International Journal of Food Microbiology 428 (2025) 110994

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Starch-octenyl succinic anhydride nanoemulsions with clove and white thyme essential oils: *In vitro* antifungal activity and application on orange (*Citrus sinensis* cv. Salustiana) preservation

Estefania Júlia Dierings de Souza[a,*], Dianini Hüttner Kringel[a], Virginia Campello Yurgel[b], Cristiana Lima Dora[b], Michele Greque de Morais[c], Eliezer Avila Gandra[d], Rufino Fernando Flores Cantillano[e], Alvaro Renato Guerra Dias[a], Elessandra da Rosa Zavareze[a,*]

[a] Department of Agroindustrial Science and Technology, Federal University of Pelotas, Pelotas, RS, Brazil
[b] LabNano - Nanotechnology Laboratory, Federal University of Rio Grande, Rio Grande 96203-900, Brazil, RS, Brazil
[c] Laboratory of Microbiology and Biochemistry, College of Chemistry and Food Engineering, Federal University of Rio Grande, Rio Grande, RS, Brazil
[d] Center of Chemical, Pharmaceutical and Food Sciences, Federal University of Pelotas, 96010-900, Pelotas, RS, Brazil
[e] Brazilian Agricultural Research Corporation, Embrapa Clima Temperado, 96010-971 Pelotas, Brazil

ARTICLE INFO

Keywords:
Penicillium digitatum
Green mold
Postharvest control
High-pressure homogenization

ABSTRACT

Starch modified by octenyl succinic anhydride (OSA) is a polysaccharide that can be used as a stabilizer in the development of emulsions added with essential oils (EOs). The objective of this study was to develop nanoemulsions based on starch-OSA containing clove essential oil (CEO) and white thyme essential oil (WTEO) and a proportional mixture of the two EOs (CWTEO) using high-pressure homogenization. The emulsions were characterized by particle size, zeta potential, polydispersity index, stability during 150 days, and antifungal activity, with inhibition of mycelial growth, against the fungus *Penicillium digitatum*. The addition of WTEO and CWTEO in concentrations of 1.5 and 2 % allowed the formation of stable nanoemulsions, with particle sizes ranging from 72 to 293 nm. These nanoemulsions presented the potential to reduce the mycelial growth of *P. digitatum* (100 % for nanoemulsion with 2 % WTEO and 80.5 % to 2 % CWTEO) up to 150 days of storage. A nanoemulsion containing 2 % WTEO was applied to orange fruits, and its antifungal potential was evaluated *in vivo*. This nanoemulsion was able to reduce the incidence of rot caused by *P. digitatum* in oranges, demonstrating their potential for application as an alternative to synthetic fungicides to reduce postharvest losses in citrus fruits.

1. Introduction

Citrus fruits are one of the most commercialized fruit crops worldwide due to their high consumption, which is attributed to their recognized nutritional value. However, during the storage period, from harvest to fresh consumption, these fruits are highly vulnerable to contamination by fungi, such as *Penicillium digitatum*, the causative agent of “green mold,” responsible for 90 % of total postharvest losses of production in tropical and subtropical regions (Guo et al., 2023; Vu et al., 2023). Currently, the control of this fungal disease mainly adopts chemical agents; however, the extensive and long-term use of these agents has evidently increased the resistance of pathogenic fungi to drugs, in addition to causing harm to the environment and human health (Yao et al., 2023). In the current context, researchers face a challenge in finding highly efficient and minimally toxic natural alternatives. This effort is aligned with the objectives outlined by the Food and Agriculture Organization of the United Nations (FAO) for the 2030 Agenda, which includes “reducing food losses along production and supply chains, including postharvest losses” and “adopting sustainable production and consumption practices” (FAO, 2023).

Thus, several studies have explored the potential use of essential oils (EOs) as an alternative to chemical control measures due to their safety

* Corresponding authors.
E-mail addresses: estefaniajulia.dierings@gmail.com (E.J.D. Souza), dianinikringel@hotmail.com (D.H. Kringel), virginia.yurgel@gmail.com (V.C. Yurgel), cristianadora@gmail.com (C.L. Dora), michele.morais@pq.cnpq.br (M.G. Morais), gandraea@hotmail.com (E.A. Gandra), fernando.cantillano@embrapa.br (R.F.F. Cantillano), alvaro.guerradias@gmail.com (A.R.G. Dias), zavareze.er@gmail.com (E.R. Zavareze).

https://doi.org/10.1016/j.ijfoodmicro.2024.110994
Received 25 July 2024; Received in revised form 10 November 2024; Accepted 24 November 2024
Available online 26 November 2024
0168-1605/© 2024 Elsevier B.V. All rights reserved, including those for text and data mining, AI training, and similar technologies.

and efficiency, in addition to their bioactive properties (Li et al., 2024). EOs are aromatic compounds of natural origin with broad-spectrum biological activities such as antioxidant, anti-inflammatory, anti-allergic, antibacterial, antiviral, and antifungal activity, among others (Chen et al., 2019; Dávila-Rodríguez et al., 2019). Among the EOs recognized for their antifungal properties, essential oils of clove (CEO) and white thyme (WTEO) stand out (Chen et al., 2019; He et al., 2016). The antifungal action of these EOs is due to the presence of their main constituents: eugenol in CEO and thymol in WTEO, responsible for inhibiting the synthesis of ergosterol, a specific component of the cell wall that is considered vital for maintaining cellular health, integrity, viability and growth of fungi (Abdi-Moghadam et al., 2023; Gengatharan and Rahim, 2023; Singh and Pulikkal, 2022).

Despite the consolidated benefits, the direct application of EOs is limited due to certain disadvantages, such as the formation of cellular injuries when applied in high concentrations, in addition to undesirable sensory changes in the products where they are used (Franklyne et al., 2019). Furthermore, due to their high volatility, the concentration of bioactive compounds shows a drastic reduction and consequent loss of their bioactive properties (Liu et al., 2021). One of the promising methods for the efficient use of EOs is their use in an emulsion system. The encapsulation of EOs in these systems is an alternative to increase their stability and solubility. Furthermore, encapsulation in nanoemulsion systems can effectively prolong the action of EOs, ensuring that the active components are preserved in the nanoemulsion matrix and promoting a slow and controlled release, which extends their activity time and, consequently, their antifungal action (Tian et al., 2023).

Nanoemulsion is a kinetically stable colloidal system in which fine droplets (in the range of 10–100 nm) of one component (dispersed phase) are dispersed in the medium of the other component (dispersion medium/continuous phase) (Franklyne et al., 2019; Singh and Pulikkal, 2022). The choice of a suitable surfactant allows the formation of nanoemulsions that are stable for an extended period of storage. In this sense, starches modified by octenyl succinic anhydride (OSA) stand out as alternatives to traditionally used surfactants. They are good candidates for stabilizing oil-in-water emulsions because of their excellent emulsifying properties, stability against environmental factors, and consumer-friendly labeling (Rehman et al., 2020; Sharif et al., 2017b; Sharif et al., 2017a). In addition to the surfactant, the method used to reduce the particle size of an emulsion can interfere with the stability of the encapsulated material. High-pressure homogenization is worth highlighting, a high-energy process used to prepare nanoemulsion (Liu et al., 2021) which has the advantage of forming nanoscale emulsions that do not depend on the hydrophilic-lipophilic balance of the components; in this way, small particles with uniform particle size distribution and good stability can be prepared (Franklyne et al., 2019; Wan et al., 2019).

In the literature, there are several reports of the *in vitro* antifungal action of CEO and WTEO against postharvest fungi, such as *Penicillium digitatum*. However, its *in vivo* efficacy in citrus fruits has been little reported; some examples are the studies of He et al. (2016), who evaluated the ability of CEO microemulsions to control green mold in navel oranges through direct contact and vapor phase; Pinto et al. (2021) who evaluated the effect of red thyme EO on the shelf life and fungal contamination of oranges during refrigerated storage; and Zhang et al. (2023) who evaluated the antifungal effects of thymol (majority compounds found in thyme EO) both *in vitro* and applied to citrus fruits.

