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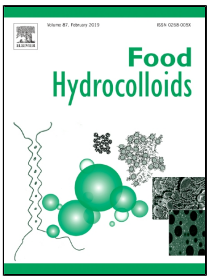

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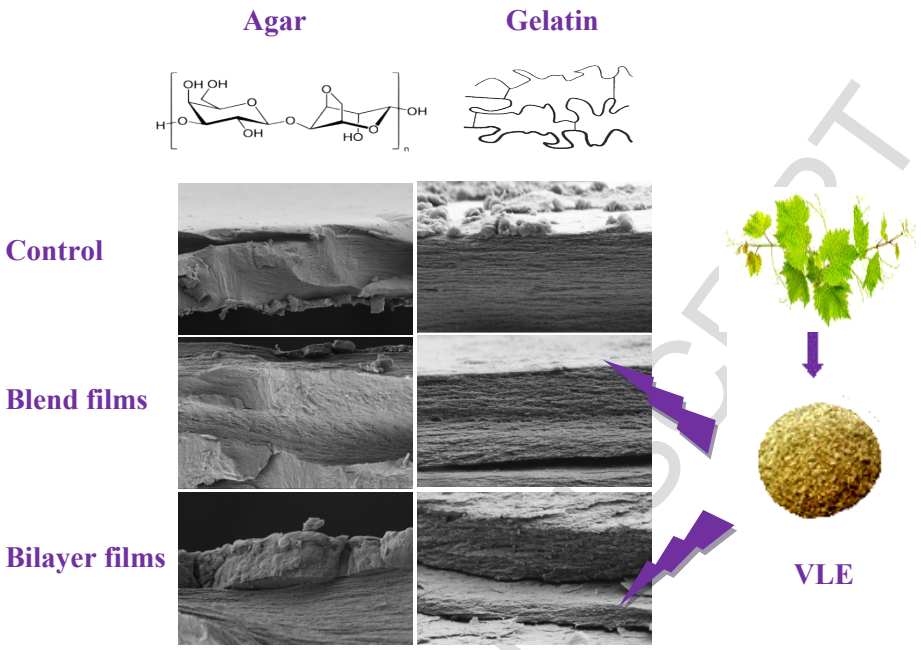

Development and characterization of grey triggerfish gelatin/agar bilayer and blend films containing vine leaves bioactive compounds

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Abstract

The present study aims to develop natural biopolymer bioactive edible films. Particularly, blend (F-Bl) and bilayer (F-Bi) films based on grey triggerfish skin gelatin (G) and agar (A), incorporated or not with vine leaves ethanolic extract (VLE), at 5 and 10 mg/mL, were prepared using the casting method. First, VLE presented an important antioxidant potential evaluated by the iron (Fe^{2+}) chelating activity, ferric (Fe^{3+}) reducing antioxidant power, β-carotene bleaching protection and DPPH•-radical scavenging activity. Furthermore, VLE presented interesting antibacterial and antifungal activities. On the other hand, the composite films were characterized by scanning electron microscopy (SEM), FTIR, physicochemical, and thermal analyses. SEM micrographs showed that agar and gelatin were compatible. Moreover, the mechanical properties results showed that F-Bl and F-Bi were mechanically stronger and more deformable than control films. This change in mechanical properties can be explained by the formation of a dense network after agar addition, due to the interactions with the gelatin matrix. After their incorporation into polymer network, VLE increased slightly the thermal stability and improved greatly the antioxidant activity of the composite films (gelatin and agar) in dose dependent manner. Thus, results encourage the further use of gelatin/agar/VLE films as active food packaging material.

Keywords: Grey triggerfish skin gelatin; Agar; Composite films; Vine leaves extract; Antioxidant and antibacterial activities; Microstructure.

1. Introduction

For environmental reasons, natural biopolymers coming from renewable bio-resources, which mostly include plants and animals (Mohajer, Rezaei & Hosseini, 2017), can serve as a potential alternative for non-biodegradable petrochemical-based plastics (Jridi et al., 2013; Tharanathan, 2003). Biodegradable materials can be made from biopolymers, including proteins, polysaccharides, and lipids or their combinations (Abdelhedi et al., 2018; Arfat, Ahmed, Hiremath, Auras & Joseph, 2017; Manivasagan & Oh, 2016; Hajji et al., 2016).

Fish gelatin films have been extensively used to protect foods against drying, while they have poor mechanical and water vapor barrier properties (Hosseini & Gómez-Guillén, 2018; Giménez, Lopez de Lacey, Perez-Santín, Lopez-Caballero & Montero, 2013). Thus, blending two polymers is one of the methods to improve gelatin film physicochemical properties, such as gelatin/chitosan (Jridi et al., 2014), chitosan/gelatin/poly-vinyl alcohol (Bento, Pereira, Chaves & Stefani, 2015), and gelatin/agar (Mohajer et al., 2017) can be a promising way for the development of a final network with high functional properties.

Likewise, Agar (A), a gelatinous polysaccharide extracted from marine red algae such as *Gelidium* and *Gracilaria* spp., is known to be one of the most promising polysaccharides for developing biodegradable packaging films (Mohajer et al., 2017; Vejdan, Ojagh, Adeli & Abdollahi, 2016; Kanmani & Rhim, 2014). Recently, Mohajer et al. (2017) reported that the combination between agar and cold water fish skin gelatin would lead to films with better properties compared to those formed by each individual material alone. On another hand, recent studies have focused on the amelioration of the biological activities of biodegradable films by adding different compounds gifted with antioxidant or antimicrobial powers. Due to safety concerns associated to synthetic active compounds, extensive research has been

performed to seek natural active compounds as alternative to synthetic ones, among them comes, the phenolic compounds (Ashwell, Moyo & Staden, 2010; Conklin, 2000).

Vitis vinifera L. is one of the most widely cultivated crops worldwide, and it is extensively cultured in many countries of the Mediterranean Basin, particularly in Tunisia, with an estimated grape production over 84 million tons in 2015 (Harb, Alseekh, Tohge & Fernie, 2015; FAOSTAT, 2017). The wine making industries and the grapevine itself lead to the production of high amounts of by-products such as leaves. Recently, several studies have reported the efficiency of grapevine leaves as an excellent source of bioactive compounds, mainly phenolic compounds and flavonoids (Farhadi, Esmaeilzadeh, Hatami, Forough & Molaie, 2016; Lima, Bento, Baraldi & Malheiro, 2016; Katalinic et al., 2013). Moreover, vine leaves have been studied due to their numerous biological activities including anti-coagulant, immune enhancing, anti-hyperglycemic, antioxidant, and antibacterial activities (Ruiz-Moreno et al., 2015; Lima et al. 2016).

