



## Promising antimicrobial and antifungal activities of free peppermint (*Mentha piperita* L.) essential oil and its conjugated form with chitosan

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### Abstract

The emergence of antimicrobial resistance has necessitated the new approaches. The peppermint (*Mentha piperita* L.) essential oil (PEO) is known for its antimicrobial and antifungal activities. However, the employing of it in workable applications is troublesome because of the sensitivity to the environmental conditions. Thus, it was encapsulated into chitosan to eliminate the difficulties in its use and increase its activity. It was observed that the immobilization of the PEO into the chitosan (PEO-Chitosan) influenced the biological activities resulting in observing less Minimum Inhibitory Concentration (MIC) values in addition to protecting the essential oil by the chitosan as environment-friendly biomaterial. The determined MIC values of the target product (PEO-Chitosan) are between 0.001–0.95 mg/mL for the studied bacterial strains and 0.006–0.36 mg/mL for the studied fungi isolates, which led us to consider them as new therapeutic alternative. *In vitro* antiviral studies gave us that even the encapsulation of the essential oil into the chitosan made the prepared product still promising candidate for the antiviral therapy.

**Keywords:** *Mentha piperita* L., encapsulation, chitosan, antibacterial, antifungal and antiviral activity

### 1. Introduction

Infectious diseases have been an important health problem for years. Since the discovery and administration of penicillin in the 1940s, antibiotics have played unique roles as invaluable weapons to combat infectious for humans and animals [1]. It is used to cure the diseases caused by microorganisms and has the ability to kill bacteria or inhibit their development by inhibiting the formation of cell membranes, protein synthesis, cytoplasmic membranes or nucleic acid synthesis [2]. In line with this importance, newer drugs as antibiotics have been introduced into clinical practice such as  $\beta$ -lactams, sulphonamides, polypeptides to prevent or treat the infectious disease over the decades [3].

Before the advent of antibiotics, these diseases were the leading cause of morbidity and death in human populations. But later, the facing with the crisis of antimicrobial resistance among pathogenic bacteria is dynamic increasing problem. This might be the because of the overuse and abuse of antimicrobial drugs posing a major threat to both human and animal health [4].

In the same way, another threat for the humanity is that the increasing resistance to antifungal compounds,

originating from drug target alteration or overexpression [5]. In a consequence of this, a number of scientific investigations have driven to the new therapeutic alternatives by the scientist to combat fungal infectious disease. Today, plant-based natural products carried out by different disciplines on the production of antibacterial and antifungal substances are increasing day by day.

Viral infections are a serious and increasing health problem and cause high morbidity, mortality and economic burden [6]. The limited availability of current therapy methods has prompted researchers to obtain new products of biotechnological importance.

The extracted essential oils from the plants have been reported as antibacterial [7], antiviral [8], antifungal [9]. Among them, peppermint (*Mentha piperita* L.) is cultivated all over the world for its use in medicinal and pharmaceutical applications in addition to using in food, herbal tea preparations due to its flavor and fragrance [10]. Because of the antimicrobial, antifungal and antiviral activities, the peppermint essential oil have been subjected in some studies [11–13]. Although the high activity of essential oils in different applications,

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some environmental conditions hinder them to use in their pure form. The conjugation to the nano-carriers or encapsulation into nano materials have been the focus of considerable interest in developing new alternatives to overcome this drawback. These strategies allow essential oils not only to preserve their bioactive components, but also to prepare new materials aimed at increasing the effectiveness of essential oils [14].

In accordance with this purpose, the studied peppermint essential oil (PEO) was encapsulated into chitosan. Chitosan as biocompatible biomaterial is known for its antibacterial and antifungal feature against bacterial and fungal pathogens that infect human hosts [15,16]. The antibacterial and antifungal activities of the obtained chitosan conjugate of oil were investigated against selected bacteria and fungus strains as compared with the pure form of peppermint essential oil. In addition to this, the prepared samples were tested *in vitro* cytotoxicity and antiviral activity, against virus's representative of herpes simplex virus-1 and Human poliovirus Type 1.