Our study hypothesizes that the starch-OSA polymer will be able to maintain stable emulsions, prepared with different concentrations of clove and white thyme essential oil. Considering the benefits already described for CEO and WTEO, combined with the advantages of encapsulation in preserving the bioactive properties of EOs, this study aimed to evaluate the stability of nanoemulsions containing CEO and WTEO, their antifungal efficacy in inhibiting *Penicillium digitatum in vitro*, and their effectiveness in controlling green on oranges fruits (*Citrus sinensis* cv. Salustiana).

2. Materials and methods

2.1. Materials

White thyme essential oils (WTEO) (thymol, 22.7 %; terpinolene, 16.4 %; linalool, 15.9 %; p-Mint-1,3,8-triene, 11.7 % and carvacrol, 8.4 %) and clove (CEO) (eugenol, 85.9 % and caryophyllene, 13.0 %) were obtained from the companies Ferquima S/A and Dierberger Essential Oils. Starch-OSA (CAPSUL®, extracted from waxy maize, suitable for food-grade use according to FCC (Food Chemical Codex) requirements) was donated by Ingredion. Tween 20 (CAS 9005-64-5), capric/caprylic acid triglyceride (TGC) (CAS 7338), Potato Dextrose Agar (CAT K25–1022, Kasvi). The fungus *Penicillium digitatum* was obtained by isolation from infected citrus fruits (Souza et al., 2024). The other reagents used for the analysis were of analytical grade.

2.2. Preparation of nanoemulsions

Initially, starch-OSA (10 %, *w/v*) was dissolved in distilled and filtered water at room temperature (22 ± 2 °C) with magnetic stirring for 30 min. Afterward, the solution was filtered with the aid of a vacuum pump (Buchi brand, model V-700) using a cellulose nitrate filter (pore size 8 μm). The aqueous phase (AP) was prepared by adding tween 20 (1 %, w/v) to the filtrate, which remained under magnetic stirring for 5 min. The oil phase (OP) was composed of different concentrations (1, 1.5, and 2 %, w/v) of clove essential oil (CEO), white thyme essential oil (WTEO), and a mixture of the clove and white thyme essential oil (CWTEO) in a 1:1 (*w*/w) ratio.

The previously prepared AP (30 mL) was poured into the OP under magnetic stirring at 1000 rpm for 30 min. After this stirring time, the formulations were subjected to a high-speed homogenizer (Ultra-turrax®T10 basic, IKA) at 14,500 rpm for 3 min, followed by 10 cycles of 40 s (totaling 6 min) in HAP (EmulsiFlex- C3, Avestin) at 10,000 psi. All formulated nanoemulsion systems were stored at 20 ± 2 °C to monitor stability and sent for subsequent analysis. The parameters described above, such as starch-OSA concentrations, % EOs, and solution homogenization times, were determined in preliminary tests (data not shown).

2.3. Stability of nanoemulsions

The stability of nanoemulsions was evaluated through analysis of phase separation, particle size distribution, polydispersity index, and zeta potential. All analyses were carried out on the day following nanoemulsion formation and after 30, 60, 90, 120, and 150 days of storage (20 ± 2 °C). Formulations that showed visual phase separation during the storage period were not evaluated in items 2.3.2 and 2.5.

2.3.1. Phase separation

Phase separation was performed according to Biduski et al. (2019), with adaptations. Nanoemulsions were evaluated by foaming or sediment formation observed. Glass tubes were filled with nanoemulsions, and the foam formed in the upper layer of the tubes or sedimented material was visually observed and recorded on macroscopic photography. The photographs were numbered from 1 to 10 according to the formulation used: (1) corresponds to the formulation with 1.5 % caprylic acid triglyceride; (2) with 1 % CEO; (3) with 1.5 % CEO; (4) with 2 % CEO; (5) with 1 % WTEO; (6) with 1.5 % WTEO; (7) with 2 % WTEO; (8) with 1 % CWTEO; (9) with 1.5 % CWTEO; and (10) with 2 % CWTEO. Caprylic acid triglyceride, which contains medium-chain fatty acids, was used as the control.

2.3.2. Particle size distribution, polydispersity index (PDI), and zeta potential (ZP)

For the analysis of particle size, PDI, and ZP, the nanoemulsions were diluted (1:30) in water (distilled and filtered) and subjected to an ultrasound bath for 2 min at room temperature (25 ± 3 °C). Droplet size distribution and PDI were determined using light scattering, while zeta potential was measured through the dynamic light scattering (DLS) method (Nano ZS-90, Malvern Instruments, Worcestershire, UK). All measurements were performed in triplicate, and the results were presented as mean and standard deviation.

2.4. Morphology of nanoemulsions

For morphological analyses by transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM), only nanoemulsion added with WTEO (2 %, *w/v*) was used after 150 days of storage. This nanoemulsion presented the best conditions of stability and antifungal activity and was chosen for *in vivo* application. To evaluate the morphology of the nanoemulsion by TEM (JEOL JEM-1400 equipped with an aperture of 20 μm at 100 kV), the methodology proposed by Lu et al. (2018), with adaptations. Samples (50 μL) were added to 200-mesh formvar-coated copper TEM sample holders (EM Sciences, Hatfield, PA, USA), then negatively stained with 50 μL of 1.5 % (*w/v*) uranyl for 10 min at room temperature (25 ± 3 °C). Excess liquid was wiped off with Whatman filter paper.

CLSM images were taken to illustrate the morphology of the nanoemulsion according to Rehman et al. (2020) and Sharif, Williams, et al. (2017) with adaptations. 2 μL of Nile red dye solution (1 mg mL^{-1} of ethanol) was homogenized with 200 μL of nanoemulsion and mixed thoroughly to stain the EO drops. Then, 5 μL of the stained sample was placed on a microscopic glass slide, covered with a coverslip, and observed under the CLSM. The excitation wavelength was 543 nm for Nile red dye. A Leica TCS SP8 confocal microscope (Leica, Heidelberg, Germany) with a 63× oil immersion objective lens was used to capture the confocal images. Images were obtained and processed using the instrument's software program.

2.5. In vitro antifungal activity of nanoemulsions

Antifungal activity was determined as described by Tao et al. (2014) and Chen et al. (2016), with some adaptations. Nanoemulsions (10 μL mL^{-1}) were added directly to the Potato Dextrose Agar (PDA) medium, which was poured into sterile Petri dishes (55 × 15 mm). PDA without nanoemulsion added was used as a positive growth control. All plates were inoculated with *P. digitatum* fungus mycelium, cultivated for 5 days in PDA medium, from the margin of a fungal colony that was cut with a cork drill (0.5 cm). The plates were sealed with parafilm and incubated at 25 °C. All treatments were performed in triplicate. After 72 h of incubation, the diameter of the colony was evaluated by measuring the growth of the fungal colony on a plate diametrically opposite, using a Mtx ® digital caliper (cm). The percentage of inhibition of mycelial growth (IMG) was calculated according to Eq. 1 (Chen et al., 2016).

$$\mathrm{IMG}(\%) = \frac{\mathrm{dc} - \mathrm{dt}}{\mathrm{dc} - 0.5} \times 100 \tag{1}$$

Where *dc* (cm) is the average colony diameter for controls and *dt* (cm) is the average colony diameter for the treatment. The results are expressed as the mean ± standard deviation.

2.6. In vivo antifungal assay

Citrus fruits (*Citrus sinensis* cv. Salustiana) were harvested at commercial maturity from an experimental orchard at *EMBRAPA Clima Temperado* in Pelotas (31°42′S, 52°24′W), and those that were healthy had a consistent size (50–70 mm), uniform color and free from bruises or diseases were chosen as experimental material. The protocol used for the *in vivo* test was adapted from Guo et al. (2023) and Chen et al. (2019). All selected fruits were dipped in 1 % (*v/v*) sodium hypochlorite solution for 2 min and then washed with running water to remove residual disinfectant and dried naturally before wounding.

