Egypt.J.Chem. **Vol. 63**, No.8. pp.3029-3039 (2020)

Egyptian Journal of Chemistry
http://ejchem.journals.ekb.eg/

Polyvinyl alcohol/thymol film for food packaging application: preparation, characterization and in vitro evaluation

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EVOLUTION of new materials that offer protection for active food ingredients is the focal point for food application research. Release of antimicrobial compounds from the packaging film is one of the common methods. In this research, an antimicrobial active packaging film based on poly(vinyl alcohol) (PVA) and thymol was prepared via freezing/thawing technique. The effect of thymol content on the property and structure of the film was characterized by Fourier transform infrared spectroscopy (FT-IR), Scanning electron microscopy (SEM), thermogravimetric analysis (TGA), X-ray diffraction (XRD), swelling and gel fraction measurements. Furthermore, the antimicrobial test shows that the prepared film has high antimicrobial effect against Gram-positive bacterial strains (*Bacillus subtilis*), fungal strain (*Candida albicans*), and Gram-negative bacterial strains (*Salmonella senftenberg* and *Escherichia coli*) in comparison with the control PVA film. The current approach verified that PVA film containing thymol could be applied as an alternative for food backing applications

Keywords: Poly (vinyl alcohol) (PVA); Freezing/thawing technique; Thymol; Antibacterial

Introduction

Currently, many studies focus on packaging materials from biopolymers to substitute plastic materials based – non-biodegradable [1,2]. Biopolymers have many properties like biodegradability, sustainability and biocompatibility [1]. Packaging material film can be acquired through two processes: a) physical crosslinking, i.e. freeze/thawing or b) chemical crosslinking, i.e. via a crosslinker agent (e.g. epichlorohydrin). The freeze/thawing method is a cost-free and safe for the environment as it does not need a crosslinking agent. Furthermore, freeze/thawing method has improved mechanical properties [3]. It includes the formation of ice crystals and phase separation together. During the freezing phase, ice crystals separate amorphous polymer pieces and polymer concentration in the surrounding environment increase, which lead to polymer microcrystalline development. The ice crystals dissolve when the gel thaws back to the room temperature, but the gel structure does not crash [4].

Poly (vinyl alcohol) (PVA) is a synthetic polymer (microcrystalline, hydrophilic). It can simply make hydrogen bonds with water molecules as it includes a big number of hydroxyl groups. PVA has been extensively used in many industrial applications for its excellent properties such as biodegradability and biocompatibility [5].

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Received ;28/04/2020; Accepted : 03/06/2020
DOI: 10.21608/EJCHEM.2020.28916.2623

PVA film formed via freezing/thawing process has gotten considerable attention in the last years, because of the notable characteristics of the freeze/thawed film like the absence of toxicity and the good mechanical properties [4, 6]. The film properties depend on the PVA concentration, the number of the freeze-thaw cycles, and the PVA's molecular weight [3, 7].

Thymol is one of the antioxidants phenolic compounds and it is categorized as GRAS by the FDA [8]. Thymol has shown several brilliant characteristics like a local anesthetic, anti-inflammatory, antioxidant, anticancer, pain relief, antifungal, and antibacterial properties [8]. Due to its extremely volatile nature and poor water-solubility lead to encapsulate of this compound by suitable methods to remove these challenges during their processing/practices [9]. The encapsulation of essential oils (EO) in different carriers can be controlled by different material design systems. Polymer-based-systems for EO encapsulation - like nanoemulsions, nanocapsules, micelles, nanoparticles, liposomes- are used to eliminate the EO shortcomings (low solubility and volatility) and to maintain its activity against environmental conditions (moisture, light, oxygen etc.) [10].

This study aims to fabricate films based on PVA and thymol via freeze/thawing method. Also, the evaluation of these films for food preservation application was done by studying its antibacterial activity against some Gram-negative, Gram-positive bacterial strains, and fungal.

