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Incorporation of Moringa leaves extract in Pectin-based edible coating as antimicrobial agent

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ABSTRACT

It is become very necessary to look for new antimicrobial substances from other sources such as plants, which the leading important things about utilizing plant-derived antioxidant are largely less dangerous than artificial ones and provide deep restoration benefits. The present study was carried out to evaluate the potential of edible pectin film that contains *Moringa oleifera* leaf extract as a new biodegradable film maintaining the shelf life of food and improve its microbiological safety. In this study, bioactive compounds of moringa were extracted by using varied solvents; ethanol 70%, acetone 95% and water. The three extracts were analyzed to determine polyphenols, flavonoids content and its antioxidant activity. Also the extracts were screened for in vitro antimicrobial potential against some probiotic bacteria strains (E-coli 0157: H7, B. Cereus, *S. aureus*, *P. aeruginosa* and *L. plantarum*) at different concentration (50, 75, 100, 150 and 200 mg/mL^{-1}) of moringa extract in dimethyl sulfoxide solution. Four concentration of glycerol (0.5, 1.0, 1.5 and 2.0%) were investigated as plasticizer agent in moringa extracts-containing pectin films to optimize the permeability to gases and mechanical properties of them. Moringa extracts-containing pectin films were evaluated by measuring optical & mechanical properties and its microbiological activities against E. coli 0157: H7 and S. aureus at varied concentration of solvents extract (0.3, 0.5 and 1.0%). The results demonstrated that moringa leaf ethanol extract, between the other solvents, had a higher amount of total phenolics (158.79 mg GAE/g), flavonoids (66.37 mg QE/g) and strong antioxidant activity (82.47%). Also results revealed that 200 mg mL^{-1} moringa leaf ethanol extract had maximum zone of inhibition against the pathogenic strains used in this study which ranged between 15.34 – 18.27 mm. The film formula of 2.0% glycerol was the best mechanical properties of it. The date confirmed that with the increase of moringa extract concentration, there are similar increase in TS, E% and decrease in O_2P and WVP of the pectin-moringa film and OP improved at the same time in addition, the films exhibited a clear antimicrobial activity against E.coli 0157: H7 and S. aureus with the increasing of moringa extract concentration. Thus, moringa is extremely valued plant, with impressive content of bioactive compounds, therefore, ME-containing pectin film can be used as antimicrobial and antioxidative packaging materials to improve the quality of food products and to prolong its shelf life.

Key words: *Moringa oleifera* leaves, pectin edible film, antimicrobial activity, physical properties

Introduction

Moringa oleifera Lam., a member of the Moringaceae family, also known as Drumstick or Horseradish-tree, it is a fast-growing tree native to South Asia and now found in many tropical and subtropical countries. It sometimes described as the "miracle tree". It grows like a weed, for those living in third-world counties, it may very well prove to be a valuable source of nutrition. One hundred grams of dry moringa leaf contains: 12 times the vitamin C of oranges, 10 times the vitamin A of carrots, 9 times the protein of yogurt, 17 times the calcium of milk (Sharama *et al*., 2012).

Moringa oleifera are rich in antioxidants including B-carotene, chlorogenic acid, V.C and high amount of polyphenols and flavonoids (such as Sitosterol, Niazin A), gallic acid, Kaempferol and Quercetin, its extracts of both mature and tender leaves exhibit strong antioxidant activity against free radicals, and prevent oxidative damage to major biomolecules (Anwar *et al*., 2007). For this reason, it is used as an alternative source for nutritional supplements and growth promoters in some countries.

Its extracts were screened for in vitro antibacterial potential against enteric pathogenic bacteria such as *S. aureus*, *P. aeruginosa*, Salmonella typhi, E. coli and several gram negative and

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gram positive bacteria (Bukar *et al*., 2018), many recent reports on disease prevention by *M. oleifera* have been reported.

Nevertheless, there are limited evidences for *M. oleifera* leaf in terms of food packaging and food preservation, despite, the phytochemical analysis revealed the presence of alkaloids, flavonoids, steroids, tannins, saponins and glycosides as major components (Malliga *et al*., 2012).

Asma *et al*., (2005) found that both 80% methanol and/ or 100% acetone extracts of *M. oleifera* leaves at concentration of 0.06% (w/w) stabilized the refined, bleached and deodorized sunflower oil samples and accelerated aging for a period of 18 days. Their results revealed that *M. oleifera* leaves might be explored as a viable source of natural antioxidants and nutraceuticals.

Ka-Yeon *et al*., (2016) improved the tensile strength, elongation at break, water vapor permeability and oxygen permeability of gelatin-based film containing *M. oleifera*. Furthermore, this film had an antimicrobial activity against L. monocytogenes and antioxidant activity such as ABTS radical scavenging activity. Pranav *et al*. (2016) studied the effect of Aloe vera Gel-based edible coating containing *M. oleifera* leaf extract on the quality of chicken bites. They found that the coating retarded moisture loss of chicken bites during storage and was successful in reducing lipid oxidation and inhibiting microbial growth during storage. They recommended that *M. oleifera* extracts could be used in processed meat products to extend shelf life and improve its quality during storage.

A recent study by Tesfay and Magwaza (2017) showed improved fruit quality and longer shelf life of avocado fruit treated with 1% CMC (w/v) containing Moringa leaf extract. Also, Fatma *et al*. 2018) used dried leaves of moringa in manufacture of soft white cheeses and revealed that 1% of *M. oleifera* dried leaves was a best ratio to give a good texture, flavor and extended the shelf life of the product. They concluded that ethanol extract of *M. oleifera* leaves should be used in manufacturing of cream cheese due to its highest content of nutritious and a variety of phenol compounds. They showed that the extract improved the probiotic strains growth in final product and enhanced the flavor and taste during storage. They mentioned that *M. oleifera* leaves may be used as natural preservative material for cream cheese due to its potential antimicrobial properties.

Antioxidants are widely used as food additives to enhance durability and prevent bad taste and protected texture of food. Antioxidants are also included in the plastic films to protect it from degradation and food oxidation control (Akbar and Monireh, 2016). Despite the above, the use of synthetic antioxidants in food due to their possible toxic effects can be questioned. The discovery of natural antioxidants is important.

Plant extracts as natural food preservatives are known. These compounds are often safe and accepted by consumers, it is therefore of great importance in industry. Moringa leaf extract as a natural preservative can be useful for industrial usage. A new generation of materials that are edible films for food storage have emerged. Discovery of edible food packaging materials of biological origin from plant sources, promises to solve the problem of environmental pollution (Bukar *et al*., 2018). Strength of these, materials and their permeability to oxygen which causes premature spoilage of food including those that led scientists to go to the new technology to solve these problems. Polysaccharides, lipids, proteins of a combination of these large molecules are polymers of edible films. Pectin-based edible films have excellent characteristics such as high resistance to oxygen, lipid, aromatic compounds diffusion and high solubility (Kang *et al*., 2007). Recently, the antimicrobial advanced packaging, in the food industry had a much improved performance. The film by release of antimicrobial substances from their surface to the food surface, make sure the health of food and as one of the most reliable tools in the package are introduced to investigate and search for the next generation.

Consumer awareness for health and environmental benefits of using edible, natural and food safe coatings has warranted more research about the natural food safe and ecologically friendly substitutes applied to reduce water transfer, gaseous exchange and oxidations of fresh products (Bill *et al*., 2014).

