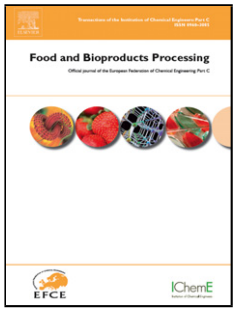

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Characterization of pea starch-guar gum biocomposite edible films enriched by natural antimicrobial agents for active food packaging

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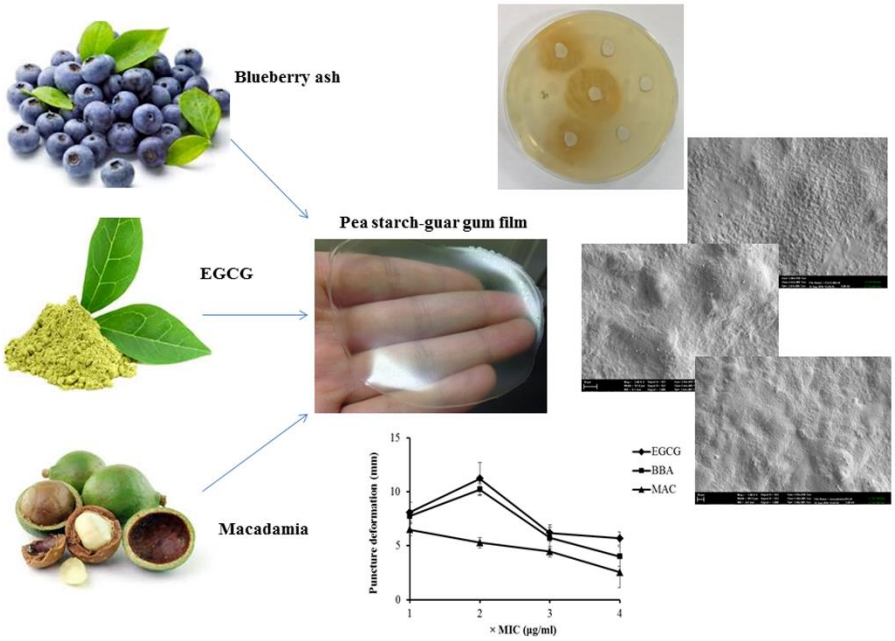
Graphical abstract



Highlights

- Active food packagings based on pea starch-guar gum films containing natural antimicrobial compounds were developed
- Plant extracts were capable to prevent an extensive spectrum of food pathogenic and spoilage microorganisms
- The mechanical and antimicrobial properties of the films changed in a concentration-dependent manner
- The resultant films can be used for preserving food safety and prolonging the shelf-life of the packaged food

ABSTRACT

Antimicrobial activity of epigallocatechin-3-gallate (EGCG) and two native Australian plants blueberry ash (BBA) fruit and macadamia (MAC) skin extracts against nine pathogenic and spoilage bacteria and seven strains of fungi, using an agar well diffusion assay were investigated. The minimum inhibitory concentrations (MIC) of these compounds were calculated using 96-well microtiter plates method. Finally, active antimicrobial packaging films were prepared by incorporation of EGCG, BBA and MAC extracts at 1-, 2-, 3-, and 4-fold of their correspondence MIC values into edible films based on pea starch and guar gum (PSGG). The antimicrobial activity of films was investigated against target microorganisms by agar disc diffusion technique and quantified using the viable cell count assay. Among the test microorganisms, *Salmonella typhimurium* and *Rhizopus sp*. were the most resistance to active films. Films containing EGCG showed the highest activity against all test strains. As the concentration of compounds increased higher than 2 × MIC, the mechanical characteristics of the films were affected considerably. The results indicated that EGCG-

PSGG, BBA-PSGG and MAC-PSGG films can be used as active food packaging systems for preserving food safety and prolonging the shelf-life of the packaged food.

Keywords: Pea starch; guar gum; natural plant extract; antimicrobial; active packaging

1. Introduction

Biodegradable films can be used to carry active substances, such as antibrowning agents, colorants, flavors, nutrients, spices, antioxidant and antimicrobial agents that provide an extra stress factor against foods' oxidative and microbial deterioration (Jouki et al., 2014; Ojagh et al., 2010; Sánchez-González et al., 2010).

Packaging materials incorporated with antimicrobial compounds have been applied as one of the most favorable active packaging systems for prolonging shelf-life of food, preserving safety, quality and stability of food during storage by destructing or preventing spoilage and pathogenic microorganisms that contaminate foods (Falguera et al., 2011; Han, 2000). A tendency to application of natural antimicrobials is progressively rising owing to consumer consciousness of the possible health risks and safety of some synthetic compounds (Gyawali and Ibrahim, 2014; Lee, 2014; Moreira et al., 2005). The incorporation of natural antimicrobials originated from plant extracts to edible films is an approach for improving the shelf life and increasing the preservation efficiency of food products since microbial spoilage is main problem influencing food quality (Moreno et al., 2015; Ponce et al., 2008).

Catechins are considered as polyphenolic compound and are derived from many plants, particularly from tea (Wu et al., 2010). Due to accessibility and moderate cost of catechins, fruitful researches have been conducted to study their properties as antimicrobial compounds (Amarowicz et al., 2000; Chou et al., 1999; Hamilton-Miller, 1995; Ikigai et al., 1993; Kim et

al., 2004; Matsumoto et al., 2012; Toda et al., 1989; Toschi et al., 2000; Vuong et al., 2010; Vuong et al., 2011; Wu et al., 2010; Yam et al., 1997). The phenolic catechins exist in green tea consist of; epicatechin (EC), epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), gallocatechin (GC) and catechin (Siripatrawan and Noipha, 2012). EGCG is the most predominant and powerful polyphenol found in green tea (Matsumoto et al., 2012). It is an influential antioxidant that presents anti-tumor, anti-inflammatory, anti-apoptotic, anti-metastatic, anti-atherogenic and antimicrobial functions (Khan and Mukhtar, 2007; Matsumoto et al., 2012). EGCG is high molecular- weight, non-volatile, water soluble, and resistance to high temperatures such as boiling water (Wang et al., 2008; Wu et al., 2010), therefore it is capable to penetrate between the packaging material, the food product and/or distributing at the interface once it is applied in an active food package (Wu et al., 2010). To the best of our knowledge, no work concerning effects of EGCG on antimicrobial activity and mechanical properties of pea starch-guar gum (PSGG) film has been reported.

The macadamia is recognized as an evergreen, native Australian tree with two more common species, the *Macadamia integrifolia* (smooth shelled) and the *Macadamia tetraphylla* (rough shelled) (Munro and Garg, 2008). Commonly, the kernel of the macadamia nut is only known as the commercial section; while, the skin and husk being roughly 80% of the nut weight, have been counted as waste (Wechsler et al., 2011). It has been pointed out that the skins of tree nuts are rich source of phenolic compounds and exhibit more antioxidant activities than the kernel and other by products of nut manufacture (Alasalvar and Shahidi, 2009). The skin of the macadamia has been reported to be a rich source of phenolic compounds (Alasalvar and Shahidi, 2009; Dailey and Vuong, 2015a; Dailey and Vuong, 2015b; Dailey and Vuong, 2016; Dailey et al., 2015). Therefore, macadamia skin is a great substance for bioactive

compound extraction and its phenolic compounds can be added to edible films for developing active packaging.

Blueberry ash (*Elaeocarpus reticulatus* Sm.) belongs to the family *Elaeocarpaceae* and is another Australian indigenous shrub or small tree growing in rainforest and coastal scrub along the east coast of Australia, from southern Queensland to Flinders Island in Bass Strait (Rickard, 2011). Blueberry ash fruits are a blue, ovoid to globose drupe with a single large endocarp. Fruits ripen over many months from December to June, which are said to contain carbohydrates and some proteins (Gosper et al., 2006). There is limited information on phytochemical and antimicrobial properties of blueberry ash fruits. In this study, the potential using of blueberry ash fruit extract as an antimicrobial compound to PSGG edible film was investigated. The present study was conducted to analyse the effect of different natural plant extracts on antimicrobial and mechanical properties of PSGG films.

