Antibacterial and Wound Healing Properties of Thymol (*Thymus vulgaris* Oil) and its Application in a Novel Wound Dressing

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Received: 9 Feb. 2014 **Accepted:** 18 Feb. 2015

Abstract

Background: In developing new products for skin care and wound treatment, biocompatibility plays a major role in the choice of ingredients. Thymol, an essential oil extracted from thyme plant, exhibits outstanding antibacterial properties, but more importantly, it proves to be much more compatible to skin cells in comparison to some conventionally used antibiotic drugs and chemicals.

Objective: The aim of the study was the use of thymol as an antibacterial and wound healing promoting agent in development of a novel wound dressing.

Methods: The antibacterial properties of thymol before and after application in the dressing were analyzed by MIC and Disk Diffusion methods respectively. To ensure biocompatibility, MTT assay was used to assess the effect of thymol on skin fibroblast cells. In addition, effects of thymol on dressing's structure and its mechanical properties were studied by SEM and tensile strength tests respectively.

Results: MIC investigation showed that thymol is capable of halting bacterial growth in concentrations as low as 156ppmdepending on the bacterial strain. Assessment of the product containing thymol by Disk diffusion method proved that the essential oil would retain its effectiveness when incorporated in the final product. Investigation of thymol's biocompatibility by MTT assay resulted in a rather unexpected outcome, thymol increased fibroblast cell growth significantly, but the exact amount could not be calculated due thymol's interference with the test material (MTT). Furthermore, increasing the concentration of thymol in the dressing increased its porosity and elongation on stress, but reduced its pore size and maximum stress.

Conclusion: The observed data backed the original claim of antibacterial and wound healing properties, but also showed that incorporating thymol into the dressing increases its elasticity and porosity, but reduces its mechanical strength.

Keywords: Antibacterial, Thymol, Wound dressing, Wound healing

Introduction

In pathology, wounds are injuries that disrupt the continuity of the dermis of the skin. To remove this rupture and restore the skin to its serene and functional status, various methods have been used throughout history. One such method is the application of naturally occurring products, such as extracts from certain plants to treat a broad range of wound and skin conditions. These medicinal plants can have a variety of compounds which show favorable medical characteristics. One group of these compounds are called essential oils [1].

Essential oils are aromatic compounds extracted from various parts of many plants [1]. Extensive research has demonstrated their wide range of favorable biological activities and thus, there has been a recent growth of interest in their application [2]. Fonget al. reported that Thyme plant (*Thymus vulgaris* L.) had exhibited favorable antibiotic properties and thus had been used since ancient times for treatment of sore throat, dry coughs, bronchitis, laryngitis and inflammatory conditions of the gastrointestinal tract, such as indigestion and gastritis [3, 4]. Cristiani et al. have demonstrated that these biological effects, especially the strong antibacterial activity are mainly due to the existence of phenolic compounds such as thymol and carvacrol [2], with thymol being the major component of the thyme essential oil [3]. Thymol has demonstrated 30 times higher antibacterial effects and 4 times lower toxicity than phenol [3], already a known antiseptic and component of many herbicides [5]. Thymol, like other essential oils is lipophilic, and thus is mostly accumulated and activated at the lipid bilayer of the cell membrane of bacteria [6]. It acts by disrupting the structure of the membrane and increasing the permeability of the cell [6], “causing a reduction in the proton motive force and an associated decrease in intracellular levels of ATP [4]”, thus killing the bacteria. This, combined with Esmaeili et al. reports of other advantageous properties such as antioxidant properties [7] makes it a great component to be added to a wound dressing, and a much superior choice to other, more conventional antiseptics (such as silver) and antibiotics.

Despite these favorable properties, attention to thymol as a wound healing and antibacterial agent is rather recent. Most, if not all current investigations have studied the properties of this compound in isolation, and its use in wound dressings is not well established. So much so that a search of the keywords “thymol” and “wound dressing” on the popular resource site “sciencedirect.com” currently yields very few results with wound dressings incorporating thymol, all of which are confined to gauze coatings [8, 9].This begs the question, how will thymol perform when incorporated in sample wound dressing? How will it change the dressing’s properties, especially the mechanical ones?

This investigation has two major objectives. The first is examining thymol for existence of antibacterial and wound healing properties, and ascertain whether it conforms to the data in literature. The second is to incorporate thymol in a sample dressing and characterize the resulting product, ensuring that it retains thymol’s favorable properties and assessing the effects of its incorporation. To reach these objectives, two sets of tests are carried out. The first are those that characterize thymol, like the MIC assay [10, 11], to test antibacterial properties and the MTT assay [12], to investigate toxicity or cell growth promoting properties. The second are those that characterize the sample product (the film containing the active pharmaceutical

ingredients or API, henceforth known as the API film) which include Disk Diffusion [13], Mechanical and SEM tests.

Materials and Methods

As noted, the MIC and MTT assays were the methods of choice to characterize the antibacterial and wound healing properties of thymol respectively.

