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Synergistic Antimicrobial Activities of Limonene with Mineral Carriers in LDPE Films for Active Packaging Application

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Abstract: The integration of essential oils (EOs) into polymers to endow antimicrobial properties has received a lot of attention. EOs are remarkable in that they have broad antimicrobial activity from a natural source while also being volatile. Their volatility, on the other hand, makes high-temperature processing techniques difficult to incorporate into polymers. In this study, active films based on low-density polyethylene (LDPE) and limonene essential oil (LEO) were prepared and characterized. Before incorporation of LEO into LDPE, vacuum pulling method was used to load the LEO into five different mineral carriers. All Carrier-LEO complexes were added into LDPE using melt compounding. The goal is to analyze the potential use of these formulations to achieve prolonged antimicrobial film packaging. The halloysite nanotubes (HNTs), kaolinite (Kao), mesoporous silica nanoparticles (MSNs), zinc oxide nanoparticles (ZnONPs), and molecular sieve type 4A (Z4A) were used as mineral carriers for limonene. The functional characterizations including mechanical, thermal, optical, barrier, and antimicrobial properties as well as limonene release behavior from the LDPE composite films were investigated. As expected, free limonene molecules acted as a plasticizer in the LDPE matrix. Thermogravimetric analysis (TGA) showed 20-25% of the initial limonene content was retained against thermal degradation in compounding and film making steps and its release from the films was efficiently delayed. A decrease in optical and oxygen barrier properties, as well as elastic modulus and tensile strength, was obtained for all developed films compared with neat LDPE. Significant antibacterial activities of the films were observed against *Escherichia coli DH5-Alpha* (*E. coli)* as a model gram-negative bacterial species. Moreover, the obtained results and the short-term and long-term release studies indicated that both HNTs and the MSNs due to their strong synergistic interactions with limonene exhibited sustained release profiles of limonene from LDPE films. Thus, these new active polymer composites present promising features in controlling microbial contamination, rendering them as excellent candidates in active packaging applications.

Keywords: Antimicrobial Properties, Limonene, Essential Oils, Antimicrobial Film Packaging, Mineral Carriers, LDPE, Melt Compounding

1. Introduction

A tremendous amount of attention has been directed towards polymeric materials with antimicrobial activity over the past decade. These polymers can greatly prevent the growth of most bacteria and fungi [1, 2]. Among these polymeric systems, polymers loaded with biocide materials or nanoparticles known as biocide-releasing polymers have been extensively studied [3, 4]. Natural active compounds like volatile essential oils are introduced as an interesting antimicrobial agent because of their diverse functionality, level of usage, and advantages in legislation and safety [5-7].

Limonene essential oil (LEO) is a volatile antimicrobial compound with an oily texture that widely exists in the citrus fruit peel and several plants [8, 9]. Limonene has major application prospects in antimicrobial packaging due to its wide-spectrum antibacterial and antifungal properties [10, 11]. Additionally, it is “generally recognized as safe” by FDA [12].

One of the challenges in the processing of polymer with essential oils is the high volatility of the oil resulted in losing the volatile compounds during processing at high-temperature and storage of the packaging products [13]. To overcome this drawback, many works have explored developing the active packaging systems with considering the retaining of these active agents in the polymer media and having control over their release for a specified time [14-16]. Moreover, different processing techniques such as solvent casting [17-19], encapsulation [20, 21], electrospinning [22-24], and supercritical CO_2 impregnation [25, 26] have been studied which are not the techniques suitable for industrial scales. Melting extrusion and compression molding are the most popular used techniques for the incorporation of active agents in the wide range of commercial polymers [26]. However, these techniques are limited because the volatile agents can be evaporated or degraded during the film production process at high-temperature condition.

