



In vitro and *in vivo* antifungal activity of chitosan-essential oils against pomegranate fruit pathogens



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ABSTRACT

This study investigated the effect of chitosan combined with essential oils (cinnamon, lemon, and oregano, respectively) as edible films against the growth of three important disease-causing pathogens of pomegranate (*Botrytis* sp., *Penicillium* sp., and *Pilidiella granati*). Essential oils (EOs) were characterised by gas chromatography analysis and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). *In vitro* antifungal activity of the EOs against *Botrytis* sp., *Penicillium* sp., and *Pilidiella granati* was conducted on agar media inoculated with fungal spores using the inverted lid and direct contact with agar techniques. Cast films were prepared from chitosan of different (low, medium, and high) molecular weights (Mw), and assessed for use as biodegradable packaging material. Thereafter, chitosan films (medium Mw) were used in combination with 0, 10, 50 or 100 g L⁻¹ concentrations of EOs (cinnamon, lemon, and oregano) for *in vitro* investigation. The use of ATR-FTIR effectively discriminated the EOs based on the energy levels of the atomic bond vibrations present, as indicated by their characteristic absorption peaks. Based on GC-MS analysis of the selected EOs, 35 volatile compounds belonging to eight different chemical classes were tentatively identified. In addition, differences in chitosan Mw were found to have significant influence on the film functional properties such as colour, thickness, contact angle, and tensile strength. Based on *in vitro* investigation, chitosan-oregano or -cinnamon EO films with the concentration of 10 g L⁻¹ exhibited complete inhibitory effect against all three pathogens. Maximum inhibitory effects of chitosan-lemongrass films was dependent on EO concentration, with *P. granati*, *Botrytis* sp., and *Penicillium* sp. inhibited completely at 10, 50, and 50 g L⁻¹, respectively. Antifungal *in vivo* study on pomegranate fruit artificially inoculated with *Botrytis* sp. and treated with chitosan-oregano EO (emulsions and films), showed that both treatment methods inhibited *Botrytis* sp. growth. However, the inhibitory effect was higher for fruit directly dipped into the chitosan-EO emulsions as opposed to samples exposed to vapour contact via the films. This study showed that chitosan-EOs applied as edible coatings or casted films can be used to control postharvest fungal pathogens associated with pomegranate fruit.

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1. Introduction

Postharvest storage duration of pomegranate fruit could be critically restricted due to fungal decay. Diseased pomegranate fruit may appear as blemishes such as black spot caused by *Alternaria alternata* or *Colletotrichum gloeosporioides*. In other instances, the fruit may present symptoms of moulding due to

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either *Botrytis cinerea* or *Penicillium* sp. infections, while other disease such as heart rot caused by either *A. alternata* or *Aspergillus niger* may only be detected when the fruit is cut open (Berbegal et al., 2014; Thomidis, 2014; Thomidis and Exadaktylou, 2011). Regardless of the symptoms, postharvest diseases cause economic losses and the diseased fruit poses a potential health risk. Pathogenic fungi contribute to foodborne outbreaks and epidemics causing illnesses from stomach cramp to fatality (Martinovic et al., 2016). Filamentous fungi, in particular, can invade and degrade food products by means of extracellular enzymes (Al-Hindi et al., 2011). Major fungal genera present along the pomegranate value chain include *Botrytis*, and *Penicillium* (Thomidis, 2014; Munhuweyi et al., 2016), which can be identified based on conidia structure and their ability to grow even in cold storage (Munhuweyi et al., 2016).

Postharvest fungal infections are predominantly controlled using fungicides. This contributes to the development of resistant pathogen populations (Rahman et al., 2014), which in turn results in the use of higher fungicide dosages and the consequent increase in toxic residues on fresh produce. Furthermore, consumers demand for safe and nutritional food, as well as the negative perception towards the use of synthetic preservatives in food (Hyldgaard et al., 2012), has heightened the research need for safer alternative antimicrobial agents. Conventional food preservation techniques, based on thermal treatments such as pasteurisation and sterilisation ensure microbiological safety at the cost of partial loss in nutritional and sensory qualities (Lado and Yousef, 2002). An alternative non-thermal preservation approach involves the use of natural antimicrobials to improve the lethal effects of the non-thermal treatments while maintaining the nutritional and sensory attributes of the produce (Severino et al., 2014). Natural antimicrobials can be applied to fruit and vegetables, as edible coatings enriched with active compounds.