Due to limited effectiveness of MIC assay to assess the antibacterial properties of our API film (since the API film was opaque itself, the results could not be determined with certainty), disk diffusion method was used as a substitute. The diameters of the zones of inhibition for the API films and the simple films of chitosan and CMC were measured and then compared.

To assess the mechanical properties of the films, four samples with varying concentrations of thymol and glycerol were prepared. These two components were experimentally found to have the most flexibility in their range of applied concentration and the most effect on the mechanical properties of the final product. An Instron Universal Mechanical Testing device with a load cell of 50N was used and the elongation versus stress curves were created.

To assess the changes in structure induced by introduction of thymol and glycerol, the same samples were cut to sizes, and a thin layer of gold was deposited onto them. They were then photographed using the scanning electron microscope of the SEM lab of University of Tehran's School of Electrical and Computer engineering at various magnification degrees.

Materials

Highly pure thymol (thymol > 99.5%), chitosan from crab shells, Silver nanoparticles (<100 nm), CMC (>99%), Muller-Hinton Agar powder and ethanol (>99.7%) were purchased from Sigma Aldrich Co. Glycerol (bp grade) and acetic acid (>99.7%) were purchased from Merck Co. Fresh *Aloe vera* leaves were provided by Institute of Medicinal plants, ACECR, Karaj, Iran. These leaves were washed and cut and their gel was removed from them. This gel was then mixed, sieved and centrifuged to remove insoluble solids. The final viscous solution was either immediately used or put in the refrigerator for up to three days before use. All of the materials required for cytotoxicity assay including MTT and the fibroblast cells were purchased from Pasteur Institute, Tehran, Iran.

Methods

Preparation of thymol solution

Thymol is an essential oil, and thus, does not solve in pure water [3]. A 60:40 ethanol-water solution was used to solve thymol in any concentration needed.

Preparation of the prototype

First, CMC was poured into a beaker with 10ml of water and stirred until solved. Glycerol and then the thymol solution were added and stirred to make a clear solution. The Silver suspension and chitosan powder were then added respectively and stirred vigorously to make a homogenous suspension. 10 ml *Aloe vera* gel was also added. Finally, a 1 ml syringe was used to inject acetic acid quickly and very close to the stirring magnet. Chitosan is protonated and quickly reacts with CMC causing the solution to begin solidifying. After the solution has reached a rather homogenous state, it was poured into casting plates to be prepared for drying. These plates were then prefreezed and put into the freeze dryer for at least 8 hours to be dried before use.

Thymol characterization tests

MIC

Solutions or suspensions of test materials were prepared for testing MIC in 96 well micro titer plates. Of each material, a 100 µl solution with an equal concentration to that used in the final product was poured into the first row of wells. Half of this solution was taken and added to the second well and water was added with equal volume so as to reduce the concentration to half of the starting concentration. This procedure was repeated for all of the wells in the micro titer plate. The last row of wells also had half of their volume removed and wasted, so that every well would have the same volume. Then, an equal volume of bacterial culture solution (50 µl) with identifier was added to each well. The plates were then incubated for a day before assessment [10, 11].

Cytotoxicity

As skin fibroblast cells are adherent to the active pharmaceutical ingredients used in this experiment, the normal MTT assay protocol for adherent cells was used [12, 14], with two different ratios of active ingredient solution to cell culture media.

Prototype characterization Tests

Disk diffusion

Muller-Hinton Agar powder was solved in water and autoclaved to ensure sterility. Then poured in plates and kept for 24 hours in the refrigerator. Bacterial samples are then passaged in each plate. Depending on the test sample's form, it is either poured over blank filter-paper disks (for liquids and solutions) and put in the culture environment or cut (for solid films) and placed in the holes made by punching the solid culture environment by a Pasteur pipette. The plates were then incubated for a day before assessment [13].

SEM

A 0.5 cm x 0.5 cm piece was cut from each of the API layers and deposed with a thin gold layer before being scanned by SEM.

Tensile strength

An Instron Electromechanical Universal Test machine was used to measure the tensile strength of the samples. Stripes of 8.0 cm x 1.5 cm are cut from each API film, and fixed in the grips of the machine. The distance between the grips of the machine was set to 4cm so that 4cm of each stripe would be under load. Tests were done with a load cell of 50 N and speed of 5 mm/min. Elongation vs. stress data were recorded until the point of break.

Results

Thymol characterization tests

MIC

Results for MIC testing of various ingredients used in the creation of API layer are listed in Tables 1 and 2. As suspected, *Aloe vera* shows no antibacterial properties, CMC shows very limited antibacterial properties, chitosan show limited antibacterial properties and both silver and thymol show excellent antibacterial properties. As it is difficult to discern the effects of these agents when used in the API by MIC tests, Disk Diffusion tests were carried out to better understand the interaction of these ingredients when used together as an antibacterial agent.