Pectin is one of the proportionally largest materials in citrus by-products. Pectin is a complex anionic polysaccharide composed of B-1, 4 linked D-glacturonic acid residues, wherein the uronic acid carboxyl are either fully (high methoxy pectin) or partially (low methoxy pectin) methyl esterified. Pectin provide strong, flexible films, which are thermally stable up to 180^{o}c, and pectin films have a good mechanical properties of packaging materials made from it (Cheorun *et al*., 2005).

Kang *et al*., (2007) examined the effect of a pectin-based edible coating containing green tea powder as antimicrobial agents and showed that the numbers of total aerobic bacteria were significantly reduced by the coating treatment.

Thus, the objective of the present study were (1) to examine the aqueous, ethanol 70% and 95% acetone extracts of *M. oleifera* lam. Leaves as a potential antioxidant and antimicrobial agent against some human pathogenic bacteria (2) to investigate the efficacy of pectin-based edible coating combined with Moringa leaf extracts as food packaging application and characterize their properties.

Material and methods:

Chemicals:

All chemicals and solvents were of analytical grade. The DPPH and Folin-Ciocalteu's phenol reagents were purchased from Merch Co. (Darmstadt, Germany).

Plant tissue extraction process:

Moringa leaves were extracted as described by Mendoza *et al*., (2013).

Aqueous extraction:

Fresh and mature *M. oleifera* leaves was collected, cleaned, chopped into small pieces and shade dried at room temperature. The dried leaves were grounded, passed through sieve no. 20 and extracted. 100 g successively with 600 ml of water in a soxhlet extractor for 20h. The extract was concentrated to dryness under reduced pressure and controlled temperature (40 – 50 ^{o}c).

Solvent extraction:

100 g of dried leaves were extracted with 1 L of ethanol 70% (v/v) for 2 h, and 100 g other of dried leaves were extracted with 1 L of acetone 95% (v/v) with constant agitation at 4 ^{o}c. The two extracts were concentrated in a rotary evaporator to get crude extracts. All extracts were kept in amber glass at -20 ^{o}c.

Preparation of the M. oleifera – containing pectin film:

The pectin-based edible film was prepared by dissolving 3 g pectin (Sigma-Aldrich Co., St. Louis, USA) in 99 ml distilled water. After addition polyvinyl alcohol 1.25% as a crosslink reagent and glycerol concentration of: 0.5, 1.0, 1.5 and 2.0% (v/v) as a plasticizer (Sigma-Aldrish Co.), the solutions were homogenized with a homogenizer (Diax900, Heidolgh. Germany) for 1h and homogenized again for 30 min. after the addition of 0.3, 0.5, 0.7 and 1.0% of concentrated extract of *M. oleifera* to each solution of various glycerol concentrate samples. The final solutions was sonicated about 1 h in order to remove air bubbles or dissolved air. All films forming solutions were cast onto the leveled Teflon film (Cole-Parmer Instrument Co., Chicago, USA) coated glass plates (24 x 30 cm) and dried at room temperature for 48 h. The films were peeled and stored in a vacuum desicator for further use.

Determination of total phenolics content of M. oleifera extracts:

The total phenolics content in *M. oleifera* extract was analyzed by the F-C method (Singieton *et al*., 1999). Briefly, 0.75 ml of Folin-Ciocaltea reagent was added to 100 µl of different dilutions of *M. oleifera* leaves extract and final volume was made 10 times with distilled water. After 5 min. 0.75 ml of a sodium carbonate solution (7.5%) was added to each tube. The tubes were kept in dark for 90 min. and absorbance (U-28000 Spectrophotometer, Hitachi, Tokyo, Japan) was measured against a black at 725 nm. A standard curve was plotted using different concentrations of gallic acid, and the amount of total phenolics content was calculated and expressed as milligram gallic acid equivalent per gram dried extract (mg GAE/g extract).

Determination of flavonoid content of M. oleifera extracts:

The total flavonoid content was determined using aluminum chloride colorimetric method according to Chang *et al.* (2002). Briefly, 100 ml of each extract were mixed with 1.5 ml of 95% ethanol, 100 µl of 10% $AlCl_3$, 100 µl of 1 M potassium acetate and 2.8 ml of deionized water. Then,

the absorbance of the reaction mixture was determined at 415nm. The total flavonoids content was expressed as mg quercetin equivalent per gram dried extract (mg QE/g extract).

Determination of antioxidant activity of M. oleifera extracts:

The antioxidant activity in terms of radical scavenging activity (RSA) of M. oleifera extract was determined by standard stable free radical DPPH method (Mensor *et al*., 2001). To an aliquot of various concentrations of the extract. 3 ml of DPPH in methanol (final concentration of 250 μm) was mixed and the mixture was vortexed vigorously. The tubes were incubated at room temperature for 30 min in the dark, and absorbance was measured at 515 nm. Butyl Hydroxy toluene (BHT, Sigma) was used as positive control (standard) while the negative control is contained the entire reaction reagent except the extracts. Inhibition % was calculated by the following equation: DPPH scavenging effect (DSE%) = $Abs_{control} - Abs_{sample} / Abs_{control}$) x 100.

Antimicrobial activity of M. oleifera extracts:

Pathogenic strains

For the antimicrobial activity of ME, *E. coli* 0157: H7, *S. aureus*, *P. aeruginosa* and *L. plantarum* were isolated and serologically identified at dairy micro. Dep. Lab., Ein-Shams Univ. *B. cereus* B-3711 was provided by the Northerm Regional Res. Lab. Illinois, USA: strains were activated in trypton soya broth, incubated at 37 ^{o}c for 24 h. *L. plantarum* strain was activated in molt extract broth, incubated at 28 ^{o}C for 72 h.

One milliliter of leaf extracts with different solvent and water were dissolved individually in 5.0 mL dimethyl sulfoxide (DMSO) to prepare different concentrations from M. oleifera extract (to get 200 mg mL^{-} solution). After that, different dilution were prepared in DMSO to get 150, 100, 75 and 50 mg mL^{-} solutions.

The antimicrobial assay of different concentration of *M. oleif*era extracts performed by agar disc diffusion method using Muller Hinton agar medium according to Singh and Sharma (2012). The medium was poured into the petri dishes and allowed to solidified, the pathogenic strains 0.1 mL (approximately 10^9 cells mL^{-1}) of the tested microorganisms was spread on the surface of the agar found in petri dish using a sterile swab. The plates were rested to 2 h at 37^{o}c to allow the agar saturated with pathogenic strains. For the agar disc diffusion method, 20 ml of each leaves concentration was impregnated on different sterile paper discs (Whatman, 106 mm) and placed on the surface of Muller Hinton agar in ptri dishes gently. According to this procedure, each disc carried a loaded of different leaves extract equals to 1.00, 1.50, 2.00, 3.00 and 4.00 mg extract. The plates were incubated at 37^{o}c for 24 h. After the incubation period the inhibition zones around the each disc was measured in millimeters.

Characterization of films:

Film thickness:

The film thickness was measured with a digital micrometer (Mitoyoto.1 – Chom, Kanagawa, Japan) with an accuracy of 0.001 mm. Five measurements were taken of each film sample and the mean values were used to calculate the mechanical properties.

Measurement of mechanical properties:

Tensile strength (TS) and percentage elongation at break (%E) of the pectin films were measured following the ASTM (1995) method using a texture analyzer (TA – XT_2, Stable Micro System Co. Ltd., Surrey, England). It was set at an initial distance between grips of 2 cm and the cross-head velocity of 3 mm/s. TS of the film was calculated by dividing the maximum strength by the initial cross-section area. The %E was calculated by dividing the initial distance between the grips from the elongated distances until the time the film breaks.