In Tunisia, grey triggerfish (*Balistes capriscus*) production was up to 106 tones in 2015 and the world captures were about 12038 tones in 2015 (FAOSTAT, 2016), but all the skin of this specie is discarded, which can causes environmental problems. Jellouli et al., (2011) extracted successfully gelatin from grey triggerfish skin.

To the best of our knowledge, no works were reported on the use of vine leaves as a source of antioxidants employed for edible bioactive film preparation. Thus, the purpose of the present study is to prepare films (blend and bilayer) based on agar (A) and gelatin (G). The effect of mixing these polymers on the physicochemical, structural, thermal and mechanical properties of films was investigated. Moreover, the effect of the incorporation of vine leaves extract (VLE), on the properties of the resulting films was also determined.

2. Material and methods

2.1. Extraction of gelatin from grey triggerfish

Skin from grey triggerfish (*B. capriscus*) was obtained from the fish market of Sfax City, Tunisia. Skin was cut into small pieces (1 cm x 1cm) and then soaked in 0.05 M NaOH (1:10 w/v). The mixture was stirred for 2 h at 4 °C and alkaline solution was changed every 30 min. The alkaline-treated skins were then washed with distilled water until a neutral pH was obtained. The alkaline-treated skin was soaked in 100 mM glycin-HCl buffer, pH 2.0 with a solid/solvent ratio of 1:10 (w/v) and subjected to collagen hydrolysis with 5 units of pepsin /g of skin, as described in our previous study (Jellouli et al., 2011). The grey triggerfish skin gelatin (G) obtained was used for films preparation.

2.2. Preparation of vine leaves powder and its ethanolic extract

Vine leaves were collected on March 2016 from the area of Sfax (Tunisia) and were washed and dried in convection oven at 50°C during 6 h (Polin A511088/AL/3125, Verona, Italy). The dried leaves were ground in a spice grinder (Black & Decker CBG100S Smartgrind, Maryland, USA), sieved through a 250 µm sieve and the obtained powder, referring to the vine leaves powder was stored at 25°C until use.

The VLP (25 g) was Soxhlet-extracted using 300 ml of ethanol during 6 h. The average yield of the vine leaves extract (VLE) was found to be 29.5% (w/w). The solvent was then evaporated under vacuum and the residual solvent was removed by flushing with nitrogen. Finally, the obtained extract was kept in the dark at 4°C until further use.

2.3. Evaluation of antioxidant activities

2.3.1. Iron (Fe^{2+}) chelating activity

The iron chelating effect of the different samples was tested according to the method of Decker and Welch (1990). Briefly, 100 µl of sample (VLE (0.1-1 mg/mL) or small pieces of each film (10 mg)), were added to 50 µl of 2 mM $FeCl_2$ and 450 µl of water. The mixtures were incubated at room temperature for 3 min and the reaction was initiated by the addition of 200 µl of 5 mM of ferrozine solution. The mixtures were then vigorously shaken and left to

stand at room temperature for 10 min. Control tubes were prepared by the same manner, substituting the sample by water and the ethylene-diamine-tetraacetic acid (EDTA) was used as a positive standard. The test was carried out in triplicate. The absorbance of solutions was measured at 562 nm and the chelating activity (%) was calculated as follows:

$$\text{Metal chelating activity (\%)} = [(OD_C + OD_B - OD_S)/OD_C] \times 100$$

where OD_C, OD_B and OD_S represent the absorbance's of the control, the blank and the sample reaction tubes, respectively.

2.3.2. Ferric (Fe^{3+}) reducing antioxidant power

The ability of sample to reduce iron was determined according to the method of Yildirim, Mavi, and Kara (2001) with slight modifications. A volume of 0.5 ml of each sample (VLE (0.1-2 mg/mL) or small pieces of each film (10 mg)), was mixed with 1.25 ml of potassium phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of 1% potassium ferricyanide solution. The reaction mixtures were incubated for 20 min at 50°C. After incubation, 0.5 ml of 10% trichloroacetic acid (TCA) was added and the reaction mixtures were then centrifuged for 10 min at 3000 rpm. Finally, 1.25 ml of the supernatant solution from each sample mixture was mixed with 1.25 ml of distilled water and then 0.25 ml of 0.1% ferric chloride was added. The absorbance of the resulting solutions was measured at 700 nm after 10 min of incubation. The butylated hydroxyanisole (BHA) was used as a positive standard. Three replicates were done for each test sample.

2.3.3. Antioxidant assay using the β-carotene bleaching method

The prevention of β-carotene from bleaching was determined according to the method of Koleva, Van Beek, Linssen, de Groot and Evstatieva (2002). First, the emulsion of β-carotene/linoleic acid was freshly prepared by dissolving 0.5 mg of β-carotene, 25 µl of linoleic acid and 200 µl of Tween 40 in 1 ml of chloroform. The chloroform was then completely evaporated under vacuum in a rotatory evaporator at 50°C; then 100 ml of

distilled water were added and the resulting mixture was vigorously stirred. Thereafter, 2.5 ml of the β-carotene/linoleic acid emulsion was transferred to test tubes containing 0.5 ml of each sample (VLE (0.05-1 mg/mL) or small pieces of each film (10 mg)). Control tubes were prepared in the same conditions by adding 0.5 ml of water to the emulsion. The absorbance of every test tube was measured at 470 nm twice, before and after incubation for 1 to 2 h at 50°C. The butylated hydroxyanisole (BHA) was used as a positive standard. Tests were carried out in triplicate and the antioxidant activity was evaluated in terms of β-carotene bleaching inhibition using the following equation:

$$\beta\text{-carotene bleaching inhibition (\%)} = [1 - (OD_0 - OD_t)/(OD_0' - OD_t')] \times 100$$

where OD_0 and OD_t are the absorbance's of the test sample measured before and after incubation, respectively; and OD_0' and OD_t' are the absorbance's of the control measured before and after incubation, respectively.

2.3.4. Free radical scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH•)

The DPPH•-radical scavenging activity was determined as described previously by Bersuder, Hole and Smith (1998). 500 µl of sample (VLE (0.5-6 mg/mL) or small pieces of each film (10 mg)) were allowed to react with 375 µl of ethanol solution and 125 µl of 0.02% DPPH•. The reaction mixtures were incubated for 60 min in the dark at room temperature and the reduction of DPPH• radical was measured at 517 nm. The butylated hydroxyanisole (BHA) was used as a positive standard. The test was carried out in triplicate and the DPPH•-radical scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [(OD_C - OD_S)/OD_C] \times 100$$

where OD_C, and OD_S represent the absorbance's of the control and the sample reaction tubes, respectively.