In this sense, mineral carriers capable of retaining bioactive molecules by physical and chemical adsorption or interaction with them are promising materials for techniques in which high-temperatures are needed [27, 28]. Different release behavior and mass transfer mechanisms can be obtained because of the interactions between the volatile additive, carrier, and polymer. Saucedo-Zuniga et al. [16] studied the incorporation of thymol and orange essential oils into porous HNTs and modified montmorillonite (MMT) for pesticide and antimicrobial applications. It was reported that MMT has a stronger potential to adsorb thyme than orange oil due to the high polarity of cymene. Following this line, the synergic antimicrobial effect of volatile vanillin and aminofunctionalized mesoporous silica incorporated in PCL polymer films via melt blending has been investigated by Stanzione et al. [29]. In this case, active polymer films containing the embedded vanillin in the aminofunctionalized mesoporous silica carrier showed a slower antimicrobial release due to interactions between amine groups of mesoporous silica nanoparticles and vanillin aldehyde. Shemesh et al. [30] investigated the advantages of HNTs as a nano-carrier for encapsulating carvacrol and incorporation of this HNTs-carvacrol complex into LDPE films using melt compounding for sustained release of carvacrol. It was further found that HNTs can be employed as a nano-carrier to improve the thermal properties of carvacrol. Following this trend, Pajnik et al. [31] reported that the strong interaction between natural zeolite and phenolic groups of thymol led to significant retention of thymol in the polymer film. Ahmed et al. [32] evaluated the synergistic antimicrobial activity of cinnamon essential oil in combination with ZnONPs and synthesized silver–copper nanoparticles (Ag-Cu NPs) towards *S. typhimurium and L. monocytogenes*. The results demonstrated that cinnamon EOs and nano-carrier have no synergistic effects against the tested bacteria.

In this work, we aim to produce films based on LDPE with limonene-loaded in different mineral carriers, with the goal of developing sustainable antimicrobial packaging. For this purpose, first, LEO was loaded into carriers in a pre-compounding step via the vacuum pulling method. In the next step, the Carrier-LEO complexes were melt compounded with LDPE, followed by a hot press in order to produce active films. A comprehensive characterization including thermal, mechanical, optical, and barrier properties was performed. Finally, the antimicrobial performance of resulted films was investigated towards *E. coli* as a function of storage time.

2. Materials and Methods

2.1. Materials

Nova Chemicals' low-density polyethylene (LDPE) resin with an MFI of 2.3 g/10min and a density of 0.918 g/cm^3 was chosen as the matrix polymer. The limonene essential oil was supplied from Sigma–Aldrich (Darmstadt, Germany). *E. coli* was obtained from the University of Montreal. VWR International, LLC provided the Luria-Bertani (LB) medium and all other reagents required in bacteria cultivation. Five commercial mineral carriers were selected. Five commercial mineral carriers were selected. Some characteristics of the selected carriers are summarized in Table 1 while provided by the carrier suppliers.

Table 1. *Summary of the carriers used in this study with some of their properties.*

Nomenclature	Supplier	Density (g/cm^3)	Specific surface area (m^2/g)	Particle size
Halloysite Nanotubes (HNTs)	American Elements	2.2	50.8	Diameter <100 nm Length: 0.5-1.2 µm
Kaolinite (Kao)	BASF	2.58	11	2 µm
Mesoporous silica Nanoparticles (MSNs)	Sigma-Aldrich	2.2	80.7	200 nm
Zinc Oxide Nanoparticles (ZnONPs)	SkySpring Nanomaterials Inc.	5.6	30-35	10-30 nm
Molecular Sieve Type 4A (Z4A)	Sigma-Aldrich	0.4	800	2-3 µm

2.2. Preparation of Limonene-loaded Carrier

Vacuum pulling treatment was used to incorporate limonene into the different carriers according to a report by Abdullayev et al. [33]. Briefly, about 10.0 mL of limonene was fully mixed with 30.0 mL of ethanol by vortexing. Two grams of the carrier were added to limonene solution and dispersed by the ultrasonic cleaner. The suspension was evacuated using a vacuum rotary evaporator for 30 min at 30°C, and then cycled back to atmospheric pressure. This procedure was repeated 3 times at 30 min intervals. The limonene-loaded carrier was then dried in a vacuum oven at 40°C for 12 hours.