2. Materials and methods

2.1. Materials

Canadian non-GMO yellow pea starch with 13.2% moisture, 0.2% protein, 0.5% fat and 0.3% ash, was used in all experiments (supplied by Yantai Shuangta Food Co., Jinling Town, China). Guar gum (E-412) was purchased from The Melbourne Food Ingredient Depot, Brunswick East, Melbourne, Australia. All other chemicals were obtained from Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia. Commercial epigallocatechin-3-gallate Teavigo™ EGCG was purchased from RejuvaCare, Sydney, NSW, Australia. It was in the form of dry powder stored at 5-8 °C until needed.

2.2. Preparation of extracts

Extraction from macadamia skin (*Macadamia tetraphylla*) was conducted using the method described by Dailey and Vuong (2015b). Briefly, the skin of nuts was removed after harvesting in the Central Coast region, New South Wales, Australia. It was frozen in liquid nitrogen and freeze dried (FD3 freeze dyer, Thomas Australia Pty. Ltd., Seven Hills, NSW, Australia). The extraction process was conducted on the dried and ground skin at Ultrasound-assisted extraction (UAE) temperature of 40 °C, UAE time of 35 min, UAE power of 200 W, sample to solvent ratio of 5:100 g/mL and a mixture of acetone: water (1:1 v/v).Blueberry ash (*Elaeocarpus reticulatus* Sm.) fruits with stems were collected in August 2015 from the Central Coast region of New South Wales (NSW), Australia (latitude of 33.4° S, longitude of 151.4° E). Fruits were then immediately frozen in liquid nitrogen and freeze dried. The dried fruit was then ground using a commercial blender (John Morris Scientific, Chatswood, NSW, Australia) and was separated from the stone seeds using a steel mesh sieve (1.4 mm EFL 2000; Endecotts Ltd., London, England). The extraction solvent (50% acetone) was then applied to extract bioactive compounds from blueberry ash fruits. UAE was applied to extract the ground dried sample at a solvent-to-sample ratio of 100:1 mL/g of dried sample. The extraction chamber was completely immersed into an ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd., The barton, Australia) with pre-set conditions for temperature of 35 °C, time of 30 min and power of 150 W. Agitation was conducted for 3 s once every 5 min using a Vortex. When the ultrasonic extraction was completed, the extracts were immediately cooled on ice to room temperature (RT), filtered using a 5 mL syringe fitted with a 0.45µm cellulose syringe filter (Phenomenex Australia Pty. Ltd., Lane Cove, Australia). The filtered extract was stored at 4 °C before further use.

2.3. Microorganisms used

Nine strains of bacteria used were *Bacillus subtilis* (ATCC 6633), *Staphylococcus epidermidis* (ATCC 14990), *Staphylococcus lugdunensis* (ATCC 43809), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas fluorescence* (ATCC 13525), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 35150), *Enterobacter aerogenes* (ATCC 49701), and *Enterococcus faecalis* (ATCC 51299). These standard strains were obtained from Thermo Fisher Scientific Pty Ltd, Scoresby, VIC, Australia. The stock culture was kept on Mueller Hinton agar medium at 4 °C. Seven fungal pathogens namely *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16888), *Geotrichum candidum* (ATCC 10663), *Penicillium italicum* (ATCC 32079), *Penicillium digitatum* (ATCC 48113), *Rhizopus sp.*, and *Mucor sp*. These fungal strains were obtained from Microbiology Laboratory (University of Newcastle, School of Environmental and Life Sciences, Ourimbah, NSW, Australia). The stock culture was maintained on Sabouraud Dextrose Agar medium at 4 °C.

Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) were used for antibacterial activity. In vitro antifungal activity was examined by using Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB) (for mycelial fungi). They were obtained from Thermo Fisher Scientific Pty Ltd, Scoresby, VIC, Australia.

2.4. Preparation of inocula

Twenty-four hours pre-activated bacterial cultures were mixed with physiological saline and the turbidity was adjusted by addition of sterile physiological saline until a Mac Farland turbidity standard of 0.5 (10^6 colony forming units (CFU) per ml) was reached.

The isolates were subcultured on SDA and incubated at 35 °C for 7–14 days. The growth was scratched aseptically, smashed and transferred to sterile distilled water and the fungal suspension was corrected spectrophotometrically to an absorbance of 0.600 at 450 nm (Chandrasekaran and Venkatesalu, 2004).

2.5. Antibacterial and antifungal activity of extracts

2.5.1. Agar disc diffusion method

The standardized microbial suspension (100 µl) was spread onto MHA for bacteria and SDA for fungi and uniformly spread. The known weight of crude extracts was dissolved in 5% dimethyl sulphoxide (DMSO) to obtain 1000 µg/ml stock solutions. Filter paper discs (6 mm in diameter, Thermo Fisher Scientific Pty Ltd, Australia) were impregnated with 20 µl of the test samples (1000 µg/ml) and placed on the inoculated plates, which were then incubated at 37 °C for 24 h (bacteria) and 28 °C for 72–96 h (mycelial fungi). The diameters of the inhibition zones were measured in millimetres. All tests were performed in triplicate. Standard discs of ciprofloxacin (CIP) (5 µg) and ketoconazole (KTZ) (25 µg) were obtained from Oxoid Ltd. and used as positive controls for antimicrobial activity. Filter discs impregnated with 20 µl of 5% DMSO served as a negative control (Cock, 2008). The percentage inhibition of the extracts was calculated using the following formula (Lee et al., 2013):

% inhibition = (diameter zone of sample/diameter zone of ciprofloxacin or ketoconazole) × 100

2.5.2. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The minimal inhibitory concentration (MIC) values of the extracts were studied using the two-fold serial dilution method for the bacterial and fungal strains which were sensitive to extracts in the disc diffusion assay. Minimum inhibitory concentrations (MIC) were determined using 96-well microtiter plates, as described by Ud-Daula et al. (2016) with minor modifications. The test extracts were dissolved in 5% dimethyl sulphoxide (DMSO) to

obtain 6000 µg/ml stock solutions. Stock solution (100 µl) was incorporated into 100 µl of appropriate broth growth medium in wells of a 96-well microtiter plate to get a concentration of 3000 µg/ml and serially diluted by double technique to achieve 1500, 750, 375, 187.5, 93.8, 46.8, and 23.4 µg/ml, respectively. Standardized suspension of the test organisms (10 µl) was transferred on to each well. The control well contained only organisms and not the plant extracts. Ciprofloxacin (0.49–1000 µg/mL) and ketoconazole (0.04–1250 µg/mL) were used as reference antibacterial and antifungal compounds, respectively. The culture tubes were incubated at 37 °C for 24 h (bacteria) and 28 °C for 48-72 h (mycelial fungi). The lowest concentrations, which did not show any growth of tested organism after macroscopic evaluation was defined as MIC.

From the wells which did not show any growth of the bacteria and fungi after the incubation period, 100 µl were subcultured on to the surface of the freshly prepared MHA (for bacteria) and SDA (for fungi) plates and incubated in at 37 °C for 24 h (bacteria) and 28 °C for 72–96 h (mycelial fungi). The MBC and MFC were recorded as the lowest concentration of the extract that did not permit any visible bacterial and fungal colony growth on the relevant agar plate after the period of incubation (Chandrasekaran and Venkatesalu, 2004).

2.6. Preparation of film-forming solution

The film-forming solution was prepared by dissolving pea starch (2.5 g), guar gum (0.3 g) and 25% w/w glycerol based on the dry film matter in 100 ml degassed deionized water with gentle heating (about 40 °C) and magnetic stirring. In another study, we determined the optimized amount of film ingredients by using Box–Behnken response surface design (BBD) (Saberi et al., 2016a). The aqueous suspension was gelatinized at 90 °C for 20 min on a hot plate with continuous stirring. After gelatinization, the film solution was cooled to room temperature with mild magnetic stirring for 1 h to decrease air bubbles (Saberi et al., 2016b).

Plant extracts at defined concentrations (×1, ×2, ×3, and ×4 of MIC) were added. Filmogenic suspensions (20 g) were cast onto Petri dishes (10 cm in diameter) and dried at 40 °C in an oven until reaching constant weight (about 24 h). Films were peeled-off carefully from Petri dishes and conditioned at 25 °C, 65% relative humidity (RH) for 72 h prior to further testing.

2.7. Film thickness

A digital micrometer (Mitutoyo Corp., Code No. 543-551-1, Model ID-F125, Japan; sensitivity= 0.001 mm) was used to calculate the thickness of the films. Measurements were randomly taken at 10 different positions for each specimen and the average value was reported.