2.4. Antibacterial activity

2.4.1. Microbial strains

Eight pathogenic bacterial strains were used for antibacterial screening of the VLE. Four Gram-positive bacteria: *Micrococcus luteus* (ATCC 4698), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778) and *Enterococcus faecalis* and four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 43972) and *Salmonella typhi* (ATCC 19430) were tested. Besides, antifungal activity was tested using *Aspergillus flavus*, *Aspergillus niger* and *Fusarium oxysporum*.

2.4.2. Agar diffusion method

The antibacterial activity assay was performed referring to the method described by Berghe and Vlietinck (1991). Culture suspensions (200 µl) of the microorganisms (10^6 colony forming units (cfu/ml) of bacterial cells and 10^8 spores/ml of fungal strains) were spread on Luria-Bertani (LB) agar and potato dextrose agar (PDA) medium, respectively. Then, 60 µl of VLE (250 µg/ml) were loaded into wells punched in the agar layer. Before incubation, all plates were stored in the dark at 4°C for 2 h, to allow the extract diffusion. At the end of incubation time (24 h at 37°C for bacteria strains) or (72 h at 30°C for fungal strains), positive antibacterial and antifungal activities were established by the presence of measurable inhibition zones. Negative controls were prepared using sterile water. Gentamycin (10 µg/well) and cycloheximide (10 µg/well) were used as positive standards for bacteria and fungi, respectively. Antimicrobial activity was evaluated by measuring the growth inhibition zone (diameter expressed in millimeters) around the wells.

2.5. Film preparation and characterization

2.5.1. Film preparation

To prepare film-forming solutions, gelatin (G) and agar (A) (Sigma-Aldrich (St. Louis, MO, USA)) powders were dissolved in distilled water to achieve a final concentration of 3% (w/v) each as previously described by Mohajer et al., (2017), with slight modifications. As

plasticizer, glycerol was added to the gelatin or agar solutions at different levels (0, 5, 10 15, 20 and 25%) and then mixed genteelly at 40 °C for 30 min. The resulting films were obtained by casting 25 ml of film forming solution (FFS) on a rimmed silicone resin plate (12 cm x 12 cm), dried at 25 °C at a relative humidity (RH) of 50% and then peeled off manually. A preliminary visual evaluation of films led to select a final glycerol concentration of 15%.

The diagram explaining the methodology used to prepare films is presented in Fig. 1. To prepare blend film (Bl-F), 12.5 ml of the G-FFS and 12.5 ml of A-FFS were gently stirred for 30 min at 40 °C. The solutions were then cast on the surface of the plate. For the bilayer film (Bi-F), casting was performed by two steps. A volume of 12.5 ml of G-FFS were cast onto the surface of the plate and dried at a temperature of 25 °C and RH of 50% until a compact surface formation. Thereafter, 12.5 ml of A-FFS were directly poured on the top of the dried gelatin layer and the system was dried again (25 °C and 50% RH). Finally, all films were peeled off manually. Resulting films from agar and gelatin were named A-F and G-F, respectively.

For the bioactive films, VLE (5 or 10 mg/mL) were dispersed in each FFS and mixed at 25 °C until complete solubility. The methodology used for films preparation is detailed in Fig. 1. Prior to characterization, all films were conditioned at 25 °C and 50% RH for at least 14 days.

2.5.2. Films characterization

2.5.2.1. Microstructure analysis of films

Microstructure of cryo-fractured cross-section of film samples was visualized using a Scanning Electron Microscope (SEM, Hitachi SU 1510). The film samples were cryo-fractured by immersion in liquid nitrogen. Prior to visualization, film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive. Samples were

photographed with an angle of 90° to the surface to allow observation of the films cross-section.

2.5.2.2. Film thickness

The thickness of films was measured using a digital thickness gauge (Schmidt, Control instrument). Ten random locations (from the centre and close to the perimeter) were taken from each film sample, and the average was used in the calculations of transparency, water permeability and mechanical properties.

2.5.2.3. Color, light transmission and transparency

Color of the film samples was determined using a chromameter (CR-200, Minolta, Japan) and expressed as L* (lightness/brightness), a* (redness/greenness) and b* (yellowness/blueness) values.

The barrier properties of composite films against ultraviolet (UV) and visible light were measured at wavelengths ranging between 200 and 800 nm, using a UV-Visible spectrophotometer (SAFAS Monaco, UVmc). The transparency value of the film was calculated by the following equation:

$$\text{Transparency value} = -\log T_{600}/e$$

where T_{600} is the fractional transmittance at 600 nm and e is the film thickness (mm). The greater transparency value represents the lower transparency of the film.

2.5.2.4. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectra of different films were determined using a PerkinElmer Spectrum infrared spectrometer equipped with an attenuated total reflection (ATR) accessory. Films were analyzed with a 32 scans per minute at a resolution of 4 cm^{-1} in the wavenumber region between 650 cm^{-1} and 4000 cm^{-1}.

2.5.2.5. Mechanical properties

Tensile strength (TS) and elongation at breakpoint (EAB) of film samples were determined using universal testing machine (Lloyd Instrument, Hampshire, UK) equipped with A/MTG tensile grips, according to the ASTM-D882 method, with slight modifications. Rectangular film samples (50 mm x 25 mm) were prepared using a precision standard cutter (Thwing-Albert JDC Precision Sample Cutter, USA) in order to get pieces with an accurate width and parallel sides throughout the entire length. Before testing, all the samples were equilibrated for two weeks at 25 °C and 50% RH. The film samples were clamped and deformed under tensile loading using a 300 N load cell with the cross head speed of 50 mm/min until the samples breaking. TS (MPa) and EAB (%) were determined from the stress-strain curves from six repetitions.

2.5.2.6. Thermal properties

Prior to experiments, samples were conditioned at 25 °C and 0% RH (silica gel) for 48 h to obtain the maximum dehydrated film samples. Conditioned films (5 mg) were then hermetically sealed in DSC Aluminum pans (PerkinElmer®) and scanned using a differential scanning calorimeter (Mettler Toledo Star). DSC measurements were carried out in duplicate for each film because of the excellent repeatability.

2.5.2.7. Water vapor permeability

Water vapor permeability (WVP) measures of the amount of water vapor passing through the surface area of a material per unit time and normalized to thickness and partial pressure differential. The WVP was determined gravimetrically using a modified ASTM E96-80 (1980) standard method, adapted to edible materials, as described by Abdelhedi et al. (2018).