## 2. Experimental

### 2.1. Chemicals and Instruments

The *Mentha piperita* L. (peppermint) sample used in this study was collected from the north of Iran (Gorgan City, Golestan Province, Iran) before flowering. Chitosan, acetic acid, acetone, diethyleter, dimethylsulfoxide (DMSO), ampicillin and fluconazole were purchased from Sigma–Aldrich. Ultra-pure water was from MilliQ water. Nutrient agar and agar bacteriological BBL Muller Hinton broth were purchased from Merck. Dulbecco's phosphate-buffered saline (DPBS) was purchased from Sigma Aldrich All the bacteria species were provided by the Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University (Istanbul, Turkey) such as *Staphylococcus aureus* (*S. Aureus*) (ATCC 6538), *Escherichia coli* (*E. Coli*) (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus epidermidis* (*S. epidermidis*) (ATCC 15442), *Candida albicans* (*C. Albicans*) (ATCC 10231), *Aspergillus niger* (ATCC 16404), *Penicillium* spp and *Methicillin-resistant Staphylococcus aureus* (MRSA) were isolated and characterized by MALDI-TOF (Bruker Microflex LT/SH).

Immortalized human keratinocytes (HaCaT) cell line, Herpes Simplex Type 1 (MacIntyre, #0810005CF, Zeptomatrix), and Human poliovirus Type 1 (LSc-2ab, RVB 1260, FLI) viruses were used (cell line and viruses were obtained from the Genetic and Bioengineering Department of Yeditepe University). GC Agilent 6890N; MS: Agilent 5973 with an HP5-MS column (30 m × 0.25 mm fused silica capillary column, film thickness 0.25

µm) was used for GC/ MS analysis. FTIR spectra were recorded on an attenuated total reflectance (ATR) apparatus on a Thermo Scientific Nicolet IS10-IZ10. SEM images were taken by a Zeiss evo 40 instrument following gold sputtering (EM ACE200, Leica). Zeta Potential of chitosan conjugate was determined using Zetasizer Nano-ZS90 (Malvern Instruments). Electronic spectra were recorded on a PerkinElmer LAMBDA 25 Series UV–vis spectrophotometer with a quartz cell of 1 cm.

### 2.2. Method of Extraction Essential oil *Mentha piperita* L.

The essential oil of peppermint (*Mentha piperita* L.) was collected from the region (Gorgan province in Iran), then the aerial parts were dried, and finally the essential oil was obtained by hydro distillation technique. The essential oil was analyzed by GC/MS method in Dr. Soltani Akhula's factory in East Azarbaijan Province, Iran. Distillation details are given in reference [17].

### 2.3. Immobilization of peppermint essential oil to Chitosan

Preparation of the encapsulation of the PEO into chitosan was carried out using a procedure reported in the literature with slight modification, namely, without precipitation with NaOH to obtain charged solution [18]. Briefly, for the preparation of PEO-chitosan, chitosan (0.2 g) was stirred in water/2% acetic acid (10 mL) until obtaining homogeneous mixture. Then 1 ml of PEO by dissolving in DMSO was added into this solution and stirred continuously for 24 h. After complete mixing, washed several times with distilled water until the pH of water was neutral. The new product was collected by centrifuging and dried by freeze-drying.

### 2.4. *In vitro* antibacterial and antifungal activities

Activity studies of PEO and its encapsulated derivative were carried out by micro-well dilution assays [19]. Briefly, the microorganisms (tested for activity were transferred from stock culture to suspend in PBS and adjusted to approx.  $1 \times 10^6$  cfu/ml per ml with the help of MacFarland turbidity curve. MIC values were determined according to the broth dilution method and performed in 96 flat-bottomed microliter plates. Each of the bacterium and the fungi suspensions was added to the first test well and mixed. Serial two-fold dilutions of the extractions were prepared in broth with a final volume of 100 µL in 96-well microplates. Then, 100 µL of bacterial suspension was inoculated in each well. The microplates were incubated at 37 °C for 24 h. The last wells including nutrient broth only and nutrient broth containing bacterium or fungi without compound was considered as negative and positive control, respectively. MIC was identified as the lowest concentration of the samples that kills 99.9% or more of the initial inoculum.

## 2.5. *In vitro* antiviral activities

The cytotoxicity and the virus-inhibitory effect of each sample was determined *in vitro* by employing MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method on human immortalized keratinocyte (HaCaT) cell line along with the cytotoxic concentration (CC<sub>50</sub>) by determining first the non-toxic concentration of the samples against HaCaT cell line [20]. All experiments were carried out three times. The data were given as logarithmic form.