Water vapor permeability (WVP):

WVP of pectin films was determined by the standard method of ASTM E96-95 (1995). The WVP measuring cup was filled with 18 ml of distilled water and placed film sample (7.5 x 7.5 cm) on

the top of cup and sealed tightly to prevent the leakage of wter vapor. The assembled WVP cup was weighed and subsequently placed in a controlled environmental chamber set at 25°c and 50% RH. Weight change of the cup was determined every 1 h for 8 h. The water vapor transmission rate "WVTR" (gm/m^2s) of the film was calculated by using the slope of the steady-state (linear) portion of weight loss versus time plot. Then, the WVP (gm/m^2 sPa) of the film was calculated as follow: WVP (WVTR x L)/ΔP
Where L was the mean thickness of the film and ΔP was water vapor partial pressure difference (Pa) across the film.

Oxygen & Carbon dioxide permeability:

Oxygen permeability (O_2P) and carbon dioxide permeability (CO_2P) were determined based on the ASTM method (1995). The film were sealed between two chambers, having each one two channels. In the lower chamber O_2 (or CO_2) was supplied at a controlled, flow rate to maintain its pressure constant in that compartment. The other chamber was purged by a stream of nitrogen, also at a controlled flow. Nitrogen acted as a carrier for the O_2 (or the CO_2). In the case of O_2P measurement, the flow leaving this chamber was connected to an O_2 sensor which measured the O_2 concentration in that flow on-line. In the case of CO_2P measurement the flow leaving this chamber was collected in a syringe for CO_2 quantification. To determine O_2 concentration 1 ml of sample was injected in a gas chromatography (Chromapack 9001, Middlelburg, Netherlands) at 110°c. A standard mixture containing 10% CO_2, 20% O_2 and 70% N_2 was used for calibration. Three replicates were obtained for each sample, in each case (O_2P and CO_2P).

Optical properties:

Hunter L. a and b value was measured with a colorimeter (CR-400, Minolta, Tokyo, Japan) at random positions of the film, and five replications were performed for each sample. The film opacity was evaluated by measuring the absorbance at 600 nm with a spectrophotometer (UV-2450, Shimadzu Co., Kyoto, Japan) and obtained using the following equation: Opacity = Abs_{600} / X
Where Abs_{600} is the absorbance at 600 nm, and X is the thickness of the film (mm).

Antimicrobial activity of *M. oleifera* containing pectin film:

Antimicrobial activity of the films were examined for their inhibitory effect on the growth of gram-negative bacteria (*E. coli* 0157: H7) and Gram-positive bacteria (*S. aureus*) at varied concentration of Moringa extract (0.3, 0.5 and 1.0%). Test microorganisms were aseptically inoculated in 20 ml of tryptone soya media broth (TSB) and subsequently incubated at 37°c for 16h. Each cultured broth was centrifuged at 2000 rpm for 10 min. and the cell pellets were suspended in 100 ml of sterile TSB and diluted 10 times with sterile distilled water. 20 ml of diluted broth (10^6-10^7 cFu/ml) was taken into 100 ml conical flask containing 100 mg of film sample and subsequently incubated at 37°c for 16 h under mild shaking. The same diluted broth without film sample was used as the control. The cell viability of each microorganism was calculated by counting bacterial colonies on the plates at 0, 4, 8, 12 and 20 h. Antimicrobial tests were performed in triplicate with individually prepared films (Shanker *et al.,* 2015).

Statistical analysis:

Measurements of each property of films were performed in triplicate with individually prepared film samples as the replicated experimental units, and mean values with standard deviation (SD) were presented. One-way analysis of variance (ANOVA) was performed and the significance of each mean property value was determined ($p<0.05$) with the Duncan's multiple range tests using the SPSS statistical analysis computer program for windows, (SPSS Inc., Chicago, IL, USA).

Results and Discussion:

Total phenolics, flavonoid contents and antioxidant activity of ME:

The results of total phenolics, flavonoid contents and DPPH radical scavenging activity of M. oleifera in aqueous, ethanol 70% and acetone 95% extracts are presented in Table (1). Ethanol extract had a higher amount of phenolics and flavonoids (158.79 mg GAE/g and 66.37 mg QE/g) respectively

among the other leaves extracts and exhibited a DPPH radical scavenging activity of about 82% while Butyl Hydroxy Toluene (BHT) as a standard had antioxidant activity of 89.12%, therefore, antioxidant from natural source can improve the antioxidant system in body for scavenging free radicals. The extract of ethanol was exhibited fine scavenging abilities against DPPH radicals due to its high polarity which can pull out more polyphenol compounds. In fact, the antioxidant activity C and E, carotene, quercetin, kaempferol, tannins and polyphenolics in Moringa leaves which remove free radicals, activate antioxidant enzymes and inhibit oxidases (Sidhuraju and Becker, 2003).

Table 1: Total phenolic, Flavonoid contents and antioxidant activity of *M. oleifera* extract:

Sample	Solvent Type	Total Phenolics (mg GAE/g dried extract)	Total Flavonoids (mg QE/g dried extract)	Antioxidant activity DPPH inhibition (%)
ME	Ethanol 70% extract	158.79 ± 0.02	66.37 ± 1.03	82.47 ± 0.07
	Acetone 95% extract	130.48 ± 0.06	58.31 ± 0.80	63.92 ± 0.01
	Aqueous extract	47.22 ± 0.01	41.45 ± 0.04	19.17 ± 0.08
BHT		--	--	**89.12 ± 0.86**
L. S. D at 0.05		**0.41**	**0.60**	**2.56**

BHT was used as the reference materials (positive control)
Values are given as mean ± standard deviation and any two means in the same column followed by the same letter were not significantly different (p>0.05)

Shah *et al*., (2015) revealed that the radical scavenging activity is positively correlated with the phenolic and flavonoid contents and found that the antioxidant activity of *M. oleifera* extract was concentration dependent and comparable to those of butylated hydroxyl toluene and α-tocopherol.

Physical and optical properties of *M. oleifera* containing pectin film:

The thickness of the films prepared was 38.21 ± 0.01 μm with no difference among the films. The TS is the most important mechanical property for many applications. Results in Table (2) showed that the increase in ME concentrations proportionally increased both TS and E values of the pectin films. TS increased from 49.78 to 79.28 MPa with the addition of 1% ME. This increase in TS can be attributed to the increase in interactions between pectin melecules and polyphenolic compounds through hydrophobic and hydrogen bonds. In particular, cross-links were formed between pectin-film and the plyphenolic compounds of moringa extract, which led to more rigid films (Schellekens & Bastiansen, 1991).

Table 2: Thickness, tensile strength (TS) and elongation at break (E%) of pectin-films incorporated with ethanolic moringa extract.

ME (%)	Thickness (μm)	TS (MPa)	F (%)
Film control	38.21 ± 1.2	49.78 ± 0.07	17.8 ± 0.11
0.30	37.86 ± 0.9	58.43 ± 1.50	35.4 ± 0.80
0.50	37.55 ± 0.6	62.48 ± 0.30	42.9 ± 0.12
0.70	37.39 ± 0.1	67.55 ± 1.90	50.5 ± 0.70
1.00	37.08 ± 0.9	79.28 ± 2.30	64.9 ± 1.00

Values was given as mean ± standard deviation and any two means in the same column followed by the same letter were not significantly different (P>0.05).