2.7. Statistical analysis

Statistical analyses were performed with SPSS ver. 18.0, professional edition using ANOVA analysis. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Antioxidant and antimicrobial activities

The antioxidant activities of the VLE were investigated by using complementary methods, such as Fe^{3+} reducing power, DPPH• radical-scavenging activity, antioxidant assay using the β-carotene/linoleate model system and Fe^{2+} chelating activity (Fig. 2). In fact, the use of several tests allows us to have a better idea about the antioxidant activity of the studied extract. Ferric reducing antioxidant power measures the reducing ability against ferric ion (Fe^{3+}), which indicates the ability of compounds to give an electron to the ion (Khantaphant & Benjakul, 2008). The Fe^{3+} reducing power of VLE at different concentrations was presented in Fig 2.A. Based on absorbance values, VLE was an effective reducing agent and its activity increased with increasing concentration between the range of 0.1 and 2.0 mg/mL to reach a maximal absorbance of 2.0 at 1.0 mg/mL. Nevertheless, the reducing power of the VLE remained significantly lower ($p<0.05$) than that of the standard butylhydroxyanisol (BHA). Besides, the scavenging effect of VLE on DPPH• radicals was evaluated and the results were presented in Fig 2.B. Data indicated that DPPH• radicals-scavenging activity of VLE increased in a dose-dependent manner and it was similar to the standard BHA. The IC_{50} value, defined as the concentrations of VLE required to inhibit 50% DPPH•, was 10 µg/ml, which was comparable to that obtained (IC_{50} = 11.18 µg/ml) by Aouey et al. (2016) for a leaf extract of the same species. Furthermore, the antioxidant activity of VLE or BHA was determined as % of inhibition of the β-carotene bleaching in an emulsified linoleic acid model system (Fig 2.C). From the obtained results, it can be clearly observed that VLE prevented the β-carotene against bleaching by donating hydrogen atoms to peroxyl radicals of the oxidized linoleic acid, in a dose-dependent manner. Indeed, the IC_{50} value, defined as the VLE concentration necessary to obtain a 50% inhibition of β-carotene peroxidation, was 0.2 mg/mL, which was similar to that published by Katalinić et al. (2009) for *V. vinifera* leaves extracts, but lower

than that of BHA. This can be explained by the fact that the VLE was a crude extract, whereas BHA was a pure molecule. This encourages to study the fractionation of the VLE and to isolate pure antioxidant molecules that could be more effective. In cells, excess free Fe^{2+} is able to catalyze the decomposition of H_2O_2 into the extremely reactive hydroxyl radical (OH•). Thus, it is important to determine the iron binding activity of the natural antioxidant molecules (Orhan, Orhan, Ozcelik & Ergun, 2009). VLE showed an important ability of chelating Fe^{2+} ion, which increased with increasing the extract concentration (Fig. 2.D). In fact, the IC_{50} value, defined as the VLE concentration necessary to react with 50% of the iron ions, was 0.23 mg/mL,

On the other hand, although the standard BHA has shown higher antioxidant activity than VLE, natural antioxidants were more interesting as compared to artificial ones. In fact, in recent years several questions have been raised regarding the safety of artificial antioxidants used in food technology. For example, the BHA commonly used in the food industry is suspected of having negative health effects on consumer health. In addition, the dietary intake of synthetic antioxidants can cause carcinogenicity and genotoxicity at high concentrations (Bouayed & Bohn, 2010; Conklin, 2000; Ito, Fukushima, Hagiwara, Shibata & Ogiso, 1983). Therefore, the consumption of food products supplemented with the vine leaves powder would potentially provide antioxidant potential and consequently health benefits.

The antimicrobial activities of the VLE (250 µg/ml) against four Gram-positive and four Gram-negative bacteria strains, and 3 fungi strains were evaluated by determining the inhibition zones (mm) on solid medium (Table 1). The VLE presented an interesting antibacterial potential against all investigated microorganisms. In fact, the values of the inhibition zones of the tested strains vary between between 16.0 and 32.0 mm. *K. pneumoniae* (Gram-negative) and *S. aureus* (Gram-positive) were the most sensitive strains (30.0-32.0 mm) for the studied extract. Jayaprakasha, Selviand, Sakariah and Krewer (2003) found that

the antibacterial effect of grape seeds extract was more effective against Gram-positive than Gram-negative strains. Our results are in line with those reported by Katalinic et al., (2013) who demonstrated antibacterial activity of extracts from different Vitis vinifera L. varieties against gram-positive and -negative bacterial strains. In another study, Orhan et al., (2009) tested vine leaves extracts against standard and isolated strains (*E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus* bacteria, as well as *C. albicans* and *C. parapsilosis* fungi) by using the microdilution method. All of the testes extracts displayed a little more antibacterial activity against gram-positive bacteria than gram-negative bacteria.

In general, the antimicrobial activity of phenolic compounds is well known. For example, the flavonoids, such as quercetin and other related compounds, have shown important antimicrobial effect acting primarily by enzymatic inhibition of DNA gyrase (Cushnie & Lamb, 2005). In addition, the antimicrobial effects of phyto-chemical extracts may be explained by the fact that they may cause disruption of cellular integrity, resulting in increased permeability of the membrane and consequently inhibition of respiration (El-Mostafa et al., 2014). However, it is difficult to attribute the antimicrobial activity of phyto-chemical extracts, characterized by a complex mixture, to a single or a particular constituent. The crude extracts may be more advantageous than the isolated compounds, since an individual bioactive component can modify its properties in the presence of other compounds present in the extract (Katalinic et al., 2013). As a result, the synergistic and antagonistic effects between several components in the phyto-chemical extract are possible and should also be taken into account (Borchers, Hackman, Keen, Stern & Gershwin, 1997). It can be said that plants can be particularly a rich source of bioactive compounds, which inhibit the growth of several pathogenic bacteria that shows marked resistance to currently available antibiotics (Syed, Prasad, Deeba, Jamil & Alshatwi, 2011).

3.2. Films characterization

3.2.1. Microstructure of films

The microstructure of the different composite (bilayer and blended) films was studied via the SEM cross-sections observations (Fig. 3). Micrographs of the blend films showed compact and homogenous structure, indicating the high compatibility between both polymers in FFSs. This structure is due to the intermolecular biopolymer associations or may be due to the good compatibility between gelatin and agar (Tian, Xu, Yang, & Guo 2011). In this context, Mohajer et al., (2017) reported that intermolecular interactions between both polymers chains is due to the hydroxyl functional groups available, which improved the miscibility of the two phases.

For bilayer films, Micrographs showed that gelatin layer could be easily identified as a homogenous and relatively compact phase (Fig. 3). A similar result for gelatin/viscera protein isolate from smooth hound bilayer films was previously reported (Abdelhedi et al., 2018). As observed in the composite films, gelatin layer can served as an excellent support for the incorporation of VLE, even though the addition of phenolic compounds induce some discontinuities in the film matrix, which could be due to the presence of hydrophilic compounds in the VLE. Similar microstructural observations have been reported with other composite films such as agar/grapefruit seed extract (Kanmani & Rhim., 2014). Thus, the obtained result suggests the improvement of the mechanical and barrier properties of the resulted composite gelatin and agar films.

