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Antimicrobial Properties of Free and Encapsulated Essential Oil of Rosemary in Chitosan

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Abstract

Pathogenic microbes are the most common cause of chronic infections, mortality in mammals, and loss of agricultural crops in the world. Antimicrobial agents, including antibiotics and antifungals, are often used in the treatment of infections due to their exceptional consequences, but they face various problems that limit their use. Therefore, herbal sources are attracting more attention due to lower side effects and, in some cases, better and faster effects. Plant essential oils (EOs) have many antimicrobial and pharmacological effects, but EOs are volatile, heat-sensitive, and water-insoluble compounds that limit their use. Encapsulating EOs can improve the properties of such compounds. Chitosan, a biodegradable nanopolymer, is very important in drug transfer due to its better encapsulation, controlled release, and low toxicity. Therefore, this research was conducted to investigate the encapsulation technique as a suitable method to preserve essential oils and increase their antimicrobial propties. First, the chitosan polymer was used to encapsulate rosemary essential oil after the obtained encapsulated product was characterized by the common techniques such as analytical techniques such as FT-IR, SEM, UV/Vis, XRD. Then, the antibacterial effect of encapsulated EO and free EO was investigated by the liquid microdilution method. The results showed that encapsulated EO had a greater antibacterial effect against gram-negative bacteria and fungi compared to free EO.

Key points

1. Using medicinal plants with antimicrobial properties and encapsulation with chitosan polymer can be a new method for treating infections caused by bacteria and fungi.
2. The results of this study show that the encapsulation of rosemary essential oil has significant results against Gram-negative bacteria and fungi and has the ability to become a new generation of herbal antibiotics.

Key word: Rosemary (Rosmarinus officinalis L.), Antimicrobial Effect, EO Encapsulated with Chitosan

Abbreviations

EO Essential oil
EOs Essential oils
GC-MS Gas Chromatograph Mass Spectrometer
RO Rosemary
ROEO Rosemary essential oil

1. Introduction

Infectious diseases are one of the great challenges of medical science in the 21st century, and as a result, the production of new antibiotics increases day by day. At the same time, the increasing spread of bacterial resistance to antibiotics has made the treatment of infectious diseases difficult and expensive. Therefore, nowadays, researchers are looking for herbal alternatives that, while having antibacterial effects, do not have side effects caused by the use of chemical drugs [1]. These problems have increasingly attracted the concern and interest of researchers, which has led to the study and use of plant essential oils with antimicrobial capabilities in various industries [2]. Increasing antibiotic resistance to bacteria and fungi and the side effects of using antibiotics have caused research on the effects of EOs on infection as a substitute for antibiotics to

be considered more than in the past [3]. In addition to the fact that the use of plant EOs has been customary for the treatment of many diseases since ancient times, today, through many research projects, the effects of EOs have been proven on many diseases. Also, it has been emphasized in various studies that plant essential oils can become a potential biological control agent against plant pathogens [4-7].

A lot of research has been done on the inhibitory effects of natural effective compounds against pathogenic microorganisms, and in this regard, it is necessary to use compounds that are non-toxic to humans and plants and do not have side effects. Using plant EOs as antibacterial and antioxidant additives is one of the methods used [8].

Plant EOs have many antimicrobial and pharmacological effects, but they are very volatile. Encapsulation of medicinal substances in polymer nanoparticles can improve the therapeutic effects of compounds that are often volatile [9]. For this reason, nanotechnology was used to increase its stability and make it water-soluble. Encapsulation is a process in which solid, liquid, and gas components are incorporated into small capsules, and their contents can be released at a controlled rate [10].

