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The physicochemical, antifungal and antioxidant properties of a mixed polyphenol based bioactive film

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Abstract

Physicochemical, antifungal and antioxidant properties of a pectin − aloe mucilage − candelilla wax − *Larrea tridentata* polyphenols based bioactive film were evaluated. Antifungal capacity was analyzed against *Botrytis cinerea, Colletotrichum gloeosporioides, Fusarium oxysporum* and *Alternaria alternata*. The main antioxidants in *Larrea tridentata* polyphenols were identified by HPLC-MS. Water vapor permeability (WVP) was measured in the film. Antioxidant capacities for $ABTS^{\cdot+}$, $DPPH^{\cdot}$, lipid oxidation inhibition (LOI) and ferric reducing antioxidant power (FRAP) (97, 92, and 57 %, 0.73 mM Fe, respectively) were evaluated. It was possible to determine the MIC_{50} for the fungi evaluated at concentrations of 558−612 ppm of polyphenols. Antioxidants identified were nordihydroguaiaretic acid (NDGA), Quercetin, and Kaenpherol. Treatment with 1.1 % of pectin, 0.16 % candelilla wax, 0.3 % glycerol, 5 % AM and 4 % extract of

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polyphenols showed values of thickness and WVP suitable to be applied on model fruits.

Keyword: Food science

1. Introduction

Edible coatings and films are an attractive alternative for extension of shelf life and quality of fruits during postharvest storage. Edible coating or film are defined as a cover material applied to foods as an effective barrier to transmission of gases, improving appearance, solving problems of migration of aromas, moisture content and oxygen and carbon dioxide diffusion, in this manner prolonging life and quality of the fruits (De Leon-Zapata et al., 2017).

The naturally functionalized packages are barriers applied on foodstuff surface which incorporate diverse natural-origin materials, in order to preserve the quality and prolong shelf-life of products. Selection of materials to formulate the film or coating has a direct impact on its function. Functional films/coatings have been top topic in the last decade because their capability of regulate moisture content, lipid migration and gas transportation (Bosquez-Molina et al., 2010) preserving thermolabile compounds like flavors, aromas and vitamins (Jimenez et al., 2010; Ochoa et al., 2011) and represent an excellent alternative to prolong shelf life of diverse foods, mainly climacteric fruits such as avocados (*Persea americana* Mill.).

Active packaging films and coatings (de Abreu et al., 2011) are designed to interact directly with environment and delay oxidation process and phytopathogenic fungi development on the fruit (López-de-Dicastillo et al., 2012; Mecitoglu and Yemenicioglu, 2007). Coatings refer materials that can be applied directly over the food surface normally in liquid form (Sathivel, 2005) by immersion, aspersion or brushing, while films are made (prefabricated) separately in solid laminates and subsequently used to cover foodstuff surface (Oussalah et al., 2004). For film formulation, are needed a film forming polymer, plasticizer (to bring flexibility) and an hydrophobic component (to retard food dehydration) (Ahmadi et al., 2012). It is also possible add a bioactive ingredient like antioxidant and/or antifungal extract to prevent microbial growth, being in this way an active packaging. Diverse bioactive ingredients have been used in food industry with the purpose of extend shelf life of products, minimize oxidation, or prevent microbial spoilage, in this sense bioactive peptides have been used in fresh meat and precooked products applied in edible coatings with successful results (Singh et al., 2018). Edible coatings and films has the property to act as a carrier for diverse additives, such as antioxidants and antimicrobials (Rojas-Grau et al., 2007), several authors observed that the incorporation of bioactive compounds into edible coatings improves their

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capacity to control microorganisms that causes spoilage in fruits and vegetables (Flores-López et al., 2015), however, specific impact depends of the bioactive ingredient and the coating system, as examples; Mohamed et al. (2013) did not observed effect on mechanical properties of chitosan based bioactive film incorporated with lactoperoxidase systems (broad spectrum antimicrobial) at different concentrations (0.5 and 1.5 %), but improve bacterial and fungal inhibitory effect. By the other hand, Ahmad et al. (2012) reported a decrease in tensile strength and elongation-at-break capacity, film transparency, solubility and water vapor permeability (WVP) of a gelatin based coating by the incorporation of bergamot and lemongrass oil.