## 3. Result Discussion

### 3.1. Structure characterization

This study was started with the extraction of the essential oil from the *Mentha piperita* L. by hydro distillation technique. The determined main components of *Mentha piperita* L. essential oil that was analyzed by GC/MS method are: menthol (43.25%), menthone (15.1%), menthofuran (9.33%), ciscarane (6.7%), 1,8-cineole (5.37%), neomenthol (5.37%), 4.1% and limonene (1.95%). The goal of our work was the encapsulation of essential oil of *Mentha piperita* L. through conjugation into chitosan in order to enhance their antimicrobial activity by the procedure as stated in the experimental part under the “Immobilization of peppermint essential oil to Chitosan” section (Scheme 1). The obtained conjugate (PEO-chitosan) was characterized by using analytical techniques such as Fourier transform infrared spectroscopy (FT-IR), SEM, UV/Vis by comparing the PEO alone and zeta potential measurement of the conjugate. The results confirmed the presence of target compounds.

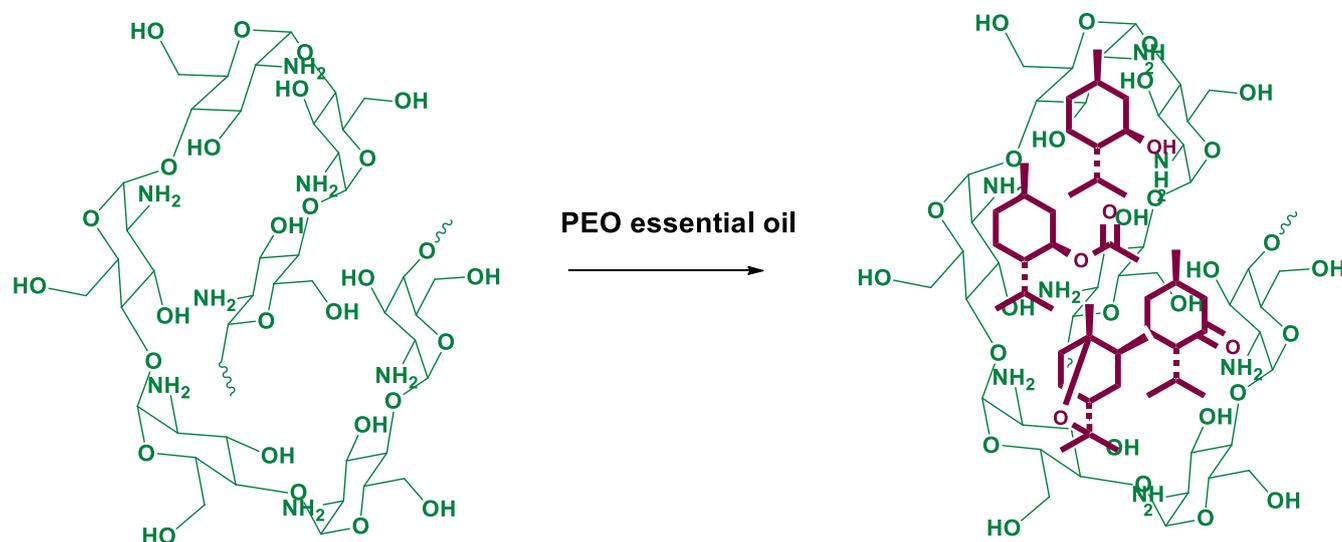
FT-IR spectrums of the studied samples show the existence of the functional groups. In the FT-IR spectrum of PEO, the band at 3398 cm<sup>-1</sup> is attributed to the OH groups coming from the alcohol derivatives which are

the some of the components of the PEO. The other characteristic peaks at 2958–2869 cm<sup>-1</sup>, 1712 cm<sup>-1</sup>, 1459 and 1370 cm<sup>-1</sup>, 1246 cm<sup>-1</sup> and 1046 cm<sup>-1</sup>, belonging to the aliph. -CH stretchings, C=O stretching, aliph. C-C stretching, -C-O asym. and -C-O sym. stretching, respectively, were seen in the FT-IR spectrum of PEO (Fig. 1B).