2.8. Film density

Film density was evaluated by dividing the weight of film by the film volume, where the film volume was determined by multiplying the film area by the thickness (Saberi et al., 2016b).

2.9. Mechanical tensile test

The mechanical properties of the films (15 × 40 mm^2) were determined using a Texture Analyzer (LLOYD Instrument LTD, Fareham, UK) with crosshead speed of 1 mm/s and initial grip distance of 40 mm. Eight film specimens of each formulation were used for mechanical tests. The parameters obtained from force–deformation curves were percent elongation at break (deformation divided by initial probe length multiplying by 100, %), Young's modulus (slope of force–deformation curve (N/mm) multiplying by initial sample length divided by film cross-section, MPa) and tensile strength (maximum force divided by film cross-section, MPa) (Saberi et al., 2016a).

2.10. Mechanical puncture test

The resistance of films to puncture was determined by using a Texture Analyzer (LLOYD Instrument LTD, Fareham, UK). A 4 cm-diameter disk of films were cut and fixed in an annular ring clamp (3 cm diameter). A spherical probe of 1.0 mm diameter was moved vertically to the film surface at a constant speed of 1 mm/s until the probe passed through the film. Force (N) and deformation (mm) values at the puncture point were then recorded to represent the puncture strength (N) and deformation (mm) of the films. For each sample, eight replicates were performed (Chen and Lai, 2008).

2.11. Scanning electron microscopy (SEM)

Scanning electron micrographs were taken by a scanning electron microscope (ZEISS, NSW, AU) at an accelerating voltage of 5 kV. Starch films were mounted on a bronze stub using double-sided adhesive tape, and the films were sputter coated with a layer of gold allowing surface visualization (Campos et al., 2014).

2.12. Antibacterial activity of films using disk diffusion

The antibacterial potential was performed based on the procedure described by Gómez-Estaca et al. (2010). For disk diffusion tests, 0.1 mL of standardized suspension of each test organisms was plated onto each appropriate agar plates. Disks (6 mm diameter) cut from the sterilized films were placed on agar plates. The plates were examined for clear zones around the discs (inhibition zones) after incubation at 37 °C for 24 h (bacteria) and 28 °C for 72–96 h (mycelial fungi). The zone diameters were measured in triplicate and the means were reported.

2.13. Antibacterial activity of films using colony counting

A sample film containing highest amount of antimicrobial compound with 30 mm diameter was placed in a 10 mL liquid culture including 10 μL standardized microbe cultures.

Subsequently, the sample was incubated at 37 °C for 24 h (bacteria) and 28 °C for 72–96 h (mycelial fungi). From the incubated samples, a 100 μL solution was taken and diluted with the proper dilution factor and the final diluted microbe solution was plated and distributed onto appropriate agar plates. All plates were incubated at 37 °C for 24 h (bacteria) and 28 °C for 72–96 h (mycelial fungi) and the numbers of colonies that formed was counted. The antibacterial efficiency of the films was evaluated according to the following equation (Maneerung et al., 2008): colony reduction (%) = ([number of colony in test samples - number of colony in control]/number of colony in test samples) × 100 (Kavoosi et al., 2013).

2.14. Statistical analysis

Analysis of variance was carried out and the results were separated using the Multiple Ranges Duncan's test ($P < 0.05$) using statistical software of Statistical Package for Social Science 16 (SPSS, Inc., NJ). All tests were performed at least in triplicate.

3. Results and discussion

3.1. Antimicrobial activity of extracts

Different extracts were first assessed directly against different microorganisms to determine their capability as antimicrobial film component. The results (Table 1) displayed that EGCG and the extracts of macadamia (MAC) skin and blueberry ash (BBA) fruit possessed antibacterial and antifungal activities against the microorganisms tested. The mean zones of inhibition were between 8 and 28 mm, when EGCG and test extracts were evaluated against the microorganisms by agar disk diffusion assays (Table 1). The blind control (dimethyl sulphoxide) did not show any zones of inhibition against the microorganisms tested. The results demonstrated that bacterial strains were more vulnerable to the extracts than the fungal strains. EGCG showed the highest antibacterial and antifungal activity than that of

extracts used for the present study. EGCG was the most effective compound against the bacterial strains *B. subtilis, S. epidermidis*, and *S. lugdunensis* with values of 28.0; 27.2 and 25.3 mm, respectively. The antimicrobial function of EGCG as abundant polyphenolic compound in green tea against fungi and Gram negative and Gram positive bacteria has been reported (Almajano et al., 2008; Chen et al., 2015; Friedman, 2007; Matsumoto et al., 2012; Radji et al., 2013; Sharma et al., 2012; Singh Arora et al., 2009; Sivarooban et al., 2008). The most resistant bacterium against EGCG, BBA and MAC was *Salmonella typhimurium*. The *Klebsiella pneumoniae* was the second most resistant bacteria against tested antimicrobial compounds. Phenolic compounds inactivate the bacterial growth by the number of mechanisms such as development of complex with cell walls, membrane distraction, and suppression of bacterial adhesion or inactivation of bacterial enzyme systems (Alkan and Yemenicioğlu, 2016; Cowan, 1999).

In the current study, Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, and *Enterococcus faecalis* were more susceptible than Gram-negative bacteria such as *Salmonella typhimurium*, *Pseudomonas fluorescence*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter aerogenes*. The reason for higher resistance of Gram-negative bacteria to plant extracts is higher complexity of their double membrane compared with the single-membrane glycoprotein/teichoic acid of Gram-positive bacteria (Emam- Djomeh et al., 2015). The rate of antimicrobial solubility in the lipid phase of the membrane and the difference in cell surface hydrophobicity may be associated with the difference in the sensitivity between Gram-positive and Gram-negative cells (Holley and Patel, 2005).

The tested agents indicated different antifungal activity for each microorganism studied. Our analysis indicated that EGCG showed almost similar effect against *Mucor sp.*, *Aspergillus*

niger, and *Candida albicans* (Table 1) with percentage inhibition of 95.9%, 93.7%, and 92.4%. However, the BBA extract showed activity against *P. italicum*, *A. niger*, and *P. digitatum* with percentage inhibition of 76.3%, 76.0%, and 70.2%, respectively, whereas against *C. albicans*, it exhibited intermediate effect with percentage inhibition of 58.7%. Further, MAC extract showed maximum inhibitory activity against *A. niger* with percentage inhibition of 63.4%. All the three compounds exhibited lowest effect against *Rhizopus sp.* with percentage inhibitions of 79.4%, 52.5%, and 39.2% of EGCG, BBA, and MAC, respectively, as shown in Table 1.

3.2. Minimum inhibitory concentration

It is essential to calculate the minimum inhibitory concentration (MIC) of added extracts into edible films, since incorporation of extracts may influence their water vapour permeability and mechanical characteristics (Emam- Djomeh et al., 2015). The MIC, MBC and MFC of EGCG and the extracts analysed for the nine bacteria and seven fungi are shown in Table 2. The MIC value for each antimicrobial compound was dependant on the target microorganism. All studied compounds inhibited the growth of Gram-positive bacteria at concentrations lower than that required for Gram-negative bacteria and fungi. In case of EGCG, lowest MIC at 93.8 μg/ml level was achieved for *Staphylococcus epidermidis* and *Bacillus subtilis*, while for *Salmonella typhimurium* and *Klebsiella pneumoniae*, 750 μg/ml were observed to result in complete inhibition of growth. MIC values of 93.8–750 μg/ml were obtained for EGCG in the tests with the bacterial and fungal agents. On the other hand, the MIC values obtained in antibacterial and antifungal assays using BBA and MAC extract were 187.5-1500 μg/ml and 375–1500 μg/ml, respectively. The values obtained in the MBC and MFC studies were higher than those of the MIC studies (Table 2). Similar results were observed by Chattopadhyay et al. (2001), Chattopadhyay et al. (2002), and Chandrasekaran

and Venkatesalu (2004) while investigating the antimicrobial activities of natural plants extracts.

