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Chitosan, chitin and chitocal; Identification, antioxidant activity and incision wound recovery

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Abstract

Aim of Research. The aim of this research is comparing Chitin and chitosan extracted from shrimp shell with the pharmaceutical chitocal. **Methods.** FT-IR spectrum has done to identify chitin and chitosan extracts. A method of antioxidant activity has been done before dressing paste for wound dressing technique has been done. **Results and Discussion.** FT IR identified N-H stretching and -C=O stretching around at 2275-2250 cm^{-1}, with a strong broad band for N-C=O for chitin acetyl group bond. These bands distinguish chitin spectrum than chitosan spectrum. In DPPH radical scavenging, vitamin C followed by chitocal was higher than chitosan and finally chitin. Reducing power compared to vitamin C the strongest, followed by chitosan, chitocal and finally chitin. Dough for incision wound consisted of chitosan-glycerol-honey. The constituted materials of the dough are synergistic together toward the rapid healing. After one week, organized collagen fiber was formed with chitosan paste. While, only chitocal or dough with oral chitocal after three weeks showed abnormal epithelial growth and dis-organized collagen.

Keywords

Chitin – chitocal – chitosan – FTIR – DPPH - reducing power – incision wound healing

Graphical Abstract

Shrimp shell
dried, grounded
demineralization, deproteination
(chitin)
deacetylation
(chitosan)

***Dough preparation*: Aqueous chitosan solution + lactic acid 1% (stir)**

+ NaHCO3 5%

glycerol 13%

honey 15% (stir)

Oven 40C at petri dish

Rinsed in 1M NaOH

Applied in rats wound incision

Abbreviation

FTIR Fourier transform infrared spectroscopy – RP reducing power – ROS reactive oxygen species – H&E hematoxylin and Eosin – DPPH 1-Diphenyl-2-picrylhydrazyl radical

Introduction

The promising carbohydrate chitosan has medicinal and biological features such as long-term stability under varied settings, progressive biodegradability, good biocompatibility, and bacteriostatic activity. Chitosan becomes a cationic polymer in acidic environments, allowing the immobilisation of ligands like glycoproteins to increase cell adhesion and proliferation **(Kim *et al.*, 2013).** Cationic chitosan could generate polyelectrolyte complexes with unique physical and chemical characteristics when combined with anionic polymers **(Yan *et al.*, 2001),**

Skin injuries have long been regarded as one of the most common types of physical harm. According to earlier studies, an ideal dressing should be able to absorb fluids well, be pleasant to touch and remove, have high flexibility while also having good mechanical strength, be transparent, and act as a barrier against germs **(Ajji *et al.*, 2005).** In our honey experiment, the chitosan with glycerol hydrogel was flexible and transparent, with strong mechanical strength and a high water absorption capacity, as previously indicated **(Yang *et al.*, 2008).**

Shamloo *et al*. (2021) investigated the effects of honey concentrations on antibacterial activities and cell activity in wound dressings. In vivo experiments were carried out, including wound healing mechanisms in a rat model and histological inspection of slice tissue samples. The results showed that adding honey to hydrogels enhanced their ultimate strain by about two times while lowering their ultimate tensile strength and elastic modulus. Moreover, the antibacterial activities of samples were increased by increasing the concentration of honey.

Chitin and chitosan produced from shrimp shell wastes proved through many research a high potential effect in antimicrobial, antioxidant, reducing power, anti-inflammatory effects. For many reasons chitosan with some additives could make potential nanomaterials and films to regenerate wound healing dress for cut or burning skin injuries.

Material and Methods

Chitosan is the most abundant natural polysaccharide after cellulose. It can be obtained from the exoskeleton of marine crustaceans as crabs, lobsters, shrimps and krill (**Varshosaz, 2007**).

Shrimp shell waste extracts

Shrimp shell waste was collected from Minia local fish market, Minia Governerate. Sample was washed and dried at hot air 35^{O}C for 2-3 days. Dried sample was finely ground in a grinding machine and analyzed with **AOAC (1990)**. Chitin extraction was done following three major steps, i.e., de-mineralization, de-proteination, and de-acetylation.

For *demineralization*, dried sample was treated with 0.25 to 2N hydrochloric acid at solid to solvent ratio of 1:15 for 2 h with constant stirring at 150 rpm in incubator shaker at room temperature **(Benhabiles *et al.*, 2012).** Sample was washed with tap water till the sample reaches neutral pH.

For *deproteination*, demineralised shrimp shell powder was treated with 0.5 to 2N NaOH at solid to solvent ratio 1:20 for 2 h with constant stirring at 150 rpm at 50°C in an incubator shaker **(Benhabiles *et al.*, 2012),** followed by thorough washing and drying to produce chitin.

For *deacetylation*, conversion will occur from chitin into chitosan **(Kurita *et al.*, 2003).** Chitin was treated with strong alkali, i.e., 1 g of chitin was added to 50% NaOH for 1 h at 121°C, and washed till it reaches neutral pH.

Chitocal as pharmacological chitosan standard;

Chitocal, the pharmaceutical industries product was used by the same ratio 1:10 w/v to compare the antioxidant study. Chitosan is the main component in chitocal drug with ascorbic acid. For this reason we used it as a reference in the biological study.

Characterization of extracted chitin and chitosan

Extracted chitin and chitosan were characterized using Fourier transform infrared spectroscopy (FT-IR) Nicholet 6700 FT-IR in which spectrum of extracted chitin and produced chitosan.

Radical scavenging of 2,2-diphenyl-1-picryl hydrazyl (DPPH) Assay

DPPH radical scavenging was done according to **(McCune and Johns, 2002)**, 100 µL (10 mg) or 300 µL (30 mg) of ethanol extract was added to ethanol 95 % to reach a 1 ml volume for the DPPH radical scavenging experiment. One ml DPPH (0.2 mM in ethanol) was mixed with the previous tube solutions and maintained at room temperature for 60 minutes in the dark. At 517 nm, the absorbance of solutions was measured and compared to that of a blank (ethanol) and a standard antioxidant (100 µL, 0.04% w/v) ascorbic acid.