Encapsulation of plant-effective compounds in polymer nanoparticles can improve the therapeutic effects of such compounds due to the volatility of the EO of plants and its instability against environmental factors. Drug-carrying nanoparticles are usually made from materials such as chitosan, albumin, polylactic acid, polyethylene glycol, etc [11]. Chitosan is a polysaccharide similar to deacetylated chitin and is found in crustacean shells [12-13]. Also, chitosan is a biodegradable nanopolymer due to its better encapsulation, controlled release, and low toxicity, and it is very important in drug delivery [14]. Encapsulation of effective compounds in polymer nanoparticles can improve the therapeutic effects of such compounds due to the volatility of the EO of plants and its instability against environmental factors. It can also significantly increase the shelf life of the EO, thus making it possible to use the antibacterial properties of the essential oil for a longer period of time. Rosemary (RO) (*Rosmarinus officinalis* L.), belonging to the Lamiaceae family, dried flowers, and flower branches have at least 1% (volume/weight) of volatile oil [15]. The biological activity of this plant is mainly related to its volatile and phenolic compounds, such as carnosol, carnosic acid, and rosmarinic acid found in the extract, and alpha-pinene, bornylacetate, camphor, and eucalyptol contained in its EO. Also, its antimicrobial activity has been shown in various studies against bacteria such as *Escherichia coli* and *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and methicillin-resistant *Staphylococcus aureus (MRSA)*. Furthermore, Hossein et al. studied the antifungal properties of rosemary essential oil against six major plant pathogen fungi: *Cylindrocarpon destructans, Alternaria panax, Fusarium oxysporum, Botrytis cinerea, Sclerotinia nivalisth*, and *Sclerotinia sclerotiorum*, and their results showed that it has been active against all studied fungal strains [16-19]. Also, showed that rosemary essential oil reduced brain fungal infections caused by *Candida albicans* by inhibiting their growth in laboratory conditions [20]. In addition, ROEO has the ability to inhibit the growth of *Penicillium spp*. [21]. The antimicrobial mechanism of EOs is due to disrupting the cytoplasmic membrane and mixing the kinetic force and flow of protons and electrons, which is cellular active transport and also causes coagulation of intracellular contents and eventually cell death [22].The antibacterial properties of chitosan and ROEO have been documented in numerous articles, as explained above. This study looked into how ROEO's antibacterial properties could be enhanced when combined with chitosan to combat various human and plant harmful microorganisms.

2. Materials and Methods

2.1 Rosemary Essential Oil Extraction and Analysis of its Compounds

The RO plant was supplied from the center of Iran (Shiraz city, Fars province). For optimal extraction of the effective compounds of RO (*R. officinalis* L.), the distillation method with water and steam was used [23]. Fig. 1 and Table 1 show the quantitative analysis of the main active compounds of rosemary essential oil by the GC-MS method (in Dr. Soltani's factory, done in this section). According to the results of GC-MS analysis of ROEO, according to the amount, the most important compounds were: 1,8-cineole (12.8%), alpha-pinene (12.6%), camphene (9.4), limonene (8.4), camphor (7.35), borneol acetate (6.25), borneol (6.20%), and Linaloal (1.06), respectively.

Comparing the results obtained from GC-MS ROEO in this study with ROEO prepared in other studies shows that despite the similarities in the chemical compounds in the EO plant, the weather conditions, growing place, and altitude can lead to differences in these compounds. As reported by the chemical composition of EOs changes with various factors such as climate, soil composition, plant organ, age, vegetative cycle stage, and distillation condition. As reported by, the chemical composition of EOs changes with various factors such as weather, soil composition, plant organ, age, vegetative cycle stage, and distillation conditions [24].

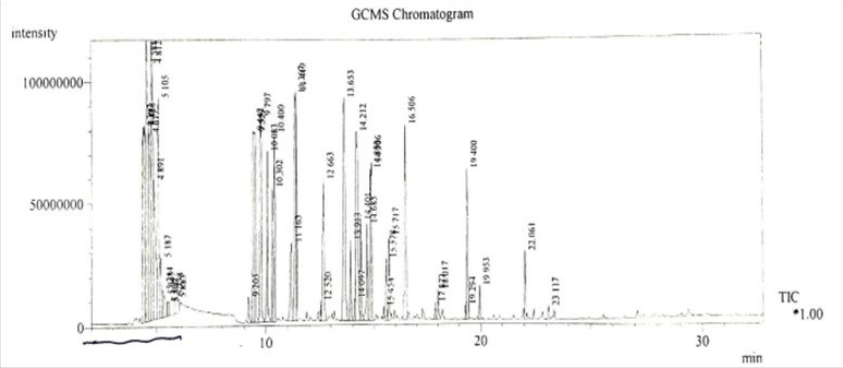

Figure 1: The Quantitative Analysis of the Main Active Compounds of ROEO By GC-MS Method