E increased from 17.8 to 64.9% when 1% ME was added to the pectin film. Lee *et al*., (2015) reported the increase in TS and E values of Jelly fish protein films with the increase in wasabi extract concentration. They affirmed that these changes in the mechanical properties must be due to non-covalent interactions between Jelly Fish protein molecules and polyphenolic compounds. Especially, in our study the pectin film had higher TS and E values, therefore, the strength and flexibility of pectin- *M. oleifera* extract films indicated that the prepared films had a substantial amount of mechanical properties compared to the synthetic films.

WVP is the most extensively studied property of edible films mainly because of the importance of the water in deteriorative reactions and low WVP values are preferable. Table (2)

shows that WVP of pectin-moringa films decreased with the increase in ME concentration. These results were similar to those obtained in puffer fish skin gelatin film containing *M. oleifera* leaf extract (Ka-Yean *et al*., 2016). The decrease in WVP of the moringa extract-containing pectin films might be because of the compact structure formed due to cross-linking between pectin molecules and polyphenolic compounds. The dense network can reduce the free volume of the film matrix, which reduced the diffusion rate of water molecules (Nie *et al*., 2015).

Table (3) shows that the values of WVP change with the use of different concentrations of glycerol in pectin-film solutions as for higher concentrations of glycerol an increase in WVP occurs than the control 2.55 x 10^{-9} (WVP of pectin-moringa film). These differences are significant when the plasticizer concentration is increased from 0.5% to 2.0%. These results indicated that a maximum of 2% would be necessary, while for values lower than 0.5% the film would be too brittle. Diab *et al*., (2001) explained that the effect of glycerol can be attributed to the hydrophilic properties of this compound which favour the adsorption of water molecules. Glycerol, through its plasticizing action, changes the polymer network creating mobile regions with water at active sites of the polymer network structure. Generally, the moringa extract-containing pectin films with low WVP can be used to prevent microbial growth caused by moisture transfer in foods.

Table 3: Values of WVP, O_2P and CO_2P of pectin-moringa films with different concentration of glycerol:

Film	WVPx10^{-9} (gm/m^2 sPa)	O2P x 10^{-13} (gm/m^2 sPa)	CO_2P x 10^{-15} (gm/m^2 sPa)
Pectin-Film 0.0% Gly	2.55 ± 0.04	0.50 ± 0.01	47.83 ± 1.20
Pectin-Film 0.5% Gly	3.80 ± 0.07	0.56 ± 0.02	34.09 ± 1.10
Pectin-Film 1.0% Gly	4.32 ± 0.03	0.61 ± 0.02	31.54 ± 1.43
Pectin-Film 1.5% Gly	5.74 ± 0.01	0.72 ± 0.08	22.35 ± 1.14
Pectin-Film 2.0% Gly	7.59 ± 0.02	0.88 ± 0.03	10.86 ± 1.60

Values was given as mean ± standard deviation and any two means in the same column followed by the same letter were not significantly different (P>0.05).

Oxygen is the key factor for oxidation, which is responsible for changes in food oder, color, flavor and nutrients deterioration. Therefore, films that provide a proper oxygen barrier can help in improving food quality and extending food shelf life. Table (3) presents the O_2P as measured for the pectin-moringa coating film. As shown in this table, the samples with higher concentration of plasticizer have higher values of O_2P than the samples with a lower plasticizer concentration. These results can be explained by the polar natural of the oxygen molecule that does not interact with the polar properties of the glycerol molecule, increasing the film permeability to the oxygen. Also the plasticizer decreases the intermolecular attractions between polymeric chains, facilitating the penetration of gas molecules (Caner *et al*., 1998). In the present case, the effect of pectin/moringa film seems to have surpassed the effect of glycerol concentration, having significant differences.

Carbon dioxide is very important to the respiration of living tissues and a higher value of CO_2P can delay fruits softening and/or food spoilage. Table (3) shows CO_2P values for the tested pectin solutions. The results seem to indicate that solutions with a higher concentration of plasticizer produce films with a lower values of CO_2P. The effect of glycerol concentration seems to be, by far, the most important one affecting CO_2P. An opposite effect of glycerol concentration has been noticed for WVP and O_2P; those films showing a lower O_2P are the ones that show a higher CO_2P. When glycerol concentration increases the WVP & O_2P increased and CO_2P decreased probably as a result of the polar and hydrogen-bonding properties of the glycerol molecule. The highest value of CO_2P was obtained with the pectin-film of 0.5% glycerol. Based on the previously presented criteria-low WVP, a low O_2P and a high CO_2P.

The effect of moringa extract addition on the optical properties of pectin-moringa films are shown in Table (4). Hunter a value did not change significantly, whereas hunter L and b value of the pectin film was affected by moringa extract addition. With increasing ME concentration, Hunter L value decreased from 93.12 to 67.95 and b value increased from 7.83 to 42.21. The reason for the change was mainly due to light brown color of ME, which made the film more yellow. An increase in Hunter b value was also reported earlier for Jelly Fish protein films with wasabi extract (Lee *et al*., 2015) and soy protein isolate films with raspberry extract (Wang *et al*., 2012). In addition, the opacity of the pectin-moringa film slightly

increased when moringa extract concentration increased. Siripatrawan & Harte (2010) also reported an increase in opacity of the chitosan film with increase in green tea extract concentration.

The increase in opacity of the pectin-moringa film can affect the appearance of food products when applied for packaging. Concisely, in this study, the pectin-moringa film had good physical and barrier properties. The addition of ME effectively improved the TS, E, WVP and optical properties of the film.

Table 4: Optical properties of the pectin films containing various concentration of ME:

ME (%)	L	A	B	Opacity
0	93.12 ± 0.14	-1.25 ± 0.40	7.83 ± 0.11	0.75 ± 0.02
0.3	84.38 ± 0.28	-8.30 ± 0.16	31.95 ± 0.25	1.04 ± 0.07
0.5	77.04 ± 0.10	-7.41 ± 0.09	38.89 ± 0.18	1.26 ± 0.01
0.7	72.88 ± 0.03	-5.87 ± 0.07	41.06 ± 0.10	1.46 ± 0.04
1.0	67.95 ± 0.15	-4.58 ± 0.03	42.12 ± 0.12	1.68 ± 0.11

Values are given as mean ± standard deviation and any two means in the same column followed by the same letter were not significantly different (P>0.05).

Antimicrobial activity of ME with different concentrations:

The antimicrobial activity of ethanol 70%, acetone 95% and aqueous extracts of *M. oleifera* leaves was determined at different concentrate solutions (50, 75, 100, 150 and 200 mg mL^{-1}) of dimethyl sulfioxide against different pathogenic strains. Ethanolic extract (Table 5) showed greatest zone of inhibition against *E. coli* 0157: H7, S. aureus, *B. cereus*, *P. aeruginosa* and *L. plantarum* at the concentration 200 mg mL^{-1}. Moreover, the 50 mg mL^{-1} concentration showed smallest zone of inhibition with all tested pathogens. The same trend of results observed when used acetone 95%, but the diameter of inhibition zone was lower than that of ethanol 70%, and these diameters of inhibition also decreased with the lowest concentration. The diameter of inhibition zone for all pathogenic strains at 200 $mgmL^{-1}$ concentration ranged from 15.34 to 18.27 mm for ethanol extract and from 9.12 to 14.28 mm for acetone extract. Unless in acetone extract (Table 5) found that *B. cereus* more sensitive to different concentration of its extract which the diameter of inhibition zone against *B. cereus* ranged from 9.14 mm at 50 mg mL^{-1} to 18.42 mm at 200 mg mL^{-1}. The data also showed that the usage water in the extraction gave the lowest inhibition zone, when compared with other solvents. Results demonstrated that *L. plantarum* more resistant to almost concentration when used aqueous extract. Ka-Yeon *et al*. (2016) reported that ethanol had the broadest spectrum of activity on the test probiotic bacteria growth and recommended with its usage. These reports corroborate the results of this study.