2.3. Preparation of the LDPE/Carrier-LEO Composite Film

LDPE/Carrier-LEO composite films were melt blended

under N_2 atmosphere at 180°C for 5 min at 50 rpm. After filling the internal batch mixer (Haake Rheocord 900, Germany) with LDPE pellets until they were completely melted (1 minute), the limonene-loaded carrier was introduced and mixed for 5 minutes. Then, for all compositions, a mixed compound was obtained, as presented in Figure 1. Neat LDPE also processed in the same conditions as a control sample. Finally, using a Carver hydraulic press, the various films were compression molded (USA). Mixed compounds were pressed for 5 minutes at 180°C under a 500 kPa pressure before cooling to room temperature over 3 minutes under a 550 kPa pressure. The final appearance of the films as presented in Figure 1 was semitransparent. For all samples, the concentration of Limonene was set at 20 wt% to meet the minimum inhibitory concentration (MIC) for pathogenic and spoilage microorganisms. The composition of the investigated blend nanocomposites and reference films is shown in Table 2. The film thickness was measured with a digital micrometre (ProGage Thickness Tester, Thwing-Albert Instrument Company, NJ, USA) at six random positions, and the mean value was reported. The average thickness of the films was discovered to be around 150 µm.

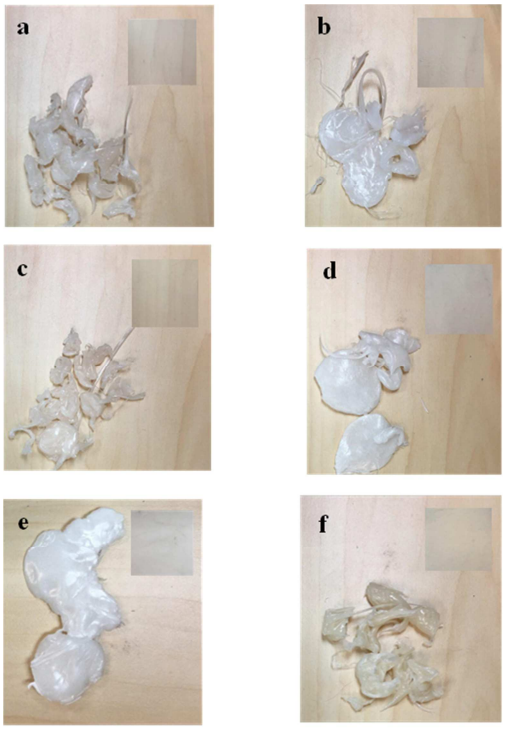

Figure 1. *The visual appearance of mixed compounds and final films: a) LDPE/LEO, b) LDPE/HNTs-LEO, c) LDPE/Kao-LEO, d) LDPE/MSNs-LEO, e) LDPE/ZnONPs-LEO and f) LDPE/Z4A-LEO.*

Table 2. *The composition of different blends.*

Films	Carrier (wt%)	Limonene (wt%)
LDPE	None	None
LDPE/LEO	None	20
LDPE/HNTs-LEO	5	20
LDPE/Kao-LEO	5	20
LDPE/MSNs-LEO	5	20
LDPE/ZnONPs-LEO	5	20
LDPE/Z4A-LEO	5	20

2.4. Thermogravimetric Analysis (TGA)

Thermal stability of the LDPE matrix and the additives content in the films were determined by TGA, using a Q500 (TA Instruments, New-Castle, DE, USA). Each LLDPE/LAM film (5–10 mg) was located in a platinum pan and heated under atmosphere containing pure nitrogen flow from 25°C to 800°C at a heating rate of 20°C /min. Universal Analysis V4.5A build 4.5.0.5 software was used to analyze the results with triplicate films for each sample.

2.5. Mechanical Characterization

Tensile tests were performed using an Instron 3365 (USA) tensile machine with a 5kN load cell at a cross-head speed of 50 mm/min, in accordance with ASTM D638 [34]. All film samples were conditioned for 24 hours at 23°C and 50% relative humidity before being tested. Five replicates of each sample were used to test tensile strength, modulus, and elongation at break. Five replicates of each sample were used to test tensile strength, modulus, and elongation at break.

2.6. Optical Properties

Optical characterization of the films in terms of haze values was carried out in accordance with the ASTM D 1003. Using a PerkinElmer LAMBDA 1050 spectrophotometer, three samples of each film were measured five times at slightly varied places on the surface.