NO	Components	Percentage of components	Retention period (min)
1	Alpha-Pinene	12.6	9.793
2	Camphene	9.4	9.205
3	Limonen	8.4	10.32
4	1,8-cineol	12.8	11.340
5	Linalool	1.06	11.40
5	Camphor	7.35	13.653
6	Borneol	6.20	14.214
8	Borneol asetate	6.25	16.506

Table 1: The Main Chemical Composition of ROEO

2.2 The Preparation of ROEO Encapsulation

Chitosan, acetic acid, acetone, diethyl ether, dimethylformamide (DMF), dimethylsulfoxide, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Sigma-Aldrich, and ultrapure water was prepared from a MilliQ device. The preparation of ROEO encapsulation in chitosan was performed with a slight change from the method reported in the literature [25]. Finally, the morphology of EO encapsulated with chitosan was characterized by using analytical techniques such as Fourier transform infrared spectroscopy (FT-IR), Scanning Electron Microscope (SEM), and UV/Vis by comparing free RO and XRD analysis of the encapsulation. The results confirmed the presence of target compounds.

2.3 The Microorganisms Used in this Study are:

Nutrient agar and agar-bacteriological BBL Muller Hinton broth were prepared from Merck. All strains of pathogenic microorganisms were provided by the Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University (Istanbul, Turkey). Human pathogen microorganisms such as bacteria *Staphylococcus aureus (ATCC 6538), Escherichia coli (ATCC10536), Pseudomonas aeruginosa (ATCC 15442), Staphylococcus epidermidis (ATCC15442),methicillin-resistant Staphylococcus aureus (MRSA)*, fungi *Candida albicans (ATCC 10231), Aspergillus niger (ATCC 16404), Penicillium* spp. and also plant pathogenic microorganisms like bacteria of *Agrobacterium tumefaciens* and *fungi, Fusarium solani, Rhizoctonia solani, Aspergillus flavus, Macrophamina phaseolina* (the microbial collections of Yeditepe University) were isolated and characterized by MALDI-TOF (Bruker Microflex LT/SH). Assays were considered using the minimum inhibitory concentration (MIC) method [26].

3. Results

3.1 Encapsulation Results

3.1.1 Spectroscopy (FT-IR) Results

Based on the obtained results, in the FT-IR spectrum of chitosan powder, the peaks at 3357 and 3290 cm^{-1} are attributed to the NH2 and OH groups as they overlap with each other. The aliph.-CH tretchings and -N-H bending vibrations of primary amine (NH2) were observed at 2924, 2871 cm^{-1} and 1649 cm^{-1}. The peak originating from the saccharide structure of chitosan arises at 1062 cm-1 for -C-O sym. stretching (Fig. 2A).

Upon encapsulation of ROEO into the chitosan (ROEO & chitosan), the FT-IR spectrum of ROEO & chitosan shows peaks at 3359 and 3280 cm-1 inrespectively, respectively, indicating -OH and -NH stretchings. The C=O stretching, which is one of the main characteristic peaks coming from the ROEO components, shifted to 1652 cm-1. Also, the shifting of the -C-O sym. stretching to 1068 cm-1 proves the interaction of ROEO with chitosan (Fig. 2B). The FT-IR spectra of the assayed samples demonstrated the existence of the functional groups. In the FT-IR spectrum of ROEO, the band at 3457 cm-1 is attributed to the OH group, which is in some of the components of ROEO. The other characteristic peaks at 2958–2877 cm-1, 1745 cm-1, and 1082 cm-1, belonging to the aliph. -CH stretchings, C=O stretching, and -C-O stretching, respectively, were observed in the FT-IR spectrum of ROEO (Fig. 2C).