Cytotoxicity

Two series of experiments were conducted to assay cell viability in the presence of API ingredients. In the first set, denominated Conc. 3/4, a 150 µl solution of API ingredient was mixed with 50 µl of cell solution with known number of living cells and in the second set;

the same volume (100 µl) was used for both solutions. The results of cell growth when subjected to the API ingredients vs. control (no API) were gathered and are shown in table 3 and figure 1 as the percentage of cells alive after MTT assay.

As apparent, silver has shown the least cell viability which is an expected occurrence for silver and is in agreement with literature.

Aloe vera shows the same cell viability as the control, or rather shows no change in cellular growth. This is a phenomenon that is referenced in literature, as acemannan and other ingredients in *Aloe vera* responsible for increasing skin cell growth have a very short life span when the leaf is separated from the root, so much so that a delay of few hours would severely reduce their effectiveness and a delay of about 2-3 days as is the case here would completely negate it. Alleviating this problem is rather a difficult task, as it requires the test to be done in very close proximity to the plant and shortening the process of preparing the *Aloe vera* gel and the test bed to a great extent. Due to the distance between the *Aloe vera* farm used as the source of this project and the laboratory in which the experiment was carried out this phenomenon could not be hindered.

Table 3- Percentage of cells alive after MTT assay

Concentration Ratio of API to total (API + Cells)	**Control** (%)	**Ag** (%)	***Aloe vera*** (%)	**Thymol** (%)	**CMC** (%)
3/4	100	12	97	-	45
2/4	100	35	98	273	153

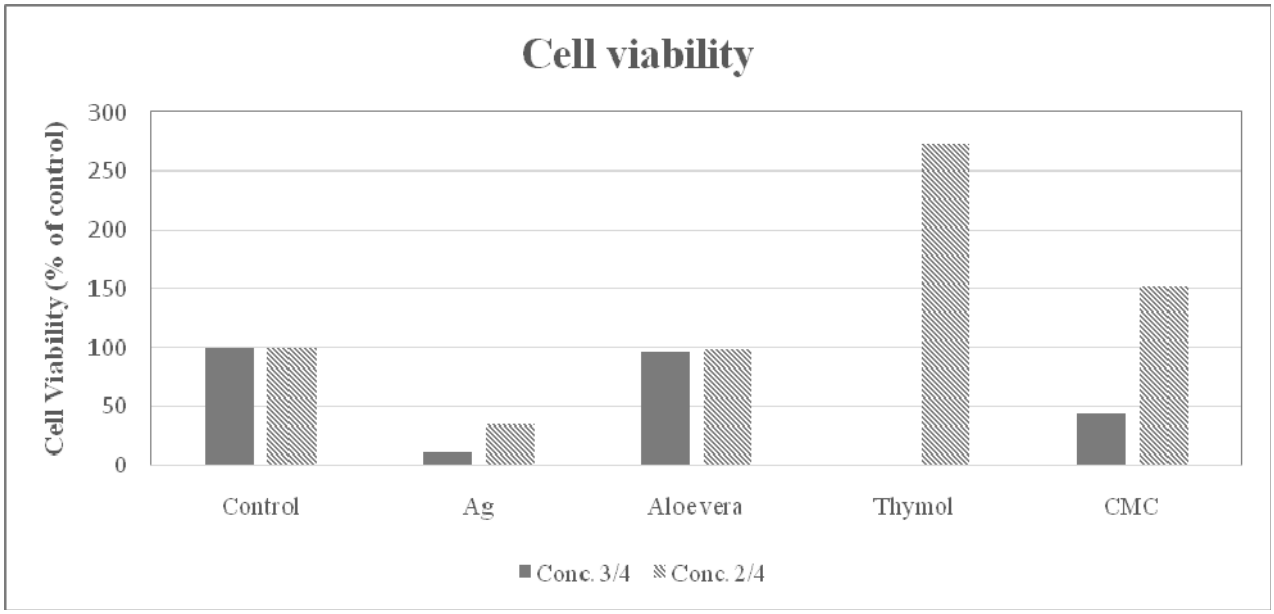

Figure 1- Percentage of cells alive after MTT assay

CMC can both delay and improve cell growth, depending on the concentration used. This puts a great emphasis on optimization of the concentration of CMC.

Thymol shows unexpectedly great cell growth, so that the results for Conc. 3/4 test could not be read due to the intensity and blackness of the solution's color. Further testing shows that at least a part of this color is due the interaction of thymol with MTT. Thus, although thymol itself has great cell growth improvement properties, the exact effect of these properties cannot be defined by MTT assay, as thymol itself causes interferences that cannot be easily isolated or removed. To better understand these effects other, more precise methods are recommended.