Essential oils (EOs) can be utilised as a natural alternative in food preservation and their usage complies with consumers' expectations for natural food with marginal chemical treatments (Arrebola et al., 2010; Hromis et al., 2016). Application of EOs is considered a safe treatment for the control of postharvest decay of fresh produce, and therefore, should not have any regulatory issues (Sivakumar and Bautista-Banos, 2014). However, when a single EO is applied on fresh produce, it is often not effective enough to prevent or delay decay. In addition, it could cause negative organoleptic effects especially at higher doses that can provide an antimicrobial effect (Hyldgaard et al., 2012). To address this drawback, various studies have investigated the synergistic effects of combining different EOs with other non-thermal antimicrobial compounds. For instance, Severino et al. (2014) demonstrated a strong antimicrobial synergism between a chitosan coating containing 0.5 g L⁻¹ mandarin essential oil and γ -irradiation treatment against *Listeria innocua* in green beans kept at 4 °C for 14 d. Similarly, Oz and Ulukanli (2012) applied 300 and 600 ppm of *Nigella sativa* (black cumin) EO into a starch-based edible coating onto "Silifke aşısı (33 N 16)" pomegranate arils kept at 4 °C for 12 d. The combination of a starch-based coating plus *N. sativa* oil significantly reduced weight loss, browning, decay rates and retained the highest overall aril quality compared with the control. Bioactive substances such as EOs can be incorporated into film-forming materials in order to create controlled-release systems (Gemili et al., 2009). However, significant losses of the volatile compounds occur during the drying stage of the film. Therefore, micro- and nano-encapsulation of EOs could improve the efficiency of the films further (Sanchez-Gonzalez et al., 2011).

Emission of bioactive vapour/volatile compounds inside packaged food product offers the advantage of preventing surface growth of microbes without having to come into direct contact with the food product. Melgarejo-Flores et al. (2013) investigated

the effects of emulsions, vapours and coatings of cinnamon leaf oil on table grape stored at 10 °C for 15 d. Application of cinnamon oil as a vapour phase was found to be most effective in reducing fungal decay and maintain antioxidant activity of the grapes, while the emulsion treatment negatively influenced odour acceptability (Melgarejo-Flores et al., 2013). The use of polymers as carriers of antimicrobials not only permits the controlled release of these antimicrobials but also prevents dramatic reductions in their antimicrobial activities through inactivation by components in foods (De Azeredo, 2013). These films also reduce the amount of active ingredient required for direct incorporation into the food, satisfying consumer demand for fewer additives. Therefore, the objectives of this study were to: (i) elucidate on the impacts of chitosan Mw on the functional properties of the casted films; and (ii) investigate the *in vitro* and *in vivo* inhibitory effects of chitosan-EOs (of varying concentrations lemongrass, cinnamon, and oregano oils) via vapour emission and direct coating against *Botrytis* sp., *Penicillium* sp., and *Pilidiella garanati* pathogens of pomegranate fruit.

2. Materials and methods

2.1. Plant material

Pomegranate fruit cv. 'Wonderful' were harvested manually under aseptic conditions at commercial maturity from an orchard located in the Wellington region (GPS S33° 39.276 E18° 59.399), Western Cape, South Africa. Fruit maturity characteristic considered at commercial harvest include red skin and deep-red arils with mature kernels, total soluble solids (TSS, %) and titratable acidity (TA, g L⁻¹). The measured TSS and TA was 16.5 ± 0.04% and 14.2 ± 1.2 g L⁻¹, respectively. Selected fruit of uniform size, shape, and free of any external defects were washed with tap water (1 min), dipped in 700 g L⁻¹ ethanol for 30 s, 3.5 g L⁻¹ sodium hypochlorite (NaOCl) for 2 min, and 700 g L⁻¹ ethanol for 30 s (Fourie et al., 2002). Thereafter, the pomegranate fruit samples were air-dried overnight under laminar airflow prior to the *in vivo* study.

2.2. Fungal cultures

A preliminary study was conducted before this study to identify fungal pathogens associated with pomegranate cv. 'Wonderful' postharvest. *Botrytis* sp., *Pilidiella granati* and *Penicillium* sp. were isolated from diseased pomegranate fruit during cold storage. Pathogenicity and virulence of these fungal isolates were investigated and confirmed according to the Koch's postulates. The isolated fungal pathogens *Botrytis* sp., *Pilidiella granati* and *Penicillium* sp were thereafter stored at the Stellenbosch University, Department of Plant Pathology (STE-U) culture collection under the given accession numbers STE-U 7866, STE-7864, and STE-U 7865, respectively. These isolated fungal pathogens were grown in Potato Dextrose Agar (PDA) (Biolab, Modderfontein, South Africa) incubated at 25 °C for 7–14 d before each trial in order to obtain spores. A solution of distilled water amended with Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.01 mL L⁻¹ was added to the PDA plates of each fungal colony. This solution was then filtered through sterile cheesecloth with two layers to obtain spore concentrations. Spores were counted using haemocytometer (Neubauer, Marienfeld-Superior, Lauda-Königshofen, Germany) and optical microscope (Leica Wild M8 Transmitted Light Stereo Microscope, Wild Heerbrugg, Switzerland). Final adjusted spore concentration of 1 × 10⁹ spores L⁻¹ was used for each fungal culture.

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