3.2.2. Mechanical and thermal properties of the composite films

Mechanical strength and elasticity are needed for a packaging film to keep its integrity and tolerate external stress (Hosseini, Javidi, & Rezaei 2016; Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015). Results of tensile strength (TS) and elongation-at-break (EAB) of the different composite (bilayer and blended) films, incorporated or not with VLE, are shown in

Table 2. Among all the films, A-F showed the lowest TS and EAB ($p < 0.05$), whereas G-F was the strongest (TS = 63.45 MPa). Bl-F anf Bi-F were mechanically stronger and more deformable than control films, with higher TS values and EAB. In fact, mixing agar and gelatin solutions led to significantly increase the values of TS and EAB ($p < 0.05$). The increase in strength value can be explained by the formation of a denser matrix after agar addition due to the interaction with gelatin matrix. No significant variations in thickness between all composite and control films were observed. Thus, the differences observed in TS and EAB couldn't be due to the film thickness. In the same context, Mohajer et al., (2017) and Suyatma, Copinet, Tighzert, & Coma, (2004) reported that the mechanical properties of the composite blended film depends on the hardness of polymer chains and the intermolecular forces, as well as the molecular symmetry of agar and gelatin.

The presence of gelatin and agar as two layers gives a softer and more flexible structure of blend films. In fact, EAB values were 4-fold higher and 3-fold higher in Bl-F and Bi-F than that recorded in gelatin film, respectively (Table 2). Thus, it may be concluded that the low EAB and TS of agar film may be improved by the addition of gelatin in blended or bilayer form.

The incorporation of VLE in Bl-F and Bl-F films decreased both the tensile strength and the elongation at break. Similar results have been reported for agar-gelatin composite based films containing green tea extract. Giménez et al. (2013) reported that the TS of agar-gelatin based films containing phenolic compounds decreased with increasing the concentration of a green tea extract in the film matrix, probably due to the loss of intermolecular interactions among agar and gelatin chains. In the same context, Kanmani and Rhim (2014) demonstrated that the addition of grapefruit seed extract into the agar based film decreased the tensile strength of the control film which was probably due to the reduction in the molecular interaction between the agar polymer and phenolic compounds.

The change in rigidity and elasticity observed in blend and bilayer films was correlated with DSC results. Indeed, a correlation between T_g, TS and EAB of different composite films is observed. In the composite films with the presence of gelatin, T_g of the composite film was higher than agar film. The highest value (71.35 °C) was obtained with gelatin film. Comparing to agar film, the increase of T_g in composite film indicates some level of blending after intermolecular interaction between gelatin and agar polymers. The incorporation of VLE into polymer matrix increases slightly the T_g value in dose dependent manner. This result indicates that the addition of phenolic compounds improves the thermal stability of the agar/gelatin composite films, contrary to the result published by Kanmani and Rhim (2014).

3.2.3. Light transmission, transparency, and color of the composite films.

Color and light transmission properties are the most important parameters defining the ability of films to be used as food surface cover, since these affect the appearance of the coated product (Rao, Kanatt, Chawla, & Sharma, 2010). Transmission of UV and visible light at 200-800 nm of gelatin, agar and the different composite (bilayer and blended) films, incorporated or not with VLE, are presented in Table 3. The transmission in the visible range (from 350 to 800 nm) of gelatin films varied from 13.67 to 91.42%. The transmission of UV light at 280 nm ranged from 0.01% to 0.29% and gelatin film had the lower transmission value. Therefore, the blend and bilayer films showed a lower light transmission than the gelatin film in the visible range, suggesting that agar and gelatin composite films were low in transparency. Thus F-A with a high UV and visible light transmission barrier contributed to limited light transmittance of the blend films. In addition, very low transmission (0.01%) was found at 200 nm for gelatin and agar films. The incorporation of VLE led to increase the transparency to be 0.04% at a concentration of 10 mg/mL for Bl-F and Bi-F, probably due to the presence of phenolic compounds in VLE. Kanmani and Rhim (2014) reported that the phenolic compounds in the vegetable material might absorb light at lower wavelength.

409 The opacity of edible films is an important factor which can affect the appearance of the
410 coated product. On the other hand, it can decrease the speed of lipid peroxidation and
411 consequently, the quality of coated product (Rao et al., 2010). Results obtained showed that
412 G-F was the lightest, while A-F was darker than the gelatin film, indicating that agar addition
413 improved the opacity of the composite films (Table 3). In addition, the transparency value
414 was higher in Bl-F than that observed in Bi-F. Similar finding was reported by Abdelhedi et
415 al. (2018) in smooth hound viscera protein isolate/gelatin blend and bilayer films.
416 Furthermore, the addition of VLE in the polymers matrix let the resulted films less bright.
417 This result is due to the color of the VLE, which absorbs at the visible range, affecting the
418 opacity of the enriched films.

419 Visually, control films based on gelatin were the most transparent and clear. However,
420 significant differences in L^*, a^* and b^* values were detected (Table 4). Gelatin film displayed
421 the highest L* (90.57) value. An increase in L^*, a^* and b^* values was revealed after mixing
422 gelatin and agar in bilayer or blend forms ($p < 0.05$). The addition of VLE makes the resulted
423 films colored (yellowish). Changes in color were more pronounced with increasing
424 concentration of VLE.

425 In fact, F-Bi-VLE (10 mg/mL) and F-Bi-VLE (10 mg/mL) have lower L* and higher a*
426 and b* values than control composite films and F-Bi-VLE (5 mg/mL) and F-Bi-VLE (5
427 mg/mL) ($p < 0.05$), indicating that the VLE addition had significant influence on producing
428 colorless films. Similar finding was previously reported by Kanmani and Rhim (2014). Du et
429 al. (2009) reported similar results when adding increasing concentration of essential oil into
430 edible apple based films.

431 *3.2.4. FTIR analysis of the composite films*

432 FTIR spectra of the control films and their composite, incorporated or not with VLE,
433 were analyzed and results are shown in Table 5. The spectrum of G-F film showed

434 characteristic bands at approximately 3298 cm^{-1} (amide-A), 2998 cm^{-1} (amide-B), 1659 cm^{-1}
435 (amide-I), 1551 cm^{-1} (amide-II) and 1190 cm^{-1} (amide-III), (Jridi et al., 2014). Additionally,
436 the band observed at 1041 cm^{-1} was detected in all films, corresponding to the glycerol used
437 as a plasticizer (Jridi et al., 2013).

