

Mueller-Hinton agar media has been chosen for this work as it has been recommended by NCCLS as it is reproducible. Disc diffusion method for filamentous fungi was tested using approved standard method (M38-A) for evaluating the susceptibilities of filamentous fungi to antifungal agents [27]. Disc diffusion method for yeasts was developed using approved standard method (M44-P) [28]. Plates inoculated at 25 °C for 48 hours with filamentous fungi as *Aspergillus flavus*; Gram (+) bacteria as *Staphylococcus aureus*, *Bacillus subtilis*; Gram (-) bacteria as *Escherichia coli*, and *Pseudomonas aeruginosa* were then incubated at 35-37 °C for 24-48 hours; whereas, yeast as *Candida albicans* was incubated at 30 °C for 24-48 hours. Diameters of the inhibition zones were measured in millimeters. Standard discs of Ampicillin (Antibacterial agent), Amphotericin B (Antifungal agent) were used as positive controls for antimicrobial activity, whereas filter discs impregnated with 10 µl of solvent (distilled water, chloroform, DMSO) were used as negative control.

Zones of inhibition have been determined for susceptibility and resistant values. Blank paper disks (Schleicher & Schuell, Spain) with a diameter of 8.0 mm were impregnated with 10µL of tested concentration of the stock solutions.

When a filter paper disc impregnated with a tested chemical is placed on agar, the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "Zone of inhibition" or "Clear zone". For the disc diffusion, the zone diameters were measured with slipping calipers of the National Committee for Clinical Laboratory Standards. Agar-based methods such as Etest and disk diffusion can be good alternatives because they are simpler and faster than broth-based methods [29].

### 2.7. Cytotoxicity assay

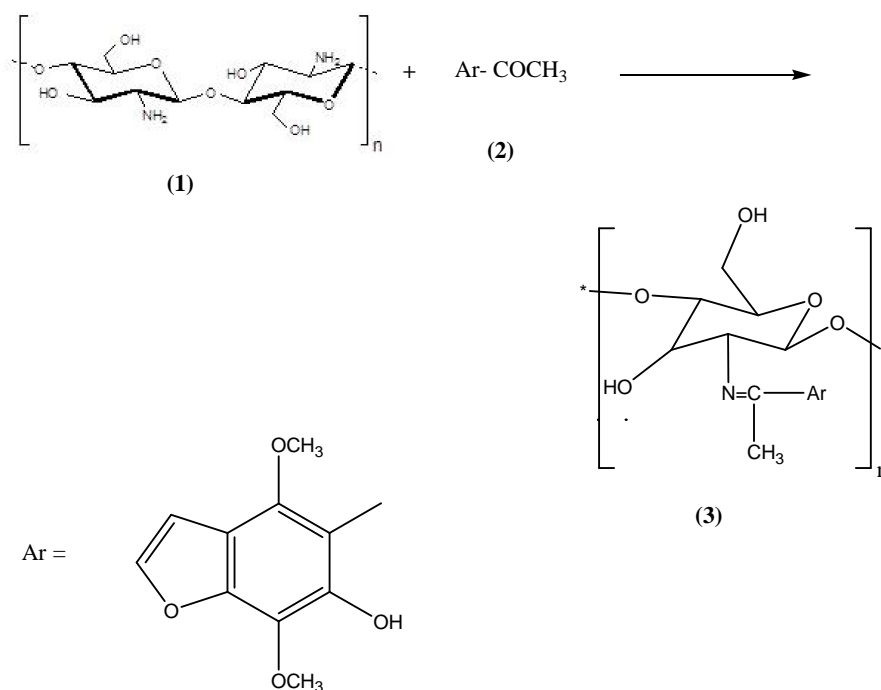
Cytotoxicity was determined using SRB method [30]. Exponentially growing cells were collected using 0.25% trypsin-EDTA and seeded in 96-well plates at 1000-2000 cells/well in RBMI-1640 supplemented medium. After 24 h, cells were incubated for 72 h with various concentrations of the tested compounds. Following 72 h treatments, the cells were fixed with 10% trichloroacetic acid for 1 h at 4 °C. Wells were stained for 10 minutes at room temperature with 0.4% SRBC (sulphorhodamine B) dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris-HCl for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, USA). The IC<sub>50</sub> values were calculated according to Boltzman equation. The sigmoidal concentration-response curve was plotted using the nonlinear regression fitting models (Graph Pad, Prism Version5).

