



PLA FILMS LOADED WITH *Achillea millefolium* - *IN VITRO* ANTIBACTERIAL EFFECTS

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ABSTRACT: Antimicrobial packaging as active food packaging represents a suitable packaging form for food in products in particular for foods where microbial contamination occurs primarily at the surface. Poly (lactic acid) (PLA) is one of the most frequently used bio-polymers because of its similarities to conventional polymeric materials used in food packaging, however its use is still limited to short-term packaging applications. This research has been focused on preparation of PLA packaging films modified with bioactive compounds from *Achillea millefolium* (AM) plant extract as possible active packaging solution. Addition of specific natural compounds could give improvements in mechanical, thermal or barrier properties, as well as the antimicrobial effect with significant impact on prolonging the food shelf-life and its quality and safety.

Accordingly, the aim of this study was to determine chemical and antimicrobial properties of crude AM ethanolic extract and PLA composite films loaded with two concentrations of AM extract expressed in weight percent (2 wt. % and 5 wt. %). The AM ethanolic extract showed very good antimicrobial activity against *E. coli* and *S. aureus*, while PLA films loaded with 5% AM extract showed significant reduction of initial *S. aureus* after 24 h contact time compared to neat PLA films (up to 90%). PLA films with 2% and 5% AM content did not show any antimicrobial activity against *E. coli*. Furthermore, the chemical composition of the ethanolic extract was determined considering its phenolic composition. These results indicated promising potential of incorporation of *A. millefolium* extract in PLA as an antimicrobial agent for food packaging applications.

Key words: antimicrobial packaging, antimicrobial activity, surface contamination, bioactive compounds, *Escherichia coli*, *Staphylococcus aureus*

INTRODUCTION

Food packaging is designed to protect food from environmental influences such as temperature, light and humidity, odors, microorganisms and dust that can lead to their degradation (Ribeiro-Santos et al., 2017). Commonly, microbial contamination is the main reason for food spoilage, therefore numerous food products require protection against microbial spoilage and lipid oxidation during their shelf-life. The growing demand of consumers

for safe and natural products, without chemical additives, has resulted in many researches with the aim to improve the quality and safety of products, while maintaining their good nutritional and organoleptic properties and, mainly, controlling foodborne pathogens (Carocho et al., 2015). Aiming to the reduction of the use of chemical additives in food industry, growing interest has been raised recently on the use of natural food additives with

antimicrobial and antioxidant properties that do not have any negative effects on the human health (Atares and Chiralt, 2016). Therefore, food industry was forced to develop new ways and technologies to satisfy the consumers' demands. In line with this, active packaging has emerged (Ribeiro-Santos et al., 2017). Innovations in food packaging include the development of active packaging solutions based on the natural active compounds aimed at increasing both the shelf-life of packed food and the sustainability of the overall product (Radusin et al., 2018).

Antimicrobial packaging presents part of active packaging concept where the antimicrobial agent is incorporated into polymer film to suppress the activities of targeted microorganisms. Depending of the active molecule and the kind of package, the bioactive compounds can be released directly to the food surface or indirectly to the package headspace. Numerous researchers have demonstrated that direct application of antimicrobial agents in foods has limited benefits (Han et al., 2014; Kashiri et al., 2017).

However, antimicrobial packaging is still an extremely challenging technology as there are only a few commercialized products found on the market (Sung et al., 2013). Bioactive plants' extracts have gained scientific and industrial attention worldwide for their application as additives in many commercial preparations, mostly in the food and pharmaceutical sectors. For the creation of antimicrobial films it is also of great importance the choice of polymer matrix that will be used for food packaging. In the recent years biopolymers have been widely used, especially polylactides (PLA). PLA represent aliphatic polyester produced from renewable resources and while it has properties similar to the conventional polymers, it is very suitable as a food packaging material (John and Thomas 2008).

According to our knowledge, no work has been reported until now in the scientific literature on the use of *Achillea millefolium* (AM) extract for the development of active antimicrobial composite films for food packaging application. Therefore, the aim

of this work was to develop an active composite material, based on a biopolymer (PLA) and bioactive natural compound (AM) extract, in specific.