438 For A-F, the band detected at 3275 cm^{-1} is attributable to hydroxyl (O-H) groups, which
439 are able to form hydrogen bonds with amino groups. Other characteristic bands at 1055, 1037
440 and 935 cm^{-1} indicates C-O stretching group of galactose (Guerrero, Kerry, & de la Caba,
441 2014; Guerrero, Garrido, Leceta, & de la Caba, 2013).

442 The combination between both polymer in blended or bilayer form caused some change
443 in different bands. Amide-I and amide II bands were shifted from 1659 and 1190 cm^{-1} (G-F)
444 to 1645-1646 cm^{-1} and 1167-1188 cm^{-1}, respectively in the composite form. The changes
445 observed in different spectra suggested the presence of protein-polysaccharide interactions via
446 hydrogen bond (Mohajer et al., 2017).

447 On the other hand, the bands observed in the FTIR spectra of the control films are
448 relatively similar to those enriched with VLE. However, some of the bands were shifted to
449 different frequency with increasing concentration of VLE. Thus, FTIR spectra results showed
450 the interaction between gelatin, agar and phenolic compounds in VLE.

451 *3.2.5.* WVP *of the composite films*

452 In this study, the WVP of composite films was evaluated with a RH differential of 54%
453 and results are illustrated in Table 5. The A-F showed a high WVP value (2.5 10^{-10} g/m.s.Pa),
454 indicating its hydrophilic character. However, blend films showed lower WVP values, (1.40
455 10^{-10} to 1.55 10^{-10} g/m.s.Pa). These values are comparable to those obtained by smooth hound
456 proteins edible films (Abdelhedi et al., 2018). However, all Bi-F showed WVP values ranged
457 between 2.12 10^{-10} and 2.75 10^{-10} g/m.s.Pa. This firmness could be due to the protein-
458 polysaccharides interaction and could prevent effectively the penetration of water vapor.

3.2.6. Antioxidant activity of the composite films

The enrichment of films with natural antioxidants allows the enhancement of nutritional and aesthetic quality aspects without affecting the integrity of the food product (Kanatt, Rao, Chawla, Sharma, 2012; Guilbert, Contard, & Gorris, 1996). Thus, the antioxidant activity of the control films and their composite, incorporated or not with VLE, were measured using three tests and results are presented in Table 6. Results obtained showed that control films and composite films (without VLE) showed a low antioxidant capacity (lower than 0.32, 25.36% and 7.25% for reducing power, chelating effect and DPPH radical-scavenging activity, respectively).

Films containing VLE had significantly higher ($p<0.05$) antioxidant activity as compared to non-enriched ones (Table 6). Regardless of the test, the increase of VLE concentration increases the antioxidant activity ($p<0.05$). For example, composite films (Bl-F and Bi-F) incorporated with 10 mg/mL of VLE, showed the highest reducing power (1.73-1.75), chelating effect (99-100%) and DPPH radical-scavenging activity (99-100%). Many previous works reported that phenolic compounds in plant extract exhibited strong antioxidant properties, and that they could act as antioxidants by donation of a hydrogen or as an acceptor of free radicals to interrupt chain oxidation reactions or by chelating metals (Giménez et al., 2013; Kanatt et al., 2012).

4. Conclusions

This study demonstrated that gelatin/agar composite films can be bioactive after the incorporation of VLE. First, the combination between gelatin and agar in blended or bilayer forms caused some change in the structure of control films. The changes observed in different FTIR spectra suggested the presence of protein-polysaccharide interactions via hydrogen bond, as well as in SEM micrographs. The agar addition improved the mechanical and thermal properties, while the extract introduced excellent antioxidant activities to the films. In

fact, composite films (Bl-F and Bi-F) incorporated with 10 mg/mL of VLE, showed the highest reducing power, chelating effect and DPPH radical-scavenging activity. Thus, this study encourages the use of gelatin/agar/VLE film as functional packaging material.

Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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647 **Table 1:** Antimicrobial activities of VLP ethanolic extract against Gram-positive and Gram-
648 negative bacteria, and fungi strains.
649

	Strains	Inhibition zone diameters (mm)	
		VLE	Antibiotics
Gram-	*E. coli*	20.0 ± 0.9	21.0 ± 1.0
	K. pneumoniae	30.0 ± 0.9	12.0 ± 2.0
	S. enterica	25.0 ± 0.4	14.0 ± 2.0
	S. typhi	24.0 ± 0.3	15.0 ± 2.0
Gram +	*M. luteus*	27.0 ± 1	18.0 ± 1.0
	S. aureus	32.0 ± 0.6	37.0 ± 1.0
	B. cereus	26.0 ± 0.8	22.0 ± 2.0
	E. faecalis	16.5 ± 0.8	18.0 ± 1.0
Fungi	*A. flavus*	23.0 ± 0.6	29.0 ± 1.0
	A. niger	21.0 ± 0.7	31.0 ± 2.0
	F. oxysporum	16.0 ± 0.4	39.0 ± 1.0

650

652 **Table 2:** Mechanical and thermal properties of the composite films.

	Thickness (µm)	TS (MPa)	EAB (%)	T_g (°C)
G-F	81.05 ± 0.10 ^{a}	63.45 ± 2.65 ^{c}	7.59 ± 0.45 ^{c}	71.35
A-F	81.04 ± 0.15 ^{a}	47.56 ± 0.45 ^{e}	3.15 ± 0.70 ^{d}	57.10
F-Bi	81.00 ± 0.10 ^{a}	68.15 ± 1.20 ^{b}	21.20 ± 1.91 ^{b}	65.15
F-Bi-VLE (5 mg/mL)	81.10 ± 0.10 ^{a}	71.58 ± 2.20 ^{a}	20.12 ± 1.27 ^{b}	67.31
F-Bi-VLE (10 mg/mL)	81.12 ± 0.10 ^{a}	69.50 ± 0.45 ^{ab}	19.5 ± 1.67 ^{b}	69.75
F-Bl	81.60 ± 0.15 ^{a}	64.50 ± 2.60 ^{c}	27.25 ± 3.01 ^{a}	63.26
F-Bl-VLE (5 mg/mL)	81.30 ± 0.10 ^{a}	62.50 ± 1.10 ^{c}	25.20 ± 1.10 ^{ab}	65.24
F-Bl-VLE (10 mg/mL)	81.14 ± 0.18 ^{a}	60.50 ± 1.80 ^{d}	22.36 ± 3.20 ^{b}	68.20

653 **TS:** Tensile strength; **EAB:** Elongation at break. G-F, A-F, Bl-F and Bi-F indicate gelatin, agar, blend and
654 bilayer films, respectively. VLE indicates vine leaves extract. All measurements were performed at 25 °C and
655 RH = 50%. Different letters in the same column indicate significant difference ($p<0.05$).