## RESULTS AND DISCUSSION

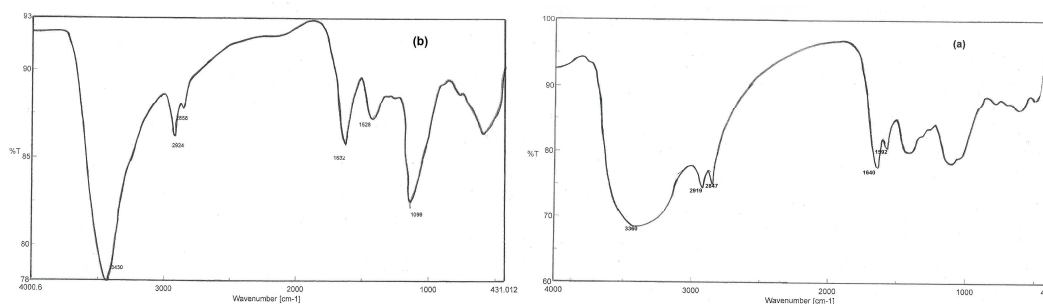
The presence of free reactive amino group in chitosan (**1**) leads to the possibility of forming a Schiff base with the benzofuran (**2**). The preparation of chitosan- benzofuran hydrogel (**3**) is shown in Scheme 1. FTIR spectroscopy was used to confirm the structure of the Schiff base of chitosan.

### 3.1. Fourier Transform Infra-Red Spectra

**Figure 1a,b.** FTIR spectra ( $\nu$ ,  $\text{cm}^{-1}$ ) were recorded on Jasco FT-IR 4100 instruments using KBr Disks, Micro analytical center, Cairo University, Cairo, Egypt. FTIR showed the spectra of chitosan and chitosan-benzofuran hydrogel, respectively. The pure chitosan spectrum in Figure 1a showed several characteristic peaks at 3360  $\text{cm}^{-1}$  (O-H stretch overlapped with N-H stretch), 2919 and 2874  $\text{cm}^{-1}$  (C-H stretch), 1640  $\text{cm}^{-1}$  (amide II band, C-O stretch of acetyl group), 1592  $\text{cm}^{-1}$  (amide II band, N-H stretch), and 1420–1377  $\text{cm}^{-1}$  (asymmetric C-H stretch bending of  $\text{CH}_2$  group) [31]. The characteristic peak's assignment of the chitosan-benzofuran hydrogel (Figure 1b) at 1632  $\text{cm}^{-1}$  is due to Schiff base (C=N) formed by a cross linking reaction between the chitosan's amino group and the khellinone's ketonic group. Furthermore, the interaction of benzofuran to chitosan has shifted some of the chitosan bands such as the O-H group from 3360  $\text{cm}^{-1}$  to 3430  $\text{cm}^{-1}$ , and the C-H stretch from 2919 and 2874  $\text{cm}^{-1}$  to 2924 and 2858  $\text{cm}^{-1}$ , respectively.