2.7. Oxygen Permeability

According to ASTM D 3985, the permeability to oxygen was evaluated using a MOCON OXTRAN 2/21 (Minneapolis, USA) at 25°C, 0% relative humidity, and 1 atm pressure. The carrier gas for these experiments was a mixture of 2% hydrogen (H_2) and 98 percent nitrogen (N_2), and the test gas was 100% oxygen (O_2).

2.8. Short-term Release Studies

The accelerated migration of limonene from the films was studied by isothermal gravimetric analysis using a TGA-Q500 (TA Instruments, New-Castle, DE, USA) at a constant temperature of 60°C under nitrogen atmosphere for a duration of 10 h, after reaching the plateau (equilibrium).

The release of limonene is calculated with the mass loss for each sample at this temperature. This method is performed to measure limonene release from the films to the atmosphere, simulating the developed film's mode of use in real applications e.g., food packaging. Several methods have been used to measure the diffusion of essential oils in polymer matrices [35-38]. In this work, the limonene diffusion coefficient $D(m^2S^{-1})$ was estimated using Eq. 1 [35, 39]

$$\frac{m_t}{m_\infty} = 4\left(\frac{Dt}{\pi l^2}\right)^{1/2} \quad (1)$$

where, m_t and m_∞ are the amounts of limonene released from the film at time t and at equilibrium $t = \infty$, respectively, and l is the overall film thickness.

2.9. Antimicrobial Assays

E. coli bacteria were tested on all developed LDPE/Carrier-LEO films. *E.coli* was cultured in LB broth at 37°C for 24 hours with continual agitation to obtain a concentration of 10^8 colony forming units (CFU)/mL. After 24 hours, the bacteria culture was diluted 1:1000 with sterile LB to achieve a density of 10^5 CFU/mL. Antimicrobial test method based on ISO 22196:2011 explained by Abdali and Ajji [40] was used to investigate the performance of the active films. In summary, both active and reference films were cut out into 50 mm × 50 mm coupons and disinfected for 20 minutes using UV light. Each sample coupon was placed in a sterile Petri dish and covered with a sterilized cover plastic thin film [40 mm x 40 mm] for distributing 400 µL of the diluted bacteria culture. Both the active and cover films are rubbed against each other, contaminating the surface. All Petri dishes holding inoculated sample coupons were placed in the incubator for 24 hours at 37°C and 90% relative humidity. The next day, the bacterial inoculums thus placed on the surfaces were washed off with 1 mL PBS solution. Then, six serial PBS dilutions (10^{-1} to 10^{-6}) were carried out. Three samples were taken from each of the six dilutions and 10 µL droplets were applied onto the LB agar plates which were incubated overnight at 37°C for counting the surviving bacteria (CFU/mL). Each sample is made in triplicate, along with the negative control. To assess the dynamics of antimicrobial properties of films, all produced films were wrapped in aluminum foil and placed at 25 ± 2°C and 50 ± 2% relative humidity. Every 2 weeks until the 8th week, samples were taken out to study the influence of storage time on the antimicrobial performance of the active films.

2.10. Statistical Analysis

The OriginPro8 software was used to do an analysis of variance (ANOVA) and a multiple comparison test (Tukey) with a 95% significant threshold ($p \leq 0.05$). The data is presented as mean ± standard deviation.

3. Result and Discussion

3.1. Thermogravimetric Analysis

As the thermal processing for film production involves high-temperature treatments and long processing time, volatile compounds such as limonene may be degraded or evaporated [41]. Therefore, the limonene retention and distribution within the LDPE matrix plays a key role in the prolonged antimicrobial performance of the active films. Total limonene content in the developed films was determined using TGA, Table 3 summarizes the limonene content in active film samples before and after melt processing and film production.

The limonene concentration is found to fluctuate between 4.1-5.1 wt% in all generated films using Carrier-LEO, showing that 20-25% of the initial limonene content is kept during the high-temperature processing. Our findings are in line with those of Ramos et al. [42, 43] who used batch melt compounding and compression molding to obtain 25-40% carvacrol retention during PP/carvacrol film fabrication. It should be highlighted that limonene entrapment in both HNTs and MSNs was shown to have a considerable impact on the residual content of the films. Thus, it could be concluded that the HNTs and MSNs preserve the volatile limonene molecules during the harsh conditions of the compounding step.