In this sense, our research group has reported that the incorporation of bioactive ingredients in emulsions with micro particles as edible coatings based on candelilla wax is a good alternative to preserve fruit products without modifications in film or coating mechanical properties, such as reported for "Fuji" and "Golden delicious" apples (De León-Zapata et al., 2016, 2017), peppers (Ochoa et al., 2011), papaya and strawberry (Télles-Pichardo et al., 2013) demonstrating that the effectiveness of edible coatings depends primarily on controlling the wettability of the coating solutions, which affects the coating thickness (De Leon-Zapata et al., 2017).

In the present study, glycerol was used as plasticizer because is cheap, efficient and generally recognized as safe (GRAS, CAS N° 56-81-5). Candelilla wax was employed as the hydrophobic agent because is a natural and cheap wax available in the Mexican semi-desert region and also is a GRAS substance (CAS N° 8006-44-8), pectin was used as structural agent, because it is an economic, safe (CAS N° 9000-69-5) and wildly available product. Aloe mucilage (AM) was added as adjuvant to the structural agent for its capacity to retard oxygen transfer and increase mechanical properties (Khoshgozaran-Abrasa et al., 2012), Creosote bush (*Larrea tridentata*) leaves extract was used as the antifungal and antioxidant ingredient. Due to the nature of ingredients, when they are mixed, resulting mixture is an emulsion, a colloid where water support rest of ingredients and represents continues phase meanwhile candelilla wax is a discontinues phase. *Larrea tridentata* belongs to the *Zygophyllaceae* family, is original of the semi-desert zones of Mexico and United States of North America, is recognized for its biological applications in traditional medicine (Lu et al., 2010) and is used actually in different sectors like agro industries, cosmetics and pharmaceuticals. In the last years, different antioxidants have been identified and isolated form *L. tridentata* leaves such as catechin derivates, quercetin and kaempherol (Arteaga et al., 2005).

Therefore, the aims of this work were to determine the antioxidant and antifungal capacity of the total polyphenols from *Larrea tridentata* (TPL) by *In vitro* assays and to estimate different quality parameters for bioactive film development such as: water vapor permeability and thickness.

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2. Material and methods

2.1. Biological material

Phytopathogenic fungi (*Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* and *Alternaria alternata*) were kindly provided by the Food Research Department-Universidad Autónoma de Coahuila, Saltillo, Coahuila, Mexico. Leaves of *Larrrea tridentata* were collected in the Lucio Blanco community which is located at the following coordinates: North: 26.16937°, West: 102.18833°, height: 1450 AMSL, in Cuatro Ciénegas de Carranza, Coahuila, Mexico.

2.2. Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH, 97 % purity), 2,2′-azino-bis (3-ethylbenzthiazoline- 6-sulphonic acid) (ABTS, >98 % purity), 6-hydroxy-2,5,7,8-tetramethyl- 2-chromanecarboxylic acid (trolox), (9Z,12Z)-9,12-octadecadienoic acid, (linoleic acid), 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ, 98 % purity), Sodium 3,4-dioxo-3,4-dihydronaphthalene-1-sulfonate (Folin-Ciocalteu reagent, 2N), 3,4,5-Trihydroxybenzoic acid (gallic acid, 90 % purity) and Amberlite™ XAD-16 were purchased from Sigma Chemical Co. (St. Louis, MO, USA.), solvents for High Performance Liquid Chromatography (HPLC) analyses were HPLC grade.

2.3. Extraction and partial purification of polyphenols from *L. tridentata* leaves

Samples of leaves (1 g), previously dried and pulverized were mixed with 20 mL of distilled water. Samples were heated at 70 °C during 30 minutes in a water-bath (Martins et al., 2012). The aqueous extract was filtered through a qualitative filter paper and stored at −20 °C until further analysis. For partial purification of the aqueous extract, it was passed through an Amberlite™ XAD-16 column several times with water to elute components of noninterest, such as sugars. The solvent used for extraction was ethanol to elute the phenolic compounds. Ethanolic extract was dehydrated in an oven at 60 °C for 12 h to obtain a powder rich in polyphenols from *L. tridentata* leaves (TPL).