Materials and Methods

Materials

Poly (vinyl alcohol) (average Mw = 72,000 g/mol, the degree of polymerization ~ 2800, the degree of hydrolysis 98.0–98.8 mol %) and thymol were obtained from Sigma–Aldrich Chemie GmbH, Riedstr., Germany. All the chemicals were used without further purification.

Methods

Preparation of PVA/thymol hydrogels membranes

PVA/thymol films were formed via freezing/thawing (F/T) technique [11]. PVA (8 g) was added to 50 mL water (distilled) and autoclaved for 20 min. at 120 °C. After that, complete the volume to 100 mL by ethanol. Different thymol concentrations were added to PVA solution at four ratios (Table 1). The blends stirred vigorously for 20 min. (at room temperature) to get a homogeneous solution. The solution was transferred to Petri dishes, subsequent by freezing at -20 °C for 20 h and thawing at 25 °C for 5 h, for four successive cycles. Finally, the films were dried at 30 °C (overnight). The films were stored in the refrigerator till used.

Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR spectra were acquired using Shimadzu FTIR-8400 S (Japan). KBr pellet method was used to collect the samples. Transmittance mode was applied to acquire the spectra at room temperature. The wavelength area was printed from 4000 to 400 cm^{-1}. Thirty scans were accrued for all spectra with a 4 cm^{-1} resolution.

X-ray diffraction (XRD) analysis

The crystalline structure of the films was investigated using X-ray diffraction (Shimadzu, USA, 7000, Cu-Kα radiation). The scanning range was 5 – 40° (2 θ) with a rate of 1.0° /min. The wavelength of radiation was 1.54 Å. The figures were obtained in the form of intensity (a.u) versus 2 theta chart.

Thermal Gravimetric Analysis (TGA)

TGA is a facility used to specify the best mass loss temperature for the material and is obtained as a function of temperature/time. The thermal performance of the films was performed using a thermal analyzer (Shimadzu 50, Japan). The TGA figure was acquired in 20 – 500 °C range under a nitrogen atmosphere with 20 mL/min as a flow rate and at 10 °C/ min as a heating rate. The chart was plotted with weight (mg) vs. temperature.

Microstructure Examination

The film surface morphology was observed via a scanning electron microscope (SEM) (JEOL, model JSM-6460LV, Japan) operated at an acceleration voltage of 10 kV. For this aim, the film pieces were covered with gold to improve the conductivity by the sputtering machine (JFC-1100E, JOEL Ltd., Japan), before being examined under the microscope.

Mechanical properties

Film thickness was measured using a digital micrometer (Mitutoyo, Japan). Six different sites on each film were measured and the average values were used for mechanical properties calculations.

Universal testing machine (Shimadzu, model AG-I 5 KN, Japan) was used to determine film elongation percentage at break (E) and tensile strength (TS) properties. The testing machine

TABLE 1. Preparation and compositions of the PVA and PVA/thymol film

Ingredient	Film designation				
	PVA	PVAT1	PVAT2	PVAT3	PVAT4
PVA (%) (w/v)	8	8	8	8	8
Thymol (%)	0	0.125	0.25	0.5	1

cross-head speed was set at 20 mm/min and initial grip separation was set at 3 cm.

Gel fraction determination

The vacuum oven was used to dry the obtained PVA film (at 50 °C for 12 h). The film -after drying- was weight (Wi). Then, it was soaked in water (distilled) for 24 h at 37 °C. Then, the film was dried another time at 50 °C and weight (Wd), which is the weight of the dry insoluble part of the sample after extraction with water. The gel fraction percentage (GF %) was measured by Eq. (1) [12].

Gel fraction (GF %) = ……… (1)

Tests were done in triplicate to reduce error and were described as an average value.

Swelling behavior

The swelling experiment is supposed to evaluate the material capacity to absorb solvents. Water was used as a solvent in this test. The film was cut into 1 cm X 1 cm parts and dried in the oven (at 40 °C for 10 h). Then, sample weights were determined (W_0) after drying. The dried samples were soaked in a falcon tube containing distilled water and incubated at 37 °C. At definite time intervals, the swollen samples were weighed (W_t) after erasure the undue surface water with filter paper. The water uptake was then calculated by Eq. (2) [13].