657 **Table 3:** Light transmission, transparency and water vapor permeability.

	Wavenumbers (nm)								Transparency	WVP (10^{-10} g $m^{-1}s^{-1}Pa^{-1}$)
	200	280	350	400	500	600	700	800		
G-F	0.01	0.01	38.62	57.91	73.57	81.92	87.39	91.42	1.06	2.05 ± 0.01^{b}
A-F	0.01	1.90	19.82	30.32	57.57	71.04	75.32	78.73	1.73	2.47 ± 0.14^{a}
F-Bi	0.01	0.12	17.08	34.39	53.49	76.91	83.66	89.83	1.32	2.12 ± 0.37^{a}
F-Bi-VLE (5 mg/mL)	0.02	0.14	13.67	28.64	53.20	75.60	83.78	86.10	1.41	2.75 ± 0.50^{a}
F-Bi-VLE (10 mg/mL)	0.04	0.16	13.87	24.23	54.96	72.33	76.99	85.21	1.63	2.33 ± 0.42^{a}
F-Bl	0.01	0.22	16.02	16.86	54.60	67.77	76.15	81.01	1.92	1.55 ± 0.07^{c}
F-Bl-VLE (5 mg/mL)	0.02	0.29	16.44	22.70	51.80	72.37	78.94	85.44	1.60	1.45 ± 0.11^{c}
F-Bl-VLE (10 mg/mL)	0.04	0.80	21.83	27.56	54.20	70.50	77.72	82.54	1.72	1.40 ± 0.05^{c}

658 Values of light transmission were measured at 25 °C and RH of 50% and expressed in %. Transparency = -log
659 (Transmission) / Thickness. G-F, A-F, Bl-F and Bi-F indicate gelatin, agar, blend and bilayer films, respectively.
660 VLE indicates vine leaves extract. WVP indicates water vapor permeability.

661

663 **Table 4:** Surface color of composite films.

	L*	a*	b*
G-F	90.57 ± 0.26 [a]	-0.36 ± 0.01 [e]	0.35 ± 0.03 [g]
A-F	80.75 ± 0.15 [b]	-1.98 ± 0.26 [f]	0.95 ± 0.01 [f]
F-Bi	75.36 ± 0.95 [c]	-0.16 ± 0.01 [d]	1.95 ± 0.16 [e]
F-Bi-VLE (5 mg/mL)	76.36 ± 0.16 [c]	2.96 ± 0.55 [c]	3.69 ± 0.21 [d]
F-Bi-VLE (10 mg/mL)	70.94 ± 0.14 [e]	5.36 ± 0.49 [b]	8.19 ± 0.42 [a]
F-Bl	72.68 ± 0.23 [d]	-0.05 ± 0.01 [d]	2.36 ± 0.15 [e]
F-Bl-VLE (5 mg/mL)	70.25 ± 0.18 [e]	3.05 ± 0.16 [c]	4.16 ± 0.23 [c]
F-Bl-VLE (10 mg/mL)	68.16 ± 0.67 [f]	6.56 ± 0.29 [a]	5.00 ± 0.19 [b]

664 Different letters in the same column indicate significant difference ($p<0.05$). Values of light transmission were
665 measured at 25 °C and RH of 50% and expressed in %.Transparency = -log (Transmission) / Thickness. G-F, A-
666 F, Bl-F and Bi-F indicate gelatin, agar, blend and bilayer films, respectively. VLE indicates vine leaves extract.

668 **Table 5:** Fourier Transform infrared spectra (FTIR) of composite films.

	Amide A	Amide B	Amide I	Amide II	Amide III	Other bonds	Glycerol
G-F (control)	3298	2998	1659	1190	1041	-	1041.9
A-F (control)	3275	-	-	-	-	1055, 1037, 935	1041.6
F-Bi	3298	2978	1645	1186	1040	1056, 845	1041.7
F-Bi-VLE (10 mg/mL)	3287	2986	1646	1167	1036	1068, 1022, 758	1041.2
F-Bl	3299	2978	1645	1188	1040	1055, 851	1041.2
F-Bl-VLE (10 mg/mL)	3286	2985	1647	1170	1032	1070, 1026, 756	1041.3

669

671 **Table 6:** Antioxidant properties of the different films

	Reducing power (A_{700})	Chelating effect (%)	DPPH radical-scavenging activity (%)
G-F	0.25 ± 0.02 ^{d}	25.36 ± 2.86 ^{d}	1.25 ± 0.10 ^{g}
A-F	0.02 ± 0.00 ^{e}	20.36 ± 1.20 ^{d}	0.02 ± 0.00 ^{h}
F-Bi	0.32 ± 0.01 ^{c}	23.56 ± 2.40 ^{d}	2.03 ± 0.90 ^{f}
F-Bi-VLE (5 mg/mL)	0.94 ± 0.08 ^{b}	78.26 ± 0.74 ^{c}	79.36 ± 2.84 ^{c}
F-Bi-VLE (10 mg/mL)	1.75 ± 0.02 ^{a}	100.0 ± 0.00 ^{a}	100.00 ± 0.00 ^{a}
F-Bl	0.23 ± 0.02 ^{d}	22.35 ± 0.96 ^{d}	7.25 ± 0.50 ^{e}
F-Bl-VLE (5 mg/mL)	1.02 ± 0.05 ^{b}	79.32 ± 0.14 ^{c}	68.95 ± 1.37 ^{d}
F-Bl-VLE (10 mg/mL)	1.73 ± 0.03 ^{a}	99.25 ± 0.07 ^{b}	99.51 ± 0.14 ^{b}

672 Results are the mean from three determinations. G-F, A-F, Bl-F and Bi-F indicate gelatin, agar, blend and bilayer
673 films, respectively. VLE indicates vine leaves extract. Different letters in the same column indicate significant
674 difference ($p<0.05$).

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692 **Figure captions:**

693 **Figure 1:** Diagram explaining the methodology used to prepare films.

694 **Figure 2:** Antioxidant activities of VLP ethanolic extract. (A) Fe^{3+} reducing antioxidant
695 power (OD 700 nm); (B) β-carotene bleaching inhibition (%); (C) DPPH•-scavenging activity
696 (%); Fe^{2+} chelating activity (%).

697 **Figure 3:** SEM micrographs of cross-sections of G-F (A), A-F (B), control blend film (C),
698 control bilayer film (D), blend films added with VLE (10 mg/mL) (E) and bilayer films added
699 with VLE (10 mg/mL) (F).