Prototype characterization tests

Disk diffusion

Plates with a diameter of 9 cm were used. API Ingredients used were *Aloe vera*, chitosan and chitosan + CMC, all at their initial concentration as the MIC test. An API layer with the same concentration of Chitosan, CMC and *Aloe vera* but with 50ppm Silver and 0.5% thymol was also tested. Silver and thymol were not tested because their effectiveness has been proved in this article and other articles in literature. The reason for using the combination of chitosan and CMC was that although CMC has little to no antibacterial properties of its own, when combined with chitosan it sometimes improves chitosan's antimicrobial properties. While the exact reasons are unknown, this effect could be related to the complex network that is created after the reaction which could physically hinder bacterial growth. After incubation, diameter of zone of inhibition for each compound or layer was measured and is represented in table 4.

SEM

Four samples were considered for SEM testing with varying glycerol and thymol concentrations for analyzing the effects of glycerol and thymol on the final structure of the API film. The glycerol and thymol composition is presented in table 5.

The SEM scans for each film is presented below.

A comparison between the figures reveals interesting results. Comparing figure 2 to figures 3 and 4 reveals that the increase in glycerol or thymol content results in a smoother surface, showing the jagged edges and the wrinkles that are abundant in figure 1 are smoothed out significantly in both figures 3 and 4. Another interesting note is that pores in figure 3 are larger than those of figure 2, whereas figure 4 pores are rather smaller. This could mean that glycerol increases pore size while thymol reduces it. Comparing figures 4 and 5, strengthens this assumption, as pores seem larger on the layer with higher glycerol content (API 3-3).

Table 4- Diameter of growth inhibition zone

Bacteria	*Aloe vera* (mm)	Chitosan (mm)	Chitosan + CMC (mm)	API (mm)
Salmonella paratyphi B	0	10	-	-
Bacillus subtilis	0	4	-	-
Pseudomonas aeruginosa	0	9	11	15
Escherichia coli	0	6	7	10
Staphylococcus aureus standard	0	7	8	11
Staphylococcus aureus wild	0	6	-	-

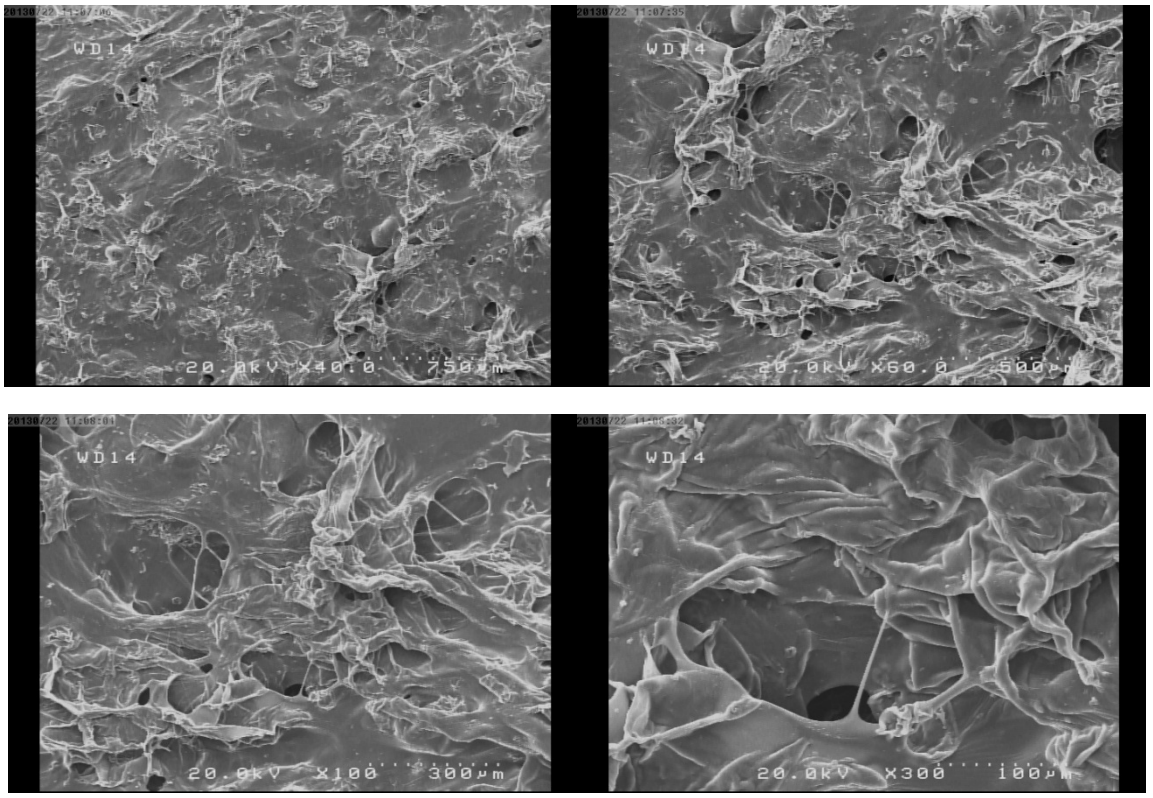

Figure 2- SEM pictures of API 1-1, with glycerol concentration of 0.2 g/dL (2 mg/ml) and thymol concentration of 0.4 g/dL (4 mg/ml), magnified 40 times (top right), 60 times (top left), 100 times (bottom right) and 300 times (bottom left)