3.3. Film thickness and density

The MIC of EGCG and the extracts were considered 750 μg/ml because at this concentration the growth of most microorganisms was inhibited completely (Tables 1 and 2). So, the antimicrobial edible films were prepared by addition of antimicrobial agents at 1, 2, 3 and 4 times of MIC (750 μg/ml) into PSGG films. The effects of different concentrations of antimicrobial compounds on thickness and density of the PSGG films were shown in Table 3. Thickness and density of the films slightly increased with the increasing concentration of antimicrobial agents, although their effects on thickness and density were slightly significant ($p < 0.05$). There is a probability that phenolic compounds may be capable to fit into film matrix; therefore, by increasing concentration, the density of the PSGG films increased (Wang et al., 2015). The higher the percentage of antimicrobial agents in the film, the greater the film thickness was, because the extract solution was denser than water (Campos et al., 2014). Similar observations were made with other edible films containing different natural plant extracts (Chana-Thaworn et al., 2011; Emam- Djomeh et al., 2015; Hoque et al., 2011; Kanmani and Rhim, 2014; Rattaya et al., 2009; Wu et al., 2013; Yuan et al., 2015). The results suggest that phenolic compounds could be distributed in the film matrix by interacting with hydroxyl groups in film molecular structure through the formation of hydrogen bonds and held the pea starch and guar gum molecules close to each other leading to increasing the film thickness.

3.4. Mechanical properties

The influences of different active compounds on tensile strength, percent elongation at break and Young's modulus of PSGG films are displayed in Table 3. The tensile strength, the

percent elongation at break, and Young's modulus of PSGG film was 27.8 MPa, 16.2%, and 175.7 MPa, respectively (Saberi et al., 2016a). Differences in the films mechanical properties were observed and could be related to different interaction of antimicrobial compounds with PS and GG and formation of new linkages affecting film structure. The results of FTIR showed that addition of EGCG, BBA, and MAC into PSGG film brought about interactions happening between polymers and active compounds (data are not shown). After cross-linking PSGG film with MAC, the tensile strength and Young's modulus considerably increased from 29.1to 43.1 MPa and from 197.8 to 531.5 MPa, but the percent elongation at break reduced from 14.8to 8.2 when MAC concentration increased from 750 μg/ml to 3000 μg/ml. Films containing EGCG and BBA had different mechanical characteristics and exhibited almost no significant change in their tensile strengths, Young's modulus, and elongation at concentration of 1× MIC; but increase in their concentration to 3× and 4× MIC showed a significant change in these parameters. Interestingly, the incorporation of 2× MIC of EGCG and BBA into PSGG films significantly decreased its tensile strength and Young's modulus ($P < 0.05$), but increased elongation. The tensile strength of PSGG film containing 3000 μg/ml EGCG was 38.8 MPa, which increased by 39.6% compared with the control film. The percent elongation at break of PSGG film incorporated with 750 μg/ml BBA was 16.2%, and there was no statistics difference comparing to PSGG film ($p < 0.05$). However, this factor noticeably decreased when the concentration of BBA was 3000 μg/ml, which was 10.5% ($p < 0.05$).

The polyphenolic compounds tested containing plentiful hydrophobic groups, which are capable to create hydrophobic interaction with hydrophobic region of film molecules. In addition, hydroxyl groups of polyphenolic compounds can form hydrogen bonds with hydrogen acceptors of polymer molecules (Gómez-Estaca et al., 2009; Hoque et al., 2011). Therefore, active compounds could interact with PSGG film via hydrophobic interaction and

hydrogen bonds, bringing about film strengthening (Wu et al., 2013). It is proposed that EGCG and BBA at concentration of 2× MIC showed a plasticizing effect by forming hydrogen bonds with polymers and increasing the free volume of film matrix, leading to decreasing the strength and increase the flexibility of the corresponding films. The plasticizing effect of phenolic compounds in film systems has been previously reported (Alkan et al., 2011; Alkan and Yemenicioğlu, 2016; Arcan and Yemenicioğlu, 2011). On the contrary, the antiplasticizing effect of phenolic compounds at high concentration could be attributed to their affinity to show polymerization and increased binding on film matrix, which reduced the molecular mobility and the free volume of film (Alkan and Yemenicioğlu, 2016). Increased concentration of active compounds destructs polymer-polymer linkages, resulting in the development of polyphenol-polymer links and deterioration of the interactions between the films components, which decreased percentage of elongation.

The maximum puncture force and deformation of PSGG films with different concentrations of natural antioxidants are presented in Fig. 1a, b and display that the puncture force and deformation were affected by antimicrobial compound concentration. The highest puncture deformation (11.2 mm) and the lowest puncture force (16.8 N) values were achieved for films formulated with 2× MIC of EGCG. The addition of EGCG and BBA at 1× and 2× MIC significantly enhanced the puncture deformation and reduced the puncture force of PSGG films, while their higher concentrations caused an increase in the puncture force and decrease in puncture deformation of films. These can be attributed to the distribution and number of inter- and intramolecular associations (Espinel Villacres et al., 2014). These interactions were increased by enhancing the crosslinks between phenolic compounds and polymer chains, which accordingly, caused the strength of edible PEGG film (Sun et al., 2014). The reduction of puncture deformation in PSGG films containing high quantity of phenolic compounds is

mostly owing to the hydrophobic interactions that maintain the polymer molecules together to keep film integrity (Alkan and Yemenicioğlu, 2016).

3.5. Scanning electron microscopy (SEM)

SEM micrographs of the surface of PSGG films incorporated with different antimicrobial compounds at low and high levels were illustrated in Fig. 2. No holes or bubbles were detected in the film surface. This proves that the diffusion of the compounds in the film were homogeneous, without accumulation and/or separation (Campos et al., 2014). The morphology of the film depended on the compound levels applied in PSGG films. After incorporating active agents, the micrographs of the films exhibited similar appearance, smoother and more homogeneous and compact surface was observed in films with low amount of compounds compared with films containing higher ones. Increased number of polymer-polyphenol interactions might contribute to the formation of rough and bumpy film structure. It was anticipated to observe more surface heterogeneity in films with MAC, resulting in increased film thickness, according to the data of film thickness (Table 3). The increased hydrophobicity of PSGG film matrix by the decreased phenolic hydroxyl groups was also considered as a main factor in increased roughness of MAC containing PSGG films (Alkan et al., 2011).

3.6. Antibacterial activity of films

Inhibition zone diameters calculated by PSGG edible film disks incorporated with different concentrations of EGCG, BBA and MAC against the tested microorganisms are demonstrated in Figs. 3a and 3b. The results of colony reduction percentage are illustrated in Table 4. The PSGG film without active compounds did not show any antimicrobial activity. When antimicrobial compounds were added into the films, these materials dispersed through agar gel and led to clear zone around the film disks. However, films containing 1 × MIC of EGCG

were not effective against *G. candidum*, *Rhizopus sp.* and all Gram-negative bacteria except *P. fluorescence*. All Gram-negative bacteria and fungi except *A. niger* were resistance against PSGG-BBA films at 1× MIC, while films with MAC at the same amount did not have antimicrobial activity. However, the antimicrobial activity of films was enhanced by increasing the concentration of added compounds. Inhibition zone diameters of active films were less than those of crude extracts, because chemical interactions between hydroxyl groups in edible films and phenolic compounds in extracts could block the active antibacterial sites. The antimicrobial films exerted the strongest influence on Gram-positive bacteria which may be related to various diffusion rates of antimicrobial agents through the surrounding external membrane of the cell walls in the gram-negative bacteria. The cell wall of Gram-negative bacteria protected with an additional external membrane constraining distribution of hydrophobic or hydrophilic substances through its lipopolysaccharide (LPS) layer (Kanmani and Rhim, 2014). The antimicrobial films had the least effect on *Rhizopus sp.* followed by *S. typhimurium*. These results were reasonable as MIC and MBC tests showed that *Rhizopus sp.* and *S. typhimurium* were the most resistant of the fungi and bacteria tested. At all assayed concentrations, the maximum zone of inhibition and colony reduction percentage were observed for films incorporated with EGCG. The effect of EGCG on the inhibition or the reduction of the microorganism growth is associated with its capability to prevent the synthesis of DNA and RNA in microbial cells (Mori et al., 1987), to inhibit the function of cytoplasmic membrane in microorganisms (Tsuchiya et al., 1994), and to impede microbial energy metabolisms (Haraguchi et al., 1998). This is the first study to investigate the incorporation of blueberry ash and macadamia extracts as antibacterial agents in edible films. The antibacterial characteristics of PSGG edible films containing blueberry ash and macadamia could be associated with the existence of bioactive compounds such as phenolic acids, flavonoids or anthocyanins derived from the extracts. However, the action behind the

antimicrobial activity of phenolic acids, flavonoids or anthocyanins is not entirely elucidated. A number of mechanisms of action have been suggested by different authors; such as alteration of the cell morphology (Sivarooban et al., 2008), inhibition of extracellular microbial enzymes (Burdulis et al., 2009; Gradisar et al., 2007), inhibition of energy metabolism (Chinnam et al., 2010), the production of hydrogen peroxide by oxidation (Arakawa et al., 2004), destabilization of cytoplasmic membrane, permeabilization of plasma membrane, and deprivation of the substrates required for microbial growth (Burdulis et al., 2009; Genskowsky et al., 2015).