A uniform wound (4 mm in diameter, 2 mm in depth) was made with a sterile punch on the equatorial surface of each fruit, and 20 μL of *P. digitatum* suspension (1.5 × 10^5 CFU mL^{-1}) was injected into each wound. After 30 min, 20 μL of nanoemulsion 2 % WTEO was injected into each wound. Fruits without nanoemulsion application were used as controls. Orange nanoemulsion-treated and control fruits were placed in containers (60 cm diameter plastic packaging) at 25 ± 1 °C, humidity 70 ± 5 %. The diameter of the lesion was measured with a digital caliper (Mtx ®) at 3, 5, and 7 days after injection. Each treatment consisted of three replications, each containing 9 fruits. The experiment was repeated twice.

2.7. Quality assessment of oranges

To evaluate fruit quality, only fruits that had not received interventions (wounding and application of nanoemulsions) to evaluate antifungal activity were used. The evaluation of quality parameters was carried out on 15 fruits at the time of harvest and after the storage period. The healthy fruits were stored under the same conditions as the fruits used in the *in vivo* experiment (25 °C for 7 days).

The color assessment was determined on the external part of the fruits (flavedo) using a Minolta CR-400 colorimeter, enabling color reading in a three-dimensional system (L*, a*, b*, Hue). The firmness of the skin was determined using a TA-XT plus 40,855 electronic texturometer. Readings were taken at 6 to 8 points on the fruit, and the result was expressed in Newtons (N). Total soluble solids (TSS) (°brix), hydrogen potential (pH), and total titratable acidity (TTA) (% citric acid) were determined from the fruit juice as described by Alvarez et al. (2023).

2.8. Statistical analysis

The data were subjected to analysis of variance (ANOVA), and the Tukey test was applied to compare means with a statistical significance level of $p < 0.05$.

3. Results and discussion

3.1. Stability of nanoemulsions

The images of the nanoemulsions after 1, 30, 60, 90, 120, and 150 days of storage are presented in Fig. 1. The zeta potential, PDI, and particle size were used to evaluate the stability of nanoemulsions and are presented in Fig. 2a, b, and c, respectively. Factors such as the proportion of the oil phase, the type and concentration of surfactant, the size of the particles, the particle polydispersity index, and the zeta potential can interfere with the stability of the nanoemulsion (Liu et al., 2021; Sharif et al., 2017b; Sharif et al., 2017a).

After 24 h, the control formulation (formulation no. 1) and the formulation with 1 % CEO (formulation no. 2) began the phase separation process. To classify a system as an emulsion, it must contain a dispersed phase within an aqueous solution. In this context, formulation 1 was designed to serve as a control or "blank" formulation. Caprylic acid triglyceride was introduced as the dispersed (non-polar) phase. However, the interaction between this compound and the other emulsion components—namely, the dispersing phase (water) and the surfactant—did not proceed as anticipated, resulting in phase separation within the formulation.

Although the 1 % CEO formulation had small particles sized (129 nm), the dispersion of these particles was not homogeneous (PDI = 0.85). The PDI value is defined as the measure of heterogeneity in the droplet size distribution. PDI values close to 0 indicate homogeneous size distributions, while PDI values close to 1 indicate heterogeneous size distributions (Frank et al., 2018; Keykhosravy et al., 2020). Therefore, in addition to the visible phase separation observed in the

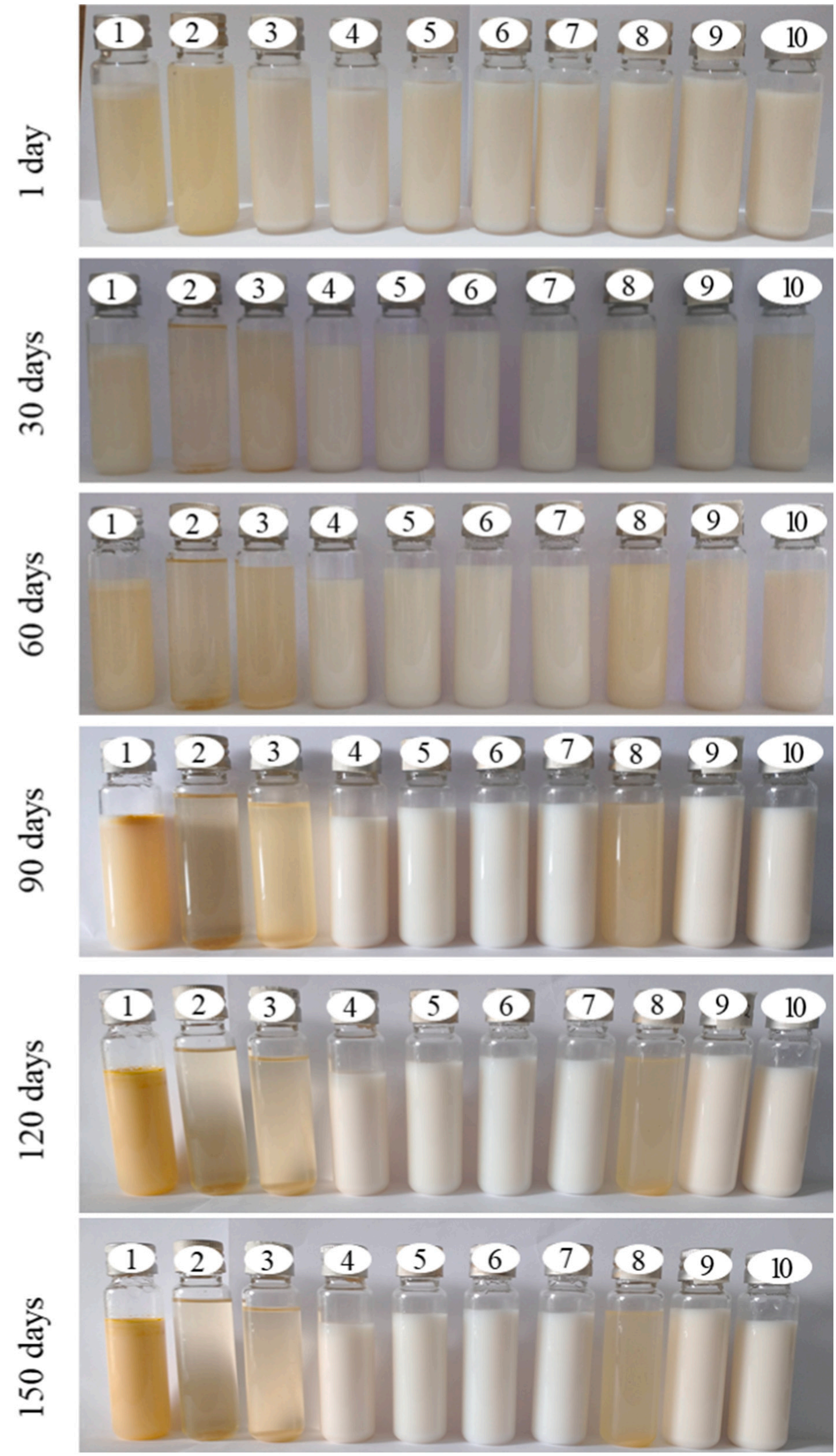

Fig. 1. - Image of the stability of nanoemulsions produced with OSA-starch and clove and white thyme essential oils during 150 days of storage. Formulations numbers: 1: caprylic acid triglyceride; 2: 1%CEO; 3: 1,5%CEO; 4: 2%CEO; 5:1%WTEO; 6:1,5 % WTEO; 7: 2%WTEO; 8: 1%CWTEO; 9: 1,5%CWTEO; 10: 2%CWTEO. CEO: clove essential oil; WTEO: white thyme essential oil; CWTEO: clove and white thyme essential oil.