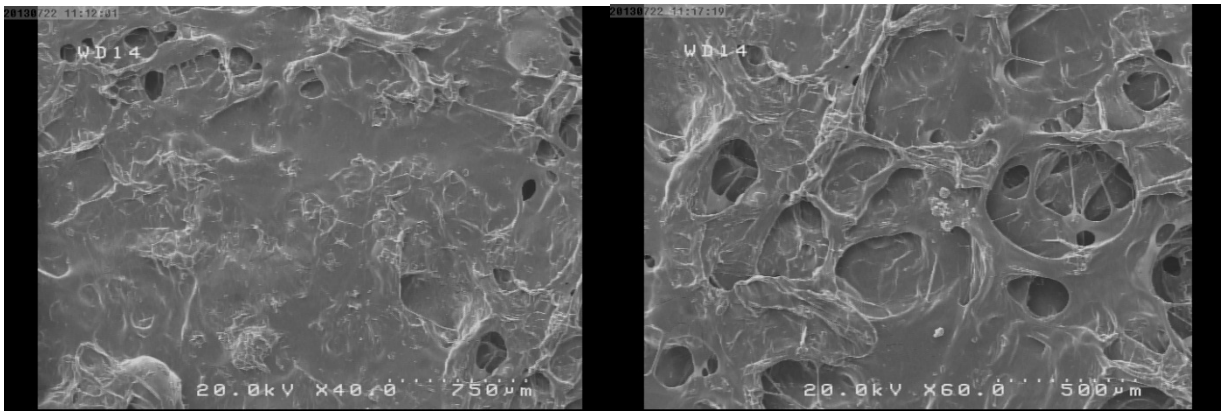

Figure 3-

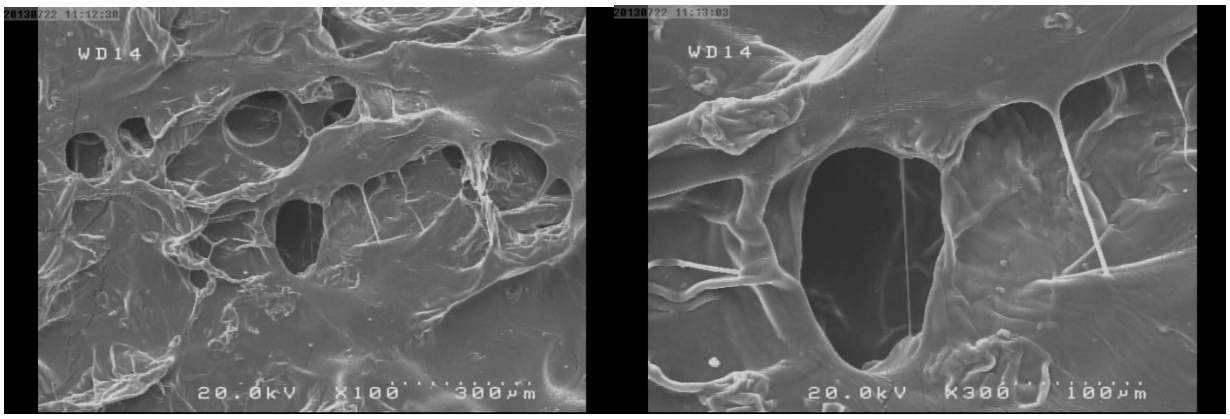

Figure 3- SEM pictures of API 3-1, with glycerol concentration of 0.6g/dL (6 mg/ml) and thymol concentration of 0.4 g/dL (4 mg/ml), magnified 40 times (top right), 60 times (top left), 100 times (bottom right) and 300 times (bottom left)