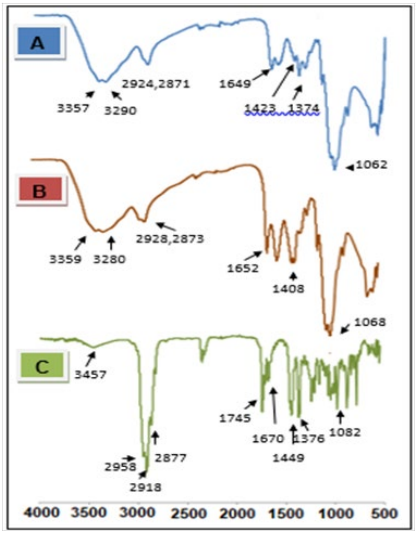

Figure 2: FTIR Spectra of A: Chitosan, B: ROEO Encapsulated with Chitosan (ROEO & Chitosan), C: Free ROEO (wavenumber cm^{-1})

3.1.2 UV-Vis Absorption Spectrum Results

The UV-vis absorption spectra of free ROEO and its chitosan capsulation were recorded in DMSO. In the UV/Vis spectrum of ROEO, there is a considerably intense band at λmax =298 nm, which can be attributed to the high contents of pinene, terpinene, and terpilonene. Upon the non-covalent immobilization of ROEO into chitosan, the absorption band was observed at 290 nm with a blue shift compared to the free ROEO, which can be explained by reducing the intermolecular electronic coupling of the ROEO in the capsulation [27].

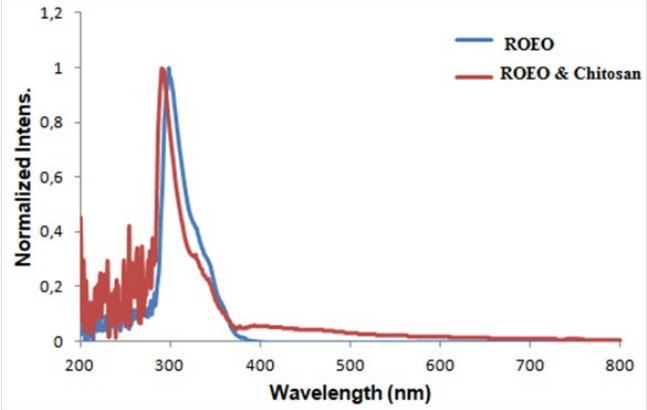

Figure 3: The Normalized Absorption Spectrum of Free and Encapsulated ROEO In Chitosan(ROEO & Chitosan)

3.1.3 SEM Electron Microscopy Results

The SEM micrograph (Fig. 4) shows an image of the dispersion of EO encapsulated by chitosan. ROEO revealed a rather uniform morphology. Minor irregularities such as air bubbles or oil Droplets significantly indicate macroscopic phase separation, and ROEO is well distributed in chitosan.

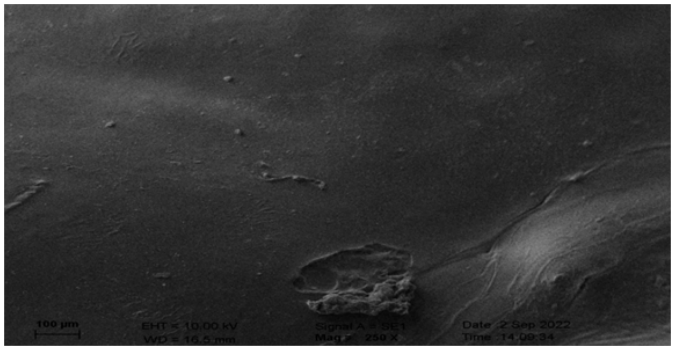

Figure 4: Scanning Electron Microscopy (SEM) Micrograph of the Encapsulated ROEO in Chitosan at a Magnification of 250x

The X-ray diffraction patterns of chitosan free in its rosemary essential oil-embedded form (RO EO&Chitosan) were recorded in Fig 5. The diffraction pattern of chitosan alone shows two peaks at 2θ=9.65o and 19.81o indicating a higher degree of crystallinity in chitosan, which fully agrees with the published report [28]. However, the diffraction spectrum of ROEO&Chitosan showed a broad peak at 2θ of 22° confirming the presence of essential oil within chitosan. The broadened peak usually results from imperfect crystal. This implied the incorporation of essential oil into chitosan, which may destroy the crystalline structure of chitosan [29].