HNTs with a tubular morphology by rolling-up of the layers can be employed as an ideal host material for loading of limonene as well as a thermal protector in case of degradation due to thermal stress. The limonene expelling from HNTs was slow because the limonene existed in narrow space between the layers. Compared to other carriers, MSNs exhibited also a higher loading capacity towards limonene due to its higher nonporous structure. Although Z4A has the highest porosity, the size and charge of the limonene as a bioactive compound are important variables in zeolite performance. These results showed that Z4A pore entrance size was not proper for limonene adsorption. The same results were derived by Shemesh et al. [30, 44] who reported that HNTs loaded carvacrol was necessary to achieve a high essential oil content in LDPE and polyamide films.

Table 3. *Limonene film samples with various carriers measured by thermal gravimetric analysis (TGA).*

Films	Pre-processing content of Limonene (wt%)	Post-processing content of Limonene by TGA (wt%)
LDPE	-	0.0 ± 0.0
LDPE/LEO	20	3.1 ± 0.1
LDPE/HNTs-LEO	20	5.1 ± 0.2
LDPE/Kao-LEO	20	4.3 ± 0.1
LDPE/MSNs-LEO	20	4.8 ± 0.1
LDPE/ZnONPs-LEO	20	4.2 ± 0.1
LDPE/Z4A-LEO	20	4.1 ± 0.1

3.2. Mechanical Characterization

Mechanical properties are important in packaging materials because they are linked to structural integrity, which is required to provide physical protection to the goods inside. The effect of Carrier-LEO incorporation on the mechanical properties of films was studied by measuring tensile strength (TS), elongation at break (% E), elastic modulus, and thickness and the results are summarized in Table 4. There were no remarkable deviation in the thicknesses of the films in these tests. The inclusion of additives has a significant impact on the mechanical properties of produced films; in particular, compared to the neat LDPE film, an overall drop in mechanical properties in

terms of TS and elastic modulus values can be observed. There was a modest increase in elongation at break in these samples. The plasticizing impact of limonene and the complex role of mineral carriers in nanocomposites are responsible for this phenomenon. Other authors have reported results that are similar to ours [45, 46]. Consequently, the mechanical performances of the produced films containing Carrier-LEO are improved by their synergistic effect with respect to LDPE/LEO films. In addition, the mechanical properties of the films containing HNTs-LEO and MSNs-LEO are improved in comparison to the other LDPE/Carrier-LEO films. The plasticizing impact of free limonene dissolved in the amorphous phase of LDPE/Carrier-LEO films is responsible for this behavior. These results revealed that the limonene fraction which is entrapped within the HNTs and MSNs carriers is higher than that entrapped within other carriers viz. LDPE/HNTs-LEO and LDPE/MSNs-LEO films contain more essential oil but are less plasticized. This research supports the findings of Krepker et al. [37] who found that polypropylene nanocomposite films containing carvacrol-loaded HNTs have superior mechanical properties to PP/carvacrol blends.

Table 4. *Mechanical properties of LDPE and LDPE/Carrier-LEO films.*

Films	Film thickness (µm)	Elastic modulus (MPa)	Tensile strength (MPa)	Elongation (%)
PE	150 ± 3	111.4 ± 15	15.1 ± 1.1	750 ± 50
LDPE/LEO	151 ± 1	90.3 ± 4	10.1 ± 1.2	800 ± 40
LDPE/HNTs-LEO	153 ± 3	104.2 ± 16	12.9 ± 0.7	780 ± 60
LDPE/Kao-LEO	150 ± 3	94.2 ± 8	11.8 ± 2.3	830 ± 80
LDPE/MSNs-LEO	152 ± 1	103.2 ± 17	12.1 ± 1.7	785 ± 20
LDPE/ZnONPs-LEO	153 ± 3	94.2 ± 5	11.1 ± 1.2	848 ± 90
LDPE/Z4A-LEO	151 ± 4	91.2 ± 6	10.8 ± 1.6	850 ± 70