In the FT-IR spectrum of chitosan powder, the peaks at 3357 and 3290 cm<sup>-1</sup> are attributed to the NH<sub>2</sub> and OH groups as overlapped with each other. The aliph. -CH stretchings and -N-H bending vibration of primary amine (-NH<sub>2</sub>) were observed at 2924, 2871 cm<sup>-1</sup> and 1649 cm<sup>-1</sup>. The peak originating from the saccharide structure of chitosan arised at 1062 cm<sup>-1</sup> for -C-O sym. stretching (Fig. 1A).

Upon conjugation of PEO to the chitosan, In the FT-IR spectrum of PEO-chitosan, the peaks at 3380 cm<sup>-1</sup> reflect the -OH and -NH stretchings that are superimposed by contributions from all the components present in the mixture. The C=O stretching which is the one of the main characteristic peaks coming from the PEO components shifted to 1702 cm<sup>-1</sup>, implying the complex formation via electrostatic interaction between NH<sub>3</sub><sup>+</sup> groups of chitosan and carbonyl group of PEO. Also, the shifting of the C-C stretching and -C-O sym. stretching to the 1457,1378 cm<sup>-1</sup> and 1066 cm<sup>-1</sup>, respectively, prove the complexation between chitosan and PEO [21] (Fig. 1C).

The UV-vis absorption spectra of PEO and its chitosan conjugate were recorded in DMSO. In the UV/Vis spectrum of PEO, considerably intense band at λ<sub>max</sub> = 311 nm, which can be attributed to the high contents of terpenes, terpenoids and phenolic compounds and might be the characteristic liquid state of aggregation [22]. Upon the non-covalent immobilization of PEO into chitosan, the absorption



**Scheme 1.** Schematic illustration of conjugation PEO to chitosan (only four of ingredient was used for illustration) (PEO: peppermint essential oil)

band was observed at 290 nm with blue shift compared to the PEO alone, which can be explained by decreasing the intermolecular electronic coupling of the PEO in the conjugate (Fig. 2) [23].

Scanning electron microscopy (SEM) has been used to study the surface morphology and the image of the conjugate is shown in Fig. 3. In the SEM images, PEO-chitosan have smooth, flat and homogeneous surfaces at its morphology. The irregularities like air bubbles or oil droplets meaning to macroscopic phase separation were not observed, showing that the PEO is well distributed in the chitosan.

Surface charge (zeta potential) ( $\zeta$ ) determination is a measure of charges that is carried by conjugates suspended in a liquid. It could considerably affect the stability of conjugates in suspension and *in vitro* efficiency of the prepared samples with the cell membrane of bacteria, through the electrostatic repulsion between particles [24]. The measurement of the chitosan conjugate (PEO-Chitosan) showed that the surfaces of sample have a positive charge about 4 mV, resulting from the dissociation of acidic groups that are on the chitosan units [25].

### 3.2. Determination of the antimicrobial activity

The antimicrobial activities of PEO alone and its conjugation with chitosan (PEO-chitosan) against *S. aureus*, *S. epidermidis*, MRSA as gram(+), *Pseudomonas aeruginosa*, and *E. coli* as gram(-) bacterial strains, *C. albicans*, *Aspergillus niger* and *Penicillium* spp. as fungi isolates were compared to investigate the effect of the conjugation of PEO with the biorganic molecule. The initial concentration of the PEO used in the study was assumed to be 100% corresponding to the 80 mg/mL while the conjugate dissolved in DMSO were first prepared at the highest concentration to be tested (7.6 mg/mL). The minimum inhibitory concentration results are presented in Table 1.

Both PEO alone and PEO-chitosan exhibited antibacterial activities against microorganisms. Pure PEO showed antibacterial activity against bacteria strains with MIC value of 2.64 mg/mL for *Pseudomonas aeruginosa*, 1.32 mg/mL for *S. aureus* and MRSA, 0.66 mg/mL for *S. epidermidis* and 2.64 mg/mL for *E. coli*. The encapsulated PEO exhibited enhancement of bactericidal activity against all the studied microorganisms with MIC value of 0.95 mg/mL for *S. aureus*, *Pseudomonas aeruginosa* and MRSA and 0.001 mg/mL for *E. coli* as compared to PEO alone. The reason why the lowest MIC value was observed for *E. coli* may be behind the better penetration of immobilized PEO molecules through the lipophilic cell wall of gram-negative bacteria [26].