Water uptake (%) = …………… (2)

Where (W_t) represents the weight of the swollen film at a time (t) and (W_0) represent the initial weight of the film. Tests were done in triplicate to reduce error and were described as an average value.

Antimicrobial activity determination

The antibacterial activity of PVA film was assessed against Gram-negative (*E. coli* and *S. senftenberg*), Gram-positive bacterial strains (*B. subtilis*), and fungal strain (*C. albicans*) by the disk agar diffusion technique as earlier defined [14]. The bacterial and fungal strains were provided kindly by Arid Land Research Institute (ALRI), City of Scientific Research and Technological Applications (SRTA-City), Alexandria, Egypt. First, a Petri dish contains nutrient agar medium was inoculated with 250 µL 10^7 cfu ml^{-1} indicator bacteria. Then, the film was cut (10 mm X 10 mm) and sterilized with ethanol (70 %) followed by drying under the laminar flow air for 2 h. The film was located on the agar plates of the bacteria and fungi, and the plates were reserved in the refrigerator (for 4 h) to let the thymol diffusion first. Subsequently, the plates were incubated for 20 h at 37 °C. Finally, Petri dishes were removed from the incubator and the inhibition zones were measured using a caliper.

Results and Discussion

Fourier transform infrared spectroscopy (FT-IR) analysis

Fig. 1 shows the infrared spectra for PVA, thymol, and PVA/thymol (PVAT4) films. The bands related to blank PVA film were: the hydroxyl (O–H) group at 3304 cm^{-1}, C–H stretching vibration at 3064.9 cm^{-1} and 2956 cm^{-1}, C=O stretching vibration at 1660 cm^{-1}, C=C stretching vibration at 1539 cm^{-1} which are related to the non-hydrolyzed acetate groups, and C–C stretching vibration at 1230 cm^{-1}. These results confirmed by work of Oliveira et al., when used PVA and silver to prepare hydrogel for wound dressings applications [15].

The FT-IR spectrum of free thymol has distinctive peaks existing at 3213 and 2931 cm^{-1} corresponding to phenolic -OH stretching [16]. The distinctive C=C stretches in aromatics were detected at 1432 cm^{-1}. Also, there is a peak at 1230 cm^{-1} corresponding to C–C stretching vibration [17, 18]. The FT-IR spectra of PVA/thymol (PVAT4) film does not show considerable differences between blank PVA and free thymol. The basic variation was observed for –OH stretching peak, which indicates the interaction between thymol and PVA and this was confirmed by earlier work [17, 18].

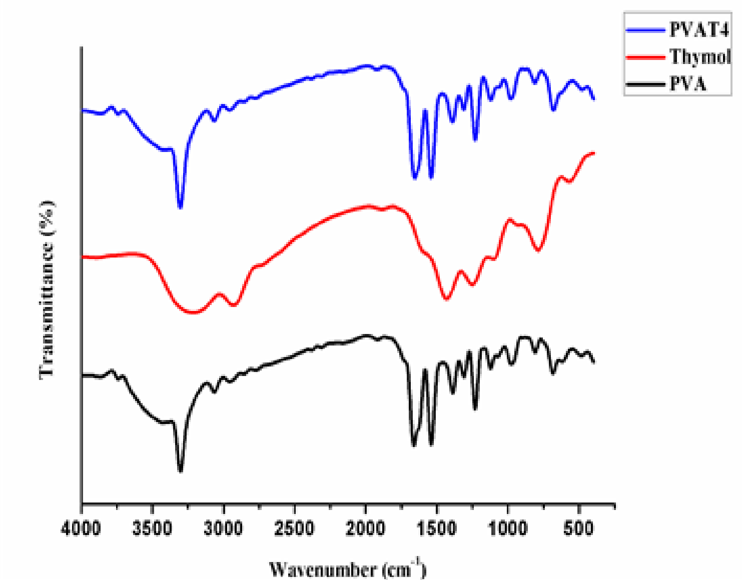

Fig. 1. FTIR spectra of PVA, PVA/thymol film and thymol (PVAT4: PVA 8% and thymol 1%).