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716 **Fig. 1.**

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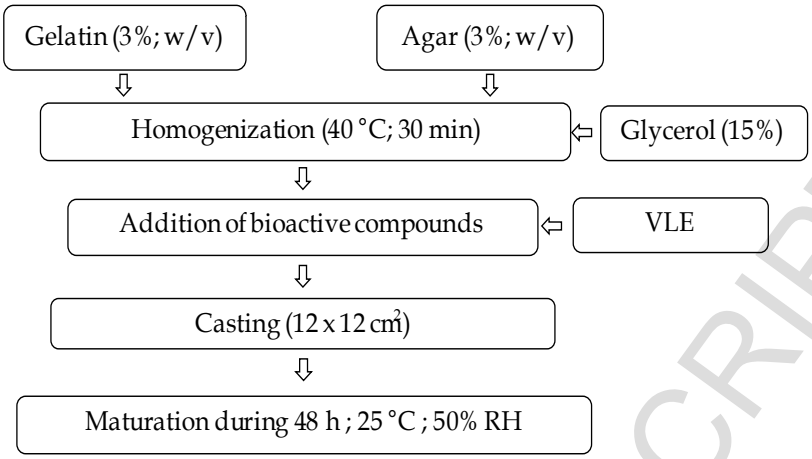

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Fig. 2.

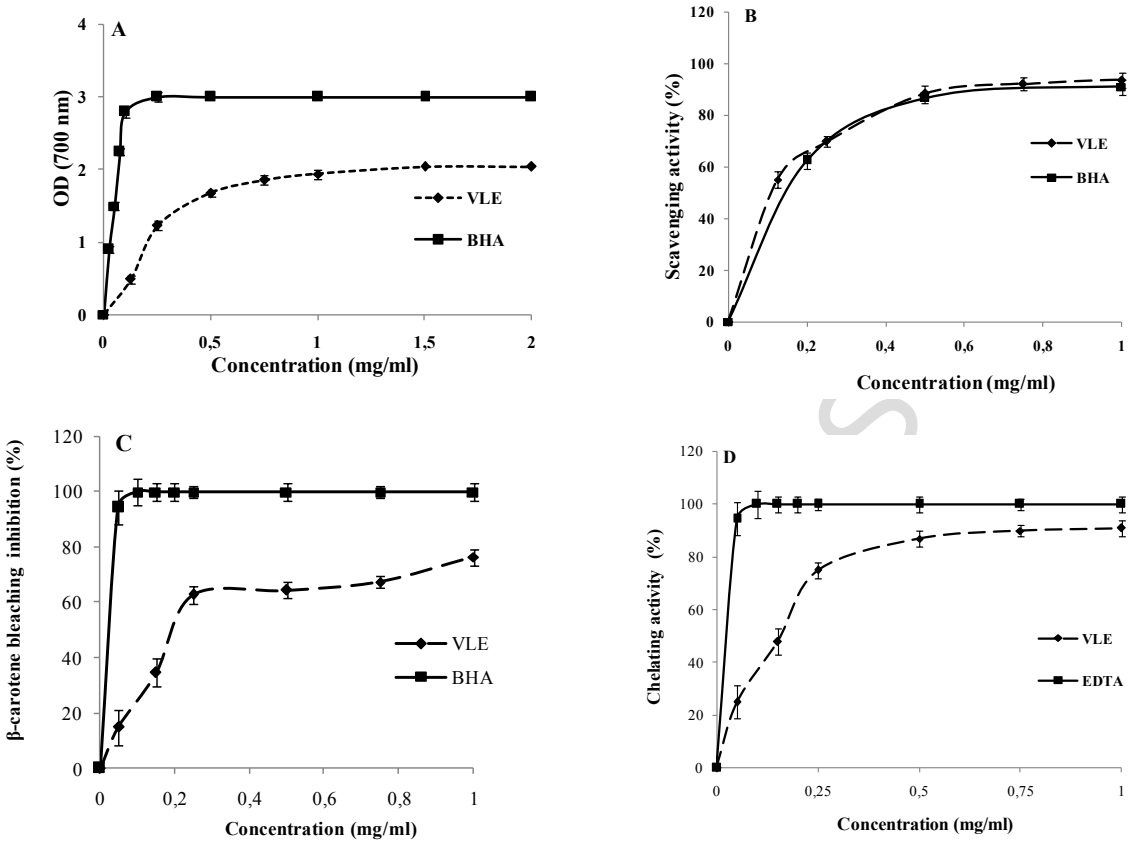
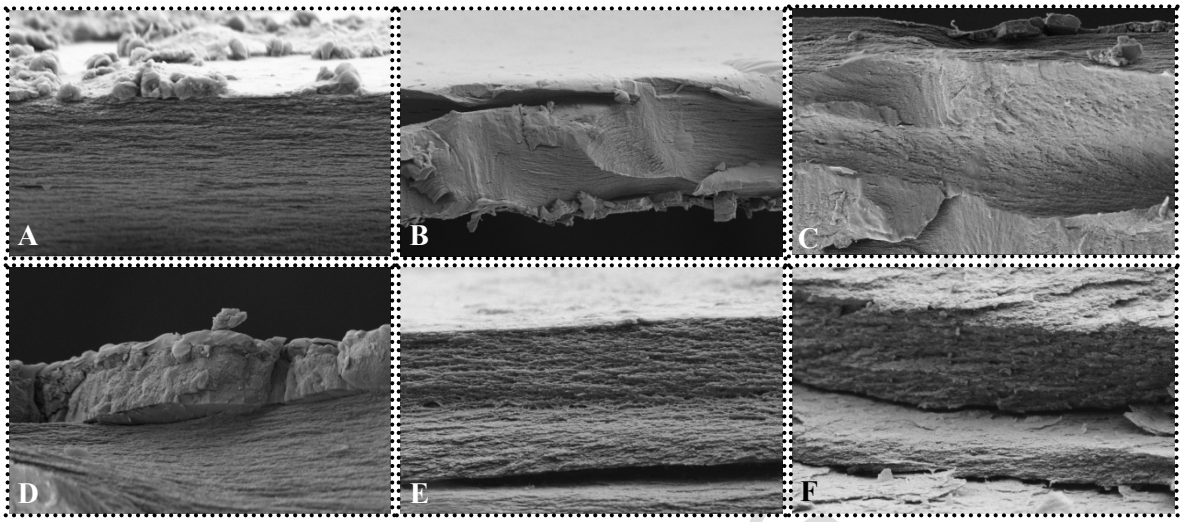


Fig. 3.



- Bio-based blend films based on fish gelatin and agar were prepared.
- Addition of agar resulted in more resistant films.
- Antioxidant power of the films increased with the incorporation of vine leaves extract.
- Cross section micrographs demonstrated the good compatibility of both polymers.
- Fish gelatin-agar/VLE films can act as a biodegradable packaging film.