2.4. Determination of polyphenols content by microplate assay

Polyphenols in the extract were estimated as follows: 20 μL of the extract with the same volume of Folin-Ciocalteu's reagent were mixed and placed in a microplate, after 5 minutes, 20 μL of sodium carbonate (0.01 mol/L) were added, after 5 minutes 125 μL of distilled water were added, too. Absorbance was read at 790 nm (Epoch, Biotek industries, Highland park, USA) (Aguirre-Joya et al., 2013). Results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g dw).

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2.5. $ABTS^{\cdot+}$ radical cation-scavenging activity assay

This analysis was performed according to Martysiak and Wenta (2012) with minimum modifications; 1 mL of the ethanolic solution of ABTS was mixed with 10 μL of sample (partially purified liquid from *L. tridentata*) in a quartz cell. Absorbance was read at 734 nm in a spectrophotometer (Thermo Spectronic, Biomate 3, Minnesota, USA). In order to determinate the percentage of inhibition of the sample, Eq. (1) was used.

$$ABTS\ inhibition\ (\%) = \frac{(Ac - As)}{Ac}\ (100) \tag{1}$$

Where: Ac is the control absorbance and As is the absorbance of the sample. A calibration curve of trolox was performed to express the result as equivalents of trolox per gram of dry weight (μET/g dw).

2.6. $DPPH^{\cdot}$ reduction

The reduction of $DPPH^{\cdot}$ radical was made following the methodology reported by Ozkan et al. (2011) with some modifications, 193 μL of DPPH solution were mixed with 7 μL of sample in each well of a microplate. After 30 minutes of reaction under dark conditions, absorbance was read at 517 nm for Enzyme-Linked Immuno Sorbent Assay (ELISA reader, Epoch, biotek industries, highland, USA). The reduction of the DPPH radical was calculated as the percentage of inhibition, with Eq. (2).

$$DPPH\ inhibition\ (\%) = \frac{(Ac - As)}{Ac}\ (100) \tag{2}$$

Where: Ac is the control absorbance and As is the absorbance of the sample.

2.7. Lipid oxidation inhibition (LOI) assay

This assay was done according to the method reported by Martínez-Ávila et al. (2012), linoleic acid was used as lipids source during the assay. The linoleic acid solution was prepared by diluting 0.56 g of linoleic acid and 1.5 g of Tween 20 in 8 mL of ethanol (96%). 50 μL of sample were mixed with the linoleic acid solution (100 μL) and 1.5 mL of 0.02 mol/L acetate buffer (pH 4.0). Controls contained distilled water instead of the phenolic sample. All the samples were homogenized in a vortex (Labnet International, Edison, NJ) and sonicated in an ultrasonic bath (Bransonic 2510R-MTH; Branson, Dambury, CT) for 3 min and incubated at 37 °C. After 1 min, 750 μL of $FeCl_2$ (50 mol/L) were added to induce reaction of oxidation. After 1 h, 1 mL of 0.1 mol/L of NaOH in an ethanolic solution (10%) was added to 250 μL of the mixture to stop the oxidation process. At 24 h of incubation 250 μL were taken again to stop the oxidation reaction. After mixing, 2.5 mL of 10% ethanol were added; absorbance was measured at 232 nm. Percentage of antioxidant activity

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was calculated by Eq. (3) (Badamus et al., 2011). Tween 20 is a non-ionic detergent wildly use to stabilize oil-in water emulsions, due that capacity is incorporated in the reagents for the assay.

$$LOI\ (\%) = \frac{(\Delta Ac - \Delta As)}{\Delta Ac}\ (100) \tag{3}$$

Where: ΔAc is the difference between 24 and 1 h of lipid oxidation in controls and ΔAs is the difference between 24 and 1 h of lipid oxidation in samples.