3.3. Optical Properties

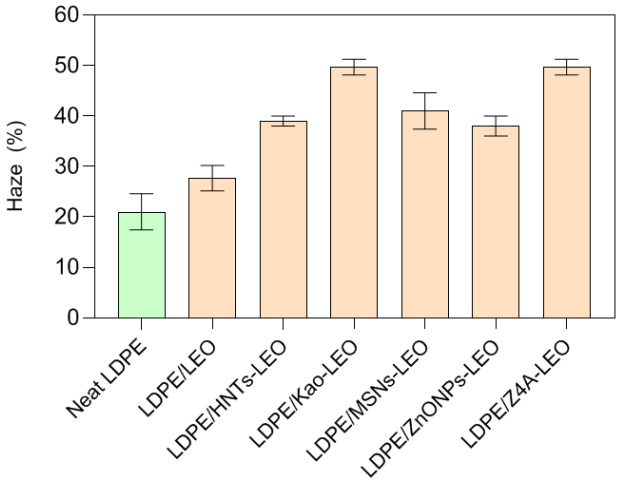

Figure 2. *Haze values for the neat LDPE and LDPE/Carrier-LEO films.*

Optical properties in nanocomposite films are of significant commercial importance. The addition of nanoparticles in a polymer matrix can negatively affect the haze value of the films, and this can affect the commercial potential of this class of materials for packaging applications [47, 48]. The haze values of all investigated films are reported in Figure 2. As it can be seen, the presence of additives raises the haze of the films. When making a comparison with the neat LDPE control film, the LDPE/LEO film sample had a little more haze as presented in Figure 2. On the contrary, both films containing Kao and Z4A showed a higher haze value than those containing other carriers and this is because of the presence of the larger particle size carries in films [49]. Moreover, films containing HNTs-LEO, MSNs-LEO, and ZnONPs-LEO showed approximately the same haze value for the same reason. Similar to our results, Druffel et al. [50] concluded that the haze of the PMMA films reduces by decreasing nanoparticle diameter.

3.4. Oxygen Barrier Properties

Oxygen diffusion into food through a package could accelerate bacterial growth; thus, barrier properties are important for protecting packaged foods from the environment and ensuring that they maintain their desired quality. The chemical structure and morphology, which are related to the composition and dispersion of fillers in the polymer matrix, have a strong influence on the gas barrier properties [51]. The results of oxygen permeability for the various developed films are presented in Figure 3. All limonene-containing films have higher OTR values than the neat LDPE reference film in general. This is probably is because of the plasticizing effect of limonene in films. Same results were reported by other authors [37, 45]. The films containing Kao-LEO, ZnONPs-LEO, and Z4A-LEO showed similar oxygen barrier properties with only a slightly higher oxygen barrier for the film containing Z4A-LEO. Conversely, films containing HNTs-LEO and MSNs-LEO presented significantly lower oxygen permeability compared with the other LDPE/Carrier-LEO films. This is most likely due to the superior efficacy of HNTs and MSNs in entrapping limonene molecules, which results in a decrease in free limonene content in films. These findings are consistent with TGA findings, which demonstrated that volatile limonene molecules are more efficiently retained into the HNTs and MSNs carriers instead of polymer matrix during the harsh conditions of film production steps. Our findings are in accordance with those of Solano et al. [46] who reported that a high concentration of oregano in the amorphous region significantly interfered with the polymer-polymer interactions, resulting in the increase in the OTR properties of the LDPE films.

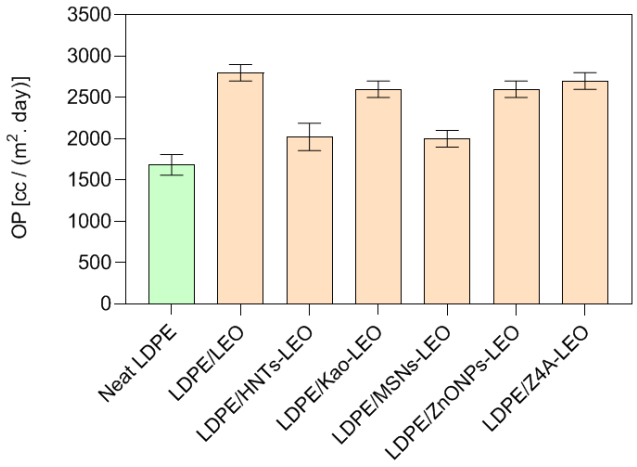

Figure 3. *Oxygen permeability of neat LDPE and LDPE/Carrier-LEO films.*

Table 5. *Estimated effective diffusivity values for limonene from developed films at 60°C.*

