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RESEARCH ARTICLE

Efficacy Of Natural Plant Extracts In Antimicrobial Packaging Systems

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ABSTRACT

Antimicrobial plant extracts used in food packaging provide a healthy alternative. They contain aromatic and phenolic compounds that are responsible for their antibacterial properties. In this study, we report the antibacterial effects of extracts obtained from sea buckthorn (Hippophaë rhamnoides L.) leaves, and inner bark of pine trees (Pinus silvestris) that were applied as coatings on paper suitable for packaging application. Extracts from sea buckthorn leaves exhibited antibacterial effect both as a solvent extract and as a coating on paper against Pseudomonas aeruginosa as test bacteria. However, coatings of pine bark extract did not exhibit antibacterial effect as coatings even though the solvent extracts exhibited antibacterial effects. Staphylococcus aureus demonstrated resistance towards both plant extracts after they had been applied as coatings on paper for packaging.

KEY WORDS

Plant extracts, antibacterial coatings, food safety, packaging, pine bark (Pinus silvestris), sea buckthorn (Hippophaë rhamnoides L.)

1. INTRODUCTION

Pathogenic bacteria in the environment cause life-threatening diseases. The presence of pathogenic bacteria in food and personal care products can lead to severe health consequences to the consumers. Excessive growth of spoilage bacteria can lead to food spoilage and render the food unsafe for human consumption while increasing food waste [1]. Proteolytic bacteria that breakdown protein in meat result in unsavoury odour, while other bacteria such as *Escherichia coli (E. coli)* can cause fatal diseases such as haemolytic uremic syndrome, kidney failure and possible death [2]. Additionally, there has been an emergence of drug-resistant bacteria strains such as *Campylobacter jejuni, E. coli, Salmonella enterica, Bacillus cereus,* and *Clostridium perfringens*, which are food borne. Several factors including overdose, drug abuse, and bacteria evolution have also been attributed to the development of antibiotic-resistant strains of bacteria [3, 4].

Synthetic food additives are commonly used for inhibiting the growth of microbes and for maintaining the shelf life of food [5]. The type of antimicrobial, whether natural or synthetic, sets restrictions for their use. For instance, food-packaging applications may require the use of natural antimicrobial agents since there is the tendency of migration into food [6]. Consequently, natural antimicrobial agents have been used in several ways such as dipping and spraying onto food, and they have also been used as coatings that help to maintain freshness of food while improving shelf life [7-10]. Metal and metal oxide nanoparticles have shown potential as antibacterial agents, but there are still unanswered questions about their long-term effects on the environment and human health [11].

Increasing need for natural food preservatives has been raised by food industry due to concern about the health impact of synthetic food additives. Consumer awareness and preferences have promoted the use of natural antimicrobials since they are presumed to be a healthier alternative [12]. Food packaging material with antimicrobial properties could therefore be an important step in the overall strategy of food preservation which aims at reducing the use of synthetic additives in food. The consequence will be an improved consumer satisfaction and a wide variety of healthy antimicrobial alternatives.

Promoting food safety and maintaining food quality has been achieved in several ways, such as the incorporation of antimicrobials into food [13]. Low or no toxicity from antimicrobials, even after prolonged exposure, is recommended for synthetic and inorganic antimicrobials. Natural antimicrobials on the other hand are generally non-toxic even after continuous usage in large doses as compared to other antimicrobial sources [14]. Although antimicrobials form natural extracts have several benefits when incorporated into food, there is a tendency for active agents to be inactivated by other molecules in the food. Shelf life of food has been increased by incorporating antimicrobials into packaging material, but there could be unwanted result since active compounds are likely to migrate away from food contact surface, hence inability to control microbial growth [15]. Consequently, barrier layers are used in packaging systems to prevent migration across the packaging material. Therefore, applying natural antimicrobials as surface coatings could be a preferred alternative.