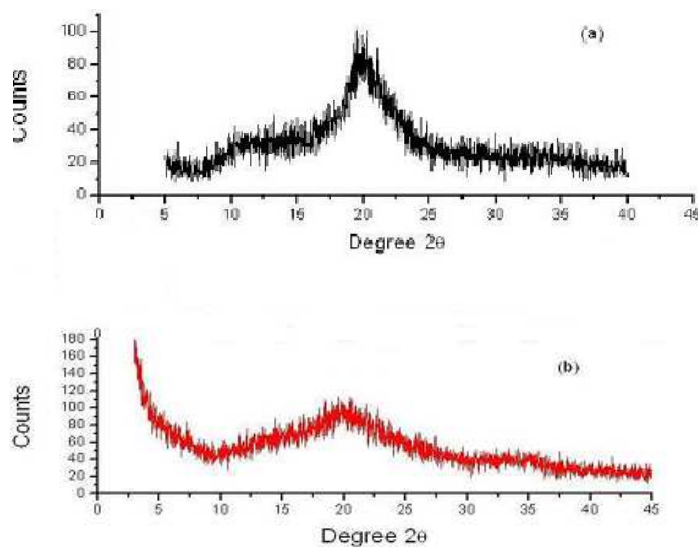
**Scheme 1 :** Schematic representation of the preparation of chitosan-benzofuran derivative gel (Schiff base).



**Figure 1:** Fourier transform infrared (FT-IR) spectra of pure chitosan (a) and chitosan- benzofuran derivative (b)

### 3.2. X-ray Diffraction Study

Study of crystalline phase were determined by the X-ray diffraction (XRD) using Philips equipment type PW, 1710 provided with a copper target and nickel filter. The X-ray diffraction patterns of the studied chitosan in both cases pure, and treated were shown in Figure 2a,b. It is clearly observed from Figure 2a that pure chitosan exhibited very broad two crystal peaks that appeared at  $2\theta = 12^\circ$  and  $2\theta = 21^\circ$ . On the other hand, the treated chitosan displayed two weak peaks at  $2\theta = 22^\circ$  and at  $2\theta = 33^\circ$ . The broad peaks for pure chitosan which was observed at  $2\theta = 12^\circ$  disappeared in chitosan-benzofuran hydrogel, while the very broad peak at  $2\theta = 21^\circ$  of pure chitosan became less broad in chitosan-benzofuran hydrogel [32].

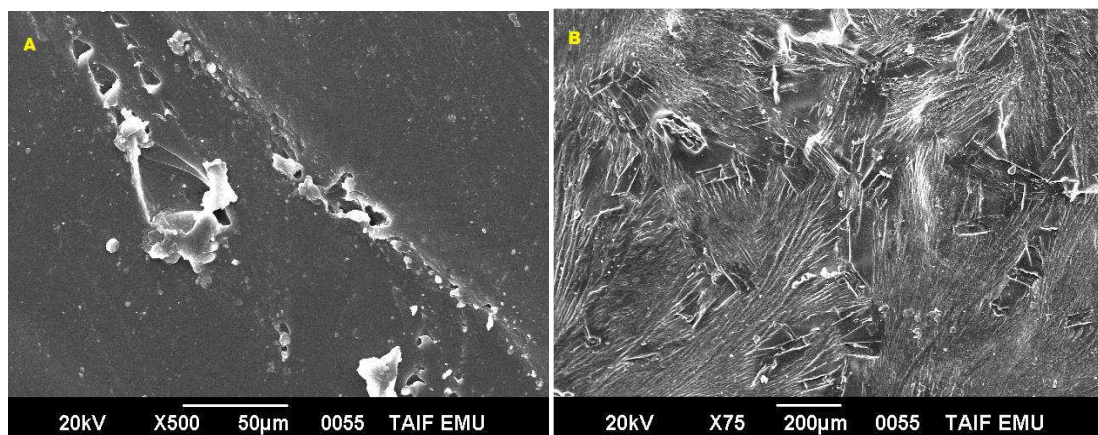


**Figure 2.** X-ray Diffraction (XRD) pattern of pure chitosan (a) and chitosan- benzofuran derivative (b)

These results suggested that chitosan-benzofuran has good compatibility, which led to the formation of a porous xerogel network. On the other hand, the X-ray diffraction patterns of the chitosan-benzofuran hydrogel displayed an amorphous (non-crystalline form), which may participate in biomedical applications [33].