2.8. Ferric reducing antioxidant power (FRAP) assay

Assay was performed as described by Benzie and Strain (1996) with slight modifications. An aliquot of 10 μL was mixed with 290 μL of FRAP reagent in a 96-well microplate. The reaction mixture was incubated at 37 °C for 15 minutes. Absorbance was evaluated at 593 nm against a blank of distilled water. A calibration curve was prepared using an aqueous solution of ferrous sulphate $FeSO_4 \cdot 7H_2O$. FRAP. The resulting values were expressed as millimoles of ferrous sulphate equivalent per gram of dry weight of the plant. (mM Fe (II)/g dw).

2.9. Main polyphenols in TPL identification

A Varian High Performance Liquid Chromatography coupled to a mass spectrophotometer (HPLC-MS) was used to identify the main components in the *L. tridentata* leaves extract. The extract was filtered through a 0.45 μm nylon membrane. A C18 column was used with the following operational conditions: 250 mm × 4.6 mm, flow rate of 1 mL min^{-1}, a sample of 10 μL, a gradient of methanol and 3 % acetic acid as the mobile phase. Mass Spectrophotometer model 500-MS with a flow rate of 1 mL/min, mass rate of 200−2000 was used for 10 minutes.

2.10. In vitro antifungal activity for MIC_{50} determination in petri dishes

It was used the technique previously reported by Bagade et al. (2013) with some modifications to determine the minimum inhibitory concentration (MIC_{50}), defined as the concentration of an extract capable to decrease the concentration at 50 % of growth in comparison to the control. Poisoned media were prepared by adding 250, 500, 1000 and 1250 ppm of TPL to a previously sterilized potato dextrose agar (PDA) medium. Control was only PDA without any TPL. After medium solidification, an explant of 3 cm-diameter of 7 days-old culture of the evaluated fungal isolates (*Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* and *Alternaria alternata*) was placed on the culture medium. Petri dishes were incubated at 37 °C; the mycelial radial growth was monitored kinetically with a Vernier until the control invaded the plate.

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2.11. Film formulation and water vapor permeability (WVP) determination

Nine films were formulated according to a Box-Behnken full factorial experimental design with 2 factors (aloe mucilage and creosote bush crude extract) at 3 levels (2.5, 5 and 10; 1, 2 and 4 % v/v, respectably). The nine treatments contained pectin, 1.1 % (w/v) candelilla wax 0.16 % (w/v) and glycerol 0.3 % (v/v). Pectin was dispersed in distilled water with continuous stirring. The resulting suspension was heated at 80 °C to add candelilla wax. The crude extract was added and homogenized at 1000 rpm/15 min. 30 mL, of coating solution, were placed into a Petri dish and dehydrated in stove at 60 °C/12 h to create a prefabricated film. WVP was evaluated as previously reported (ASTM-e96, 1995).

2.12. Statistical analysis

All experiments were performed in triplicate, treatment mean values are presented. Results were analyzed by one-way analysis of variance (ANOVA) in SAS statistical program (SAS 7.0). When it was needed, means comparison was performed using the Tukey's range test ($P \leq 0.05$).

3. Results and discussion

The total phenolic content from creosote, after partial purification through a column of Amberlite™ XAD-16, permitted obtain 0.348 ± 0.02 g of TPL from 12 g of dry material (leaves), which represents a recuperation yield of 2.9 %. The main antioxidants present in the aqueous extract from *L. tridentata* leaves were identified by HPLC-MS. Three major signals were identified in the extract (Fig. 1), corresponding to the antioxidants previously reported by Martins et al. (2012). The antioxidants identified were the norlignan: nordihydroguaiaretic acid (NDGA), and the flavonoids: kaempferol and quercetin. These compounds are directly related to the antioxidant and antifungal activities (Osorio et al., 2010).