The calculation of Inhibition percentage = (Ab – As) / Ab x 100 was used to calculate DPPH scavenging activity. **(Deng *et al*., 2011)**

Where Ab is the blank absorbance at 517 nm and As is the sample absorbance at 517 nm.

Reducing Power RP Assay

For determination of RP response based on a method outlined by **Athokorala *et al.* (2006)** with some modifications, 100 µL (10 mg) of extract diluted appropriately in ethanol was mixed with destilled water until 1 ml solution. Ascorbic acid (0.03% w/v) was used as standard reducing agent and blank without antioxidants. Phosphate buffer (0.2 M) pH 6.6 was added before $K_3Fe(CN)_6$ potassium ferricyanide and incubation before trichloroacetic acid 10% (w/v) **(Athokorala *et al.*, 2006)**. Then, absorbance was measured at 700 nm.

Increase in reducing power (%)=$A_{test} - A_{blank} / A_{blank} X 100$

Where A_{test} is absorbance of test solution; A_{blank} is absorbance of blank at 700 nm

Bioactive wound dressing preparation using chitosan honey film

Dough or paste preparation. The films were prepared using casting technique, modification of the method described by **Rathinamoorthy and Sasikala (2019)**. Briefly, a chitosan aqueous

solution was prepared in distilled water with 25% concentration, with lactic acid 1% (w/v) as solvent and stirred for 2 h. Then drop wise addition of 5 ml of 5% sodium bicarbonate was carried out. The solution was stirred using magnetic stirrer for 15 h after addition of glycerol (13% concentration) and honey (15% concentration) based on previous research results **(Sasikala *et al.*, 2018)**. The previous dresses contained the honey (Isis plain honey 100%; SEKEM group, Egypt) was applied on the wound daily. The isotonic saline (2 ml) was also applied daily if needed.

The resultant solution was filtered out and left to stand until the air bubbles get disappeared. Then required quantity of the solution was casted onto the petri dish. The petri dish was maintained in the oven at 40°C for 24h. The prepared films were rinsed with 500 ml of 1M NaOH solution and then washed with distilled water **(Zhong and Xia, 2008)**. Then, these transparent, flexible films were stored at 25 ± 1°C and relative humidity 60–65% in an airtight glass container until further use.

Animals. This study was conducted in the Histology department of faculty of Medicine of El-Minia University. The study was dealing with the skin tissues of adult male albino rats. In this study, sixty male Sprague Dawley albino rats at the age 6-8 weeks, weighting 150-200 grams, specific pathogens free were used. Animals were housed in a clean plastic cage in an air conditioned room under a 12 h light dark cycle. All animals became acclimatized for 14 days before the outset of the study and were given food and water ad-libitum and were kept at constant humidity and temperature.

Incision Wound Model

No systemic antimicrobials were used throughout the experimental study. A longitudinal incision of 1 cm length and 2mm depth was made through the skin using a surgical knife.

Randomly distributed 15 rats in each group;

First group (control) was injured as the incision wound as explained.

Second group (chitosan film) was covered daily the cutting wound area with 0.5 g experimental film.

Third group (oral chitocal) was only treated the cut injured group by oral injection with 0.5 ml chitocal.

Fourth group (chitosan film and chitocal oral) was covered the cutting wound with 0.5g film and oral injected with chitocal 0.5 ml.

The experimental protocol is considering the wounding day as Day 0. Each week five rats were sacrificed in each group to measure the skin histological investigation.

Histological Study.

The Paraffin Technique and Staining with Hematoxylin and Eosin (Hx & E) Theory of **(Bancroft and Gamble, 2008):**

The specimens of the wounded skin were rapidly fixed in 10% formal saline for 48 hours. Formalin was used because it has a beneficial hardening effect and causes little shrinkage of tissues. Specimens were washed by tap water and processed to prepare tissue sections for morphological studies.

After that, dehydration in ascending grades of alcohol (50%, 70%, 90%, and three changes of absolute alcohol) was done, Clearance with xylene, impregnation in three successive changes in soft paraffin at 55°- 60°C and finally embedding in hard paraffin wax to obtain solid blocks containing the tissue.

Staining: The sections were stained with hematoxylin and eosin (H&E) reagent to examine under light microscope. The de-waxed paraffin sections were put in Hematoxylin stain for 2-20 minutes then washed well in running tap water for 2-3 minutes. The excess stain was removed by decolorizing in 0.5-1% HCl in 70 % alcohol for few seconds, then the sections were put in 1% aqueous eosin for 1-3 minutes and the surplus stain was washed off in water. The section were dehydrated in alcohol and cleared in xylene and then mounted. The cytoplasm appeared red to pink while the nuclei took a blue color.

Results and Discussion

Different yield ratios have been varied between studies, The study found that the yield ratio was 35% for chitin and 23% for chitosan. In a closed extrated method, **Vilar Junior *et al.* (2016)** showed that yields percentages % for chitin and chitosan were 49 and 33, respectively. The variation between these and other studies could be due to difference in the age of the shrimps from which the samples were taken.

I. Identification of chitin and chitosan structure using FT-IR

Figures 1, and 2 showed the FTIR spectrum of the absorbance for extracted chitin and chitosan.