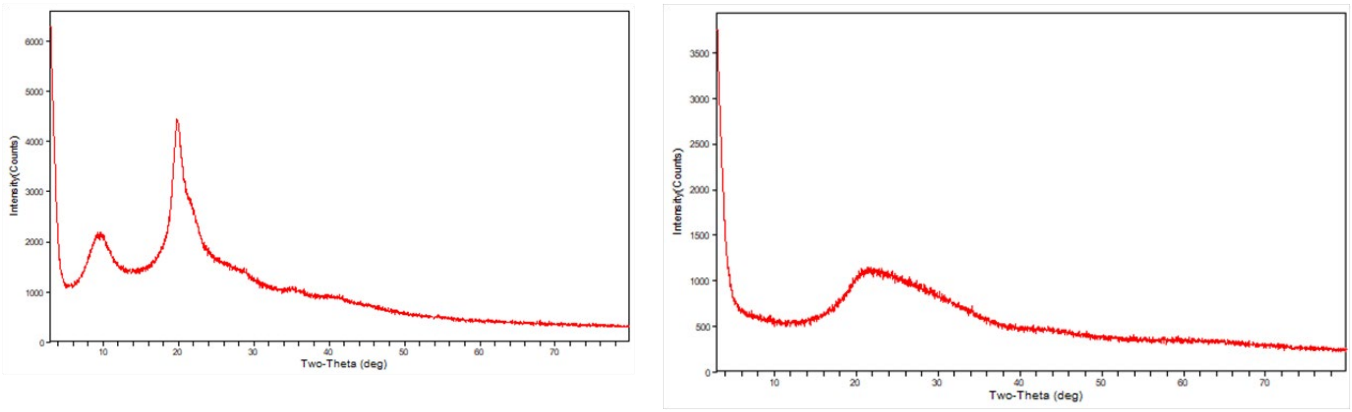

Figure 5: X-ray Diffraction Patterns of Chitosan (a) and ROEO&Chitosan (b).

3.2 The Results of the Antimicrobial Effect of Free Roeo and Roeo Encapsulated by the Minimum İnhibitory Concentration Method (Mıc)

According to the results, the most antimicrobial effect of free ROEO was obtained on the bacteria S. epidermidis (0.001 mg/ml), A. tumefaciens (0.003 mg/ml), and the pathogenic fungus C. albicans (0.009 mg/ml), respectively. Also, the lowest antimicrobial effect of ROEO was observed on bacteria such as P. aeruginosa and E. coli (0.78 mg/ml) and plant pathogenic fungi such as M. phaseolina (12.5 mg/ml), respectively. The results of the antimicrobial effect of the ROEO encapsulated on the studied pathogenic microorganisms showed the most antimicrobial effect on E. coli, A. tumefaciens (0.3 mg/ml), P. aeruginosa (0.78 mg/ml), and the plant pathogenic fungus R. solani (0.007 mg/ml), respectively. Also, the lowest antimicrobial effect was identified on S. epidermidis (14.84 mg/ml) (Tables 2 and 3).

The results of the antimicrobial effect of free ROEO and ROEO encapsulated by the minimum inhibitory concentration method (MIC).

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Sample	MIC (mg/ml)	
	Free ROEO	ROEO & chitosan
Staphylococcus aureus (+)	0.39	0.95
Staphylococcus epidermidis (+)	0.001	14.84
Methicillin-resistant taphylococcus aureus (MRSA) (+)	0.09	0.95
Pseudomona aeruginosa (-)	0.78	0.475
Escherichia coli (-)	0.78	0.30
Agrobacterium tumefaciens (-)	0.003	0.30
*(+) A positive sign indicates gram-positive bacteria * (-) A negative sign indicates gram-negative bacteria		

Table 2: The Results of Minimum Inhibitory Concentration (Mic) for Free ROEO, and ROEO Encapsulated in Chitosan on Some Pathogenic Bacteria's