Table 5: Effect of antimicrobial activity of ME concentration by varied solvents on pathogenic strains.

Solvent	Pathogenic strains	Diameter of Inhibition zone (mm)				
		50	75	100	150	200 $mgmL^{-}$
Ethanol 70%	*E. coli 0157:H7*	8.21 ± 0.02	10.27 ± 0.02	13.31 ± 0.07	15.00 ± 0.02	18.00 ± 0.01
	B. cereus	7.33 ± 0.04	10.51 ± 0.10	14.52 ± 0.01	16.20 ± 0.09	18.27 ± 0.02
	S. aureus	9.29 ± 0.09	11.25 ± 0.08	14.45 ± 0.01	17.33 ± 0.02	15.34 ± 0.08
	P. aeruginosa	5.51 ± 0.09	7.34 ± 0.03	10.10 ± 0.08	13.41 ± 0.06	16.16 ± 0.08
	L. plantarum	4.22 ± 0.08	6.29 ± 0.01	9.73 ± 0.10	11.51 ± 0.03	15.59 ± 0.01
Acitone (95%)	*E. coli 0157:H7*	6.20 ± 0.10	7.30 ± 0.02	9.40 ± 0.02	11.42 ± 0.03	14.28 ± 0.01
	B. cereus	9.14 ± 0.09	10.28 ± 0.06	14.19 ± 0.01	16.14 ± 0.05	18.42 ± 0.09
	S. aureus	7.30 ± 0.06	8.35 ± 0.02	10.10 ± 0.07	12.33 ± 0.03	14.10 ± 0.02
	P. aeruginosa	4.32 ± 0.02	5.27 ± 0.09	7.25 ± 0.05	9.16 ± 0.07	11.56 ± 0.10
	L. plantarum	2.16 + 0.05	3.44 ± 0.03	5.19 ± 0.05	7.25 ± 0.10	9.12 ± 0.08
Aqueous phase	*E. coli 0157:H7*	0.00	2.17 ± 0.05	4.15 ± 0.06	7.15 ± 0.02	9.24 ± 0.03
	B. cereus	2.11 ± 0.04	4.28 ± 0.03	6.62 ± 0.01	7.19 ± 0.09	10.33 ± 0.08
	S. aureus	0.00	2.11 ± 0.01	4.34 ± 0.05	6.25 ± 0.01	7.38 ± 0.03
	P. aeruginosa	0.00	0.00	2.15 ± 0.09	4.18 ± 0.08	6.27 ± 0.10
	L. plantarum	0.00	0.00	0.00	0.00	2.14 ± 0.07

P value is statistically highly significant at the 0.001 level.

Antimicrobial activity of ME – containing pectin film:

The antimicrobial activity of ME-containing pectin film were analyzed by the total colony count method against food-borne pathogenic bacteria, *S. aureus* and E. coli 0157: H7 at varied concentration of aqueous, ethanol 70% and acetone 95% extracts (0.3, 0.5 and 1.0%) and the results were shown in Tables (6, 7, 8) and figures (1, 2, 3). Results exhibited clear antimicrobial activity against Gram-negative bacteria (E. coli 0157:H7) after 12 h, and increased by increasing the concentration of ME in the pectin film and also exhibited clear antimicrobial activity against Gram-possitive bacteria (*S. aureus*) but, after 8 h. only and increased by increasing ME concentration. It has been frequently observed that ME-containing pectin film exhibited stronger antimicrobial activity against Gram-negative bacteria than Gram-positive one (Shanker & Rhim, 2015). The difference in the antimicrobial activity of pectin-ME films could be explained by the difference in the stracture and thickness of cell wall of Gram-positive and Gram-negative bacteria (Shanker & Rhim, 2015). The Gram-negative bacteria are composed of the negatively charged after membrane of thin peptidoglycan layer (7-8 nm) which facilitates the effect of pectin-moringa film on the bacterial cells. On the other hand, Gram-positive bacteria are composed of thick peptidoglycan layer (20-80 nm) consisting of linear polysaccharide chains cross linked by short peptides, forming a complex structure that makes more difficult effect of pectin-moringa film on the bacterial cells. Furthermore, an obvious results have demonstrated that ethanol 70% was the effective one for the extraction between the three tested solvents as indicated by the population reductions. A decrease of approximately 1.0 – 1.5 log 10 cfu/g in populations of E. coli 0157:H7 and / or *S. aureus* bacteria at the end of inhibition time (20 h), when compared with the population reductions by the other solvents (figure 1, 2, 3). Briefly, ME is considered as more environmentally friendly method than the conventional chemical methods for adding to food packaging material as antimicrobial agent for maintaining the safety and extending the shelf life of packaged food.

Table 6: Antimicrobial activity of pectin film incorporated with ethanol M. extract against two strains bacteria at varied concentration.

Probiotics strains bacteria	Conc. of extract (%)	Cell viability (Log CFU/g)					
		Inhibition time (h)					
		0	4	8	12	16	20
S. aureus	Zero	3.82	4.69	5.55	6.38	7.14	8.70
	0.3	3.82	4.42	5.27	6.27	5.61	4.54
	0.5	3.82	3.95	5.03	6.02	5.30	4.23
	1.0	3.82	3.65	4.65	5.88	4.26	3.45
***E. coli* 0157: H7**	Zero	3.95	4.83	5.38	6.42	8.09	3.32
	0.3	3.95	4.45	5.00	4.41	3.55	3.07
	0.5	3.95	4.21	4.89	4.37	3.25	2.34
	1.0	3.95	4.33	4.70	4.23	3.08	2.00

Table 7: Antimicrobial activity of pectin film incorporated with acetone M. extract against two strains bacteria at varied concentration.

Probiotics strains bacteria	Conc. of extract (%)	Cell viability (Log CFU/g)					
		Inhibition time (h)					
		0	4	8	12	16	20
***S. aur*eus**	Zero	3.90	4.88	5.42	6.98	8.62	10.03
	0.3	3.90	4.71	5.37	6.90	6.14	6.00
	0.5	3.90	4.54	5.20	6.43	5.77	5.28
	1.0	3.90	4.29	5.00	6.00	5.15	4.22
***E. coli* 0157: H7**	Zero	3.98	4.50	6.42	7.60	9.53	11.58
	0.3	3.98	4.32	6.22	5.60	4.52	3.37
	0.5	3.98	4.10	6.01	5.46	4.30	3.15
	1.0	3.98	4.02	5.89	5.28	4.11	3.09

Table 8: Antimicrobial activity of pectin film incorporated with aqueous M. extract against two strains bacteria at varied concentration.