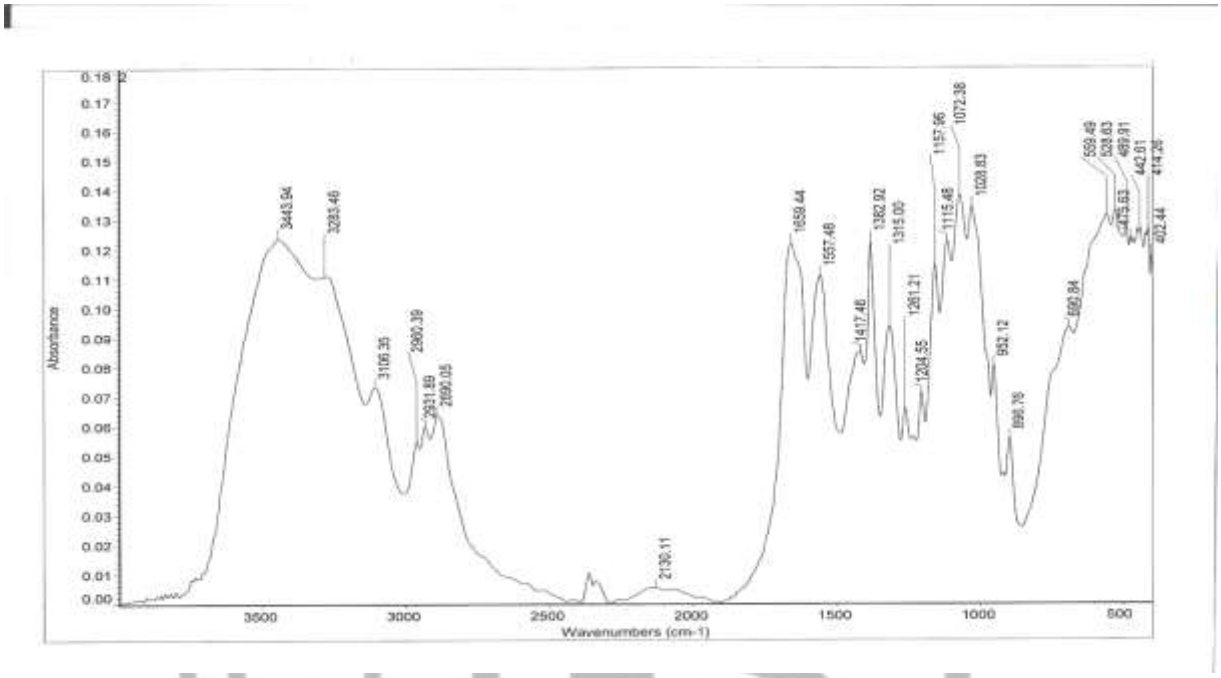


Figure 1: IR spectrum for obtained Chitin from the waste shrimp shell

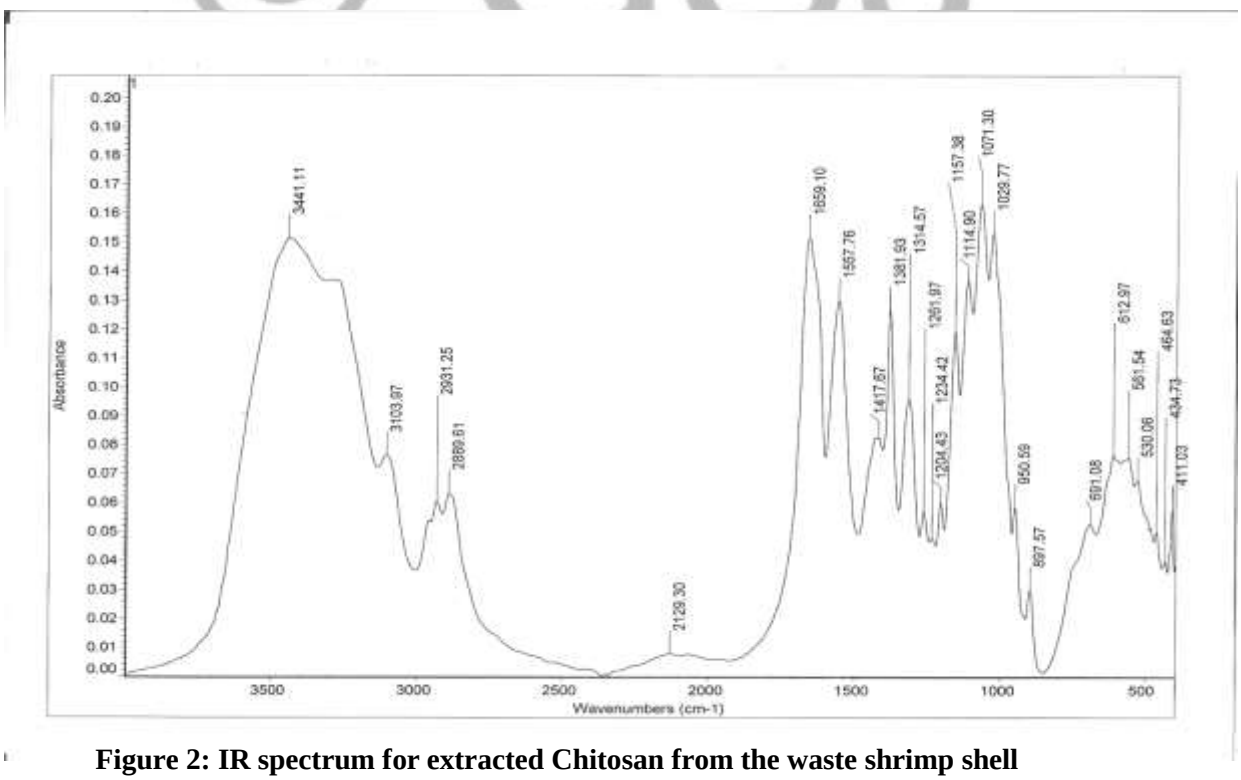
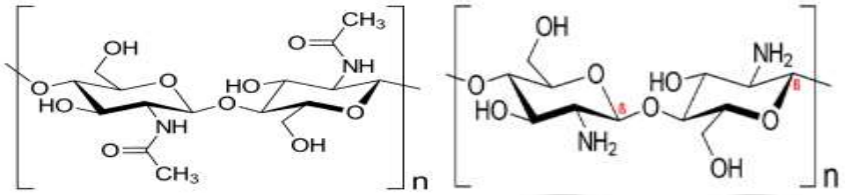