X-ray diffraction (XRD) analysis

X-rd data of PVA and PVA/thymol (PVAT4) films and thymol is illustrated in Fig. 2. The figure demonstrates that the PVA has an amorphous structure [19]. The X-rd pattern show a peak at 2θ = 21.6° related to the (110) reflection. These results agree with the previous X-rd pattern for PVA [20]. The X-rd pattern of free thymol powder confirm the crystalline nature of the thymol powder as there are intense crystalline peaks at 2θ equals to 7.7°, 11.8°, 16.4°, 18.6°, 20.5°, and 25.3° [20]. X-rd graphs for PVAT4 film indicates that there are many peaks related to free thymol disappeared. This refers to thymol molecules are detached from each other by the implication in PVA polymer network and that prevent the development of thymol crystals. Moreover, X-rd graphs for PVAT4 film indicates that it has two peaks one related to amorphous part and one related to the crystalline part. These results strongly suggested the inclusion complexation between thymol and PVA molecules and these agree with work of Celebioglu et al., when used Thymol with cyclodextrin [18].

Thermal gravimetric analysis (TGA)

TGA demonstrates the stability issue of the substance at a high temperature in the absences or the presence of gas [21]. Free thymol TGA curve show one-step thermal degradation pattern as the thymol starts to evaporate at 50 °C and evaporated at around 163.1 °C (Fig. 3 and Table 2). In contrast, PVA and PVA/thymol (PVAT4) films have four phases: (1) in the range of 27.5 - 176.5 °C; mass loss corresponding to the water loss and thymol evaporation [17], (2) in the range 160.3 - 235.2 °C; mass loss due to decomposition of PVA molecules [18], (3) in the range of 235.7 - 411.6 °C and (4) in the range of 411.6 - 598.4 °C, refers to the complete degradation of PVA molecules. These results exhibit that the adding of thymol to the PVA polymer does not affect the thermal degradation behavior of it in an inert nitrogen atmosphere. But it would be predictable that a definite amount of thymol may be lost through the processing since materials are subjected to temperatures over the decomposition point of this additive. Therefore, the processing variables, in specific the temperature and the time, would be optimized to avoid the extreme evaporation of this additive incorporated to PVA and the consequent loss of it. These results are in agreement with work of Jimenez et al., when added carvacrol and thymol to polypropylene (PP) film for fresh food packaging [22].

Microstructure examination (SEM)

Morphology of the film (surface and cross-section) was explored by SEM which are displayed in Fig. 4. The surface of the PVA film (thymol free) is unsmooth as there are many rods shapes (Fig 4a), This roughness may come from drying conditions. While using the thymol change the film surface roughness as there are many micropores in the film (Fig. 4b). The size of these pores is a few microns and spread non-uniform in the film surface. These pores refer to the evaporation of thymol bubbles and bursting

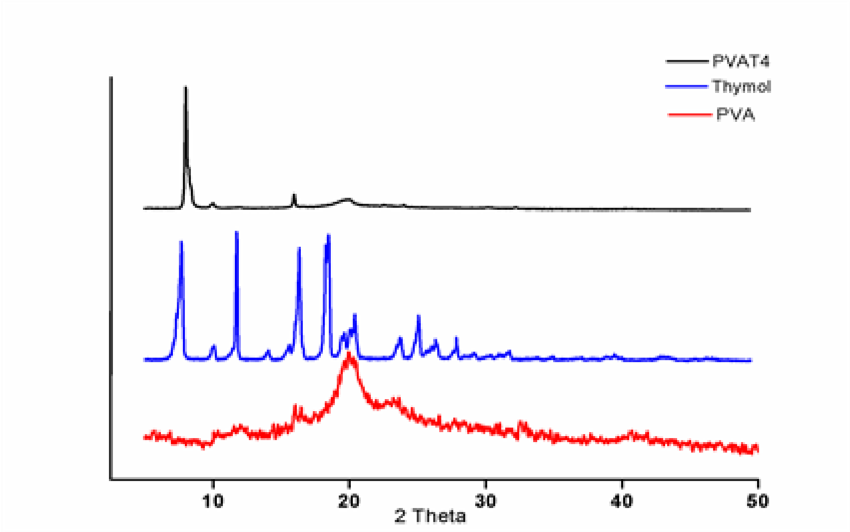

Fig. 2. X-ray diffraction (XRD) patterns of of PVA, PVA/thymol film and thymol (PVAT4: PVA 8% and thymol 1%).