For this purpose, PLA films with addition of two border concentrations of AM extract (2 and 5 wt. %) were prepared and characterized in order to understand the influence of the extract addition on PLA films properties, as well as its potential for antimicrobial packaging application. This preliminary research should point out potential substitution of synthetic additives as antimicrobial agents, possibly broadening the use of biopolymers from renewable sources as sustainable and eco-friendly solutions for food packaging application.

MATERIAL AND METHODS

Standards and reagents

Methanol (HPLC, grade), quercetin and formic acid (HPLC grade) were supplied by Merck KGaA (Darmstadt, Germany). Standard substances including protocatechuic acid, caffeic acid, chlorogenic acid, rutin, rosmarinic acid, were purchased from Sigma-Aldrich GmbH (Sternheim, Germany). Water used throughout the experiments was purified using a Millipore, Elix UV and Simplicity Water Purification System (Milford, MA, USA)

Samples

Achillea millefolium was collected during the flowering period and the vegetative phase (July 2016) in Pale area, Bosnia and Herzegovina. The specimen was air dried mixed to obtain a homogenous sample and kept at +4 °C until further analysis.

Preparation of the extract

Before extraction, the plant material was grounded in a blender. Crude plant extracts were obtained by maceration using ethanol/water mixture (80:20, v/v), with the ratio of raw materials to ethanol solution of 1:10, for 24 h at room temperature and subsequently extracted in an ultrasonic bath at room temperature for 10 min. The obtained extracts were filtered through the filter paper (Whatman No. 4). The solvent (EtOH) was removed by rotary evaporator

at 40 °C (Büchi, Switzerland). The obtained extract was stored at +4 °C. The relevant dried residues were redissolved in EtOH prior to analysis.

Preparation of PLA film

Semi-crystalline PLA was provided from Shenzhen Esun Industrial Co., Ltd (Shenzhen, China), characterized by a number-average molecular weight $M_n = 60520 \text{ g mol}^{-1}$, weight-average molecular weight $M_w = 160780 \text{ g mol}^{-1}$, and polydispersity index (PDI) = 2.66. Pure PLA film and PLA films with 2 and 5 wt% of AM extract were prepared by solution casting method. Appropriate amounts of AM extract were added in chloroform and stirred in an ultrasonic bath for 10 min. PLA was added to extract dispersion and stirring continued with magnetic bar for 4 h at room temperature. After complete PLA dissolution, samples were poured into glass Petri dishes (10-cm diameter) and vacuum dried at room temperature.

Preparation of bacterial suspensions

Antimicrobial properties were tested against two bacterial microorganisms: one Gram negative (G-) strain, *Escherichia coli* ATCC11105, and one Gram positive (G+) strain *Staphylococcus aureus* ATCC 6538p. Pure bacterial strains were subcultured on nutrient agar slants at 37 °C for 18 h. Thus, obtained culture was centrifuged at 7000 rpm for 10 min, the pellet was washed in sterile phosphate buffer solution (PBS) and suspended in saline solution in order to obtain a cell suspension of 10^8 CFU/mL .

Antimicrobial activity extracts

For further procedures, 96-well microplates were used to each well and 100 μl of extract (10 wt. % extract), 100 μl of nutrient broth, and 50 μl of bacterial suspensions were added and incubated at 37 °C for 24 h. In order to count viable bacterial cells, 1.8 ml of PBS was added to the tube, thus obtaining a 10^{-1} dilution, which was then serially diluted and 0.1 ml plated on Plate Count Agar (PCA). After incubation of the plates at 37 °C for 24 h, the number of colonies, corresponding to the number of viable cells, was counted as CFU/ml. The percentage of reduction of

viable cells was determined, after averaging the triplicate counts, through the equation:

$$\% \text{ reduction} = (a - b) \times 100 / a$$

where a - is the number of viable cells in the control (non- treated cells), and b - is the number of viable cells in the specimens containing 10% extract.