4. Conclusion

The outcomes of the current research demonstrated that EGCG, BBA and MAC are capable to prevent an extensive spectrum of food pathogenic and spoilage microorganisms. Antimicrobial films were produced by addition of EGCG, BBA and MAC as natural antimicrobial compounds with various concentrations into PSGG film for the application of active food packaging. The antimicrobial activity was determined by the amount of natural antimicrobial compounds in the film and microbial strains. Micrographs of films presented a continuous and smooth structure, with no development of cracks in the film. Furthermore, addition of active agents caused a significant increase in tensile strength and Young's modulus and a reduction in percentage of elongation at break of films. Thus, these results proposed the potential application of PSGG-based edible films containing phenolic compounds to improve the safety of foods and food products by delaying microbial spoilage. Further studies are suggested to investigate chemical and pharmacological properties of BBA and MAC extracts, to discover the exact mechanisms of microbial growth inhibition by their phenolic compounds, and to determine the function and stability of developed PSGG edible coatings on fruit and vegetables, and alternative contaminated food surfaces.

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Conflict of Interest

The authors declare no conflict of interest.

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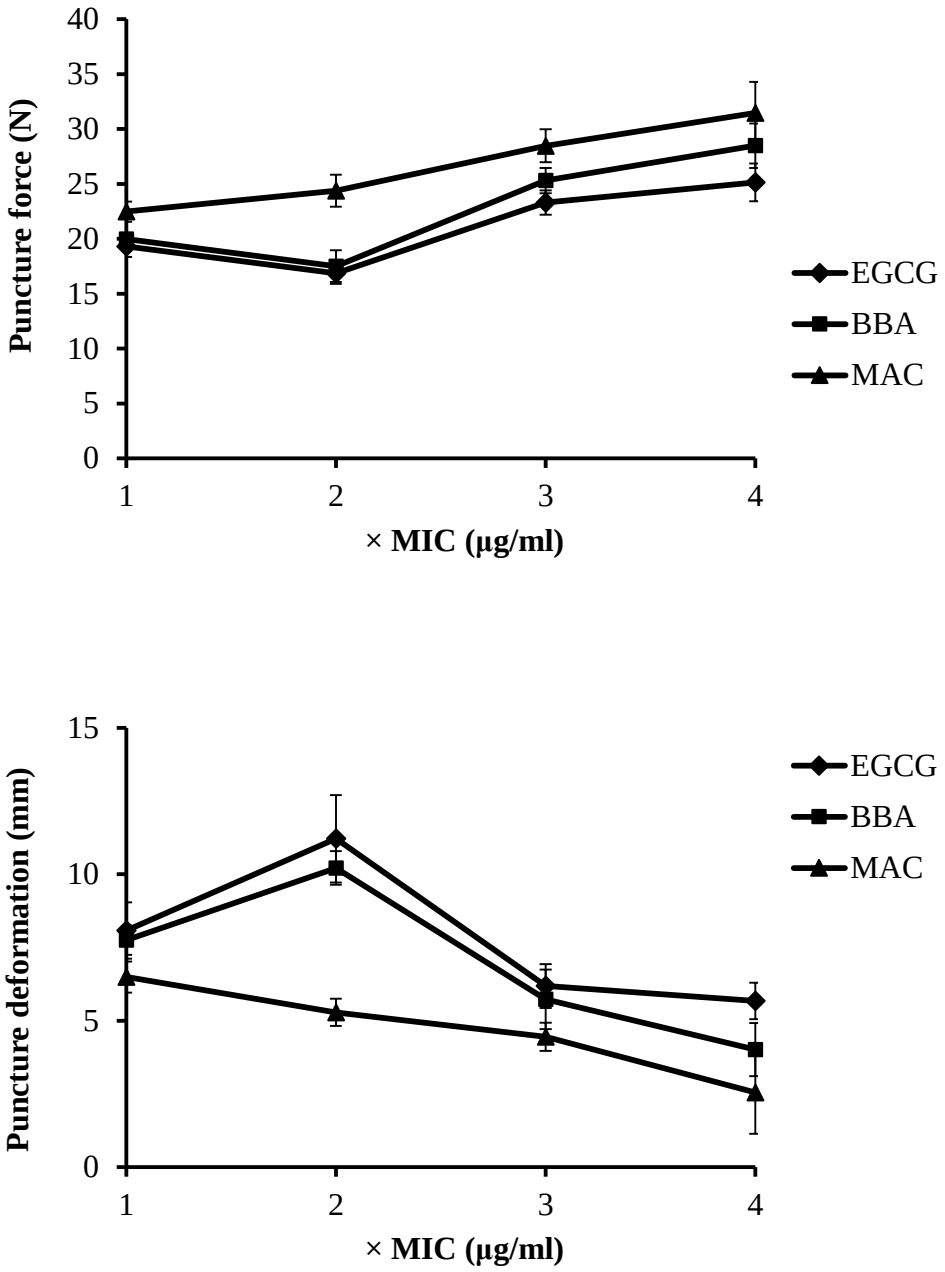

Fig. 1. The puncture mechanical properties of edible PSGG films as a function of natural antimicrobial compound concentrations.