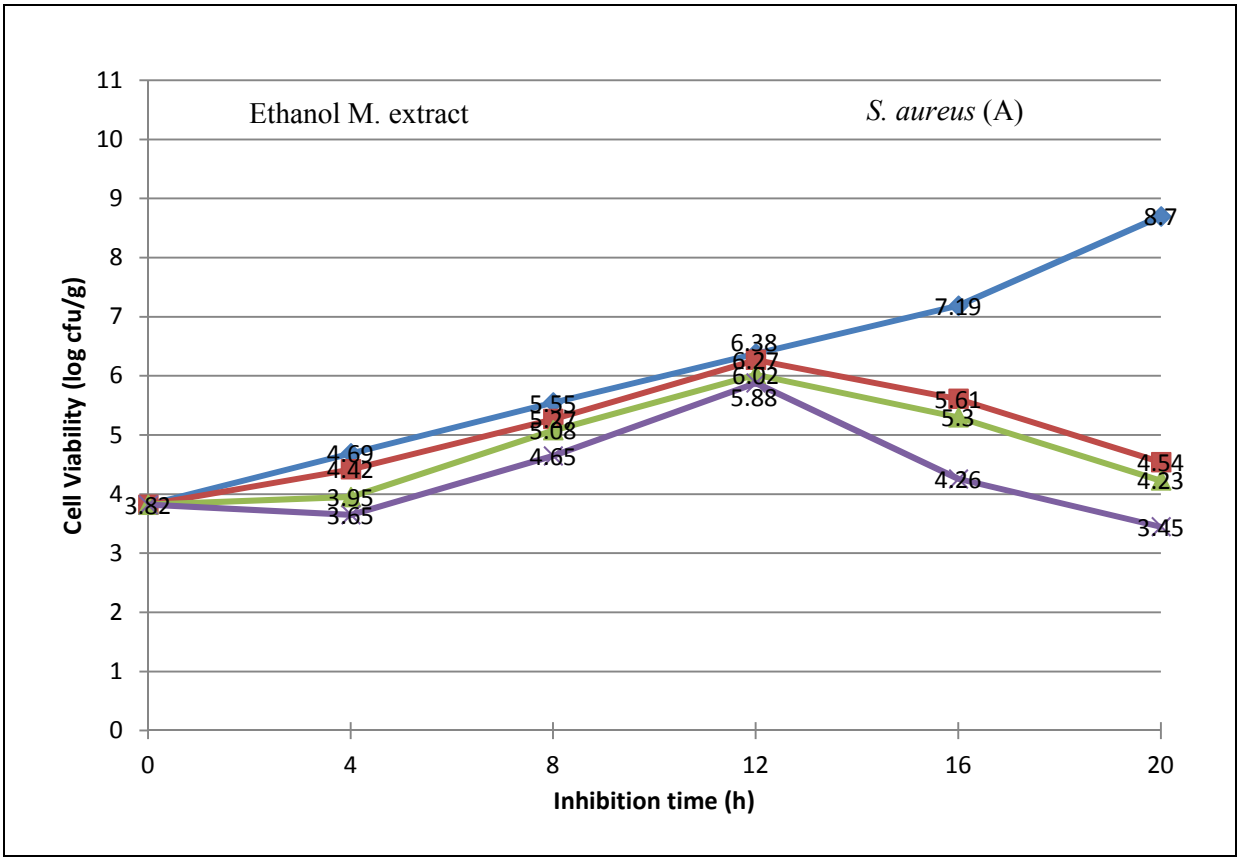
Probiotics strains bacteria	Conc. of extract (%)	Cell viability (Log CFU/g)					
		Inhibition time (h)					
		0	4	8	12	16	20
S. aureus	Zero	4.45	5.82	6.76	7.83	8.55	10.69
	0.3	4.45	5.58	6.61	7.70	7.32	7.11
	0.5	4.45	5.32	6.45	7.51	7.06	6.15
	1.0	4.45	5.19	6.28	7.33	6.49	5.92
E. coli 0157: H7	Zero	4.51	5.98	6.89	7.96	9.36	11.72
	0.3	4.51	5.60	6.51	5.36	5.01	4.64
	0.5	4.51	5.43	6.25	5.12	4.93	4.30
	1.0	4.51	5.20	6.02	5.00	4.70	4.12

Conclusion

In this study, a biodegradable film was developed using pectin as a film base material and incorporated with ME as antimicrobial agent. The investigation have shown the potentials of ME as sanitizers and / or preservatives by inhibiting the growth of the test organisms which range from food-borne pathogens to spoilage causing organisms in foods and may be successfully incorporated into pectin edible film. Furthermore, the ME-containing pectin film had antioxidant activity such as DPPH radical scavenging activity.

ME-pectin film had good physical barrier properties which improved the TS, E, WVP and OP of the film. Results revealed that moringa ethanolic extract had the highest effect on microbial count when compared with those of acetone or aqueous extracts.

Based on these results, the ME-containing pectin film can potentially be used as an alternative to synthetic plastic to secure the food safety, to maintain food quality, and to extend the shelf life of packaged food.