Antimicrobial activity film

The antimicrobial activity of PLA films (neat, with 2 wt. % and 5 wt. % of AM extract) against *E. coli* and *S. aureus* was tested according to the Japanese Industrial Standard JIS, Z. 2801:2000 (2000). Microorganism suspension containing about $5 \times 10^5 \text{ CFU/ml}$ was applied onto the active multilayers of $3 \times 3 \text{ cm}$ area and covered by an inert piece of LDPE of $2.5 \times 2.5 \text{ cm}$ and 90 mm of thickness. After incubation at room temperature and 95% R.H. for 24 h, bacteria were recovered with PBS, and the viable cells determined by the conventional plate count method. This method is designed to evaluate the efficiency against bacteria on the surface of finished polymer products, including films and pieces. Pure PLA films were used as negative controls, whereas the tested bacterial inoculum without PLA films was used a positive control. All samples were analyzed in triplicate. The antibacterial activity was taken as the test surface reduction (R) using the expression:

$$R = [\log (B / A) - \log (C / A)]$$

where A - is the mean of bacterial counts of the control sample immediately after inoculation, B is the mean of bacterial counts of the control sample after 24 h, and C is the mean of bacterial counts of the test sample after 24 h. Antimicrobial activity was evaluated with the following assessment according to manufacturer procedure: no significant ($R < 0.5$), slight ($R \geq 0.5$ and < 1), significant ($R \geq 1$ and < 3), and strong ($R \geq 3$).

Phenolic compounds composition

Phenolic compounds were determined by HPLC (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies) as

previously described by the authors (Mišan et al., 2011). The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in mg/g of dry weight (d.w.) extract.

RESULTS AND DISCUSSION

Antimicrobial properties

The present study has revealed the antibacterial activity *A. millefolium* extracts against G- *E. coli* and G+ *S. aureus* bacteria (Figure 1, 2). The percentage of reduced viable cells for each bacterium was calculated referring to the control, that is, a bacterial suspension without antimicrobial agent. According to the presented results, AM extracts (1.5%, 2%) were effective against *E. coli* and *S. aureus*, showing 100% reduction of viable cells compared to control.

According to Stojanović et al. (2005) antibacterial activity of the examined extracts (hexane: ether: methanol = 1:1:1) of the aerial parts of *Achillea* species: *A. clavennae*, *A. holosericea* Sm., *A. lingulata* and *A. millefolium* showed to possess a broad spectrum of antimicrobial activity against five bacteria (*S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Salmonella enteritidis*) and two fungi (*Aspergillus niger* and *Candida albicans*). Conversely, in another study, the ethanol extract of Turkish *A. millefolium* did not show any antibacterial activity (Unal et al., 2008). These differences may be attributed to the genotypic variation and/or climatic conditions.

Antimicrobial properties of PLA loaded with 2 and 5 wt. % AM extract against *S. aureus* are shown in Figure 3. According to the presented results both polymer composites loaded with 2 and 5 wt. % AM extract showed antimicrobial activity against *S. aureus*. PLA films loaded with 2 wt. % AM extract resulted with test surface reduction value, $R=0.92$, which indicates slight antimicrobial activity. While the samples loaded with 5 wt. % AM extract

showed significant ($R \geq 1$ and < 3) antimicrobial activity ($R= 1.9$). However, the PLA films loaded with 2 and 5 wt% AM extract did not show significant ($R < 0.5$) antimicrobial activity against *E. coli*. In general, analysis showed that PLA films with AM extract was inactive against *E. coli*.

These results are in accordance with previous studies where ethanolic extract *A. millefolium* from Siberia and the Brazil was examined (Kokoska et al., 2002; Holetz et al., 2002). This and many previous studies (Smith-Palmer et al., 1998; Lopez et al., 2005; Shan et al., 2007) indicated that AM extract was more active against G + bacteria than G - bacteria. This is likely due to the significant differences in the outer layers of G- and G+. G- possesses an outer membrane and a unique periplasmic space unlike in G + bacteria (Nikaido 1996; Duffy and Power 2001).

Additionally, the resistance of G - bacteria toward antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules, and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside (Nikaido 1994; Gao, et al., 1999). G + bacteria do not have such an outer membrane and cell-wall structure therefore antibacterial substances can easily destroy the bacterial cell-wall and cytoplasmic membrane and result in a leakage of the cytoplasm and its coagulation (Shan et al., 2007).