The Folin-Ciocalteu's reagent was used to quantify the polyphenols present in the sample (crude aqueous extract). Under the assay conditions, 300 mg of gallic acid equivalents per gram (GAE/g) were obtained. Martins et al. (2012) determined a concentration of 62.55 mg GAE/g when used only water as extraction solvent and a concentration of 487.13 when they use 90 % (v/v) acetone. The differences with the present work may be due to nature of plant growing area and month of the year where were collected also to physiological conditions of the plant. The radical cation-scavenging activity assay of the 2,2′-azino-bis (3-ethylbenzthiazoline- 6-sulphonic acid) is based on the single electron transfer (SET) (Tan and Lim, 2015), that involves a simple redox reaction, where the oxidant substance becomes the indicator for endpoint measurement (Huang et al., 2005). This technique is

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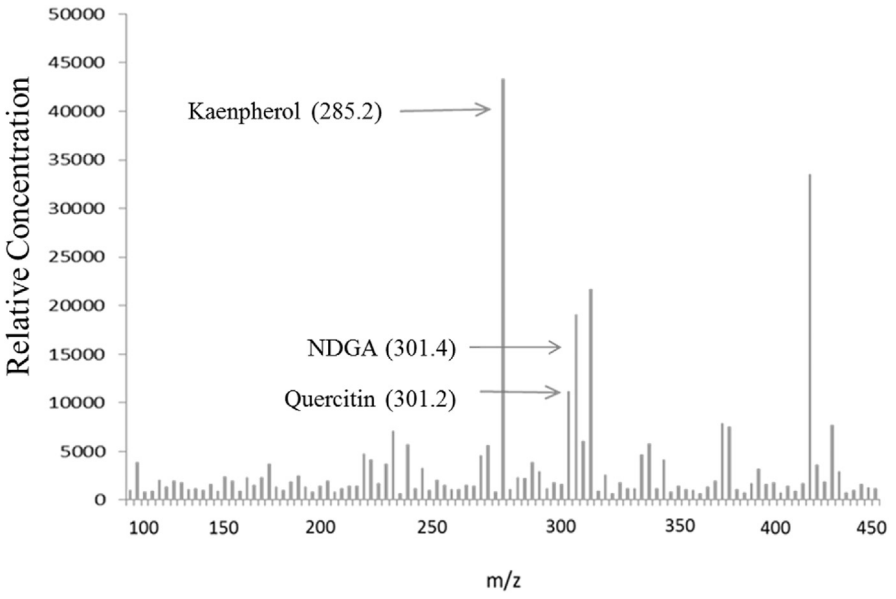

Fig. 1. Identified phytocompounds from Total Polyphenols of *Larrea* (TPL) by High Performance Liquid Chromatography coupled to a Mass Spectrophotometer.

usually compared with the standard Trolox. The crude, aqueous extract showed anti-radical scavenger capacity against $ABTS^{\cdot+}$ since it almost completely inhibited the radical 97 %, which represents a concentration of 4.11 μET/g.

The assay of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity is based on the single electron translate reaction, is one of the most popular assays to determinate antioxidant capacity of the sample because is a highly sensitive method with reproducible results. The crude aqueous extract showed good antioxidant capacity according to this technique, like the observed in the ABTS assay. The sample almost inhibits the total of the free radical present in the assay (92 %) similar to reported by Martins et al. (2012), who worked with different solvents in different concentrations, with practically no differences on DPPH reduction for each solvent. In the present work is corroborated that the extraction with only water presents a high antiradical activity, with the advantage that is used a nontoxic and economic solvent (water).

In order to evaluate a global antioxidant activity of phenolic samples more than one assay must be used (Cíz et al., 2010). The LOI assay is a hydrogen atom transfer (HAT) based assay (Tan and Lim, 2015). In spite of the HAT and SET based assays the final result (stabilized free radical) is the same (Power et al., 2013), the LOI assay is a better analog of the *in vivo* antioxidant capacity because it evaluates the antioxidant capacity against a peroxyl radicals. Peroxyl radicals, have biological significance because are involved in lipid peroxidation and autoxidation (Prior et al., 2005). In this assay, 57 % of LOI was achieved, that is lower than the reported by

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Turner et al. (2011), who obtained 80 % of LOI. It may be due to they worked with *Larrea divaricata*, that present different phenolic compounds to Kenpherol and Quercetin (NDGA prevalence as the main phenolic compound).