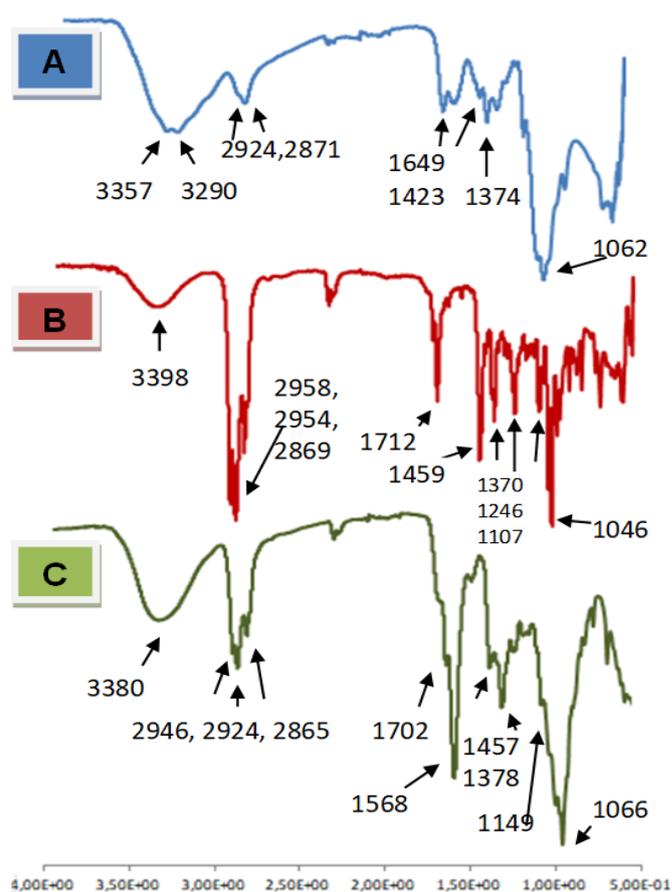


Figure 1. FTIR spectra of A: Chitosan, B: Peppermint oil alone C: PEO-Chitosan (wavenumber  $\text{cm}^{-1}$ ) (PEO: peppermint essential oil)

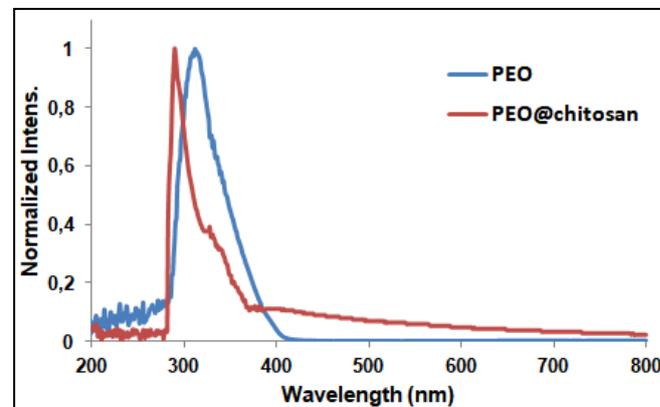


Figure 2. Normalized absorption spectra of PEO and its chitosan conjugate (PEO-chitosan) (PEO: peppermint essential oil)

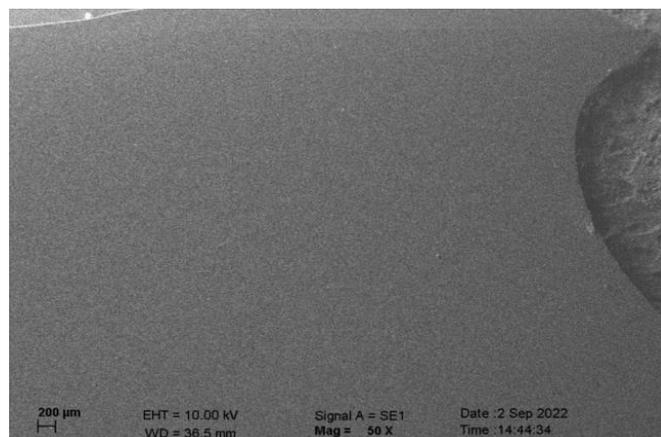


Figure 3. SEM of the surface image of PEO-chitosan film viewed at a magnification of 1000x. (PEO: peppermint essential oil)