The recent surge in the use of natural antimicrobials can also be associated with the biocompatibility and nontoxicity within the environment. Extracts from medicinal plants that have antitumour, anti-inflammatory, and antioxidant effect have also demonstrated antimicrobial properties [16]. Thus, antimicrobials from natural extracts that are used in food packaging application could fulfil the primary goal of maintaining food quality while delivering additional health benefits, such as nutritional supplements. Natural antimicrobials

that have exhibited health benefits include essential oils, pomegranate peel extracts [17], and these have subsequently demonstrated a positive antimicrobial effect against selected organisms [18, 19]. The antimicrobial effects of natural plant extracts have been attribute to the presence of compounds such as flavonols, phenolic acids, terpenes, anthocyanins, stilbenes, and tannins [20, 21]. The exact mechanism of microbial control is not yet clear, but it is suggested to be a result of cytoplasmic rapture while other compounds present could act differently. Unlike cellulose, lignin, and hemicellulose that give mechanical support to plants, these extractives are secondary metabolites that may act as catalysts in biosynthesis, and protect the organism against microbial damage and insect attack.

Several studies that have confirmed the antibacterial effects of plant extracts have been investigated using solvent extracts [22] or as coatings using film solutions [23, 24]. In comparison with solvent extracts, there is considerable increase in surface area when these extracts are applied as coatings onto a substrate, and there exist a possibility that volatile active compounds of the extractives will be evaporated. The bioactive compound may also be oxidized, or other surface reactions could occur, which could result in inactivation of natural antimicrobial agents although the bulk solution may exhibit antibacterial effects. It is therefore imperative to investigate and establish the exact ways in which different natural antimicrobials can be applied; whether as solvent extract, coatings or other method of application [25]. There have been successful attempts at incorporating natural antimicrobials into packaging material [26, 27]. However, there has been limited success with incorporating natural antimicrobials directly onto paper surface for packaging applications.

In this study, natural extracts from sea buckthorn *(Hippophaë rhamnoides L.)* leaves and the inner layer of pine bark *(Pinus silvestris)* are examined for their antibacterial properties, applied as coatings suitable potentially for packaging applications. Efficacy of the natural extracts as antibacterial agents is compared to an industrial antimicrobial blend with a known antimicrobial effect. Rod coating is used to apply the natural extracts as thin films onto paper surface, which are then dried. The results show that the sea buckthorn extract is effective against *P. aeruginosa* but it is ineffective against *S. aureus*. Pine bark extract did not show significant antibacterial effect against either of the tested bacteria when applied as a coating.

2. MATERIALS AND METHODS

2.1. Preparation of extracts

Both pine bark (PB) and sea buckthorn (SB) extracts were prepared using a similar procedure. The inner layer of PB and SB leaves were ground into powder with the aid of liquid nitrogen. A portion of 20 g pine bark powder was mixed with 400 mL of aqueous ethanol (70 %). For extraction of sea buckthorn leaves, 40 g of fresh leaf powder was mixed with 400 mL aqueous ethanol (70 %). Both mixtures were stored overnight in a refrigerator, after which they were sonicated for 30 mins, stirred for 20 mins followed by centrifugation for 10 mins. This procedure was repeated with four different batches of powder samples. Supernatants were pooled separately for PB and SB extracts. Ethanol was then removed from the supernatant with a rotatory evaporator at 35°C. To completely recover the extracts, a small amount of autoclaved water was used to flush the glassware. The total volume of PB extract was 249.6 mL whereas the total volume of SB extract was 361 mL after the evaporation of ethanol. Both values include the water used to flush the glassware. The anti-microbial blend (AB) was a CO_2 extract from a mixture of herbs consisting the following: 30% sage leaf *(Salvia fruticosa)*, 20%

hop *(Humulus lupulus),* 15% licorice root *(Glycyrrhiza uralensis),* 15% curcuma *(Curcuma xanthorrhiza),* 10% clove bud *(Syzygium aromaticum),* 5% oregano leaf, and 5% ajowain seed *(Trachyspermum ammi),* obtained from Flavex Germany.

2.2. Coating of extractives on paper

SB and PB extracts and the industrial AB were coated on specialty paper (MLPC), which has multiple coating layers, including a barrier, which prevents penetration of the extract coatings into the paper [28]. 4 ml of carboxymethyl cellulose (CMC, Finnfix 2G, CPKelco, FI) was added as a thickener to 10 ml of SB and PB extracts separately. Before coating, the AB was diluted using ethanol as a thinning agent by mixing 2 mL of AB with 10 mL of ethanol. The extracts were rod coated (K202 Control Coater) as thin films on paper followed by drying.