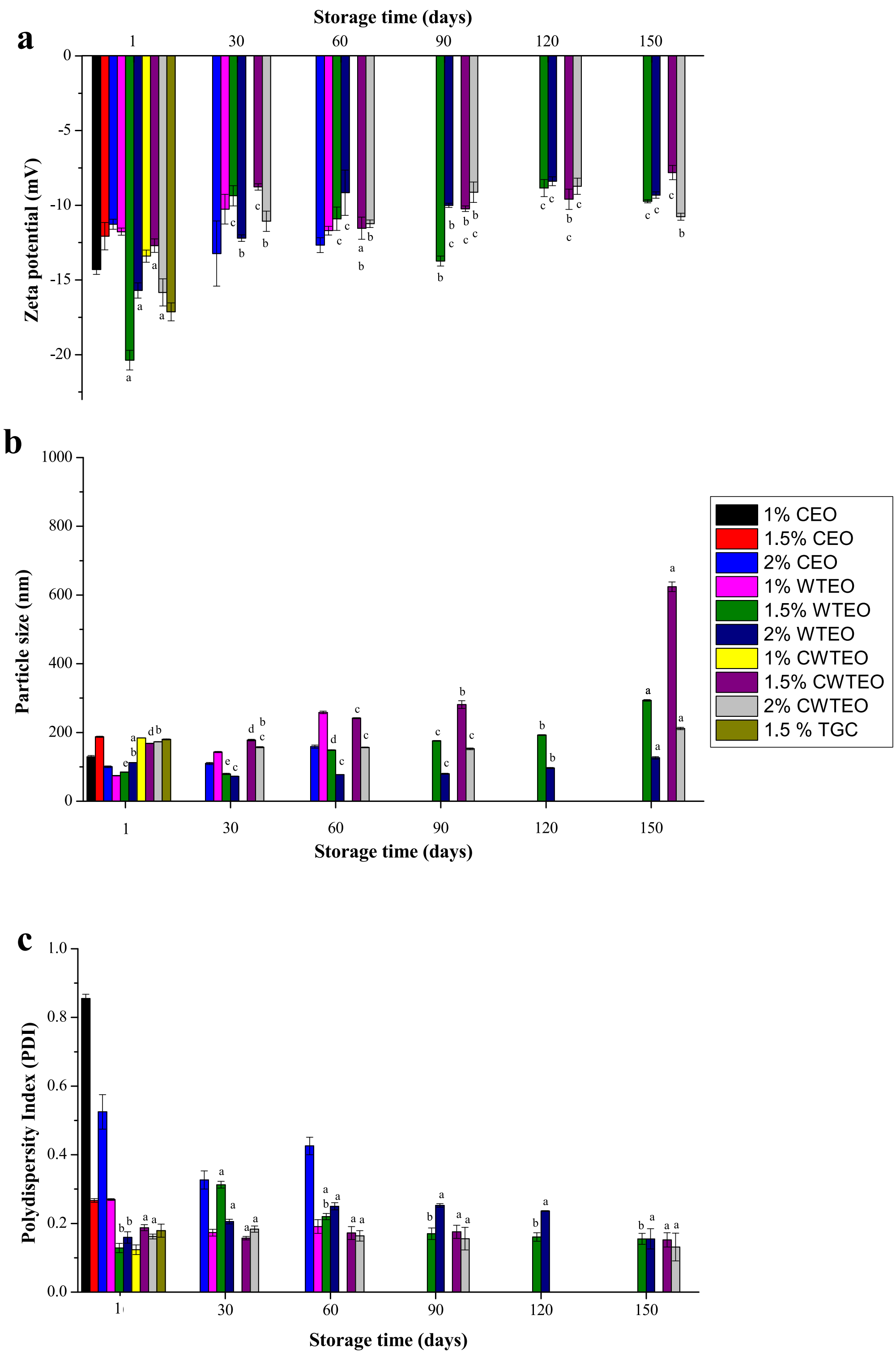

Fig. 2. - Zeta potential (a), particle size (b), and polydispersity index (c) of starch-OSA with clove and white thyme essential oils nanoemulsions during 150 days of storage.
CEO: clove essential oil; WTEO: white thyme essential oil; CWTEO: clove and white thyme essential oil; TGC: caprylic acid triglyceride; The 1 % CEO, 1.5 % CEO, 1 % WTEO and 1.5 TGC formulations were not evaluated after day 30. After day 60, the 2 % CEO and 1 % WTEO formulations were also no longer evaluated. Different letters represent differences between storage times for the same formulation ($p < 0.05$).

photos, the emulsion of 1 % CEO can be considered heterogeneous. The addition of a small amount of CEO (1 %) did not result in a favorable interaction within the formulation, rendering it unstable compared to the higher concentration (2 %). CEO is primarily composed of eugenol, and the amount of this compound at the lower concentration was likely insufficient to promote the necessary interactions for the formation of a stable emulsion.

After 30 days of storage, the formulations with 1.5 % CEO (formulation no. 3) and 1 % CWTEO (formulation no. 8) also showed phase separation (Fig. 1). During this storage time, all visibly stable formulations had a particle size <200 nm (Fig. 2b), with emphasis on the formulations with 1.5 % and 2 % WTEO (formulations no. 6 and 7), which had particle sizes below 100 nm. Despite showing a slight increase in particle size, the formulation with 2 % WTEO remained stable for up to 150 days of storage, recording the smallest particle size among the formulations (126 nm). The reduced droplet size to the nanoscale, combined with the presence of a stabilizing surfactant, inhibits droplet coalescence and allows nanoemulsions to be stable for many months or years (Singh and Pulikkal, 2022).

From 90 to 150 days of storage, only the formulations with 1.5 % WTEO, 2.0 % WTEO, 1.5 % CWTEO, and 2.0 % CWTEO were stable. The PDI, zeta potential, and particle size values showed small fluctuations during the storage time of 90 to 150 days. Still, they were not enough to interfere with the stability of the emulsions. A PDI value close to 1 and a higher particle size value (~850 nm) were observed during a storage time of 120 days in the formulations with 1.5 % WTEO and 2 % WTEO. This fact is related to a deprogramming in the equipment due to a power outage that occurred during the analysis, which consequently interfered with the reported values, which do not report the actual particle size and dispersion of these emulsions and, therefore, were not included in Fig. 2.

The zeta potential can also be used to evaluate the stability of emulsions, as it provides a relationship with the electrostatic repulsion between dispersed droplets with similar charges in order to avoid coalescence or aggregation. Zeta potential values on day 1 of emulsions