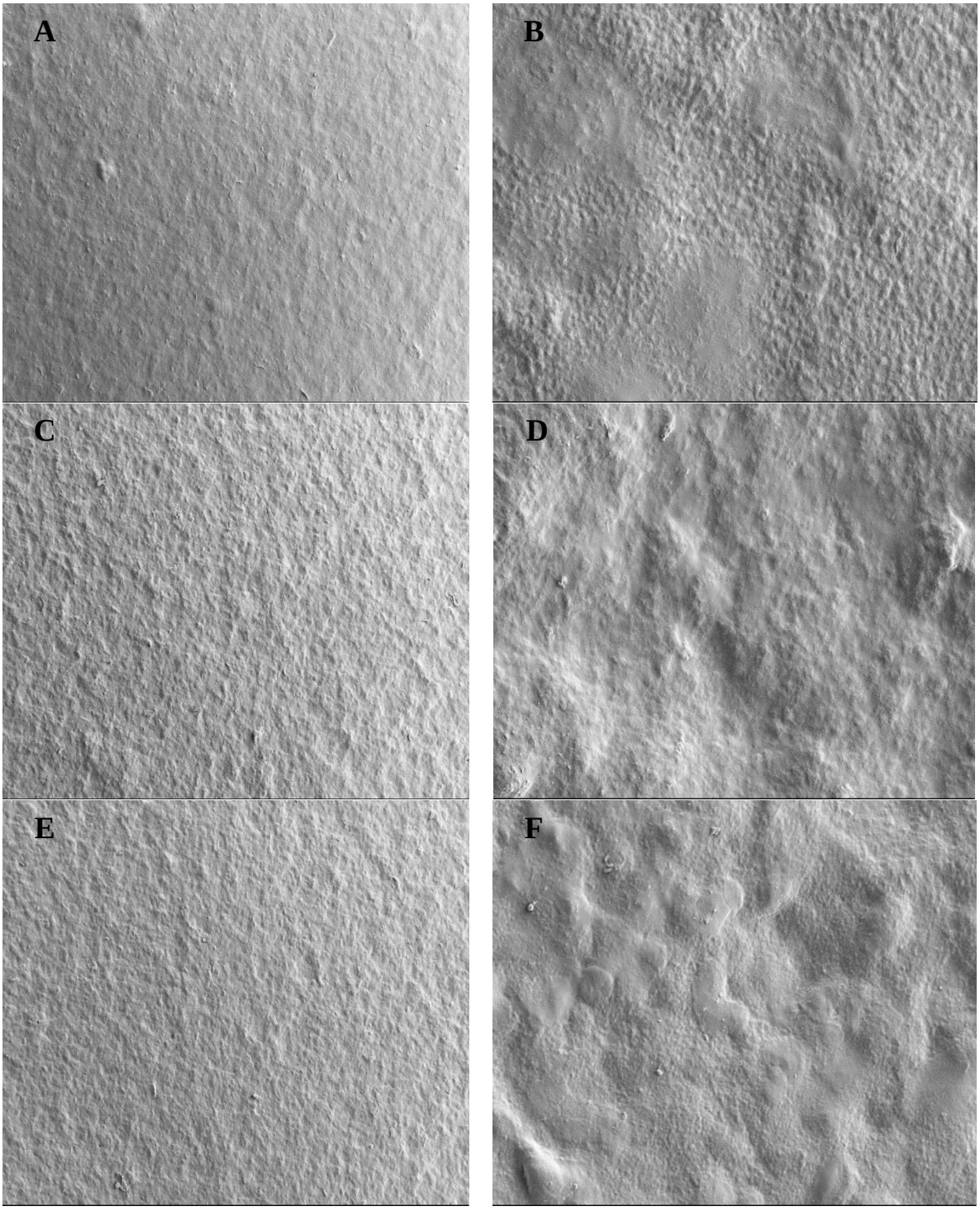

Fig. 2. Micrographs of the PSGG films (× 1000). A: film surface with 1× MIC of EGCG; B: film surface with 4× MIC of EGCG; C: film surface with 1× MIC of BBA; D: film surface with 4× MIC of BBA; E: film surface with 1× MIC of MAC; F: film surface with 4× MIC of MAC.

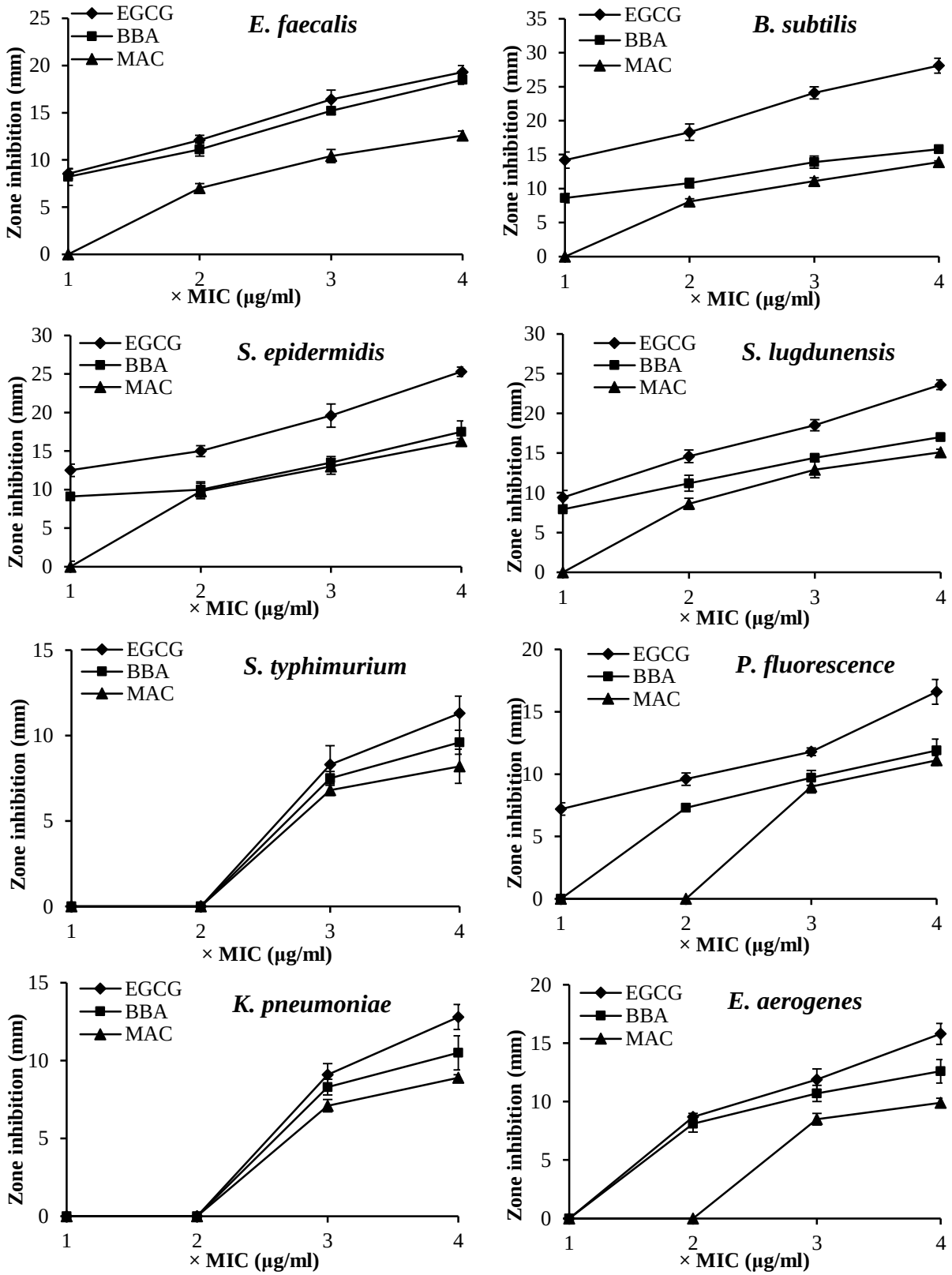

Fig. 3a. Antimicrobial activities of edible PSGG films as a function of natural antimicrobial compound concentrations.

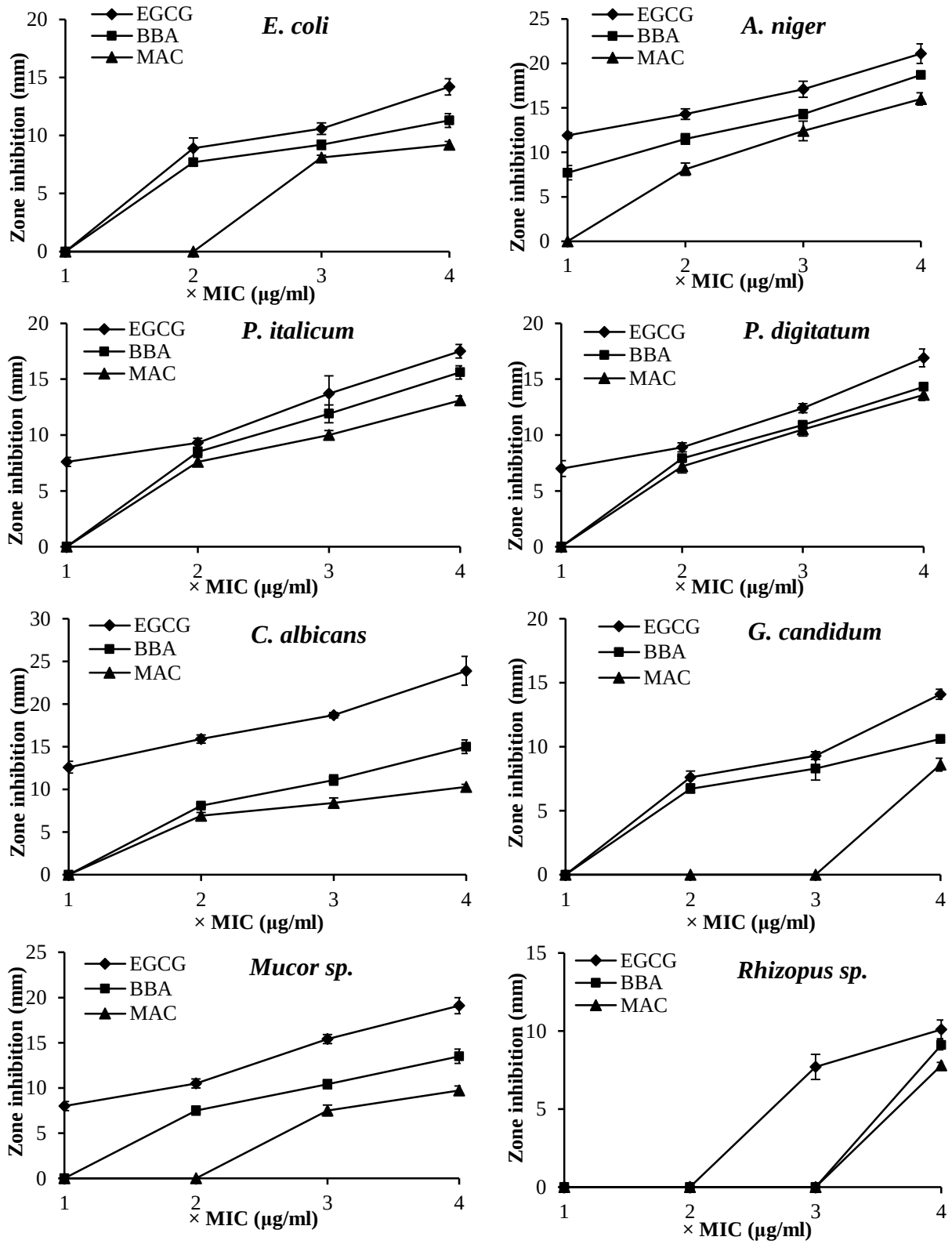

Fig. 3b. Antimicrobial activities of edible PSGG films as a function of natural antimicrobial compound concentrations.