Two different metering rods (MR) were used for the coatings: MR-12 and MR-24, producing wet film thicknesses of approximately 12 µm and 24 µm, respectively. The metering rods were cleaned thoroughly after each coating. The coated samples were dried at either room temperature (RT 25 º C, 50% relative humidity) or using infrared (IR) irradiation. IR drying was carried out using three 30 cm long 2 kW strip light bulbs (IRT systems, Hedson Technologies AB, SE) at a distance of approximately 20 cm from the sample. The samples dried at RT were allowed to dry for at least 24 hours until no moisture was observed by visual inspection. Coat weight was measured after drying, and coating surface was imaged with scanning electron microscope (Jeol JSM-6335F) with accelerating voltage of 5.0 kV.

2.3. Antibacterial measurements

Antimicrobial efficacy of the samples was examined with agar diffusion method – modified from EN1104 'Paper and board intended to come into contact with foodstuffs - Determination of the transfer of antimicrobial constituents'. *Staphylococcus aureus* VTT E-70045 and *Pseudomonas aeruginosa* E-96728 were used as target bacteria. Liquefy sterile test agar was cooled to below 60°C and the inoculating inoculum was added resulting in a concentration of 104 cells/mL test agar. The suspension was distributed evenly by careful shaking. Aliquots of 15 mL were dispensed into sterile petri dishes with a diameter of 90 mm. Three test pieces from each extract-coated sample were laid on the still semi-solid nutrient medium using sterile forceps. Test pieces were slightly pressed down to ensure that no air remained between the agar plate and the test pieces. The extract-coated surface was placed downwards facing the surface of the agar plate, and the diffusion zone on the agar plate around the test piece was measured. For each analysis, a negative control (blank) nutrient agar plate without the test pieces was prepared. A known antibiotic, penicillin G 0.03 units from Abtekbio UK, was used as positive control in the antibacterial testing.

3. RESULTS AND DISCUSSION

3.1. Coat weights

The amount of extract delivered to the surface of the multilayer pigment coated (MLPC) paper was measured as the coat weight. A piece of extract-coated paper with a surface area of 50 cm^2 was cut, and the coat weights were estimated after each coating cycle. Average weight of the reference paper was 0.63 g, and the measured coat weight for each sample is shown in table 1. The solid content estimated for CMC was 2 % while that of SB and PB extracts were 4.2 % and 5.6 % respectively. The multilayer coated paper with sufficient barrier properties was used as preferred substrate in this study. It had a thin layer of latex coating on paper surface, which ensured extract that coatings stayed on the surface of the paper. As a result the problem of

Table 1: Measured coat weights of SB, PB, and AB. RT and IR label the different drying conditions at room temperature and infrared drying, respectively.

Extract	RT MR-12 (g/m^2)	RT MR-24 (g/m^2)	IR MR-12 (g/m^2)	IR MR-24 (g/m^2)
SB	2.1	2.2	2.2	2.5
PB	2.3	2.7	2.4	2.8
AB	1.5	3.7	1.6	2.9 (oven)

extracts migrating into the base paper of the packaging material, away from the surface in contact with food, was avoided. Thus, there was little or no penetration into the base paper.

As expected, MR-24 produced higher coating weights compared to MR-12. However, the increase in coating weight with MR-24 was smaller than expected. A reason for this can be the surface roughness and waviness as apparently shown in SEM images from figure 1, which seems to have controlled the wet metered coating amounts. This could affect the spreading of extracts on the surface. Hence, it is important to have as uniform surface as possible to enhance uniform spreading of the extracts on the package surface.

Viscosity of the extracts was increased by adding CMC as a thickener to SB and PB while ethanol was added as a thinning agent for AB. This improved the uniformity of the coating. After the coating, some aggregation of the extracts was observed on the surface for both PB and SB coating. These appeared to be circular structures with diameters up to about 1 mm. In comparison, coatings with AB appeared to be and remained uniform. In figure 2, SEM images that show the aggregate of extracts on the surface of MLPC paper are presented.