Films	Limonene diffusivity × 10^4 (m^2s^{-1})
LDPE/LEO	510 ± 63
LDPE/HNTs-LEO	360 ± 20
LDPE/Kao-LEO	430 ± 33
LDPE/MSNs-LEO	380 ±11
LDPE/ZnONPs-LEO	460 ± 25
LDPE/Z4A-LEO	470 ± 28

3.5. Short-term Release Studies

Perhaps the residual amount was actually below the minimum inhibitory concentration (MIC), insufficient to remove the bacteria examined, due to the release of active volatile chemicals from the LDPE films over time. As a result, knowing the limonene release kinetics and controlling its release from the film is crucial for establishing its antimicrobial effectiveness and prospective application as a packaging material [6, 52, 53]. TGA was utilized to characterize the limonene release from the various manufactured films by measuring weight loss over time at a constant temperature. The calculated diffusion coefficient values are presented in Table 5. The diffusivity of limonene in the LDPE/HNTs-LEO and LDPE/MSNs-LEO films is lower by 30% and 25% in comparison to LDPE/LEO, respectively. Thus, LDPE/HNTs-LEO and LDPE/MSNs-LEO films were observed to keep significantly higher limonene content than LDPE/LEO and the other LDPE/Carrier-LEO films. Results presented that the diffusivity of limonene in the LDPE/Carrier-LEO films is strongly influenced by the carrier type used to produce the films. The LDPE/LEO film had the highest effective limonene diffusivity, while the addition of Carrier-LEO reduced limonene out-diffusion from the developed films. These results are ascribed to the reduction of limonene desorption rate from the carrier's surface and forming a tortuous diffusion path in presence of mineral carriers in the LDPE matrix [54-56]. Krepker et al. [57] examined the effective diffusion coefficient of carvacrol in the presence of HNTs in the LDPE matrix and found similar results. Furthermore, the effective role of HNTs and MSNs as mineral nano-carriers, hindering the release of confined limonene molecules and reducing the effect of plasticizing, is attributed to the slower out-diffusion kinetics of limonene from the LDPE/HNTs-LEO and LDPE/MSNs-LEO.

3.6. Antimicrobial Property of Films

Antimicrobial studies were initially carried out using the ISO 22196:2011 method to verify the limonene content within the films. The results obtained are summarized in Table 6. Fresh films containing limonene had strong antimicrobial activity against *E. coli* bacteria, as expected. Because some strains of *E. coli* are widely spread foodborne pathogens, it was selected as a model bacterial species. All studied films reduced the *E. coli* density by approximately 5-6 logs (CFU/mL) in comparison with neat LDPE film. This output is consistent with the TGA results, which confirmed the presence of limonene in the studied films.

Table 6. *Log reduction of LDPE/Carrier-LEO films towards E. coli.*

Films	Log reduction by viable cell count (CFU/mL)
LDPE	None
LDPE/LEO	6.0 ± 0.20
LDPE/HNTs-LEO	6.0 ± 0.10
LDPE/Kao-LEO	5.0 ± 0.10
LDPE/MSNs-LEO	5.5 ± 0.10
LDPE/ZnONPs-LEO	5.0 ± 0.20
LDPE/Z4A-LEO	5.5 ± 0.30