Table 1. Antibacterial and antifungal activity of different natural antimicrobial compounds measured as zones of inhibition (mm).*

Bacteria	EGCG		Blueberry ash		Macadamia		CIP
	1000 μg/ml	% inhibition	1000 μg/ml	% inhibition	1000 μg/ml	% inhibition	5 μg per disc
Enterococcus faecalis	24.5±0.5^{c}	92.8±1.9^{abc}	23.7±1.4^{a}	89.7±5.3^{a}	13.9±1.4^{cd}	52.8±5.4^{de}	26.4±0.3
Bacillus subtilis	28.0±0.6^{a}	87.6±1.9^{bcd}	21.4±1.2^{bc}	66.9±3.6^{de}	14.9±1.1^{bc}	46.7±3.5^{efg}	32.0±0.8
Staphylococcus epidermidis	27.2±1.7^{ab}	96.4±5.9^{ab}	20.5±1.5^{bc}	72.7±5.3^{bcd}	17.3±1.2^{a}	61.4±4.1^{ab}	28.2±1.0
Staphylococcus lugdunensis	25.3±0.6^{bc}	92.1±2.1^{abc}	21.9±1.6^{ab}	79.7±5.8^{b}	15.6±1.3^{b}	56.6±4.5^{bcd}	27.5±1.4
Salmonella typhimurium	18.0±1.4^{ghi}	76.1±6.0^{de}	14.1±1.7^{hij}	59.4±7.1^{ef}	8.3±0.6^{ij}	34.9±2.7^{i}	23.7±0.6
Pseudomonas fluorescence	21.7±0.8^{de}	84.8±3.1^{bcd}	17.8±0.8^{def}	69.7±3.0^{cd}	12.2±0.4^{ef}	47.5±1.4^{efg}	25.6±1.2
Klebsiella pneumoniae	19.3±1.5^{efgh}	78.6±6.2^{de}	16.9±1.7^{efg}	68.9±6.8^{cd}	9.7±0.6^{hi}	39.6±2.6^{hi}	24.6±2.3
Enterobacter aerogenes	21.2±1.8^{ef}	83.7±6.9^{cd}	19.7±1.1^{bcd}	78.0±4.3^{bc}	11.4±0.7^{fg}	45.1±2.7^{fgh}	25.3±0.8
Escherichia coli	20.4±0.6^{efg}	85.4±2.6^{abcd}	18.1±0.9^{de}	75.6±3.8^{bcd}	10.0±1.0^{gh}	41.8±4.2^{gh}	23.9±0.6
Fungi	EGCG		Blueberry ash		Macadamia		KTZ
	1000 μg/ml	% inhibition	1000 μg/ml	% inhibition	1000 μg/ml	% inhibition	25 μg per disc
Aspergillus niger	23.8±0.8^{hij}	93.9±3.0^{abc}	19.3±0.4^{cd}	76.0±1.4^{bcd}	16.1±0.7^{ab}	63.2±2.8^{a}	25.4±0.6
Penicillium italicum	18.4±1.2^{ghi}	87.2±5.7^{abcd}	16.1±0.9^{efgh}	76.3±4.1^{bcd}	11.5±0.5^{fg}	54.5±2.4^{cd}	21.1±1.0
Penicillium digitatum	17.4±2.3^{f}	77.3±10.0^{de}	15.8±1.1^{fgh}	70.1±4.8^{bcd}	13.4±1.0^{de}	59.5±4.3^{abc}	22.5±0.3
Candida albicans	24.4±0.8^{c}	92.3±3.0^{abc}	15.5±0.5^{ghi}	58.6±1.9^{ef}	10.3±0.5^{gh}	38.9±1.7^{hi}	24.4±1.1
Geotrichum candidum	15.2±1.0^{j}	71.5±4.6^{e}	12.4±1.6^{jk}	58.5±7.7^{ef}	9.5±0.5^{hij}	44.7±2.4^{fgh}	21.2±0.6
Mucor sp.	18.9±1.7^{fgf}	97.2±8.8^{a}	13.3±1.5^{ij}	68.7±7.8^{cd}	9.9±1.1^{gh}	51.0±5.7^{def}	19.4±2.1
Rhizopus sp.	16.2±2.8^{ij}	79.5±13.6^{de}	10.7±0.6^{k}	52.6±3.2^{f}	8.1±0.6^{j}	39.9±3.0^{hi}	20.4±0.7

*Diameter of inhibition zone including disc diameter of 6.0 mm. Values are the means of triplicates ± standard deviations, CIP: ciprofloxacin antibacterial control, KET: ketoconazole antifungal control. Means at same column with different lower case are significantly different ($P < 0.05$).

Table 2. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of different natural plant extracts.*

Bacteria	EGCG		Blueberry ash		Macadamia	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
Enterococcus faecalis	187.5	375.0	93.8	187.5	750.0	750.0
Bacillus subtilis	93.8	93.8	187.5	187.5	750.0	750.0
Staphylococcus epidermidis	93.8	187.5	187.5	375.0	375.0	375.0
Staphylococcus lugdunensis	187.5	187.5	187.5	375.0	375.0	750.0
Salmonella typhimurium	750.0	1500.0	1500.0	1500.0	1500.0	3000.0
Pseudomonas fluorescence	375.0	375.0	750.0	750.0	750.0	1500.0
Klebsiella pneumoniae	750.0	1500.0	1500.0	1500.0	1500.0	3000.0
Enterobacter aerogenes	750.0	750.0	750.0	750.0	1500.0	1500.0
Escherichia coli	375.0	750.0	750.0	750.0	1500.0	1500.0
Fungi	EGCG		Blueberry ash		Macadamia	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
Aspergillus niger	93.8	187.5	187.5	375.0	375.0	750.0
Penicillium italicum	375.0	375.0	375.0	750.0	750.0	750.0
Penicillium digitatum	375.0	375.0	375.0	750.0	750.0	750.0
Candida albicans	93.8	187.5	375.0	750.0	750.0	750.0
Geotrichum candidum	750.0	750.0	750.0	750.0	750.0	1500.0
Mucor sp.	187.5	375.0	375.0	750.0	750.0	750.0
Rhizopus sp.	750.0	1500.0	1500.0	3000.0	1500.0	3000.0

* Values are the means of triplicates ± standard deviations.