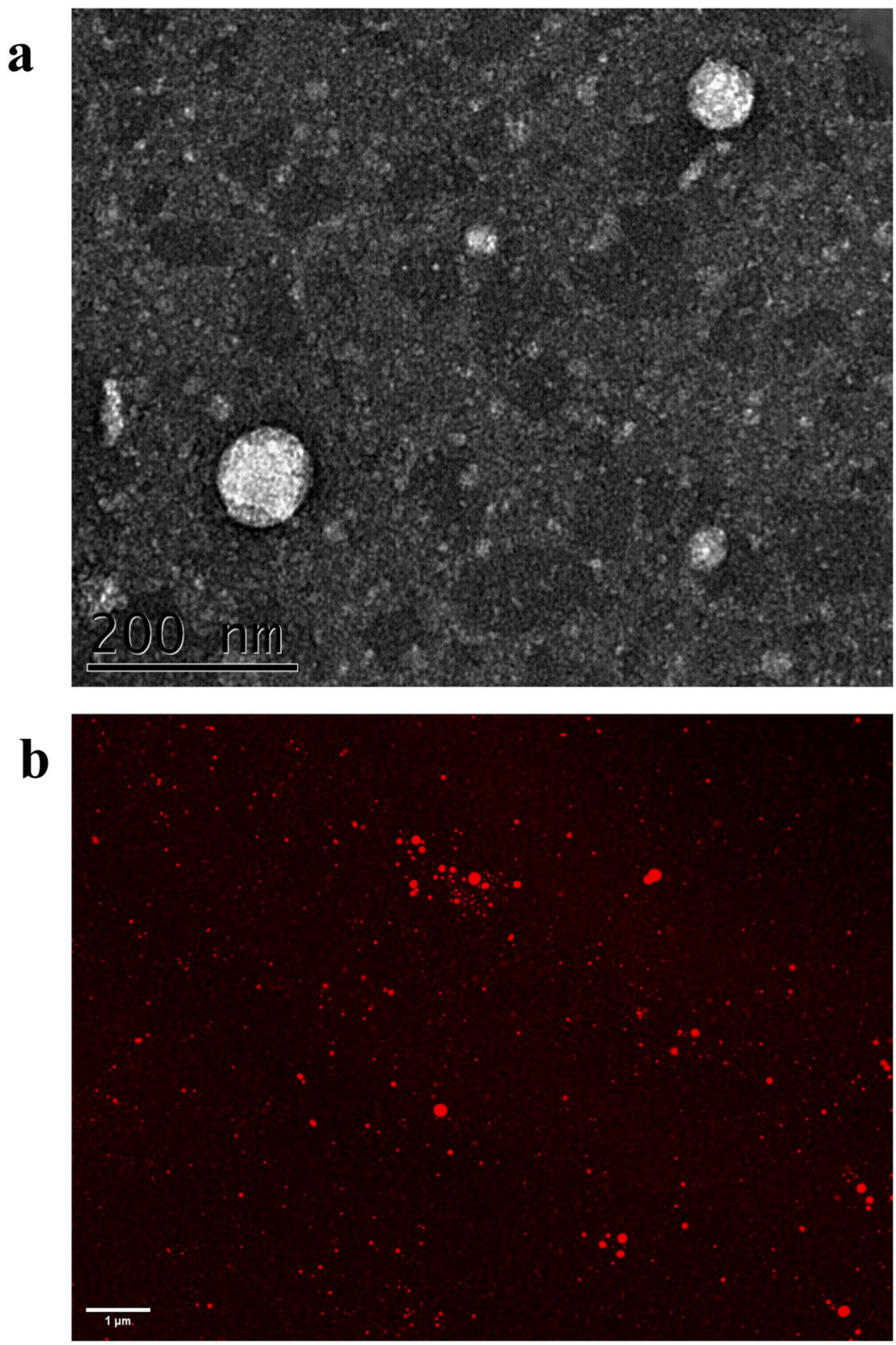

Fig. 3. - Morphology of the nanoemulsion with 2 % white thyme essential oil observed by transmission electron microscopy (a) and confocal laser scanning microscopy (b).

storage ranged from −11 to −20 mV. At the end of the storage time, a reduction in zeta potential was observed in all stable emulsions with a minimum value of −7.8 mV observed for the formulation with 1.5 % WTEO. The negative values found in our study for all emulsions are due to the carboxylic groups present in starch-OSA (Sharif et al., 2017b).

At the nanoscale, higher zeta potential values confer physical stability through electrostatic repulsion. Zeta potential values > |20| mV are desired when combined steric and electrostatic stabilizations are required, and values > |30| mV are crucial to maintaining the stability of nanoemulsions when spherical stabilization is not present. Furthermore, when zeta potential presents values above |30| mV, repulsion forces are sufficient to overcome the attractive van der Waals forces, preventing flocculation from occurring (Engelmann et al., 2023; Sharif et al., 2017b). In our study, the zeta potential reached a maximum value of −20 mV for formulation no 6 (1.5 % WTEO, 1 day of storage) and − 10.8 no 10 (2 % CWTEO, 150 days of storage). Since the zeta potential cutoff is only a partial index of physicochemical stability, it is essential to monitor long-term stability. If the values found in our study (ZP < |20| mV) were evaluated in isolation, they would indicate that no emulsion would remain stable; however, the formulations no 6, 7, 9, and 10 nanoemulsions were stable for up to 150 days of storage (Fig. 1).

The development of starch-OSA emulsions added with CEO proved to be lacking in terms of long-term stability compared to other formulations. On the other hand, the use of WTEO in concentrations of 1.5 and 2 % showed satisfactory results in terms of stability for up to 150 days of storage, and with the highest concentration of WTEO added, emulsions with small particle sizes were obtained. The combination of CEO and WTEO for emulsion production was also acceptable, especially at concentrations of 1.5 and 2 % EO. Possibly, the stability of emulsions 1.5 % WTEO, 2 % WTEO, 1.5 % CWTEO, and 2 % CWTEO is linked to their reduced particle size and low PDI value (<0.3). Furthermore, the chemical composition of the EO may have interfered in terms of stability.

3.2. Morphology of nanoemulsion

The morphology of nanoemulsion with 2 % WTEO, characterized by TEM and CLSM, is presented in Fig. 3. In the TEM (Fig. 3a), the contrast performed with uranyl allowed us to observe that the droplets were tiny, spherical, and dispersed, which is characteristic of a nanoemulsion. Similar droplet characteristics (precisely spherical and uniformly dispersed) were observed by Huang et al. (2021) when developing nanoemulsions from starch-OSA and cedar essential oil. The spherical nature of nanoemulsions particles is attributed to the reduction in interfacial area due to the sizeable Laplace pressure ($\Delta P = 2\gamma/r$) as a result of the relatively high interfacial tension (γ) and small particle radius (r) (Singh and Pulikkal, 2022).

The CLSM image (Fig. 3b) was captured to evaluate the structural fluctuations in EO droplets in the nanoemulsion. When the Nile red dye comes into contact with the oil phase of the nanoemulsion, it emits fluorescence (red dots, Fig. 3b). Very small, evenly dispersed EO droplets were observed. In previous studies, Rehman et al. (2020) and Sharif, Abbas, et al. (2017) also confirmed the uniform distribution of peppermint and black cumin EO droplets in starch-OSA stabilized nanoemulsions. The droplets observed by TEM and CLSM confirm the results obtained by DLS. As a result of high pressure, high-velocity impact, cavitation, and intense shear rate, high-pressure homogenization is consolidated as a promising system for producing stable starch-OSA and EO nanoemulsions, with nanoscale droplet size and antifungal activity against *P. digitatum*.

3.3. In vitro antifungal activity of nanoemulsions

When evaluating the antifungal capacity of nanoemulsions throughout their storage, it was observed that, in addition to remaining physically stable, the compounds present in EOs also remain active, showing action against *P. digitatum*. In an ideal scenario, total inhibition of fungal growth and proliferation is desired from the initial nanoemulsion preparation stage to the final storage stage. The percentages of inhibition of *P. digitatum* mycelial growth of nanoemulsions evaluated every 30 days during 150 days of storage are shown in Fig. 4.

At time 0 (1 day after preparing the formulations), the difference in the percentage of inhibition between the formulations is notable. Although phase separation of 1 % CEO and 1.5 % TGC nanoemulsions has already been observed (Fig. 1), they were also evaluated for antifungal activity for characterization purposes. As expected, nanoemulsion with 1.5 % TGC did not show significant antifungal action, as there was no bioactive compound present. Nanoemulsion with 1 % CEO, despite not being physically stable, showed a 40.8 % reduction in the mycelial growth of *P. digitatum*. The formulations made with the same EO (1.5 % CEO and 2 % CEO) showed higher inhibition percentages, 55.3 and 60.5 %, respectively, possibly due to the addition of a higher concentration of EO and, consequently, the greater availability of bioactive compounds. Up to 60 days of storage, nanoemulsion 2 % CEO was stable; however, a decrease in its potential to inhibit the mycelial growth of *P. digitatum* was observed, with values of 45.8 % and 23.9 % on the 30th and 60th day of storage, respectively. After this period, due to the destabilization of the emulsion, the antifungal activity was also not evaluated. The antifungal action of CEO is linked to the potential of its primary compound, eugenol, to bind to ergosterol, a natural constituent of fungal mycelial membranes. As a result, the production of fungal biomass is inhibited, and mycelial growth is affected, even at low concentrations of added EO (Abdi-Moghadam et al., 2023).