3.2. Antibacterial activity of coatings

Prior to the coating application on paper, antibacterial effects of both SB and PB extracts were demonstrated in test tubes where these extracts were added to liquid culture media. The minimal effective dosage that showed inhibitory effect for PB extracts was about 6.7% in a 300 µL liquid culture medium. Similarly, SB extract and AB showed minimum inhibitory effects at a concentration of 3.3-6.7 % and 0.05 % in a 300 µL liquid culture medium respectively. After the coating

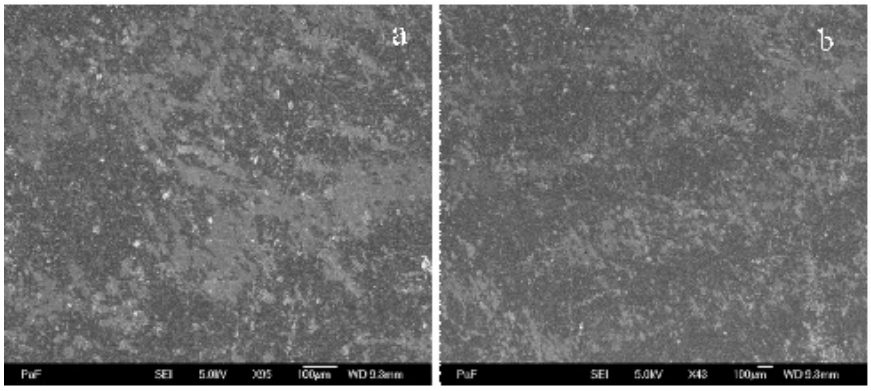

Figure 1: SEM images of surface roughness (a) and waviness (b) of MLPC paper.

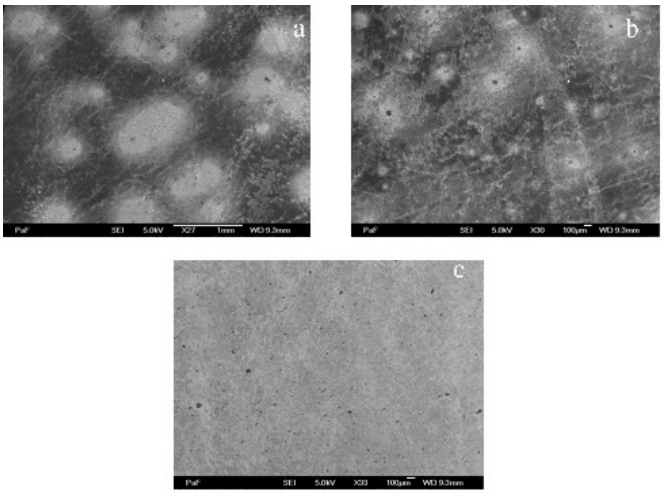

Figure 2: SEM image showing aggregation of sea buckthorn (a) and pine bark (b) on paper surface. Uniform coating of antimicrobial blend is shown in (c).

process, both gram-positive Staphylococcus aureus (S. aureus) and gram-negative Pseudomonas aeruginosa (P. aeruginosa) were used as model test organisms to investigate the inhibitory effects after the coating process. In the current study, our focus was to evaluate whether these effects could be maintained in application as coatings. Disk diffusion method was used to estimate the efficacy of natural plant extracts as antibacterial agents after the coatings had been sufficiently dried. For each sample, measurements were carried out from three pieces and the standard deviation was calculated. The inhibition zone diameter was 12 mm for the control disk, and considered as having no antibacterial effect as shown in table 2. Therefore, measurements having a diameter greater than 12 mm would suggest antibacterial effects of the coated surface. The diffusion zone obtained from the control disk could have resulted from biocides that were included during the papermaking process. Penicillin G 0.03 units with disk diameter of 6.5 mm was used as a positive control, which resulted in an inhibition zone diameter of 11.5 mm. The results are presented in Table 2.

In the antibacterial measurements of the extracts coated onto paper, neither SB nor PB showed positive results against *S. aureus* when coated with MR-12 or for MR-24. MR-12 coatings of AB did not show antibacterial effect for *S. aureus,* whereas MR-24 coatings of AB showed antibacterial effect. *S. aureus* was more resistant towards the natural extracts while *P. aeruginosa* was found more susceptible to the natural extracts especially with both SB coatings from MR-12 and MR-24 showing antibacterial effect. The maximum diffusion zone measured for SB coatings is around 15.4 mm and 16.6 mm for MR-12 and MR-24 respectively. This implies that higher SB amounts coated on the surface can result in higher antibacterial effect. At the same time, PB coatings did not show significant antibacterial activity against *P. aeruginosa* for either MR-12 or for MR-24. Similar to the results obtained from *S. aureus*, AB coatings did not show antibacterial effect with coating from MR-12 whereas coatings from MR-24 showed inhibitory effects on the growth of *P. aeruginosa*.