Sample	MIC (mg/ml)	
	Free REO	ROEO &
Candida albicans	0.09	0.23
Aspergillus niger	0.39	0.02
Peniciliyum spp	0.39	0.01
Fusarium solani	0.39	0.01
Rhizoctonia solani	0.39	0.007
Aspergillus flavus	0.39	0.11
Macrophamina phaseolina	12.5	0.6

Table 3: The Results of Minimum Inhibitory Concentration (Mic) for Free ROEO, and ROEO Encapsulated in Chitosan on Some Pathogenic Fungi

Discussion

In general, according to the results of the present research, it is possible to point out the effect of free ROEO and ROEO & chitosan on inhibiting the growth of gram-negative and gram-positive bacteria, as well as on fungi. However, the antimicrobial effects of free ROEO and ROEO & chitosan were different against pathogenic microorganisms.

Based on the results of the MIC, the highest antimicrobial activity of ROEO & chitosan was obtained against gram-negative bacteria such as E. *coli* and *P. aeruginosa,* while free ROEO was more effective against gram-positive bacteria such as *S. epidermidis*. The results of the studies of Stojiljkovic et al. on the effect of different EOs on pathogenic microorganisms showed that the effect of essential oils on gram-positive bacteria is greater than that on gram-negative bacteria [30]. Also, showed the strong antibacterial effects of ROEO against a number of gram-positive bacteria, such as S. epidermidis *and* S. Aureus [31].

In general, ROEO has been more effective on gram-positive bacteria. Similar results were reported on *Salvia* L. species by Pierozan et al.[32]. However, RO contains phenolic compounds including carnosol, rosmarinic acid, caffeic acid, flavonoids including diosmin, luteolin, and genquanine, and monoterpenes such as camphor, cineole, and borneol. The antimicrobial activity of ROEO is due to the presence of volatile compounds 1, 8-cineole, camphor, ignol, and alpha-pinene in its structure, as well as the phenolic compounds carnosic acid and linalool [33]. According to the results of this research, the antibacterial activity of ROEO can be due to the presence of phenolic compounds and camphor, 1.8 cineole, and borneol derivatives, or the synergistic effect of the complex or part of its constituents may cause its microbiological effect. In various studies, the mechanism of action of phenolic compounds has been pointed out: through increasing the permeability of the cell membrane, they are able to destroy the outer membrane of bacteria (LPS) and cause the release of lipopolysaccharide, which protects against various stress conditions and antibiotics [34-36]. But the encapsulation of essential oil can destroy the integrity of the membrane of gram-negative bacteria and thus help to kill the cell, and compared to the free EO, it acts better to destroy them [37].

Bacteria appear to be less sensitive to the penetration of phenolic compounds (Burt 2004) due to the specific structure of their cell wall [38]. The findings of this research revealed the antimicrobial property of ROEO capsuled well; also, the encapsulated ROEO has a greater antibacterial effect against gram-negative bacteria and studied fungi compared to the free ROEO. It can be said that the transformation of EOs into the form of capsules containing them has increased the effectiveness of these compounds, especially in the long term when gradual release can be useful.

By encapsulating ROEO, its antifungal effect was improved against the pathogenic fungi of *A. niger, Penicilium* spp. *F. solani, R. solani, A. flavus* and *M. phaseolina* compared to free essential oil. Because of the conversion of bioactive compounds to the nanoscale, reducing their size and increasing their surface area, their effectiveness will increase [39].

Also, an important point in the synthesis of such compounds is the stability of the final product due to the various ROEO phytochemicals that are attached to the surface of chitosan as a covering agent.

Ethics Approval and Consent to Participate.

The study was approved by Department of Genetics and Bioengineering, Yeditepe University, Istanbul, Türkey. ALL participants signed informed consent before enrolment.

Competing Interests: The authors have no conflict of interest to declare

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Availability of data and material

Availability of Data and Materials: Some data that support the fndings of this study are available from the corresponding author upon reasonable request. The strains used in this work can be shared by exchanging MTA. Declarations Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

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