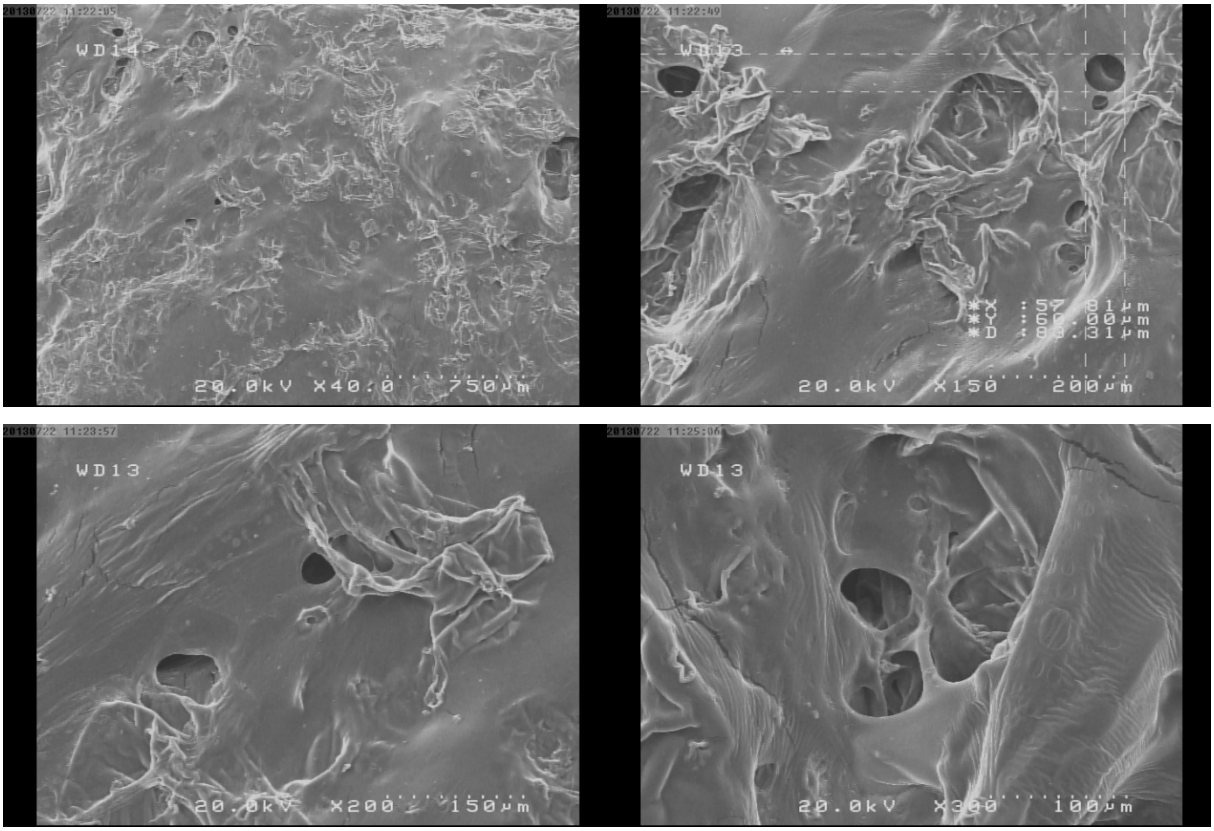

Figure 4- SEM pictures of API 1-3, with glycerol concentration of 0.2 g/dL (2 mg/ml) and thymol concentration of 0.6g/dL (6mg/ml), magnified 40 times (top right), 150 times (top left), 200 times (bottom right) and 300 times (bottom left)