Nanoemulsion with 1 % WTEO evaluated at 0, 30, and 60 days of storage showed *P. digitatum* mycelial growth inhibition values of 69.7, 67.4, and 61.3 %, respectively. The lower values compared to nanoemulsions 1.5 % and 2 % WTEO are possibly due to the lower concentration of EO added. Despite being stable for up to 150 days of storage, nanoemulsion with 1.5 % WTEO showed total inhibition of mycelial growth of the fungus only up to 90 days of storage. After this period, a loss of ~25 % of its potential to inhibit fungal mycelial growth was observed. The reduced bioactive action is probably associated with the gradual breakdown of the micelles surrounding the EO. Over time, the surfactant weakens, leading to the release of the encapsulated EO into the dispersing medium. As a result, the EO, previously shielded within the micelles, is exposed to degradation processes, which in turn reduces its bioactive activity.

In addition to presenting the formation of a stable emulsion up to 150th of storage, the highest concentration of WTEO (2 %) provided total inhibition of *P. digitatum* mycelial growth throughout the entire storage period. WTEO also inhibits ergosterol in fungi (Abdi-Moghadam et al., 2023). Furthermore, components present in WTEO, such as thymol and carvacrol, target efflux pumps, which prevent the overexpression of genes associated with these pumps (Abdi-Moghadam et al., 2023; Singh and Pulikkal, 2022; Zhang et al., 2023). Furthermore, WTEO may also exhibit an inhibitory effect on fungal spores (Zhang et al., 2023). For this emulsion (2 % WTEO), the active ingredients were continuously released in the process, with some of them expressing their fungal inhibition potential at the beginning of the storage time due to the higher concentration added to the formulation. Other compounds were released slowly as the destruction of the emulsion structure occurred. This result demonstrates the promising application of this nanoemulsion in more extended storage periods.

When combining CEO and WTEO in nanoemulsions, inhibition of 51.3 % was observed; 52.6 % and 78.9 % of fungal mycelial growth at time 0 of storage at respective concentrations 1, 1.5, and 2 % of CWTEO. At 30 and 60 days of storage, 1.5 % and 2 % CWTEO showed 100 % inhibition of the mycelial growth of the fungus. Possibly, the lower percentage of inhibition observed at the beginning of storage could be related to the more significant entrapment of EOs in the matrix and that, as storage time passed, the compounds gradually released into the aqueous phase of the emulsion, thus increasing the potential antifungal

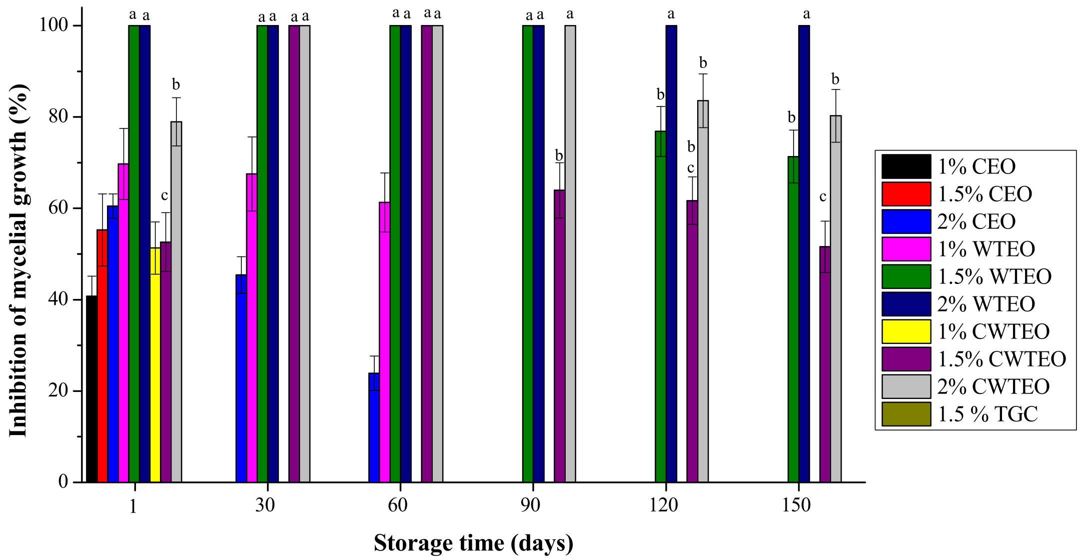

Fig. 4. - Antifungal activity against *Penicillium digitatum* of nanoemulsions developed with starch-OSA and clove and white thyme essential oils evaluated for up to 150 days of storage.
CEO: clove essential oil; WTEO: white thyme essential oil; CWTEO: clove and white thyme essential oil; TGC: caprylic acid triglyceride; The 1 % CEO, 1.5 % CEO, 1 % WTEO and 1.5 TGC formulations were not evaluated after day 30. After day 60, the 2 % CEO and 1 % WTEO formulations were also no longer evaluated. Different letters represent differences between storage times for the same formulation ($p < 0.05$).

action. Rapid release in the initial storage stage is essential to inhibit fungal proliferation completely; however, for promising long-term results, it is more interesting that a gradual release occurs during storage (Zhang et al., 2023). In this sense, the formulation prepared with the combination of CEO and WTEO, at a concentration of 2 %, would be indicated to control the growth of *P. digitatum* up to 90 days of storage; after this period, its efficiency would be reduced, and therefore not indicated.

3.4. *In vivo antifungal assay*

The influence of the application of starch-OSA nanoemulsion added with 2 % WTEO on the incidence of fungal disease and the diameter of the lesion in oranges inoculated with *P. digitatum* is presented in Table 1. The diameter of the lesion increased with storage time for both treatments evaluated ($p < 0.05$) but more markedly for fruits subjected to the control treatment, which were not treated with nanoemulsion. The incidence of disease in the control group after 3 days of injection was 22 %, while in the group treated with nanoemulsion, it was only 4 %; at 7 days, the incidence of the disease reached 100 % of the fruits in the control group and 33 % in the group of treated fruits. These results confirm the *in vitro* antifungal action of nanoemulsion with 2 % WTEO (item 3.3). The antifungal activity of the 2 % WTEO nanoemulsion can be attributed to compounds present in EO, such as thymol (22.7 %) and carvacrol (8.4 %) that can damage the structure of the fungal cell wall, make its membrane more permeable, and reduce the chances of cell survival, interrupting enzymatic reactions and intercepting electron transport (Abbasi et al., 2023; Zhang et al., 2023).

Abbasi et al. (2023) reported promising results in an *in vivo* experiment for the control of *P. digitatum*. They achieved maximum inhibition in lemons covered with carboxymethylcellulose (CMC, 1.5 %) coatings added with WTEO (0.2 %). The authors reported changes in the morphology of fungal cells and suggested that WTEO can attack the cell wall to inhibit infection and hinder the progress of fungal diseases (Abbasi et al., 2023).

The *in vivo* experiment confirmed that the application of 20 μL of 2 % WTEO nanoemulsion caused a reduction in the incidence of oranges infected with *P. digitatum*. It is worth highlighting that a series of studies demonstrate the antifungal potential of numerous EOs; however, studies on the evaluation of this activity *in vivo* studies are scarce. Furthermore, when these EOs are applied to a matrix, they are not always able to perform the same antifungal action as reported in *in vitro* studies. Thus, our results confirm the antifungal action of emulsions containing WTEO both *in vitro* and *in vivo* and thus provide a promising approach capable of helping to control the development of green mold and reduce post-harvest losses of citrus fruits.

Table 1
Lesion diameter and incidence of infected fruits (%) in oranges treated with starch-OSA nanoemulsion added with 2 % white thyme essential oil infected with *Penicillium digitatum* on different days post-inoculation.