### 3.3. Scanning Electron Microscopy

High resolution and magnification of surface morphology and microanalysis for various spots of selected samples of both pure and treated chitosan was observed using JEOL JSM – 6390 LA operating at 20 kV, Taif University, Taif, Kingdom of Saudi Arabia. The SEM images of the pure chitosan are shown in Figure (3A to D). The images exhibited fine morphology definitely compact, a non porous, smooth membranous phase which consisting of homogeneous dome shaped, micro fibrils and crystallite feature. Moreover, the figure showed chitosan of having few crystal structures on surface with straps and shrinkage, in some spots it exhibited rough surface and prominent sheath – like layers it may attributed to the low degree of deacetylation [34].



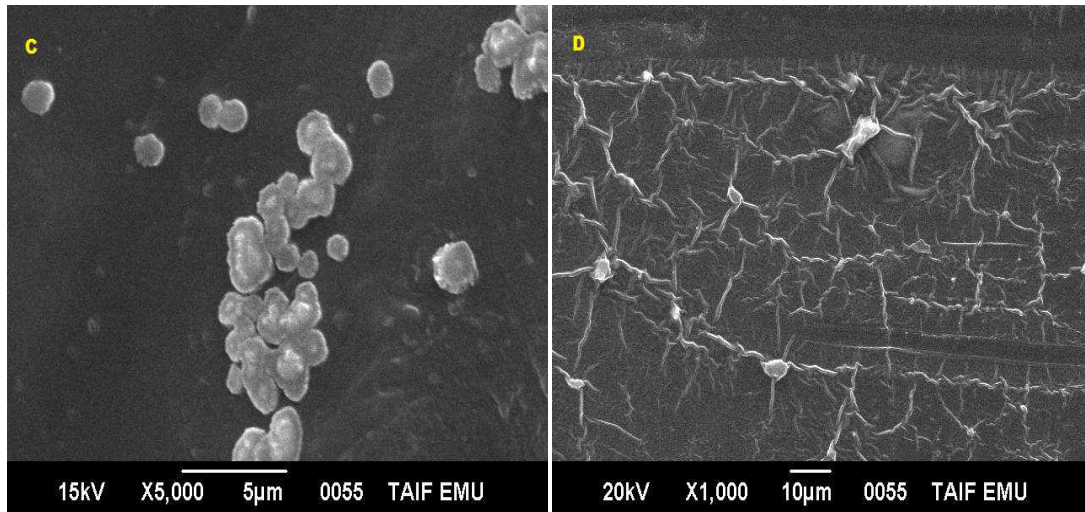
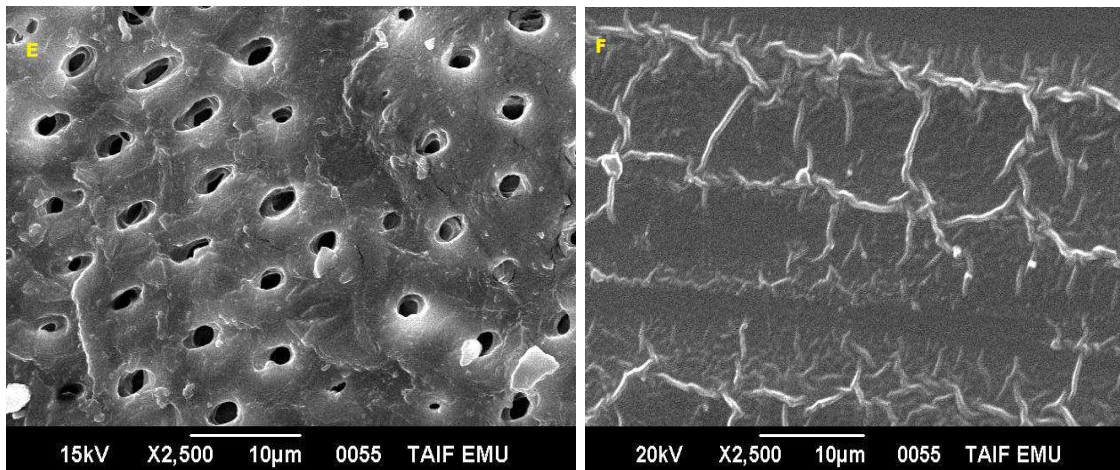