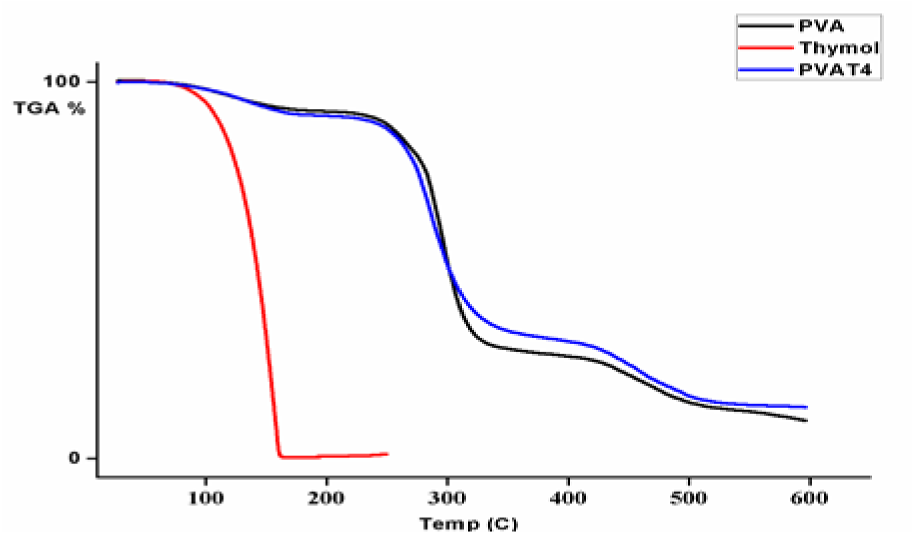

Fig. 3. TGA curve for of PVA, PVA/thymol film and thymol with heating Rate =10°C/min with continuous argon flow = 200 ml/mi (PVAT4: PVA 8% and thymol 1%).

TABLE 2. TGA phases (°C) for PVA and PVA/thymol film and thymol.

Film	TGA phases (°C)			
	1	2	3	4
PVA	27.5 - 160.3	160.3 - 235.9	235.9 - 422.6	422.6 - 599.9
PVAT4	27.8 - 176.5	176.5 - 219.7	219.7 - 411.6	411.6 - 599.4
Thymol	50.5 – 163.1	----	----	-----

in these locations [23]. The cross-section of PVA film is smooth and has no pores (Fig. 4c). But PVA/thymol (PVAT4) film cross section illustrates much microporosity which has non-uniform distribution as a result of evaporation of thymol bubbles (Fig. 4d). These results demonstrate that using thymol change the morphology of the film by making their surface rough. These results are in agreement with work of Koosehgol et al., when added thymol to chitosan [24].

Mechanical properties

The mechanical properties of PVA and PVA/thymol films are presented in Fig. 5. All films have low tensile strength compared to the great elongation percentage. The film tensile strengths are in the range from 35.3 to 40.7 N/mm^2 and elongation percentage between 269 and 351 %. The elongations at the break reduced with increasing thymol concentration as the thymol molecules affect the PVA crystallization and therefore samples with high thymol concentrations expected to present a lower elongation [25]. In conclusion, the elongation of PVA films was decreased and tensile strength nearly does not change by increasing thymol content. The addition of active materials into polymeric matrices showed different effects as published by previous studies. Sometimes there was a reduction in the mechanical properties like in case of starch/ clove /cinnamon powder and sometimes the mechanical properties improved [26, 27]. All results indicated that the active materials effect on the membrane's mechanical properties relies on kind and concentration of active materials, the interactions among the components, and the polymer matrix type [28].