Figure 2: IR spectrum for extracted Chitosan from the waste shrimp shell

IR spectrum has been analyzed chitin and chitosan extracted from shrimp shell wastes. Bands characterized the functional groups in both materials. The characteristic OH stretching bands obtained at 3440 cm^{-1} is strong and broad as has been investigated. Band O-H stretching vibration is observed as broader and shifted as in the spectrum, while, N-H stretching is medium and broad appeared about 3400-3300 cm^{-1} **(Varun *et al.*, 2017)**. At 3350-3310 cm^{-1} is showing a middle beak for secondary amine N-H stretching for chitin. At 3200-2700 cm^{-1}, O-H stretching appeared once again in a weak broad band. N-H stretching appeared as well at 3000-2800 cm^{-1} in a strong broad band.



Chitin **Chitosan**

Figure 3: Structural formula for chitin (N-acetyl-D-glucosamine) and chitosan (β 1-4 linked D-glucosamine).

At 2349 cm^{-1}, -C=O stretching has kind of strong band and at 2275-2250 cm^{-1}, a strong broad band for N-C=O are identified for chitin acetyl group bond with amino group (**Teli and Sheikh, 2012**). Another prove, 1557 and 1659 cm^{-1} band for chitin and in the vicinity of 3103 and 3441cm^{-1} are found (**Teli and Sheikh, 2012**) for acetyl amide stretching vibration C=O and NH ($NHCOCH_3$), and at 1654, 1646 cm^{-1} (**Varun *et al.*, 2017**).

A weak band for C-H bending appears at 2000-1650 cm^{-1}, and at 1818 strong band appears for C=O stretching. Secondary amide might appeared at 1680 cm^{-1} in strong band for C=O stretching. Medium band appears at 1650-1580 cm^{-1} for N-H bending of amine group. In methyl group, C-H bending shows medium band at 1450 cm^{-1}. O-H bending for alcohol shows medium band at 1420-1330 cm^{-1}. C-N stretching for amine appears in medium band at 1250-1020 cm^{-1}. Secondary alcohol shows strong band for C-O stretching at 1124-1087 cm^{-1}, and primary alcohol has also strong band for C-O stretching at 1085-1050 cm^{-1}. While 1620 cm^{-1} bending vibration band appears for amine NH_2 group in chitosan indicating the degree of deacetylation. Chitosan the intermolecular hydrogen bonding contributed to some extend to the broad band at

3600-3000 cm^{-1}. Both figures, has been showed bands at 2931, 2890 cm^{-1} for C-H stretching vibration and bending vibration at 1417 and 1381 cm^{-1}. At 1381 cm^{-1}, methyl group was detected for $NHCOCH_3$ **(Varun *et al.,* 2017).** The peak appeared at 1028 cm^{-1} is for C-O stretching.

Chitin and chitosan are highly basic polysaccharides, and their poly oxysalt formation gives them ability to form films, chelate metal ions with optical structural characteristics (**Austin *et al.,* 1981**). Chitin is highly hydrophobic and is insoluble in water and organic solvents. On the other hand, chitosan the deacetylated product of chitin for its free amino groups makes it soluble in aqueous acids such as formic, acetic, and lactic acids.

II. The antioxidant and reducing power activity for chitin and chitosan

The antioxidative activity measured by scavenging DPPH radical or inhibition % was illustrated in Figure (4). Comparing to vitamin C, 100 µl chitosan dramatically exceeded than chitocal followed by chitin in the inhibition of DPPH radical. At 300 µl, all the antioxidant activity were increased in a variable amounts than 100 µl. At 300 µl, comparing to vitamin C chitocal activity was gradually increased than chitosan followed by chitin activity.

Saddam *et al.* (2022) investigated that chitosan is an effective material for heavy metal adsorption from polluted water, and that increased the antioxidant activity. Chitosan proved to adsorb Mn^{++} ions from aqueous solution. Moreover, **(Liu *et al.,* 2020)** synthesized and determined the phenolic acid-g-chitosan. As compared with chitosan, phenolic acid-g-chitosan exhibits enhancing antioxidant, antimicrobial and acetylcholinesterase inhibitory activities. The ability to donate a hydrogen atom to DPPH or reducing ability has been found in chitin, chitocal and chitosan. At 300 µl, between 35 to 85% DPPH radical inhibition have been detected for the carbohydrate extracts. Inhibition oxidative processes mean

the free radical scavenging as primary mechanism. The stable free radical (DPPH) reagent can be reduced by the proton donating substrate, causing the decolorization of DPPH and the reduction of the absorbance at 517 nm. The decrease in absorbance is taken as a measure for radical scavenging activity.