**Table 1.** *In vitro* antimicrobial and antifungal activity of the studied samples (MIC in mg/mL)

| Microorganisms                                     | MIC  |              |            |              |
|--|------|--------------|------------|--------------|
|  | PEO  | PEO-chitosan | Amp.       | Fluc.        |
| <i>Staphylococcus aureus</i>                       | 1.32 | 0.95         | 0.01 [31]  | —            |
| <i>Pseudomonas aeruginosa</i>                      | 2.64 | 0.95         | 0.05 [31]  | —            |
| <i>Staphylococcus epidermidis</i>                  | 0.66 | 0.03         | 0.05 [32]  | —            |
| <i>Methicillin-resistant Staphylococcus aureus</i> | 1.32 | 0.95         | 0.05 [33]  | —            |
| <i>Escherichia coli</i>                            | 2.64 | 0.001        | 0.025 [31] | —            |
| <i>Candida albicans</i>                            | 0.33 | 0.36         | —          | 0.0023 [34]  |
| <i>Aspergillus niger</i>                           | 0.33 | 0.022        | —          | > 0.58 [34]  |
| <i>Penicillium spp</i>                             | 0.09 | 0.006        | —          | 0.00025 [35] |

PEO: peppermint essential oil,

MIC: Minimum Inhibitory Concentration

Amp.: Ampicillin, Fluc.: Fluconazole

Also, this could be attributed to the contribution of the chitosan units due to already known antibacterial properties [27,28].

The studied samples (PEO alone and PEO-Chitosan) exhibited also antifungal activity. When compared to the PEO alone, the results showed that the PEO-Chitosan has more potent antifungal activity against *Aspergillus niger* with the MIC value of 0.022 mg/mL and *Penicillium spp* with the MIC value of 0.006 mg/mL than that of PEO alone (Table 1). This could be attributed that the bioactivity of PEO-Chitosan enhanced through the activation of passive mechanisms of cell absorption [29]. However, the antifungal activity of PEO-Chitosan is not as potent as that of PEO alone with the MIC value of 0.36 mg/mL against *C. albicans*. The resistance of PEO-Chitosan to *C. albicans* may be the result of the weak interaction between cell membrane components (phospholipids) leading to the fungal plasma membrane impermeability [30]. The results coming from the currently used an antibiotic (applied concentration:0.124 mg/mL) and antifungal drug (applied concentration:1,4 mg/mL) were shown in Table 1 to compare with the studied samples [31–35].

### 3.3. Determination of the antiviral activity

The antiviral activities of the studied samples (PEO alone and PEO-Chitosan) against Herpes Simplex Type 1 and Human poliovirus Type 1 were compared to investigate the effect of the encapsulation of the essential oil and the results are summarized in Table 2. As can be seen from Table 2, it was found that the samples showed antiviral activities. PEO alone was found the more active than PEO-Chitosan with log reduction values of 3.75 for Human poliovirus Type 1 and 4.5 for Herpes Simplex Type 1. The same experiments exhibit the antiviral activity for the encapsulated PEO (PEO-Chitosan) as 3.25 log for Human poliovirus Type 1 and 3 log for Herpes Simplex Type 1. We might conclude that the PEO alone activated the immune system of host to some extent and induced the releases of immune factors [36].

**Table 2.** Antiviral activity of the studied samples

| Microorganisms        | Log reduction |              |
|-----------------------|---------------|--------------|
|                       | PEO           | PEO-chitosan |
| Herpes Simplex Type 1 | 4.5           | 3            |
| Polio                 | 3.75          | 3.25         |

PEO: peppermint essential oil

## 4. Conclusions

In the present study, a successful preparation of the conjugate of PEO into the chitosan via the immobilization method was carried out to improve the antibacterial and antifungal activity of PEO against bacterial and fungal pathogens. The biological actions of the PEO alone and its conjugate were determined separately. Results displayed the encapsulation process promoted an increase in antibacterial and antifungal activity *in vitro* by disrupting membrane integrity.

This research proved that combining biologically active components resulted in better efficacy. In summary, this conjugate is biologically active macromolecules that might be used as for new antimicrobial and antifungal agent and could be considered potential options instead of the currently available synthetic drugs. We observed that the employed essential oil had better activity against the studied viruses compared to the encapsulated derivative.

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