Figure 3. illustrate the Scanning electron microscopy (SEM) images of the fraction surface of the studied samples of pure chitosan

On the other hand, the SEM images of the chitosan-benzofuran as shown in Figure 4 E-H exhibited porous and chain like structure, which is different than the pure chitosan hydrogel. It also exhibited cross-section of randomly oriented grains. Moreover, the SEM image confirmed the point that the chitosan-benzofuran hydrogel has a near spherical morphology, which may participate into biomedical applications.



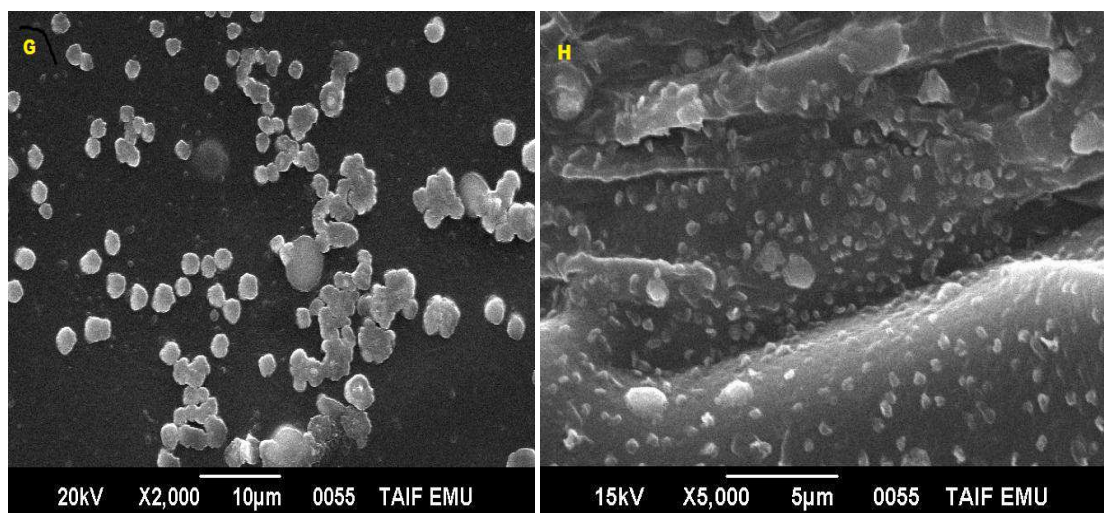
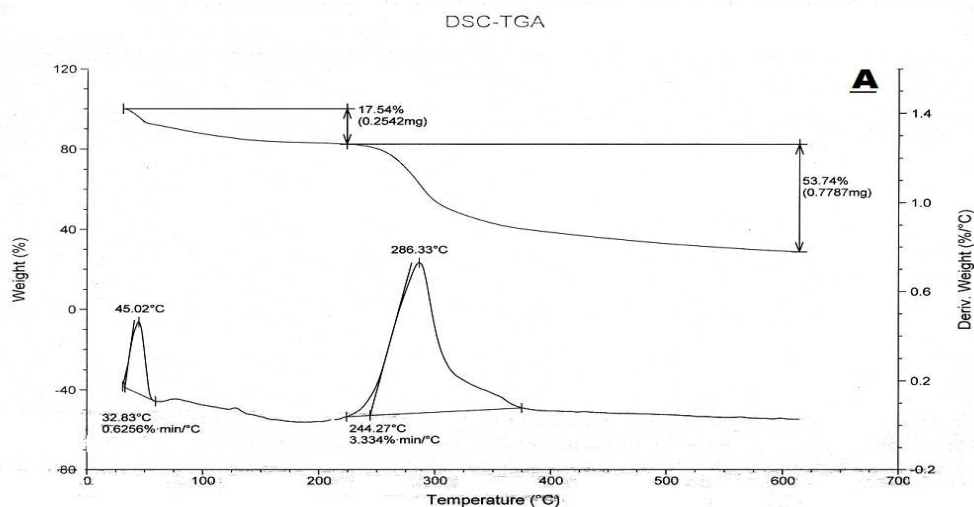