Parameter	Storage (days)	Treatment	
		Control	Nanoemulsion
Lesion diameter (mm)	3	$4.69^{Ca} \pm 0.01$	$4.23^{Ca} \pm 0.01$
	5	$4.82^{Ba} \pm 0.02$	$4.33^{Ba} \pm 0.01$
	7	$4.90^{Aa} \pm 0.02$	$4.39^{Aa} \pm 0.01$
Infected fruits (%)	3	22	4
	5	67	26
	7	100	33

A, B, C represent differences between storage days for the same treatment.
a, b, c show differences on the same day for different treatments.

3.5. *Quality assessment of oranges*

The quality attributes of oranges without fungal contamination, evaluated before and after storage, under conditions similar to those used in the *in vivo* experiment, are presented in Table 2. Internal quality parameters, such as TSS, TTA, and pH, showed subtle differences from the beginning to the end of the storage time, which is typical for this type of fruit. Alvarez et al. [24] evaluated the quality of oranges *Citrus sinensis* cv. 'Valencia' with and without the application of bioactive coatings to control sour rot caused by *Geotrichum citri-aurantii* also reported that the storage time did not interfere with internal quality parameters.

External parameters, such as the color and firmness of the shell, were also evaluated. For the color parameters (L*a*b and Hue), no differences were observed depending on storage time. The fact that no significant changes were observed in fruit quality parameters during storage can be justified by the fact that oranges are classified as a non-climacteric fruit,

Table 2
Internal and external quality parameters of orange fruits.

Parameter	Storage time (days)	
	0	7
TSS (° brix)	12.23[a] ± 1.29	11.63[a] ± 1.09
pH	3.51[a] ± 0.16	3.71[a] ± 0.15
TTA (%, citric acid)	1.19[a] ± 0.17	1.13[a] ± 0.29
L*	69.87[a] ± 1.63	67.99[a] ± 1.43
a*	18.55[a] ± 3.15	21.05[a] ± 2.12
b*	72.37[a] ± 1.83	71.98[a] ± 2.34
Hue angle (°)	75.62[a] ± 2.49	73.70[a] ± 1.50
Firmness (N)	28.96[a] ± 4.53	19.23[a] ± 4.22

"a" in the same line indicates that there are no significant differences ($p < 0.05$) between the treatment means, using the t-test.
[*] TSS = total soluble solids; TTA = total titratable acidity.

and for this reason, they do not present a peak in ethylene production, primarily responsible for changes in fruit characteristics, such as color, hardness, and texture (Sun and Nasrullah, 2022).

Regarding firmness, a reduction in this parameter was observed after 7 days of storage. Evaporation and transpiration losses that result in weight loss and decreased firmness occur as a natural process during fruit storage. To minimize these losses, the addition of bioactive coatings is a promising alternative (Abbasi et al., 2023; Alvarez et al., 2023).

In our study, the quality parameters of the oranges did not show significant changes during storage time. Thus, these parameters do not interfere with the proliferation of the fungus, and the result of the *in vivo* application of nanoemulsion can be attributed solely to its action.

4. Conclusion

The source and concentration of essential oils significantly impact the stability of nanoemulsions during storage. Nanoemulsions with white thyme essential oil (WTEO) were more stable over 150 days compared to those produced with clove essential oil (CEO). The 2 % WTEO nanoemulsion had the smallest particle size (< 100 nm) on the first day and maintained a low size (126 nm) after 150 days. It showed excellent stability, with favorable zeta potential and polydispersity index results. Additionally, the 2 % WTEO nanoemulsion completely inhibited the mycelial growth of *Penicillium digitatum* throughout the storage period, demonstrating its strong antifungal activity. In contrast, the 2 % CEO nanoemulsion only inhibited 60.5 % of fungal growth and remained stable with antifungal properties for up to 60 days.

In vivo results further confirmed the antifungal efficacy of WTEO, reducing infection in oranges when 20 μL of 2 % WTEO nanoemulsion was applied. These promising findings suggest that WTEO can effectively control *P. digitatum* in citrus fruits without compromising quality, offering a natural, viable alternative to reduce postharvest losses in citrus farming.

CRediT authorship contribution statement

Estefania Júlia Dierings de Souza: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Dianini Hüttner Kringel:** Writing – original draft. **Virginia Campello Yurgel:** Formal analysis. **Cristiana Lima Dora:** Writing – review & editing, Supervision. **Michele Greque de Morais:** Supervision. **Eliezer Avila Gandra:** Writing – review & editing, Supervision. **Rufino Fernando Flores Cantillano:** Writing – review & editing, Project administration. **Alvaro Renato Guerra Dias:** Writing – review & editing, Resources, Project administration. **Elessandra da Rosa Zavareze:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

This study was financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Finance Code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPQ (306378/2015-9) and Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (BR) – FAPERGS (17/255100009126). We thank CEMESUL (FURG) for the SEM and TEM analysis, Ingredion S.A for the donation of starch-OSA, and Dieberger S.A for the donation of clove essential oil.

Data availability

Data will be made available on request.

References

Abbasi, M., Dastjerdi, A.M., Seyahooei, M.A., Shamili, M., Madani, B., 2023. Postharvest control of green and blue molds on Mexican lime fruit caused by *Penicillium* species using *Thymus vulgaris* essential oil and carboxy methyl cellulose. J Plant Dis Protect 130, 1017–1026. https://doi.org/10.1007/s41348-023-00773-1.

Abdi-Moghadam, Z., Mazaheri, Y., Rezagholizade-shirvan, A., Mahmoudzadeh, M., Sarafraz, M., Mohtashami, M., Shokri, S., Ghasemi, A., Nickfar, F., Darroudi, M., Hossieni, H., Hadian, Z., Shamloo, E., Rezaei, Z., 2023. The significance of essential oils and their antifungal properties in the food industry: a systematic review. Heliyon 9. https://doi.org/10.1016/j.heliyon.2023.e21386.

Alvarez, M.V., Pérez-Gago, M.B., Taberner, V., Settier-Ramírez, L., Martínez-Blay, V., Palou, L., 2023. Postharvest application of novel bio-based antifungal composite edible coatings to reduce sour rot and quality losses of 'Valencia' oranges. Coatings 13, 1412. https://doi.org/10.3390/coatings13081412.

Biduski, B., Kringel, D.H., Colussi, R., Hackbart, H.C. dos S., Lim, L.T., Dias, A.R.G., Zavareze, E. da R., 2019. Electrosprayed octenyl succinic anhydride starch capsules for rosemary essential oil encapsulation. Int. J. Biol. Macromol. 132, 300–307. doi: https://doi.org/10.1016/j.ijbiomac.2019.03.203.

Chen, C., Cai, N., Chen, J., Wan, C., 2019. Clove essential oil as an alternative approach to control postharvest blue mold caused by *Penicillium italicum* in citrus fruit. Biomolecules 9. https://doi.org/10.3390/biom9050197.

Chen, C.Y., Zheng, J.P., Wan, C.P., Chen, M., Chen, J.Y., 2016. Effect of carboxymethyl cellulose coating enriched with clove oil on postharvest quality of "Xinyu" mandarin oranges. Fruits 71, 319–327. https://doi.org/10.1051/fruits/2016019.

Dávila-Rodríguez, M., López-Malo, A., Palou, E., Ramírez-Corona, N., Jiménez-Munguía, M.T., 2019. Antimicrobial activity of nanoemulsions of cinnamon, rosemary, and oregano essential oils on fresh celery. LWT 112. https://doi.org/10.1016/j.lwt.2019.06.014.

Engelmann, J.I., de Farias, B.S., Igansi, A.V., Silva, P.P., Cadaval, T.R.S., Gelesky, M.A., Crexi, V.T., de Almeida Pinto, L.A., 2023. Chitosan-based nanocapsules by emulsification containing PUFA concentrates from tuna oil. Food Sci. Technol. Int. https://doi.org/10.1177/10820132231153496.

FAO, 2023. Food and Agriculture Organizations of the United Nations: Database. Available at: www.fao.org/home/en/.