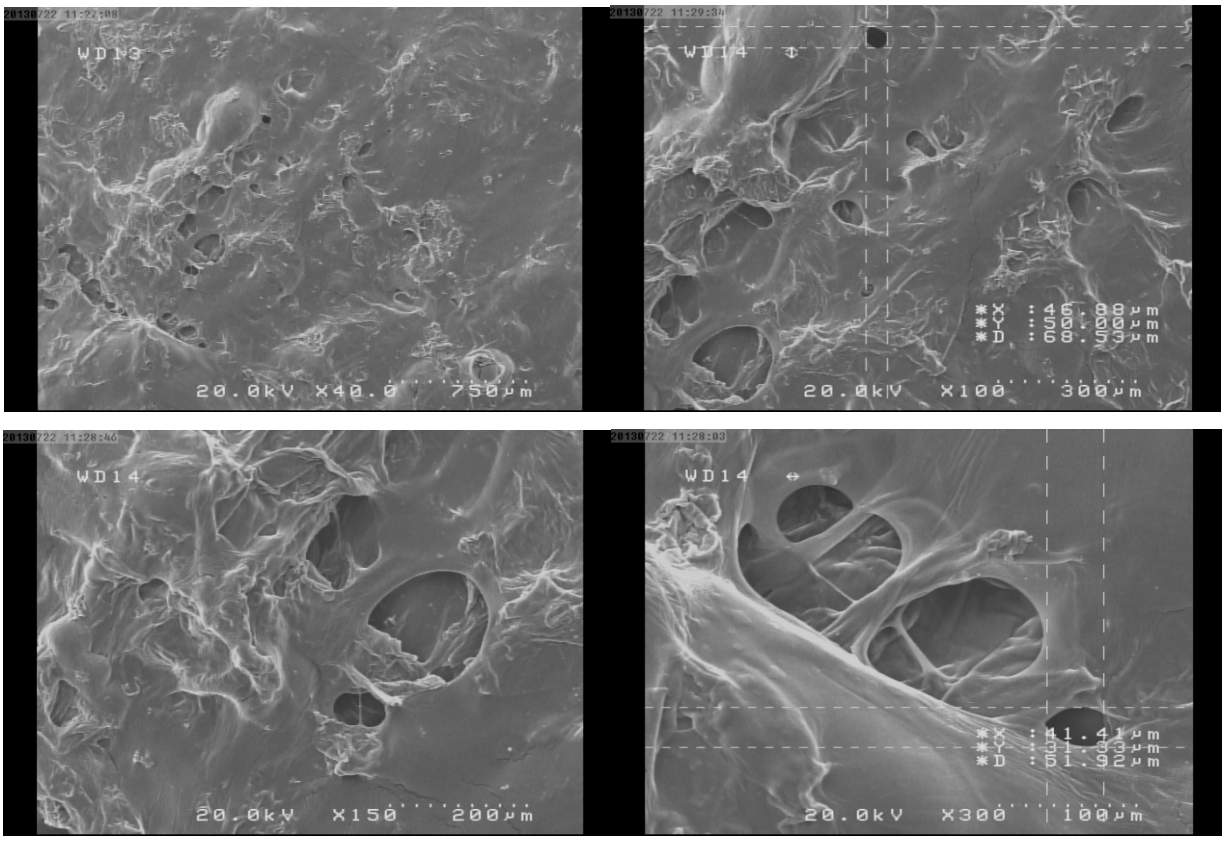

Figure 5- SEM pictures of API 3-3, with glycerol concentration of 0.6 g/dL (6 mg/ml) and thymol concentration of 0.6 g/dL (6 mg/ml), magnified 40 times (top right), 100 times (top left), 150 times (bottom right) and 300 times (bottom left)

Tensile strength

The effects of both thymol and glycerol on mechanical properties of the dressing were analyzed. Figure 6 shows the elongation rate of the dressings vs. the load applied. By comparing the figures several points can be made: increasing the concentration of thymol (API 1-1 (a) to API 1-3 (b) and API 3-1 (c) to API 3-3) (d) causes both the maximum elongation and elongation vs. stress to increase whereas increasing glycerol concentration (API 1-1 (a) to 3-1 (c) and 1-3 (b) to 3-3 (d)) results in a decrease in maximum elongation and an increase in elongation vs. stress. Also worthy of note is the small dependency of maximum stress to the concentration of thymol and instead its much greater dependency to the concentration of glycerol, as increasing thymol concentration results in a comparatively small decrease in maximum stress (increasing thymol concentration from API 1-1 to API 1-3 sees a the maximum stress change from 0.45N to 0.38N (about 15.5%) and the change from API 3-1 to API 3-3 sees it change from 0.15N to 0.14N (about 6.6%)) whereas the increase in glycerol concentration causes a much more dramatic change to the maximum stress of the dressing (increasing glycerol concentration from API 1-1 to API 3-1 sees a the maximum stress change from 0.45N to 0.15N (about 66.6%) and the change from API 1-3 to API 3-3 sees it change from 0.38N to 0.14N (about 63%)).

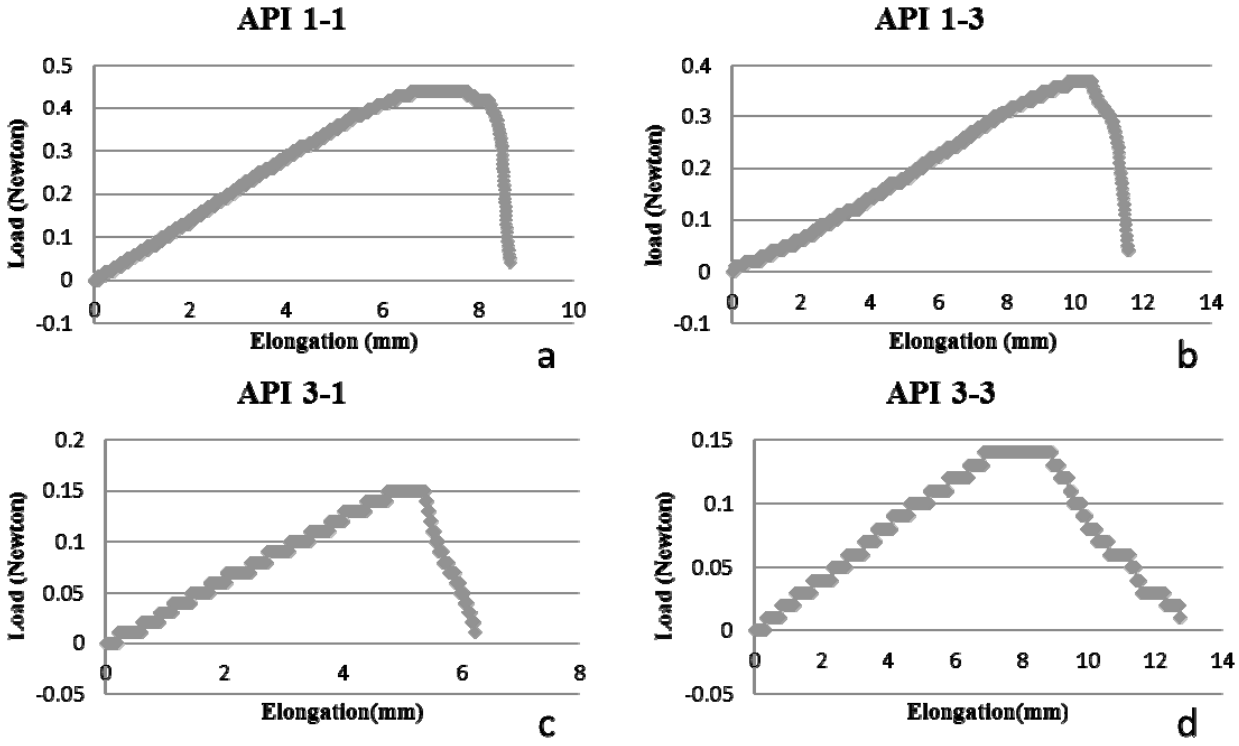

Figure 6- Elongation (in mm) vs. stress (in Newton), API 1-1 (a), API 1-3 (b), API 3-1 (c) and API 3-3 (d)