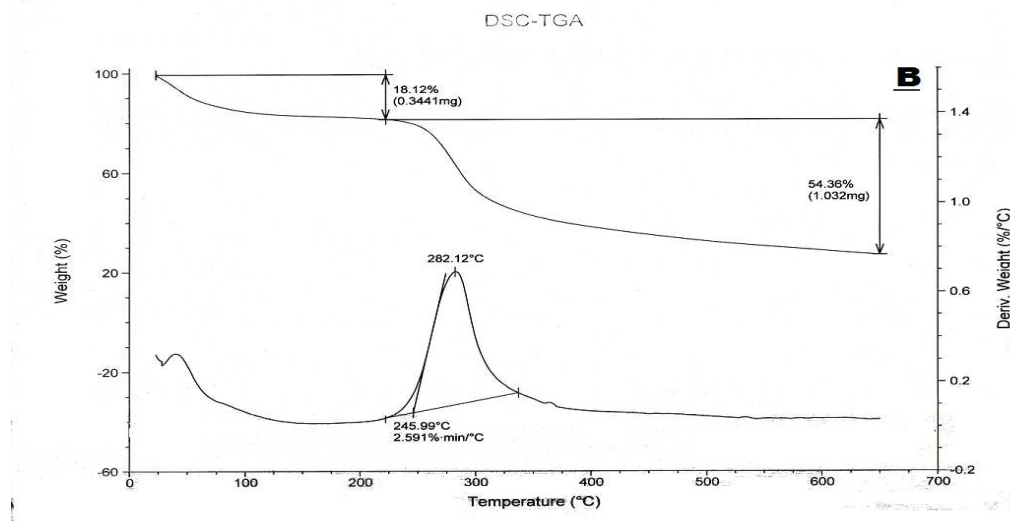
Figure 4. illustrate the Scanning electron microscopy (SEM) images of the fraction surface of the studied samples of chitosan- benzofuran derivative

### 3.4. Thermal Analysis

The thermal gravimetric analysis (TGA) is commonly used in research to determine characteristics of materials. TGA technique is very helpful to understand the degradation temperature, moisture, content and percentage of inorganic and organic components in materials. Thermal gravimetric analysis (TGA) was carried out using thermal analyzer model SDTQ -600 America, heating rate of 10°C/min under nitrogen, National Research Center, Dokki, Egypt. TGA was used for pure chitosan and chitosan-benzofuran hydrogel.

Figure 5A illustrated the TGA spectrum of pure chitosan, which indicated that the two stages of weight loss were in the range from 25 to 225 and 225 to 650 °C. Considering the first stage of degradation, 17.54% w/w weight loss, which is related to water loss, therefore, variations on the peak area and/or position related to water loss are expected to reflect physical and molecular changes. There are differences in peak area and position, indicating that these systems differ in their water holding capacity and strength of water-polymer interaction. Considering the chitosan structure, it can be seen that water molecules can be bound by two polar groups, hydroxyl and amine, present in this macromolecule [35]. Since a considerable amount of water is released at temperatures below 100 °C, it might be thought that, in the case of pure chitosan, water molecules bound to amine groups could be more easily removed (at lower temperature) than those molecules bound to hydroxyl groups [35]. On the other words, the second degradation of pure chitosan started at 225°C and it was degraded at about 650°C with weight loss of about 53.74 % [36].





**Figure 5. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of pure chitosan and chitosan- benzofuran derivative. (A and B respectively)**

On the other hand, the TGA of chitosan-benzofuran as in Figure 5B showed two different stages of weight loss. The first stage of weight loss, started from 25 to 220 °C with 18.12% weight loss, which may correspond to loss of adsorbed water. The second decomposition stage occurs due to thermal degradation with a weight loss of about 54.36%. The results demonstrated slight loss of the thermal stability of the chitosan-benzofuran hydrogel compared to chitosan gel.