According to the obtained results it might be suggested that the antimicrobial activity of the AM extract is also dependent on the tested bacterial species and their difference in cell - wall structure. Additionally, antimicrobial activity of extract depends on part of the plant used for extraction, the type of solvent, the extraction method, as well as the strain of microorganism examined (Lupoae et al., 2015). However, the effects observed for sample also strongly depend on the specific mixture of compounds present in the sample,

their interaction and action of their metabolites that induce specific and often unexpected cellular responses.

Chemical composition regarding bioactive compounds

The chemical composition of *A. millefolium* regarding the bioactive *folium*

compounds of its ethanolic extract is presented in Table 1.

Phenolic compounds are particularly potent natural products with a wide range of biological activities (Bais et al., 2002; Rahman and Moon 2007; Ayaz et al., 2008).

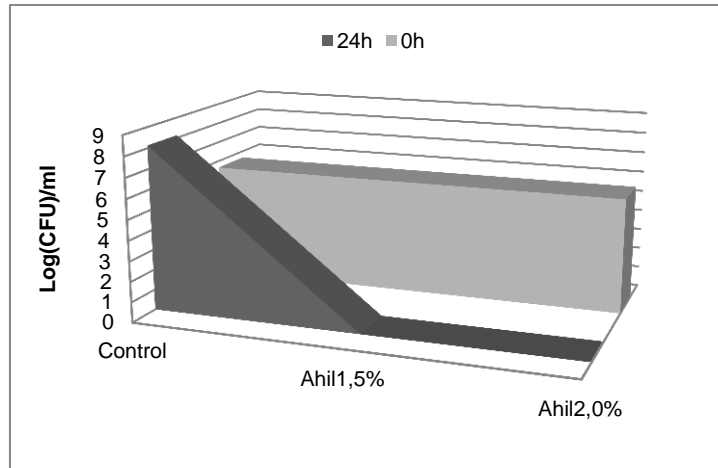


Figure 1. Antibacterial activity of AM extract against *E. coli*

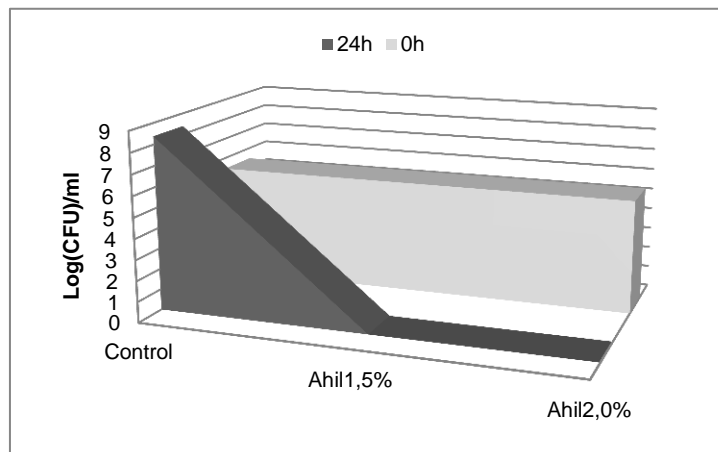


Figure 2. Antibacterial activity AM of extract against *S. aureus*

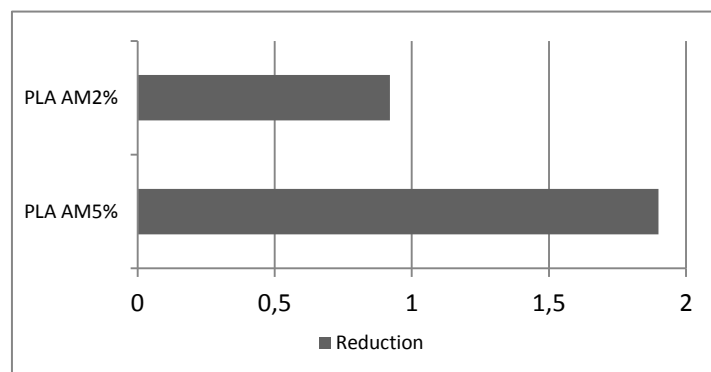


Figure 3. Antibacterial activity of PLA loaded with AM extract against *S. aureus*

Table 1.
Content of phenolic compounds in crude AM ethanolic extracts

Phenolic compounds	Content mg/g
Caffeic acid	11.2480
Rosmarinic acid	2.3067
Protocatechuic acid	0.0597
Chlorogenic acid	0.0764
Rutin	6.9223
Catechin	0.8892

The antimicrobial activity of phenolic acids is well documented in the literature (Heleno et al., 2013). Extracts of various medicinal plants (Cowan, 1999; Rios and Recio, 2005) containing phenolics including flavonoids have been previously reported to possess antimicrobial activity.