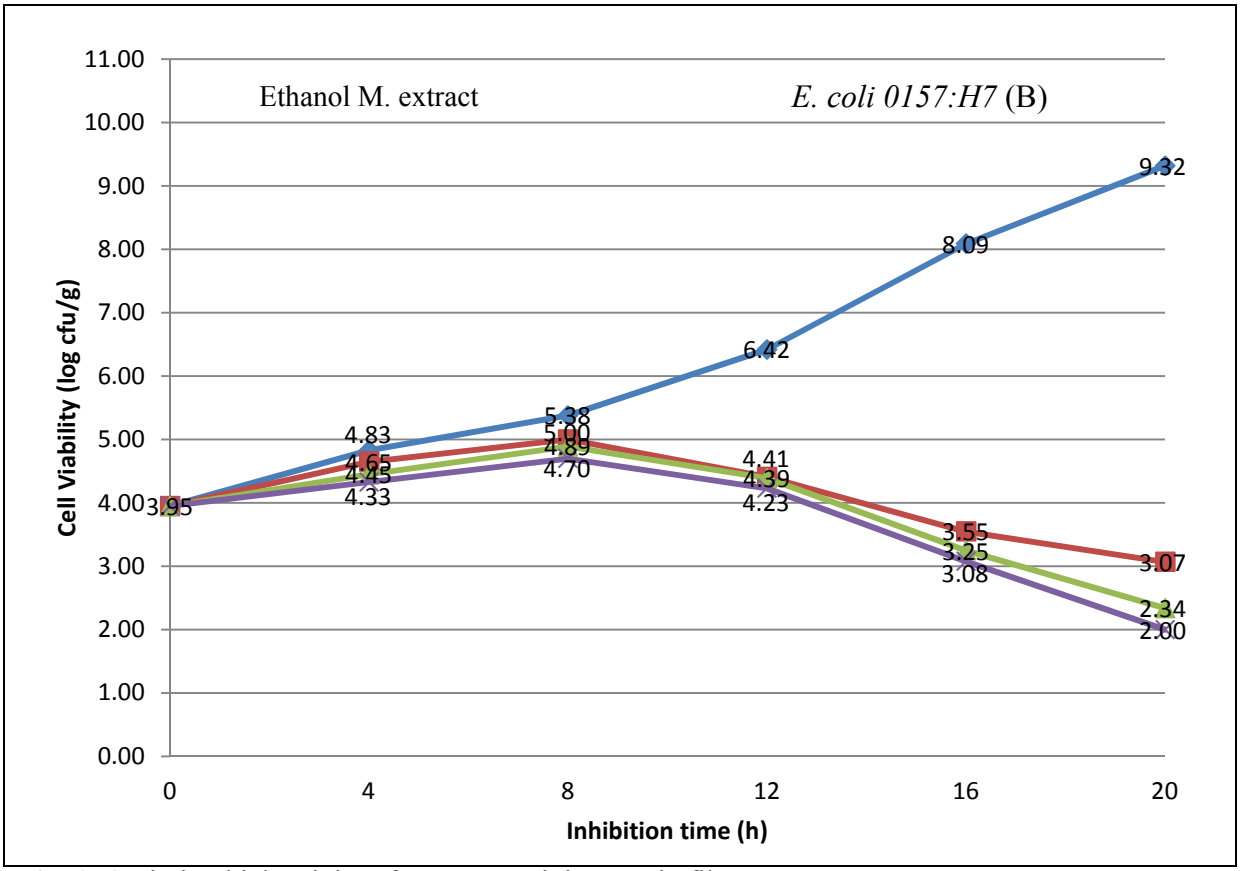
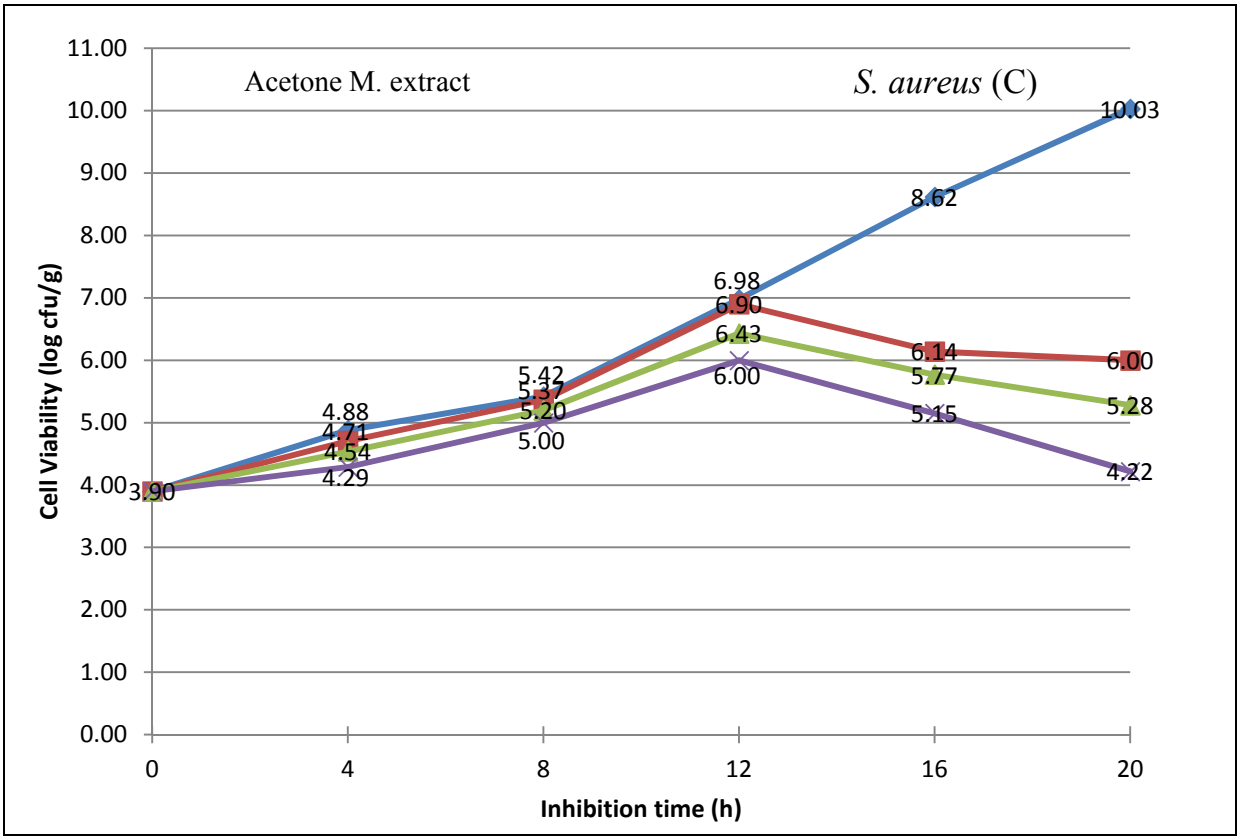