The differential scanning calorimetric analysis (DSC) is the best analytical technique to find the polymer crystallinity, which measures the physical nature of the sample. The DSC thermogram of chitosan as in Figure 5A showed two broad exothermic peaks at 45 °C and 286.33°C. The first peak may be due to water vapor, while the latter may be attributed to the molecular arrangement of chitosan chain. DSC of chitosan- benzofuran hydrogel as in Figure 5B showed characteristic exothermic peak at around 40 °C due to the loss of water molecules [37]. There is one broad exothermic peak at 282.12°C corresponding to the thermal decomposition of chitosan-benzofuran hydrogel. The results indicated that the structure of chitosan chains have been changed due to the formation of Schiff base which reduced its ability to crystallize.

The TGA and DSC results are in accordance with that of the SEM. The SEM showed that more and larger pores were observed in case of chitosan-benzofuran (Fig 4E) compared to chitosan (Fig 4A). These larger and more abundant pores could be the reason behind the decrease in the thermal stability of the modified chitosan with benzofuran.

### 3.5. Antimicrobial Study

The antibacterial activity of chitosan was dependent on its molecular weight [12]. In this concern, previous studies reported that for chitosan with MW below 300 kDa, the antimicrobial effect on *S. aureus* was strengthened as the MW increased. In contrast, the effect on *E. coli* was weakened. The antifungal activity of chitosan is dependent on its molecular weight, degree of acetylation and type of fungi [27]. The inhibitory concentration of chitosan depends on several factors such as molecular weight of chitosan, targeted pathogen and the pH [19]. For example, the antimicrobial character of grafted chitosan with chromone (chitosan-chromone) hydrogel was due to the fact that it has a positively charged amino group which can interact with the negatively charged microbial cell membranes to cause the leakage of intracellular constituents of the microorganisms, thereby resulting in microbial death [38]. They also mentioned that the activity of the chitosan could be depend on the electrostatic interaction of the charged amino groups of chitosan with the negatively charged cell wall surface of the target microorganism which can lead to disruption of the cell wall and therefore death of the cell.



As shown in Table 1, benzofuran showed antibacterial effect against gram positive bacteria as staphylococcus aureus and Bacillus subtilis and it also had antibacterial effect against gram negative bacteria as Echericheia coli and pseudomonas aeuroginosa and the inhibitory zones diameter formed were 12, 11, 11 and 11 (mm/mg sample), respectively. Benzofuran exhibited antifungal activity against Candida albicans but it had no effect on Aspergillus flavus. These results are in agreement with other studies [39&40]. Reaserchers reported that novel derivatives of benzofuran showed various antimicrobial against various strains [39]. Novel benzofuran derivatives had variable antifungal activities [40]. Antifungal activity of newly synthesized benzofuran derivatives included changes in cytoplasmic calcium concentration [41]. Analyses of these benzofuran derivatives suggest that some structural features are important for antifungal activity. Antifungal activity drastically increased on converting methyl 7-acetyl-6-hydroxy-3-methyl-2-benzofurancarboxylate into its dibromo derivative, methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2-benzofurancarboxylate.

**Table (1) : Antibacterial and antifungal activity of chitosan (1), benzofuran derivative (2) and chitosan-benzofuran Schiff base (3)**