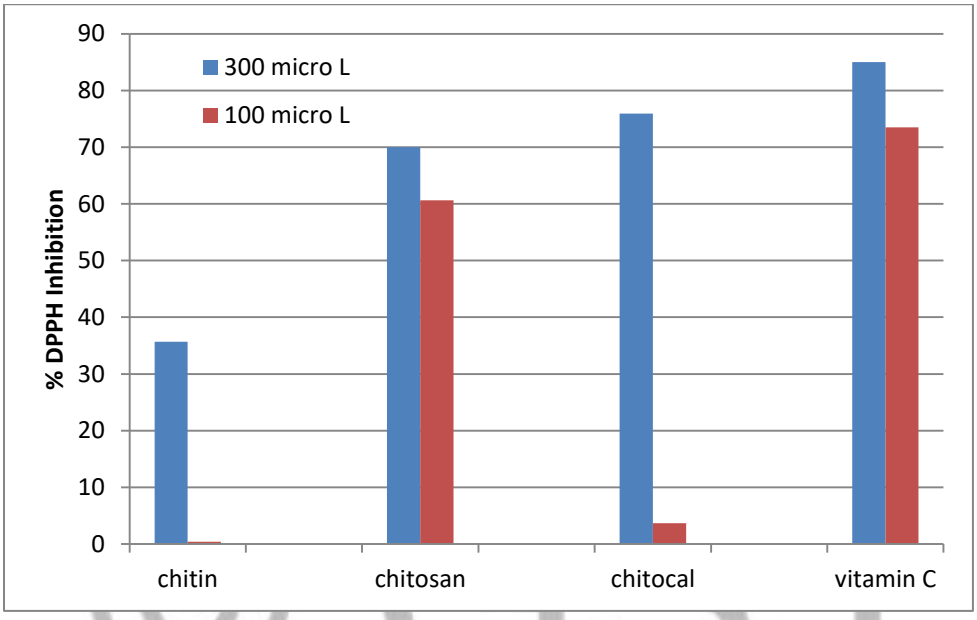


Figure 4: Inhibition of radical DPPH % at 100 and 300µl from chitin, chitosan comparing to chitocal

Reactive oxygen species ROS and reactive nitrogen species RNS have been shown to cause oxidative damage in the human body and that converted into harmless species by enzymatic antioxidants of living cells. Consumption of the foods containing antioxidants is exerting beneficial health effects against the oxidative stress factors. The antioxidative activity measured in this work using the reducing power activity (Figure 5). Comparing to control, vitamin C at 100 µl showed higher reducing activity than the three studied samples. Chitosan showed higher reducing power than chitocal and followed by the chitin extract. In unpublished results, there was correlation with that cleared from the inhibition of DPPH radical result.

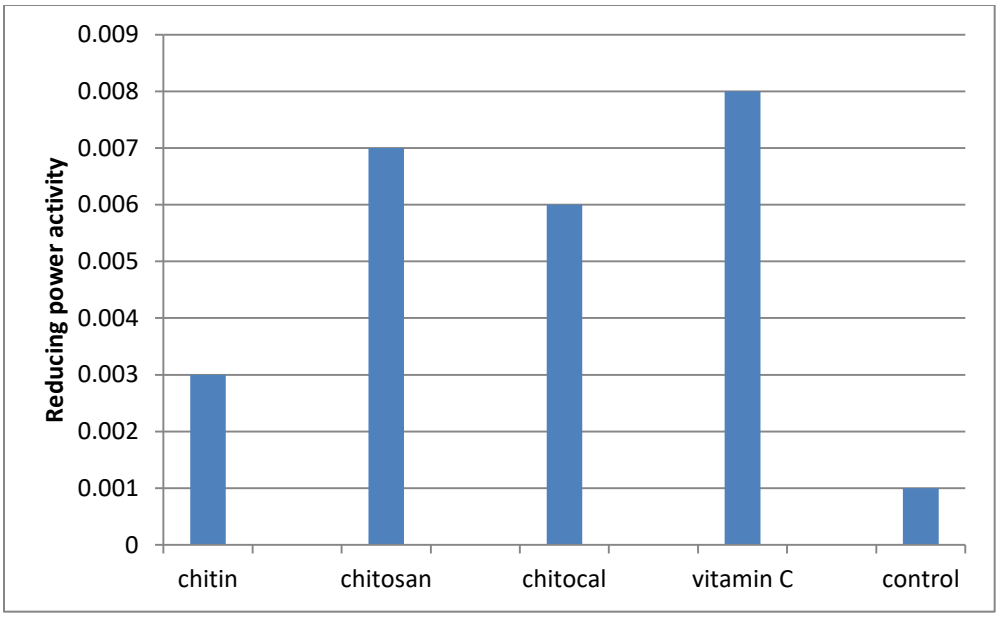


Figure 5: Reducing power % for 100 µl from chitin, chitosan and chitocal

Reducing power assay could be used to evaluate the ability of antioxidant to transfer electron or hydrogen **(Yildirim *et al.*, 2001).** Presence of antioxidants as reducing agents in tested samples results in the reduction of Fe^{3+} / ferric cyanide complex to ferrous form. It said that there is a direct correlation between antioxidant activities and reducing power or certain bioactive compounds. In spite of, chitosan showed high scavenging activity and reducing power, **Younes *et al*. (2014)** proved that chitosan showed relatively low reducing power, as they declared that chitosan was not able to reduce Fe^{3+} to Fe^{2+} by donating electron.

III. The wound healing effect of chitosan film

The healing combination of chitosan - honey has been used before by Indian honey (**Sasikala *et al.*, 2018)** for vast variety of wounds. Our investigated study, using solvent evaporation technique, chitosan-honey films were tested and compared with chitocal treatment. Chitosan, honey and glycerol were optimized and followed for the biological recover experiment. Achieving optimum responses to be used as a wound contact layers would be discovered as in our research to be chitosan concentration of 25%, honey concentration of 15% and glycerol concentration of 13%.

After one week, control untreated wound (figure 6) showed blood clot and cell debris, with epithelial loss, dermal inflammation and disorganized collagen. For chitosan paste covered wound group showed new formation of epithelium, more organized collagen fibers, and inflammatory cell infiltration. On the other hand, wound after one week with oral chitocal (the commercial chitosan used for the diet or reduction body weight) had abnormal epithelial growth with excessive keratin deposition, disorganized collagen in the dermis and inflammatory cell infiltration. Finally, using chitosan paste with chitocal oral injection noticed excessive keratin and disorganized collagen.