Fig. 1: Antimicrobial activity of ME – containing pectin film



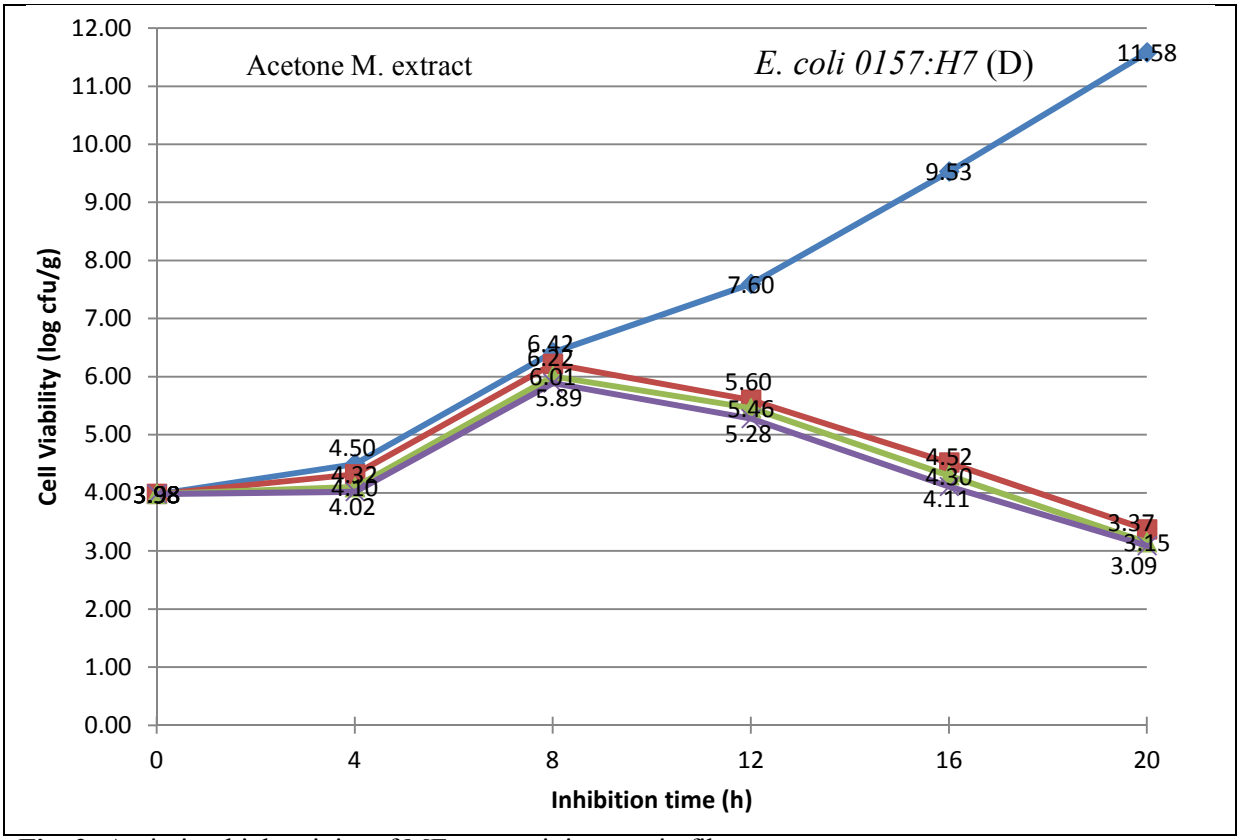
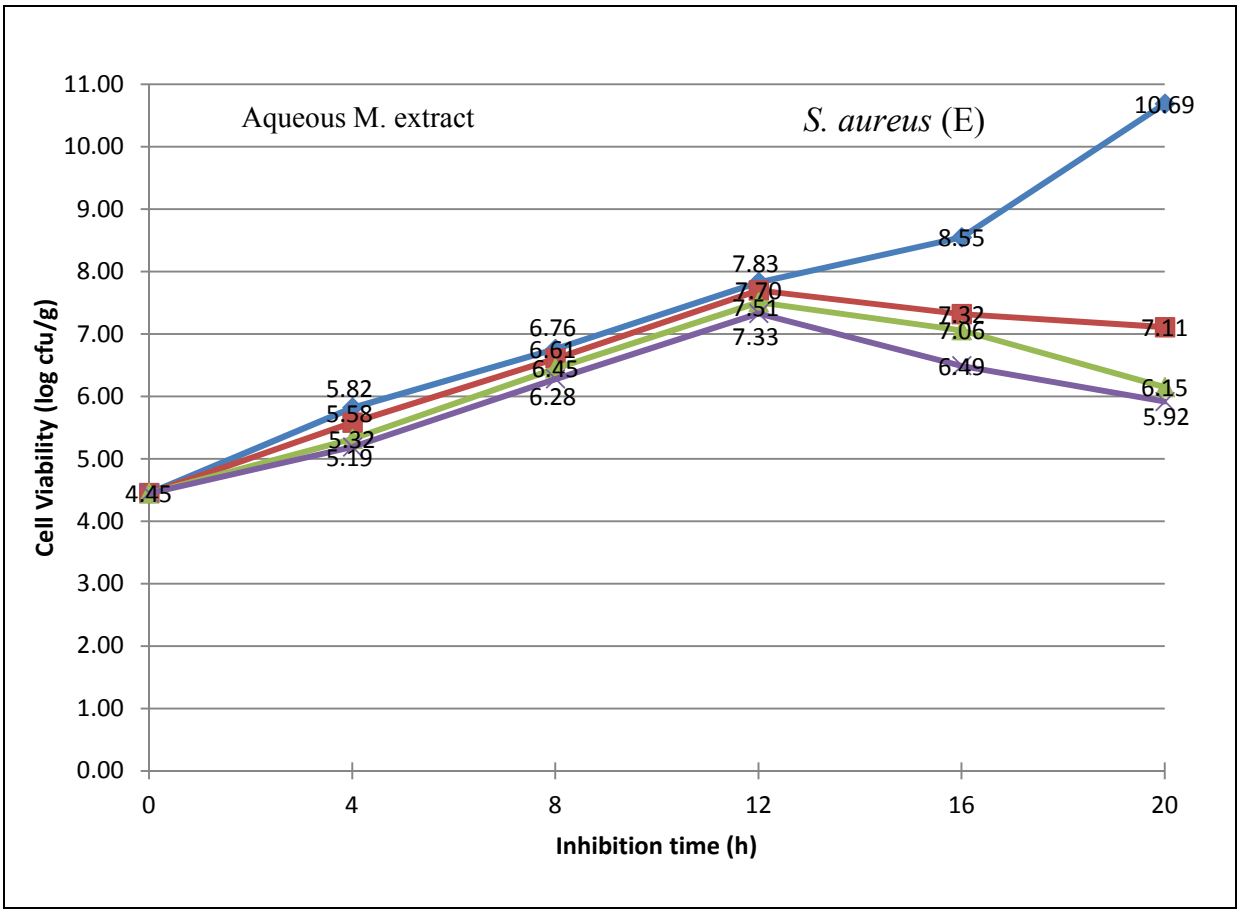


Fig. 2: Antimicrobial activity of ME – containing pectin film



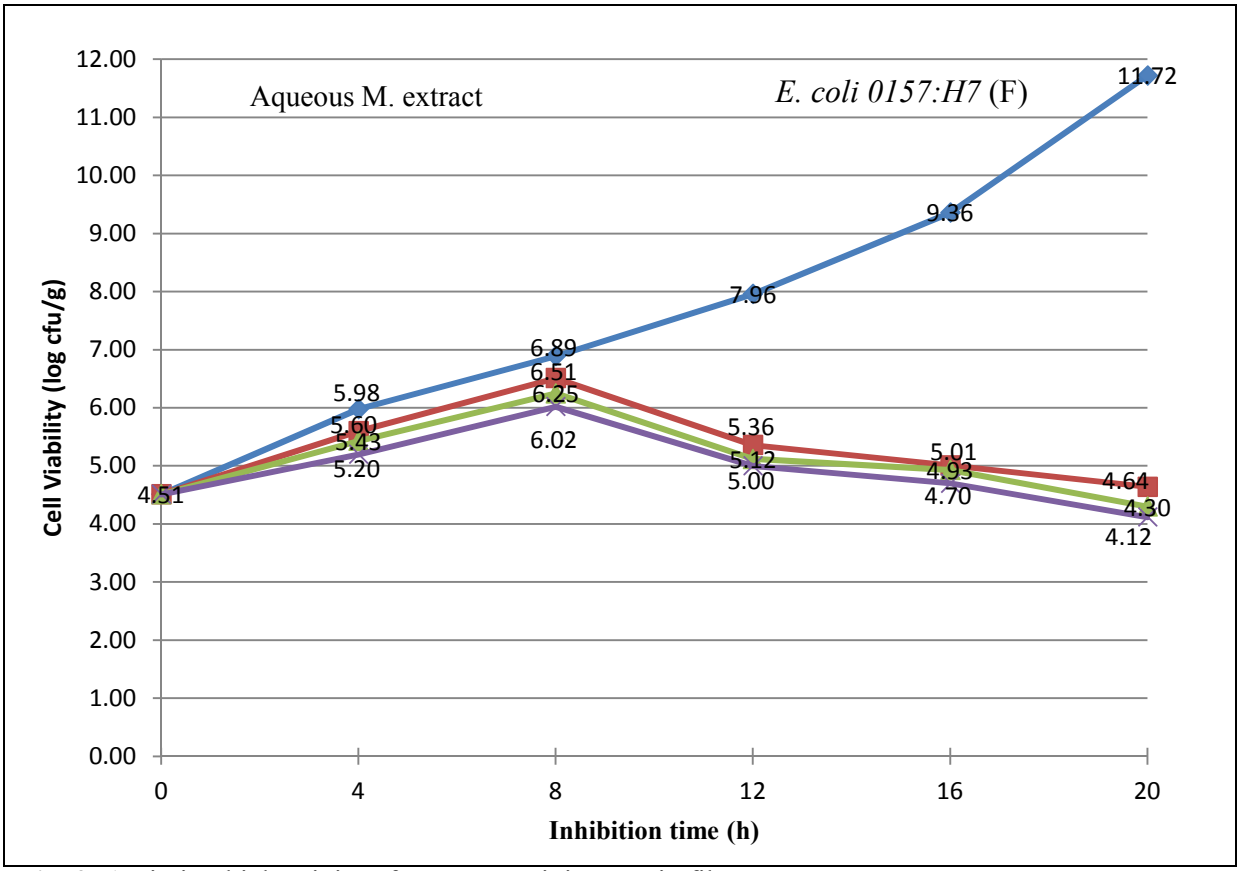


Fig. 3: Antimicrobial activity of ME – containing pectin film

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