3.7. Effect of Storage Time as Long-Term Release Study

These tests are being carried out to see how storage time affects the antimicrobial efficiency of the produced films and to evaluate the bio-functionality of limonene after processing. Figure 4 demonstrated the results of these experiments for studied films versus storage time. All fresh films registered 5-6 logs (CFU/mL) reduction for *E. coli*. Antimicrobial activity of LDPE/LEO, LDPE/Z4A-LEO, and LDPE/Kao-LEO films decreased by two logs (CFU/mL) after two weeks of storage, while LDPE/HNTs-LEO and LDPE/MSNs-LEO films remained unchanged. After 6-week storage, LDPE/LEO film has completely lost its antimicrobial activity; while, the films containing MSNs-LEO and HNTs-LEO have kept their activity by 2 and 3 logs (CFU/mL) reduction respectively, and the rest of the films showed less than 1 log reduction. Finally, all developed films lost their antimicrobial activity within 8-week of production, while LDPE/HNTs-LEO film has preserved its antimicrobial activity. The film remained active for another 30 days, but with a 2 log reduction in *E. coli*. Thus, these results showed that the storage time has an extreme effect on the antimicrobial potency and further emphasize the advantages of using HNTs-LEO and MSNs-LEO synergistic mixtures rather than the individual limonene or other Carrier-LEO combinations to achieve a controlled release rate over a prolonged period. Similarly, In comparison to LDPE-based films containing the individual essential oils, Krepker et al. [58] found that LDPE films containing this composition exhibited excellent enduring antimicrobial activity against *E. coli* due to

synergistic interactions between carvacrol and thymol essential oils entrapped within HNTs. These results are also evident that the entrapment of limonene into HNTs and MSNs enables slow release of limonene function over long periods of time and are consistent with the results from short-term release analysis of the studied films.

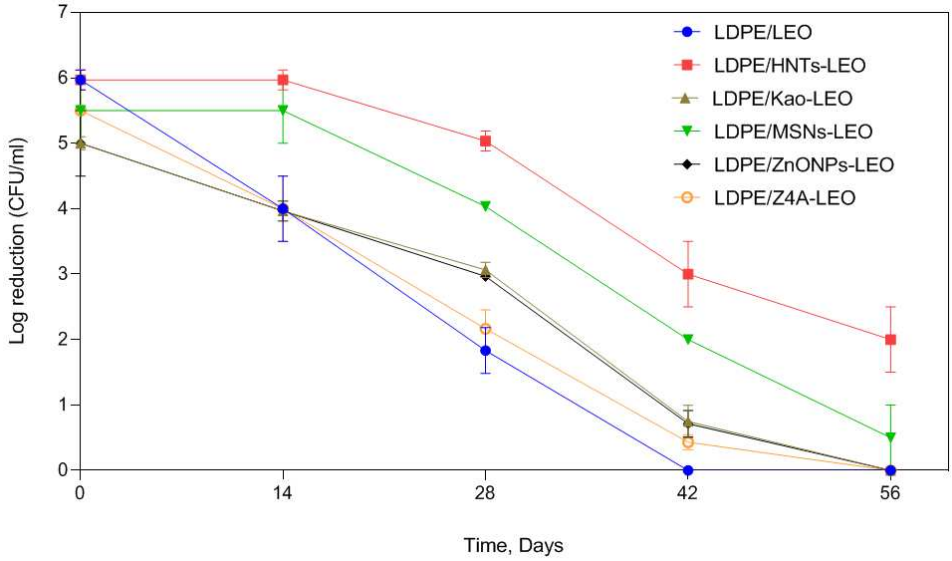

Figure 4. *Antimicrobial properties of LDPE/Carrier-LEO film samples towards E. coli as a function of storage time.*

4. Conclusion

In the present study, LDPE/Carrier-LEO films were produced by incorporating limonene, loaded into the different mineral carriers, via batch melt compounding. To assess the effect of these Carrier-LEO combinations in the LDPE matrix and their material stabilization performance during processing, different analytical techniques were used to characterize active films. The addition of these Carrier-LEO systems was found to affect the optical, oxygen barrier, mechanical, and long-term antimicrobial properties. The inclusion of free limonene in the LDPE matrix as a plasticizer causes poor mechanical and barrier properties, as well as rapid limonene release from the films. The high amount of limonene retained within HNTs and MSNs carriers produced superior prolonged antimicrobial films against *E. coli* in comparison with other carriers, revealed strong synergistic interactions between these carriers and limonene due to their morphological characteristics and high porosity. As a result of this synergistic interaction, desired antimicrobial performance can be achieved with lower active component concentrations and slower limonene molecule out-diffusion, thus leading to the potential use of these systems for numerous applications, such as antimicrobial active packaging, medical and hygiene.

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