Frank, K., Garcia, C.V., Shin, G.H., Kim, J.T., 2018. Alginate biocomposite films incorporated with cinnamon essential oil nanoemulsions: physical, mechanical, and antibacterial properties. Int J Polym Sci 2018. https://doi.org/10.1155/2018/1519407.

Franklyne, J.S., Iyer, S., Ebenazer, A., Mukherjee, A., Chandrasekaran, N., 2019. Essential oil nanoemulsions: antibacterial activity in contaminated fruit juices. Int. J. Food Sci. Technol. 54, 2802–2810. https://doi.org/10.1111/ijfs.14195.

Gengatharan, A., Rahim, M.H.A., 2023. The application of clove extracts as a potential functional component in active food packaging materials and model food systems: a mini-review. Appl Food Research 3. https://doi.org/10.1016/j.afres.2023.100283.

Guo, L., Mao, X., Li, Y., Zhou, Z., 2023. Polymethoxylated flavonoids (PMFs)-loaded citral nanoemulsion controls green mold in citrus by damaging the cell membrane of *Penicillium digitatum*. Fungal Biol. 127, 854–864. https://doi.org/10.1016/j.funbio.2022.12.003.

He, S., Ren, X., Lu, Y., Zhang, Y., Wang, Y., Sun, L., 2016. Microemulsification of clove essential oil improves its in vitro and *in vivo* control of *Penicillium digitatum*. Food Control 65, 106–111. https://doi.org/10.1016/j.foodcont.2016.01.020.

Huang, K., Liu, R., Zhang, Y., Guan, X., 2021. Characteristics of two cedarwood essential oil emulsions and their antioxidant and antibacterial activities. Food Chem. 346. https://doi.org/10.1016/j.foodchem.2020.128970.

Keykhosravy, K., Khanzadi, S., Hashemi, M., Azizzadeh, M., 2020. Chitosan-loaded nanoemulsion containing *Zataria multiflora* Boiss and *Bunium persicum* Boiss essential oils as edible coatings: its impact on microbial quality of Turkey meat and fate of inoculated pathogens. Int. J. Biol. Macromol. 150, 904–913. https://doi.org/10.1016/j.ijbiomac.2020.02.092.

Li, S., Jiang, S., Jia, W., Guo, T., Wang, F., Li, J., Yao, Z., 2024. Natural antimicrobials from plants: recent advances and future prospects. Food Chem. 432, 137231. https://doi.org/10.1016/j.foodchem.2023.137231.

Liu, X., Chen, L., Kang, Y., He, D., Yang, B., Wu, K., 2021. Cinnamon essential oil nanoemulsions by high-pressure homogenization: formulation, stability, and antimicrobial activity. LWT 147. https://doi.org/10.1016/j.lwt.2021.111660.

Lu, W.C., Huang, D.W., Wang, C.C.R., Yeh, C.H., Tsai, J.C., Huang, Y.T., Li, P.H., 2018. Preparation, characterization, and antimicrobial activity of nanoemulsions incorporating citral essential oil. J. Food Drug Anal. 26, 82–89. https://doi.org/10.1016/j.jfda.2016.12.018.

Pinto, L., Cefola, M., Bonifacio, M.A., Cometa, S., Bocchino, C., Pace, B., De Giglio, E., Palumbo, M., Sada, A., Logrieco, A.F., Baruzzi, F., 2021. Effect of red thyme oil (*Thymus vulgaris* L.) vapours on fungal decay, quality parameters and shelf-life of oranges during cold storage. Food Chem. 336. https://doi.org/10.1016/j.foodchem.2020.127590.

Rehman, A., Jafari, S.M., Tong, Q., Karim, A., Mahdi, A.A., Iqbal, M.W., Aadil, R.M., Ali, A., Manzoor, M.F., 2020. Role of peppermint oil in improving the oxidative stability and antioxidant capacity of borage seed oil-loaded nanoemulsions fabricated by modified starch. Int. J. Biol. Macromol. 153, 697–707. https://doi.org/10.1016/j.ijbiomac.2020.02.292.

Sharif, H.R., Abbas, S., Majeed, H., Safdar, W., Shamoon, M., Khan, M.A., Shoaib, M., Raza, H., Haider, J., 2017a. Formulation, characterization and antimicrobial properties of black cumin essential oil nanoemulsions stabilized by OSA starch. J. Food Sci. Technol. 54, 3358–3365. https://doi.org/10.1007/s13197-017-2800-8.

Sharif, H.R., Williams, P.A., Sharif, M.K., Khan, M.A., Majeed, H., Safdar, W., Shamoon, M., Shoaib, M., Haider, J., Zhong, F., 2017b. Influence of OSA-starch on the physico chemical characteristics of flax seed oil-eugenol nanoemulsions. Food Hydrocoll. 66, 365–377. https://doi.org/10.1016/j.foodhyd.2016.12.002.

Singh, I.R., Pulikkal, A.K., 2022. Preparation, stability and biological activity of essential oil-based nano emulsions: a comprehensive review. OpenNano 8, 100066. https://doi.org/10.1016/j.onano.2022.100066.

Souza, E.J.D. De, Kringel, D.H., Lima Costa, I.H. de, Hackbart, H.C. dos S., Cantillano, R. F.F., Ueno, B., Dias, A.R.G., Zavareze, E. Da R., 2024. Antifungal potential of essential oils from different botanical sources against *Penicillium digitatum*: chemical composition and antifungal mechanisms of action by direct contact and volatile. Nat. Prod. Res. https://doi.org/10.1080/14786419.2024.2405865.

Sun, L., Nasrullah F., Ke, Nie, Z., Xu, J., Huang, X., Sun, J., Wang, P., 2022. Genome-wide identification and transcript analysis during fruit ripening of ACS gene family in sweet orange (*Citrus sinensis*). Sci. Hortic. 294. https://doi.org/10.1016/j.scienta.2021.110786.

Tao, N., Jia, L., Zhou, H., 2014. Antifungal activity of *Citrus reticulata* Blanco essential oil against *Penicillium italicum* and *Penicillium digitatum*. Food Chem. 153, 265–271. https://doi.org/10.1016/j.foodchem.2013.12.070.

Tian, X., Wang, D., Li, Y., Xu, Z., Ren, X., Kong, Q., 2023. Preparation and characterization of emulsions of soy protein isolate-chitosan quaternary ammonium salt complexes and peppermint essential oil with extended release effect. Food Hydrocoll. 142. https://doi.org/10.1016/j.foodhyd.2023.108779.

Vu, T.X., Tran, T.B., Tran, M.B., Do, T.T.K., Do, L.M., Dinh, M.T., Thai, H.D., Pham, D.N., Tran, V.T., 2023. Efficient control of the fungal pathogens *Colletotrichum gloeosporioides* and *Penicillium digitatum* infecting citrus fruits by native soilborne *bacillus velezensis* strains. Heliyon 9. https://doi.org/10.1016/j.heliyon.2023.e13663.

Wan, J., Zhong, S., Schwarz, P., Chen, B., Rao, J., 2019. Physical properties, antifungal and mycotoxin inhibitory activities of five essential oil nanoemulsions: impact of oil compositions and processing parameters. Food Chem. 291, 199–206. https://doi.org/10.1016/j.foodchem.2019.04.032.

Yao, Y., Li, Y., Zhao, L., Li, S., Zhou, Z., 2023. Citrus lemon (Citrus Limon (L.) Burm. f. cv. Eureka) essential oil controls blue mold in citrus by damaging the cell membrane of Penicillium italicum. LWT 188. doi:https://doi.org/10.1016/j.lwt.2023.115456.

Zhang, Y., Tan, Y., OuYang, Q., Duan, B., Wang, Z., Meng, K., Tan, X., Tao, N., 2023. γ-Cyclodextrin encapsulated thymol for citrus preservation and its possible mechanism against *Penicillium digitatum*. Pestic. Biochem. Physiol. 194. https://doi.org/10.1016/j.pestbp.2023.105501.