Ferric reducing antioxidant power evaluates the reducing capacity of the sample (Benzie and Strain, 1999). In this study a value of 0.73 mM Fe (II)/g of dried weight was obtained. This value was similar than the reported by Martins et al. (2012) when using only water as extraction solvent, who achieve 0.77 mM Fe (II)/g of dried weight, which is a similar methodology as used in this paper. This result demonstrates that is possible extract the major antioxidant compounds from *L. tridentata* leaves with good antioxidant capacity by using only water as solvent. Table 1 summarizes the antiradical/antioxidant techniques evaluated and the response obtained in the present work.

The antifungal activity of TPL was tested against well-known phytopathogenic fungi. The results of the biological activity of this extract showed that TPL presented a high fungi-static activity. However, a fungicide effect at the concentrations assayed was observed. Osorio et al. (2010) reported a fungicide effect of *L. tridentata* leaves extract at a concentration of 0.7 ppm against of some *Oomycetes* and *Deuteromycetes*. These differences can be attributed at the phenolic extraction process.

Fusarium oxysporum, is a very persistent and disseminated phytopathogenic fungus in crops around the word. In this work inhibition of 50 % of the growth of this phytopathogen was achieved by using TPL. The inhibitory concentrations of TPL observed were lower than reported by Lira-Saldivar et al. (2002), who used 4000 ppm to inhibit the growth of *F. oxysporum*. The minimum inhibitory concentration for the 50 % of the fungal growth (MIC_{50}) was defined as the lower concentration need to delay in a 50 % the fungal growth, compared with the control. Table 2 shows the MIC_{50} values for each fungus strain used in the present work. The film thickness was measured with a hand-held micrometer (Mitutoyo, Tokyo, Japan). The WVP and thickness of the nine treatments are shown in Table 3. Treatments 6, 8 and 9, presented ideal values of thickness and WVP to be used as film over a fruit or vegetable with the intention to prolong the shelf-life of these foods, keeping organoleptic quality intact. Because as previously reported these values

Table 1. Antioxidant activity of TPL evaluated by 4 different methods.

Assay	Value
Folin-Ciocalteu	300 mg GAE/g
DPPH	92 %
ABTS	97 % (4.11 μET/g)
LOI	57 %
FRAP	0.73 mM Fe(II)/g plant

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Table 2. MIC values.

Fungal strain	MIC_{50} (ppm)
Alternaria alternata	566
Fusarium oxysporum	558
Botrytis cinérea	612
Colletotrichum gloeosporioides	579

Table 3. Thickness value for each treatment of formulated film.

Treatment	Thickness (μm)	WVP (g/m.s.Pa),
7	230 ± 20	3.85E-10 ± 1.16E-11
5	190 ± 90	2.75E-10 ± 1.3E-10
8	100 ± 30	1.07E-10 ± 1.0E-11
4	10 ± 3	8.18E-10 ± 2.7E-10
3	60 ± 1	8.6E-10 ± 1.4E-10
9	90 ± 3	1.21E-10 ± 4.81E-11
2	50 ± 3	1.95E-10 ± 3.83E-11
1	50 ± 1	4.66E-10 ± 5.65E-11
6	80 ± 9	1.2E-10 ± 1.1E-11

does not affect the respiration rate and maturity process in a negative way (Bosquez-Molina et al., 2010).

4. Conclusions

The crude extract of *Larrea tridentata* leaves, showed an excellent *in vitro* free radical-scavenging and antioxidant activities, corroborated by four different techniques (ABTS, DPPH, LOI and FRAP). The three major polyphenolic compounds identified with the HPLS-MS were NDGA, Kenferol and Quercetin. TPL showed high fungistatic effect against some of the most important phytopathogenic fungi. At least 3 formulations of the films have an ideal WVP value to be applied on a model fruit to prolong shelf-life, keeping organoleptic quality intact. Future researches are focused on the *in vivo* applications over a model fruit with the TPL concentration against the phytopathogenic fungi previously evaluated.

Declarations

Author contribution statement

Jorge A. Aguirre-Joya, Lorenzo Pastrana-Castro, Diana Nieto-Oropeza, Janeth Ventura Sobrevilla, Romeo Rojas-Molina, Cristobal N. Aguilar: Conceived and

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designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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