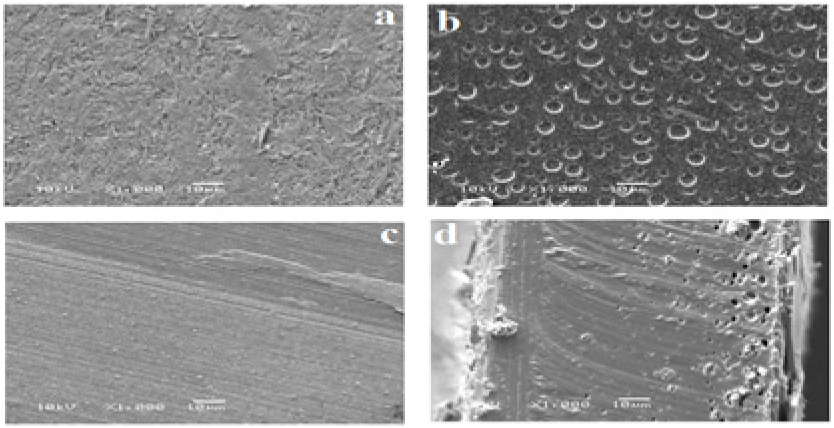

Fig.4. SEM micrograph of PVA, PVA/thymol film (PVAT4) (PVAT4: PVA 8% and thymol 1%).

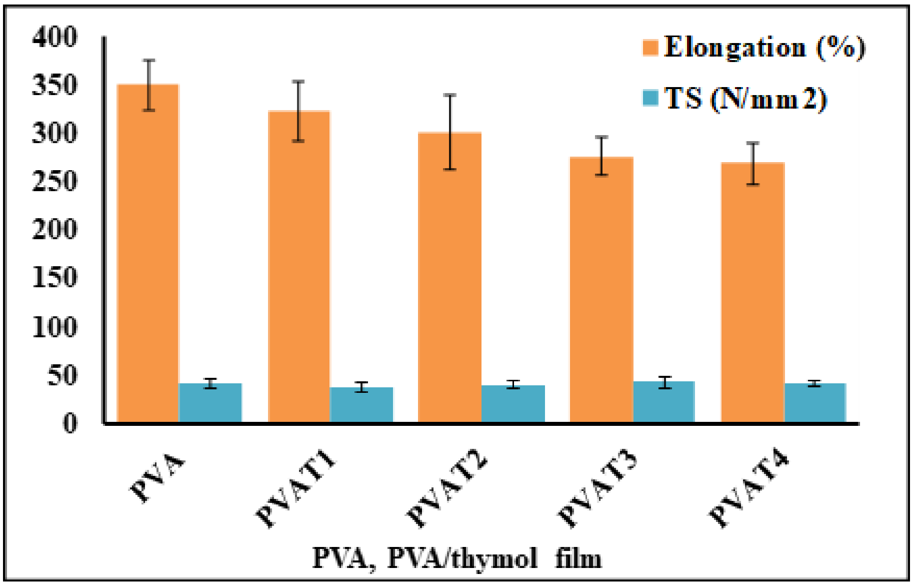

Fig. 5. Mechanical properties of of PVA, PVA/thymol film (PVA: PVA 8%; PVAT1:PVA 8% and thymol 0.125%; PVAT2:PVA 8% and thymol 0.25%; PVAT3:PVA 8% and thymol 0.5%; PVAT4:PVA 8% and thymol 1%)

Gel fraction determination

The variety in the gel fraction percentage of the prepared film with different thymol concentration (0.125, 0.25, 0.5, and 1 %) is presented in Fig. 6. The variation in gel fraction is insignificant with all concentrations of thymol, for example the gel fraction is 88.1 %, and this percentage become 89.4 % by using 0.125 % thymol. Moreover, the increase of thymol concentration more than that has no significant effect on the gel fraction percentage. For instance, the gel fraction percentage change from 89.4 to 81.8 % when using 1 % thymol. These results demonstrate that the addition of thymol to PVA film does not change the cross-linking network and this agrees with earlier work about PVA and k-carrageenan [4].