For instance, chlorogenic acid and catechin, detected also in examined AM extract, are known to exhibit a great inhibitory effect on *E. coli* and *Listeria innocua* (Muthuswamy and Rupasinghe, 2007). In the examined extract of AM, caffeic acid was detected as dominant and the most abundant compound. This acid is one of the most popular food additive regarding requirements of thermal processing reduction of heat sensitive foods (Bowles and Miller 1994). In addition, rosmarinic acid, protocatechuic acid and rutin were identified. According to the chemical profile of phenolic compounds in examined AM extract, the observed antimicrobial activities of AM extract and PLA loaded with AM extract might be due to the presence of phenolic compounds.

CONCLUSIONS

Crude extract of *A. millefolium* possessed high antimicrobial properties against *E. coli* and *S. aureus*. Films loaded with 2 wt. % AM extract showed slight antimicrobial activity; while PLA film loaded with 5 wt. % AM extract showed significant antimicrobial activity against *S. aureus*. Antimicrobial activity of both samples is related to AM content into PLA polymer matrix compared with neat PLA, pointing out the clear antimicrobial activity of the AM extract.

Antimicrobial activities observed in this study might be due to the presence of

phenolic compounds. This research presents a preliminary study on AM extract as suitable natural additive for improvement of PLA material properties as well as potential antimicrobial compound for use in prepared composite as active packaging solution.

ACKNOWLEDGMENTS

Provincial Secretariat for Higher Education and Scientific Research, Republic of Serbia contract grant number: 142-451-2771/2017-01-01

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ПЛА ФИЛМОВИ СА ДОДАТКОМ *Achillea millefolium* - IN VITRO АНТИМИКРОБНИ ЕФЕКАТ

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Сажетак: Антимикробно паковање као врста активног паковања, представља одговарајућу амбалажу за паковање прехранбених производа, посебно за храну која је склона микробиолошкој контаминацији. Полимлечна киселина (ПЛА) је један од најчешће заступљених полимера због њене сличности са кновненционалним полимерним материјалима који се користе као амбалажни материјал. Међутим примена ПЛА још увек није довољно развијена. Ово истраживање је фокусирано на примени ПЛА филмова за паковање који су модификовани додатком биактивних компоненти из екстракта *Achillea millefolium* у циљу добијања нових активних амбалажних филмова. Додатком одређених једињења природног порекла може доћи до побољшања механичких, термалних и/или баријерних особина, као и до антимикробног деловања, што значајно продужава рок трајања и чува квалитет и безбедност упакованог производа.

Циљ овог истраживања био је да се утврде хемијска и антимикробна својства сирових АМ етанолних екстракта и ПЛА филмова са додатком две концентрације АМ екстракта (2% и 5%). Етанолни екстракт АМ показао је веома добру антимикробну активност против *E. coli* и *S. aureus*, док је ПЛА са 5% екстракта показао значајну антимикробну активност против *S. aureus* након 24 сата контактеног времена у поређењу са контролним узорком ПЛА (до 90%). ПЛА филмови са садржајем 2% АМ и 5% АМ нису показали антимикробну активност против *E. coli*. Поред тога, хемијски састав етанолног екстракта одређен је с обзиром на његов фенолни састав. Ови резултати показују обећавајући потенцијал укључивања екстракта *Achillea millefolium* у ПЛА као антимикробног агенса за паковање хране.

Кључне речи: полимлечна киселина (ПЛА), *Achillea millefolium*, антимикробно паковање, антимикробна активност

Received: 16 May 2018

Received in revised form: 31 May 2018

Accepted: 7 June 2018