Discussion

To ascertain the favorable properties of thymol two sets of tests were carried out, the MIC assay, to test antibacterial properties and the MTT assay, to investigate toxicity or cell growth promoting properties.

The antibacterial properties of thymol is well documented in literature [6, 15, 16], but to make sure of their existence in the sample at hand, a preliminary set of MIC antibacterial testing was carried out. The MIC assay was done for two bacterial strains, Escherichia coli and Staphylococcus aureus, two of the most abundant bacteria in human environment. Results found the MIC to be around 0.3 mg/ml for Escherichia coli and 0.15 mg/ml for Staphylococcus aureus, which is in agreement with literature [17]. These results make further antibacterial testing relevant.

Wound healing properties of thymol itself has been under less scrutiny, and so although these properties have been known to exist their exact effects are less known [18]. Here the MTT assay, a simple in-vitro test, was performed to have an understanding of thymol's effects on fibroblast cells. The assay was performed in two sets, one with 150µl of test media and 50µl cell culture media denominated Conc. 3/4 and the other with both at 100µl denominated Conc. 2/4. In initial testing thymol exhibited outstanding performance, as it improved the growth rate of skin fibroblast cell by an impressive 173% in Conc. 2/4 test, and the samples were so dark in the Conc. 3/4 test that the exact percentage could not be read. Further testing proved that some of this is due to thymol's interaction with MTT, and so the interference would prevent us

from getting an exact read on the thymol's cell growth promotion properties.

To assess the effect of thymol when used on a dressing, a simple prototype was created as described in the methods section. This prototype then underwent various investigations to characterize its antibacterial, structural and mechanical properties.

Due to limited effectiveness of MIC assay to assess the antibacterial properties of our API film, disk diffusion method was used as a substitute. The final product includes *Aloe vera* as a wound healing agent as well, but *Aloe vera* has shown a zone of increased bacterial growth around it in place of the inhibition zone normally seen around antibacterial agents, which may reduce the effectiveness of any antibacterial agent added to the mix. Because the final product should have antibacterial properties, this barrier must be overcome, so a mixture of thymol and nanosilver was used as the antibacterial agent. Thymol and silver with concentrations close to their MIC were used and the radii of the zones of inhibition for the API films and the simple films of chitosan and CMC were measured. The API films produced better results overall, which is exactly what is needed.

To assess the mechanical properties of the films, four samples with varying concentrations of thymol and glycerol were prepared. These two components were experimentally found to have the most flexibility in their range of applied concentration and the most effect on the mechanical properties of the final product. An Instron general mechanical testing device with a load cell of 50N was used and the elongation versus stress curves were created. Evidently, an increase in thymol content increases elongations before break and elongation vs. stress, but reduces the maximum stress the product can endure before breakage. On the other hand, an increase in glycerol content reduces maximum stress and maximum elongation before break, but increases elongation vs. stress.

To assess the changes in structure induced by introduction of thymol and glycerol, the same samples were scanned with an SEM. As is apparent by figures 2-5, increasing the content of both glycerol and thymol smoothens wrinkles and jagged edges, but an increase in glycerol content makes the pores rather larger while but an increase in thymol content makes the pores rather smaller.

Conclusion

There were two goals for this investigation. The first goal was to analyze thymol's antibacterial and wound healing properties for a wound dressing. The second goal was to incorporate it in a prototype dressing and put it through its paces to first make sure that it retains its properties and second, to assess the effects of thymol on the properties of the dressing.

Antibacterial investigation by MIC assay backs up the claim provided by literature of thymol having antibacterial properties on its own. Further assessment of the product containing thymol by Disk diffusion method ensures the effectiveness of the essential oil when incorporated into the final product. MTT assay has shown thymol to have outstanding cell growth promotion properties, although the exact amount could not be interpreted due to a conflict between thymol and MTT. In addition, SEM and mechanical testing show that incorporating thymol into the dressing increases its elasticity and porosity, but reduces its mechanical strength.

Acknowledgement

The authors would like to thank the Measurement Unit of Sharif University, Institute of Biophysics and Biochemistry of University of Tehran and The Polymer Properties Lab of Tarbiat Modares University for their support. They would also like to extend their gratitude to Ms. Razieh Khosraviani and Ms. Leila Sadegh Zadeh for their assistance in realization of the project.

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