After two weeks, figure 7 showed the photomicrograph of skin wound; in control untreated wound has less blood clot and cell debris, with beginning of epithelial regeneration, less dermal inflammation and collagen disorganization. In chitosan paste covered wound showed new formation of epithelium, more organized collagen fibers, and no-inflammatory cell infiltration. In the oral chitocal group, abnormal epithelial growth was noticed with excessive keratin deposition, and disorganized collagen in the dermis. Finally, both local paste and oral chitocal treatment, was showed excessive keratin and disorganized collagen. The quick recovery with chitosan treatment in our experiment was compared in another study which showed no abnormal findings in the exudate formation and skin reaction. The wounds were completely reconstructed at the end of 21 days (**Kimura. 2011**).

Figure 8 showed photomicrograph of skin wound after three weeks. The untreated control wound showed regeneration of the epithelial covering, and collagen reorganization as correlated with **Kimura (2011)**. In positively results, more recovering has appeared in chitosan paste adherence the previous weeks and nearly normal epithelium arrangement has been occurred. Organized collagen fibers, no-inflammatory cell infiltration have been showed. On the other hand, injured rats treated with oral chitocal have abnormal epithelial growth, and disorganized collagen in the dermis. Skin wound after three weeks with both chitosan paste and oral chitocal treatment had been noticed excessive keratin and disorganized collagen.

As been found from our results, chitosan-honey baste was faster in recovery after two weeks from injury. The biological reason for using chitosan with honey hydrogel has been focused in our research. Comparison to commercial oral injection chitocal in our research showed the high efficient of using that practical paste. Recently, **Bagher *et al.* (2020)** used alginate and chitosan hydrogels in the wound healing, with different concentrations of hesperidin were loaded to the hydrogels. Chitosan for the high antibacterial effect as shown in unmentioned results is a common agent in wound dressing to accelerate the wound healing process (**Boateng *et al.*, 2008**).

In another view, chitosan has biocompatibility, biodegradability and hemostatic feature (**Kumar *et al.*, 2016**), but it has not enough flexibility. Adding honey or alginate help to absorb large amounts of fluid. **Khanmohammadi *et al.* (2019**) suggested that polyanionic polymer as carboxyl group in alginate can interact with the amino group of chitosan. Honey as well stimulates the proper healing of dermal wounded for its intrinsic antimicrobial activity, and the synergetic effect might be happen to accelerate the wound healing. Honey is composed of enzymes, minerals, oligosaccharides, carbohydrates, and phytochemicals (**Nazeri *et al.*, 2015**), and it eliminates wound infection, reduces inflammation, enhances granulation and epithelization, and promotes wound healing as been found in our results and correlated with (**Mohd Zohdi *et al.*, 2012**).

We attributed the high efficiency for the high osmolarity, pH, H_2O_2 production, and presence of other phytochemical components (**Molan and Betts, 2004**). That may removes water from bacterial cells and prevent the growth of bacteria (**Molan and Rhodes, 2015**). Some researchers have revealed that honey stimulates fibroblast proliferation and angiogenesis (**Rossiter *et al.*, 2010**). Moreover, honey reduces the pH of the wound, and thus, leads to increased oxygen release from hemoglobin and promotes wound healing (**Al-Waili *et al.*, 2011**).

Answering how to act honey, **Krishnakumar *et al.* (2020)** stated that honey acts in synergy with its immunologic modulators and physiologic mediators in multitude of signalling pathways and molecular targets offering beneficial vigor effects in wound healing. Lactic acid as a solvent was used in our paste to increase the properties of the developed wound dressing and provided the required fundamental properties for the wound dressing than the acetic acid **(Sasikala and Durai, 2015).** Out of the selected methods, hydrogel (film) dressing showed better performance in terms of handling and performance **(Sasikala *et al.*, 2017)**.

Our work comparing to that for **Marei *et al.* (2017)** showed same trend where the experiment was extended to three weeks. They showed earlier granulation tissue formation, angiogenesis, epithelization and thicker epidermis formation comparing to control. While, **Azuma *et al.* (2015)** found that skin wound –in the beginning- healing growth factors act as stimulators of fibroblast proliferation *in vivo* and synthesis extra cellular matrix proteins. Many research could be investigate more successful wound dressing for their wide uses for stop bleeding and form skin tissue in many diseases.