Swelling behavior

The rate of solvent permeation into the membrane matrix determines the swelling behavior of the film [29]. Table 3 demonstrates the water uptake percent for PVA and PVA/thymol films. It illustrates that all films behave similarly to the spongy material. For instance, after 8 h, all the films display good water uptake percent values from 60.9 to 84.4 % for PVA and PVAT4 film, respectively. These results attribute to the polymer chains that form strong hydrogen bonding between each other [30]. These intensive interactions between the polymer chains suggest a massive capacity to absorb big amounts of water and swell without dissolving in the solution [31, 32]. After 8 days, the water uptake percent nearly unchanged for all films. This means the water uptake reaches the equilibrium state after 8h [30, 31].

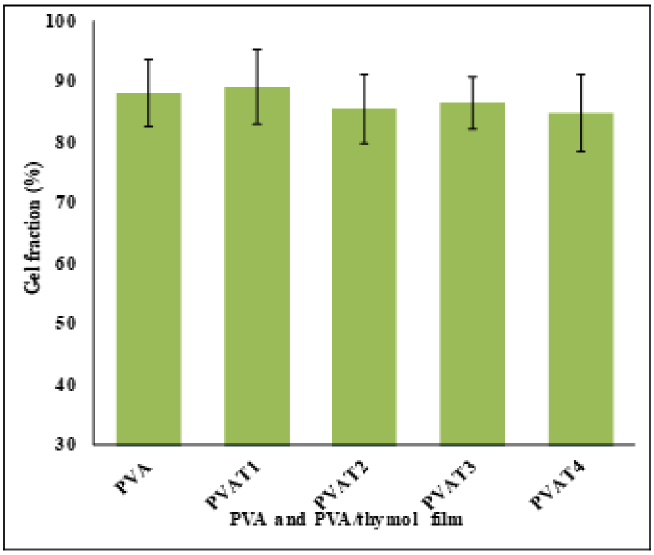

Fig. 6. Gel fraction of PVA, PVA/thymol film (PVA: PVA 8%; PVAT1:PVA 8% and thymol 0.125%; PVAT2:PVA 8% and thymol 0.25%; PVAT3:PVA 8% and thymol 0.5%; PVAT4:PVA 8% and thymol 1%)

TABLE 3. Swelling behavior of the PVA and PVA/thymol film

film	Time (h)				
	8	24	48	72	192 (8 days)
PVA	60.9 ± 0.76	66.5 ± 2.3	65.5 ± 2.6	66.5 ± 2.8	67 ± 2.9
PVAT1	63.3 ± 4.3	70.5 ± 5.2	66 ± 5.8	67.5 ± 5.8	71.5 ± 4.7
PVAT2	68.6 ± 4.6	74.7 ± 2.9	71.4 ± 3.7	73.9 ± 4.9	75.8 ± 4.70
PVAT3	74.9 ± 4	81.8 ± 44.9	79.7 ± 4.5	79.9 ± 3.9	80.5 ± 4.2
PVAT4	84.4 ± 2.3	84.6 ± 3.6	83.4 ± 5.3	85.1± 2.6	86.8 ± 2.8

Antimicrobial properties

The antimicrobial activities of the PVA and PVA/thymol are presented in Fig. 7 and Table 4. No inhibition zone is detected for the PVA membrane (control). Adding thymol to PVA film, even at the smallest concentration (0.125 %), causing inhibition of pathogenic bacteria and fungal. As the concentration increase, the antimicrobial activity improves significantly in a concentration-dependent manner. Fig. 7 shows the inhibition zones obtained from PVA/thymol film -containing 1 % thymol - against Gram-positive (*B. subtilis*), Gram-negative bacterial strains (*S. senftenberg* and *E. coli*.), and fungal strain (*C. albicans*). At all concentrations examined, *C. albicans* display the greatest inhibition zones values between 13.5 and 52 mm (including the film disc), followed by *E. coli* and *S. senftenberg*. The results demonstrate that the thymol can be magnificently added to the films and therefore released, thereby inhibiting the pathogenic microorganisms. These results agree with previous work about pullulan and Polypropylene films containing thymol [33, 34].