Table 3. Thickness, density and tensile mechanical properties of edible PSGG films as a function of natural antimicrobial compound concentrations.*

Natural antimicrobial compound × MIC (μg/ml)		Thickness (mm)	Density ($\times 10^{-3}$ g/mm^3)	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (MPa)
PSGG**		0.129±0.042^{bcd}	1.2±0.3^{h}	27.8±2.2^{fg}	16.2±1.7^{bc}	175.7±14.2^{fg}
EGCG	1	0.121±0.007^{d}	1.5±0.1^{g}	27.2±3.1^{fg}	17.5±1.5^{b}	168.1±15.3^{fg}
	2	0.124±0.002^{cd}	1.6±0.2^{ef}	21.8±1.1^{h}	21.9±1.0^{a}	100.1±9.1^{h}
	3	0.129±0.002^{bcd}	1.7±0.1^{de}	33.9±3.1^{cd}	14.0±1.1^{cd}	242.6±27.3^{de}
	4	0.133±0.002^{abc}	2.3±0.1^{b}	38.8±2.3^{b}	13.2±1.6^{de}	295.4±27.5^{cd}
BBA	1	0.123±0.008^{cd}	1.6±0.1^{fg}	28.5±0.6^{f}	16.2±1.6^{bc}	162.9±12.8^{fgh}
	2	0.127±0.004^{cdef}	1.8±0.3^{de}	24.0±3.0^{gh}	20.0±1.7^{a}	121.4±23.4^{gh}
	3	0.132±0.003^{abc}	1.8±0.1^{d}	37.1±2.3^{bc}	12.4±0.5^{def}	300.8±27.9^{cd}
	4	0.137±0.001^{ab}	2.3±0.2^{ab}	40.7±0.9^{ab}	10.5±1.2^{fg}	389.9±38.6^{b}
MAC	1	0.126±0.003^{cd}	1.7±0.1^{de}	29.1±2.2^{ef}	14.8±1.0^{cd}	197.7±27.9^{ef}
	2	0.129±0.005^{bcd}	1.8±0.2^{d}	32.7±1.9^{de}	12.7±1.7^{def}	261.7±43.1^{d}
	3	0.134±0.001^{abc}	2.0±0.1^{c}	39.1±1.5^{b}	11.2±1.2^{ef}	351.9±51.6^{bc}
	4	0.141±0.003^{a}	2.5±0.3^{a}	43.1±1.4^{a}	8.2±1.0^{g}	531.5±78.7^{a}

* Values are the means of triplicates ± standard deviations. Means at same column with different lower case are significantly different ($P < 0.05$).

** Please refer to Saberi et al. (2016a).

Table 4. Antibacterial and antifungal activity of edible PSGG films as a function of natural antimicrobial compound concentrations.*

Bacteria	Colony reduction (%)											
	EGCG × MIC (μg/ml)				BBA × MIC (μg/ml)				MAC × MIC (μg/ml)			
	1	2	3	4	1	2	3	4	1	2	3	4
E. faecalis	54.8±10.5^{bc} de	73.3±10.0 b	88.7±9.9^{ab}	99.0±1.0^{a}	47.5±7.5^{b} c	62.0±10.4 ab	76.3±9.5^{a}	91.0±3.0^{a}	0.0±0.0	23.7±7.2^{c}	43.7±10.0^{cd} ef	51.0±7.0^{cde}
B. subtilis	78.1±8.7^{a}	87.4±7.3^{a}	94.4±7.0^{ab}	100.0±0.0^{a}	54.7±10.1^{a}	67.4±12.8 a	72.1±5.4^{ab}	80.0±7.5^{abcd}	0.0±0.0	47.4±12.8 ab	53.1±3.9^{abcd}	66.7±13.1^{a} bc
S. epidermidis	63.2±7.0^{c}	80.5±7.7^{ab}	98.6±1.2^{a}	100.0±0.0^{a}	50.9±3.2^{a} b	63.9±7.8^{ab}	70.3±4.1^{ab}	82.7±8.1^{abc}	0.0±0.0	50.2±1.9^{a}	60.3±4.1^{ab}	77.3±8.4^{a}
S. lugdunensis	57.3±1.1^{bcd}	77.0±9.7^{ab}	98.3±1.5^{a}	100.0±0.0^{a}	42.7±4.2^{c}	65.0±6.2^{ab}	75.0±10.4^{a}	85.3±6.5^{abc}	0.0±0.0	48.3±10.0 a	60.0±10.0^{ab}	75.3±6.5^{ab}
S. typhimurium	0.0±0.0^{a}	0.0±0.0^{f}	47.5±9.4^{gh}	66.5±6.4^{de}	0.0±0.0^{d}	0.0±0.0^{f}	40.5±8.0^{e}	58.5±9.6^{fg}	0.0±0.0	0.0±0.0^{d}	31.5±8.9^{f}	43.5±4.2^{def}
P. fluorescence	47.8±8.3^{cde}	56.8±7.8^{d}	67.8±6.5^{cde}	84.2±12.7^{a} bc	0.0±0.0^{d}	37.5±8.2^{de}	47.5±3.0^{de}	70.8±11.6^{cde} fg	0.0±0.0	0.0±0.0^{d}	44.2±2.9^{cdef}	50.8±8.9^{cde}
K. pneumoniae	0.0±0.0^{f}	0.0±0.0^{f}	50.4±1.4^{fgh}	72.4±8.6^{cd}	0.0±0.0^{d}	0.0±0.0^{f}	43.8±12.0^{e}	61.8±7.6^{fg}	0.0±0.0	0.0±0.0^{d}	36.8±10.0^{ef}	46.4±10.9^{d} ef
E. aerogenes	0.0±0.0^{f}	53.7±9.4^{d}	61.1±6.8^{defg}	77.4±4.2^{bcd}	0.0±0.0^{d}	43.7±9.2^{cd} e	55.1±5.9^{cde}	66.1±10.4^{def} g	0.0±0.0	0.0±0.0^{d}	40.4±8.4^{def}	48.4±7.3^{de}
E. coli	0.0±0.0^{f}	55.1±7.0^{d}	64.7±7.9^{def}	74.7±7.9^{cd}	0.0±0.0^{d}	40.1±6.3^{cd} e	50.7±7.8^{cde}	63.1±8.4^{efg}	0.0±0.0	0.0±0.0^{d}	38.4±10.6^{ef}	45.7±9.3^{def}
Fungi	EGCG × MIC (μg/ml)				BBA × MIC (μg/ml)				MAC × MIC (μg/ml)			
	1	2	3	4	1	2	3	4	1	2	3	4
A. niger	61.0±9.8^{bc}	70.0±4.5^{bc}	81.0±13.0^{bc}	92.7±7.0^{ab}	54.7±6.0^{a}	66.7±7.3^{a}	75.0±4.7^{a}	87.0±8.0^{ab}	0.0±0.0	53.4±12.4 a	65.7±5.3^{a}	73.7±13.9^{a} b
P. italicum	44.2±8.6^{de}	56.2±2.4^{d}	64.5±12.5^{de} f	84.5±12.3^{a} bc	0.0±0.0^{d}	51.5±2.7^{bc}	63.2±10.2^{a} bc	78.2±10.9^{abc} de	0.0±0.0	45.2±5.9^{ab}	55.5±3.7^{abc}	60.2±10.3^{b} cd
P.	41.9±15.2^{e}	52.2±4.7^{d}	61.5±11.5^{de}	80.9±15.9^{b}	0.0±0.0^{d}	47.9±12.4	58.9±6.9^{bc}	73.9±8.6^{bcdef}	0.0±0.	41.5±6.8^{ab}	50.5±10.4^{bc}	57.5±9.5^{cd}

digitatum			fg	cd		cd	d		0		de	
C. albicans	65.4±10.1^{ab}	77.8±14.1 ab	87.8±3.5^{ab}	98.3±2.1^{a}	0.0±0.0^{d}	61.4±5.8^{ab}	70.1±5.4^{ab}	81.3±6.0^{abcd}	0.0±0.0	36.4±10.5 b	45.1±6.1^{cdef}	51.3±8.1^{cde}
G. candidum	0.0±0.0^{a}	35.9±7.3^{e}	54.6±8.8^{efgh}	66.3±12.3^{d} e	0.0±0.0^{d}	31.6±6.8^{e}	44.6±8.7^{de}	57.3±5.8^{g}	0.0±0.0	0.0±0.0^{d}	0.0±0.0^{a}	35.3±8.2^{ef}
Mucor sp.	47.1±10.8^{cd} e	59.7±8.4^{cd}	71.1±7.9^{cd}	86.4±9.7^{abc}	0.0±0.0^{a}	37.1±6.0^{de}	52.7±12.7^{c} de	66.8±8.2^{defg}	0.0±0.0	0.0±0.0^{d}	37.7±6.7^{ef}	45.8±7.0^{def}
Rhizopus sp.	0.0±0.0^{f}	0.0±0.0^{f}	40.3±8.7^{h}	57.0±6.6^{e}	0.0±0.0^{d}	0.0±0.0^{f}	0.0±0.0^{f}	43.3±6.5^{h}	0.0±0.0	0.0±0.0^{d}	0.0±0.0^{g}	31.0±1.8^{f}

* Values are the means of triplicates ± standard deviation. Means at same column with different lower case are significantly different ($P < 0.05$).