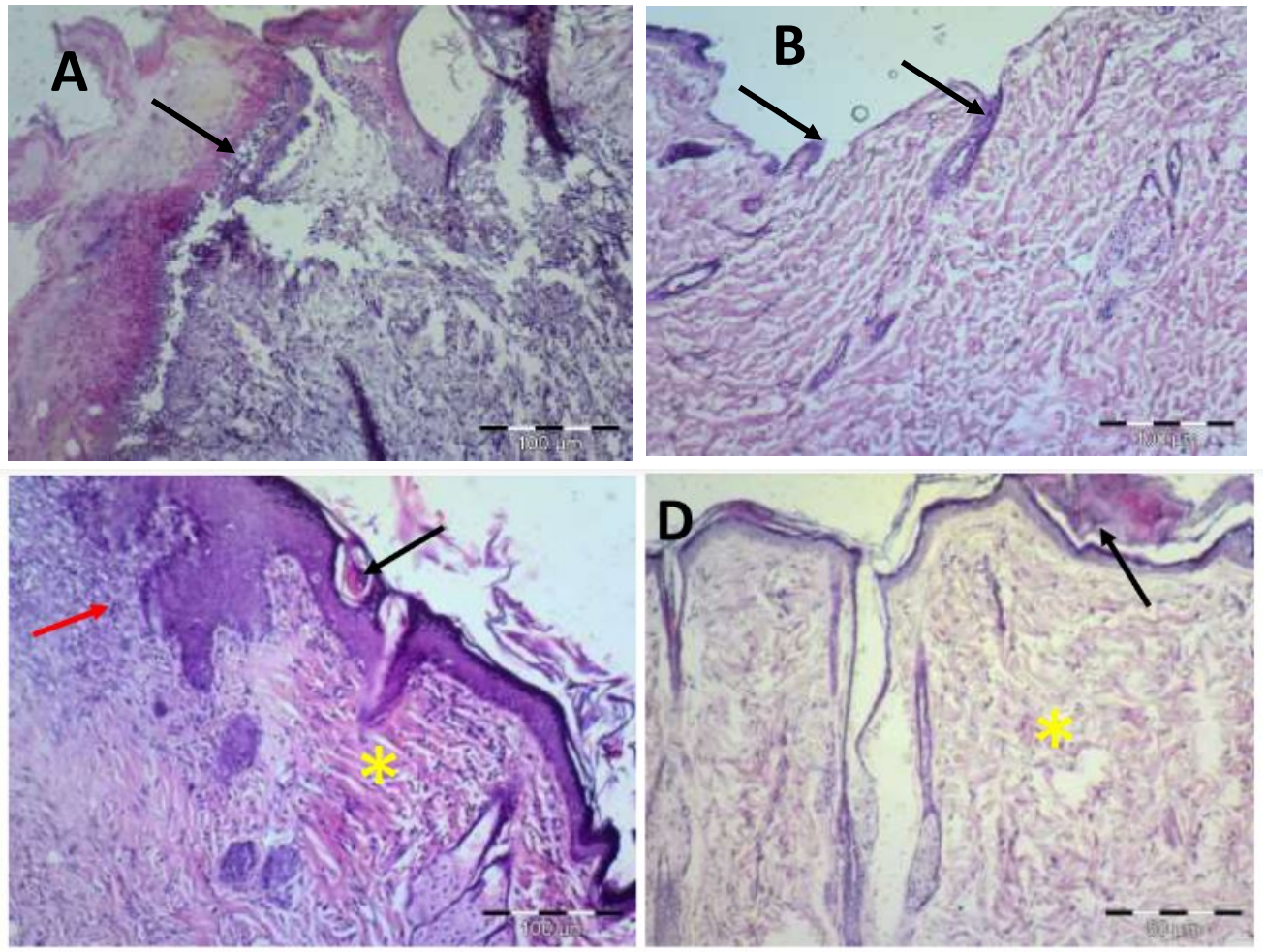
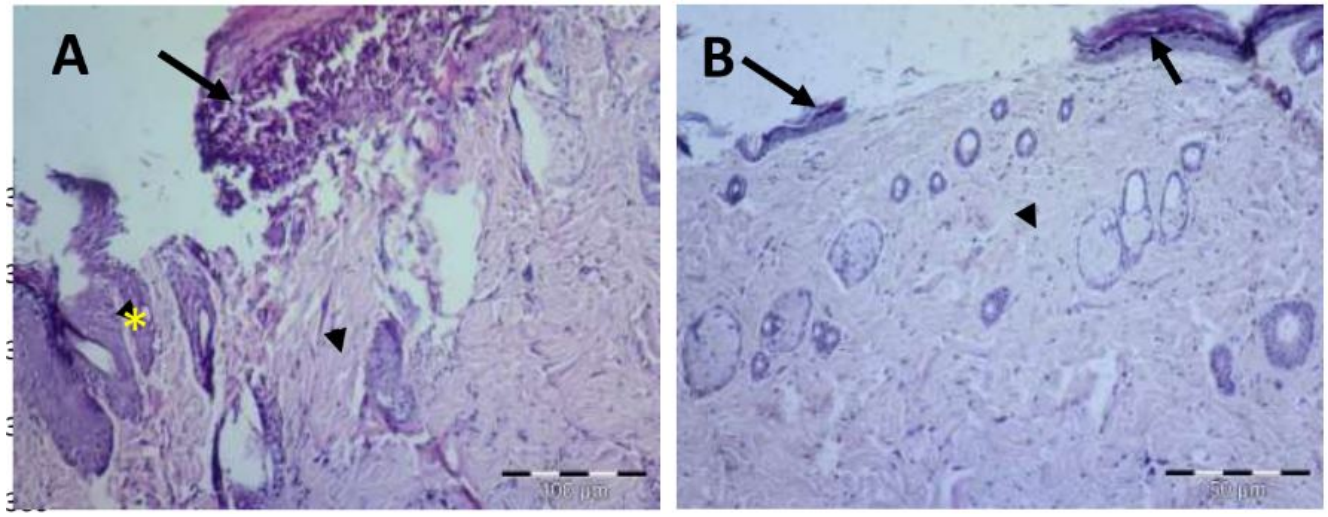


Figure 6: Photomicrograph of skin wound after one week (A) Untreated wound (B) wound with chitosan coating (C) wound with treated orally with chitocal (D) wound with chitosan coating and treated orally with chitocal H&E x 40