| Sample                              | Inhibition zone diameter (mm / mg sample) |                |                      |                  |                                       |                                     |     |
|-------------------------------------|---|----------------|----------------------|------------------|---------------------------------------|-------------------------------------|-----|
|                                     | <i>B. subtilis</i>                        | <i>E. coli</i> | <i>P. aeruginosa</i> | <i>S. aureus</i> | <i>Aspergillus flavus</i><br>(Fungus) | <i>Candida albicans</i><br>(Fungus) |     |
| Control: DMSO                       | 0.0                                       | 0.0            | 0.0                  | 0.0              | 0.0                                   | 0.0                                 |     |
| Standard                            | Ampicillin<br>Antibacterial agent         | 20             | 22                   | 17               | 18                                    | 0.0                                 | 0.0 |
|                                     | Amphotericin B<br>Antifungal agent        | 0.0            | 0.0                  | 0.0              | 0.0                                   | 17                                  | 19  |
| chitosan (1)                        | 0.0                                       | 0.0            | 0.0                  | 0.0              | 0.0                                   | 0.0                                 |     |
| Benzofuran derivative (2)           | 11  | 11             | 11                   | 12               | 0.0                                   | 10                                  |     |
| chitosan-benzofuran Schiff base (3) | 10  | 0.0            | 11                   | 10               | 0.0                                   | 0.0                                 |     |

### 3.6. Cellular Cytotoxicity and Viability

Results of table 2 showed that the increase in the concentration of chitosan (1), benzofuran derivative (2) and chitosan-benzofuran Schiff's base (3) decreased the viability percent of baby hamster kidney cells. The cellular cytotoxicity percent of baby hamster kidney cells culture by adding different concentrations of chitosan-benzofuran hydrogel were greater than those obtained from benzofuran or chitosan alone. Inhibitory 50% concentration of chitosan (1), benzofuran derivative (2) and chitosan- benzofuran hydrogel (3) were 9.43±4%, 5.27±2% and 4.81±2%, respectively.

**Table (2): Effect of different concentrations of chitosan (1), benzofuran derivative (2) and chitosan-benzofuran Schiff base (3) on the viability percent of baby hamster kidney cells culture**

| Drug used                           | Concentration used | Viability percent | Inhibitory concentration 50% |
|-------------------------------------|--------------------|-------------------|------------------------------|
| Chitosan (1)                        | 0                  | 100               | 9.43                         |
|                                     | 6.25               | 70.73             |                              |
|                                     | 12.25              | 42.86             |                              |
|                                     | 25                 | 22.04             |                              |
|                                     | 50                 | 13.28             |                              |
|                                     | 100                | 9.75              |                              |
| Benzofuran derivative(2)            | 0                  | 100               | 5.27                         |
|                                     | 6.25               | 39.45             |                              |
|                                     | 12.25              | 14.75             |                              |
|                                     | 25                 | 14.35             |                              |
|                                     | 50                 | 13.82             |                              |
|                                     | 100                | 12.83             |                              |
| Chitosan-benzofuran Schiff base (3) | 0                  | 100               | 4.81                         |
|                                     | 6.25               | 51.17             |                              |
|                                     | 12.25              | 33.48             |                              |
|                                     | 25                 | 24.07             |                              |
|                                     | 50                 | 21.76             |                              |
|                                     | 100                | 19.55             |                              |

That might be regarded to the changes in chitosan concentration affected the polymer surface topography, which has a direct effect in the growing cell behavior [42]. Also K562 cells cultured in three dimensional gels might be

inhibited by increasing proportion of chitosan concentration [43].

### CONCLUSION

Chitosan with its wide range of application was used as a promising material in the biomedical field. In this work, we incorporated benzofuran to low molecular weight chitosan in order to improve its antimicrobial activity. The new hydrogel has good compatibility, which led to the formation of a porous xerogel network, and it displayed an amorphous structure with large pore sizes, which increases its possible chance to be used in biomedical applications. On the other hand, it showed antibacterial and antifungal properties. Furthermore, the effect of different concentrations of chitosan, benzofuran, and chitosan-benzofuran hydrogel on the viability percent of baby hamster kidney cells culture were for the favor of the new hydrogel. In brief, we are recommending the new chitosan-benzofuran hydrogel for the use in biomedical applications due to its physical and biological properties.

### Acknowledgments

This work was financial supported by Taif University, under the project number (1/435/3291). The authors gratefully acknowledge Chemistry Department, Faculty of Science, Taif University, KSA for all facilities provided in terms of the use of the available chemicals and equipment.

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