The mechanism of how thymol effect on the microorganism's growth is not completely well-known. But some investigations have exposed the effect of thymol on the microorganism's growth includes outer and inner membrane disruption, and interface with membrane proteins and intracellular targets [35]. Microorganism's membrane permeability is affected by these interactions. Also, cell membrane damaging is demonstrated through ethidium bromide cellular uptake, carboxyfluorescein and leakage of potassium ions [36, 37].

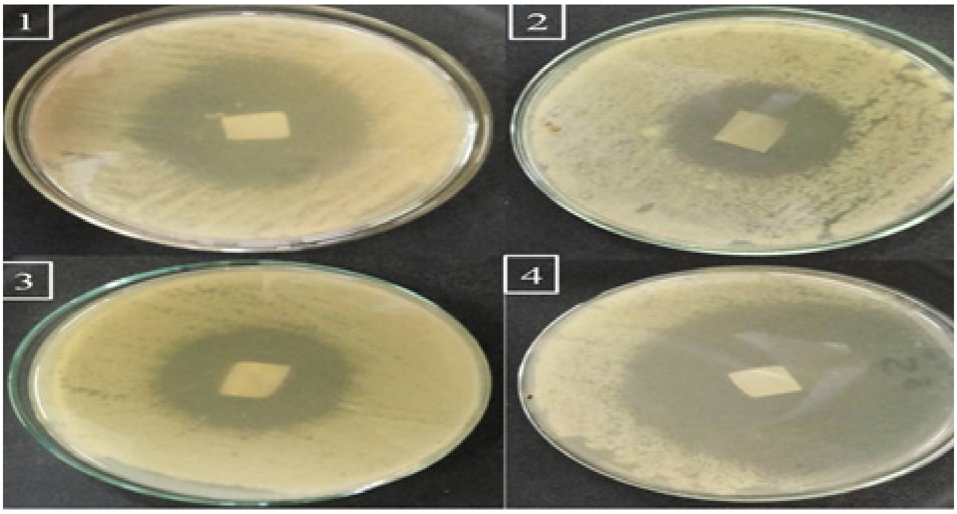

Fig.7. Antibacterial activities of of PVA, PVA/thymol film against S. senftenberg (1), B. subtilis (2), E. coli (3), and C. albicans (4).

TABLE 4. Antimicrobial activity expressed as the inhibition zone (mm) of the PVA and PVA/thymol film

Film	**Inhibition zone (mm)**			
	S. senftenberg	***C. albicans***	***E. coli***	***B. subtilis***
PVA	0	0	0	0
PVAT1	0	13.5 ± 0.9	11 ± 0.6	0
PVAT2	18 ± 0.4	18 ± 1.8	21 ± 1.3	0
PVAT3	28 ± 0.5	34 ± 0.8	29 ± 1.4	18 ± 0.8
PVAT4	33 ± 1.1	52 ± 1.5	40 ± 1.1	30 ± 0.5

Conclusion

The freeze/thawing method was successfully used as an easy method for preparing PVA/thymol film. XRD results indicated the presence of thymol in the film and the thymol inhibits the crystallization of PVA. The FT-IR analysis indicated no significant chemical interaction between thymol and PVA in the film. The SEM micrographs showed that by adding thymol there are many pores formed on the surface of the film than a control film. TGA showed higher thermal stability for PVA/thymol film than thymol alone. The films had antimicrobial activity against various bacterial and fungal strains. The antibacterial ability was significantly enhanced by increasing thymol content. It is concluded that the film containing thymol is suitable for food packaging application.

Acknowledgement

This research was supported by Shanghai Science and Technology Committee Project (18490740400), Open Foundation of Key Laboratory of Science & Technology of Eco-Textile (Eco-KF-201612), Opening project of Zhejiang provincial preponderant and characteristic subject of key university (traditional Chinese pharmacology), Zhejiang Chinese Medical University (ZYAOX2018035).

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