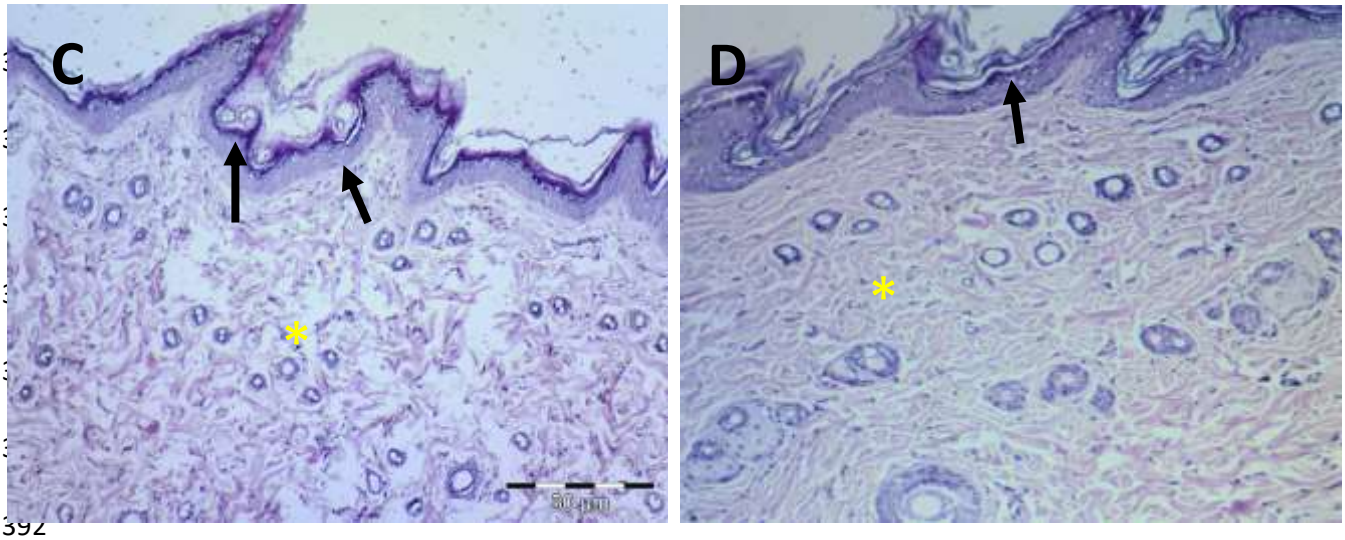


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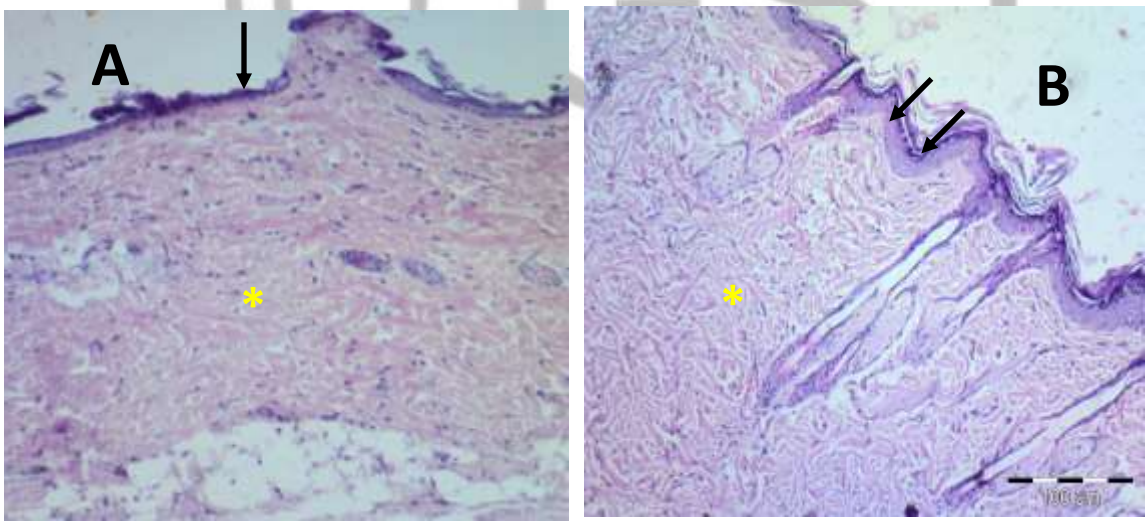
Figure 7: Photomicrograph of skin wound after two weeks (A) Untreated wound (B) wound with chitosan coating (C) wound with treated orally with chitocal (D) wound with chitosan coating and treated orally with chitocal H&E x 40



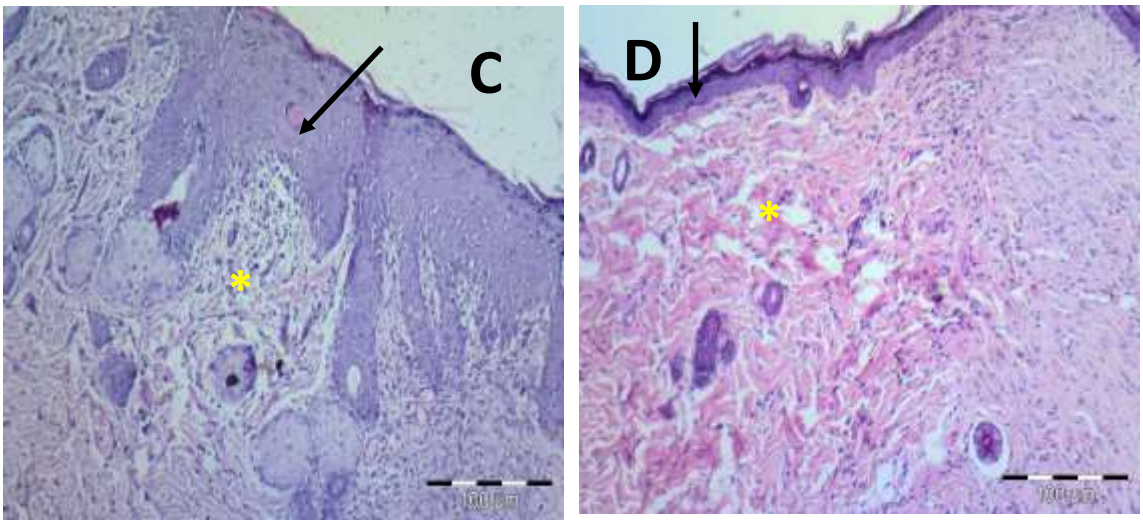


Figure 8: Photomicrograph of skin wound after three weeks (A) Untreated wound (B) wound with chitosan coating (C) wound with treated orally with chitocal (D) wound with chitosan coating and treated orally with chitocal H&E x 40

Ethical approval. The experiment was conducted in accordance with the guidelines laid down as animal ethics committee for animal handling by Faculty of Medicine, Minia University.

Authors agree for publication. They declared that all data and materials are available. The authors declared that they have no conflict of interest. No fund is accepted from any other parts. Authors would like to say that as their specialization they contribute in the research work and writing with reviewing.

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