Berry extracts have been investigated as natural antimicrobial agents for many years, and phenolic compounds have been reported to be among the key

Table 2: Size of diffusion zone in (mm) obtained from antibacterial measurements with standard deviation (SD).

S. aureus				
	RT MR-12	RT MR-24	IR MR-12	IR MR-24
SB	12 ±0	12 ±0	12 ±0	12 ±0
	12 ±0	12 ±0	12 ±0	12 ±0
PB	12 ±0	12 ±0	12 ±0	12 ±0
	12 ±0	12 ±0	12 ±0	12 ±0
AB	12 ±0	14.8 ±0.7	12 ±0	17.3 ±2.4
	12 ±0	16.4 ±1.5	12 ±0	16.8 ±0.7
P. aeruginosa				
SB	15.3±0.5	15.4±0.4	15.4±0.8	16.6±0.9
	14.3±0.3	16.4±0.8	15.2±0.4	16.2±0.5
PB	12 ±0	12 ±0	12 ±0	12 ±0
	12 ±0	12 ±0	12 ±0	12 ±0
AB	12 ±0	12 ±0	12 ±0	17.2±0.9
	12 ±0	12 ±0	12 ±0	18.5±0.9

antimicrobial components of berry extracts [29]. Further studies have confirmed that berry extracts contain different groups of phenolic compounds, which include anthocyanins, flavonols, tannins, and phenolic acids [30, 31]. For sea buckthorn (SB) berries, it has been shown that flavonols represent up to 87% of the total content of phenolic compounds [32]. The solvent used for extraction can affect the yield of phenolic compounds in the extracts. Even though extraction with ethyl acetate produces the highest amount of phenolic compounds in SB as determined using the Folin–Ciocalteau method, 70% ethanol as the extraction solvent is commonly recommended in literature [33]. Phenolic compounds that have been observed in extracts of SB leaves include gallic acid, glycosides of isorhamnetin and quercetin [16]. Genetic factors (species and cultivars), growth location and conditions as well as harvesting time affect the phenolic constituents present in sea buckthorn [34-37], and this is likely to be valid for pine bark as well. Pine bark (PB) extracts have been used traditionally as medicinal components, and the phloem (inner bark) of pine trees have been used in food such as bread, after baking and other processing. Studies have confirmed the antioxidant activity of phenolic constituents of pine bark [38]. The inner bark of pine contains phenolic compounds, which include monoarylic phenols, stilbenes, lignans, and flavonoids [39]. Tannis have been reported in pine bark extracts [40, 41], while extractives from the phloem have exhibited antibacterial effects against selected microbes [42].

There have been few reports in literature about the antibacterial properties of pine bark extracts, and it has not been extensively investigated. In this study, it is observed that while the solvent extracts from the inner layer of pine bark has antibacterial effects, it not effective as an antibacterial coating on paper. On the other hand, antibacterial properties of sea buckthorn have been extensively demonstrated in literature [43, 44]. Currently, there are limited reports where it has not been used as the main constituent for achieving antibacterial effects in packaging application. It is generally combined with other extractives, which also possess inhibitory effect against bacteria [45]. As a result, antibacterial effects may be due to synergistic effect from all components present. In this study, it is shown that extract from sea buckthorn leaves can be exclusively used to inhibit bacterial growth since the antibacterial effect is maintained when applied as a coating on paper that is intended for packaging application. The minimum coat weight of sea buckthorn that resulted in antibacterial effect after it was coated on paper for packaging was 2.1 g/m^2.

Different drying methods were used after coatings had been applied to paper surface. IR drying was significantly faster with exposure time of 3-5 s. At a distance of 20 cm from the radiation source, drying more than 7 sec destroyed the coated samples with burns on the surface and wrinkles. For samples with higher coat weights (AB MR-24), infrared drying caused wrinkles within 3-5 sec, therefore these samples were dried in an oven at 60 ºC for 3-5 mins. In this study, there was no significant difference resulting from the different drying methods. For samples coated with MR-24, the estimated coat weights were similar irrespective of the drying method. Similar result was obtained from MR-12 coated samples as coat weights were comparable. For sea buckthorn extracts coated with MR-12 and MR-24, little variation was observed in the size of diffusion zone measured for both groups of coatings. The results suggest that irrespective of the drying method used, room temperature or infrared drying, the antibacterial activity was not reduced for sea buckthorn and antimicrobial blend since the results are obtained for repeated experiments.

Even though pine bark extracts exhibited antibacterial effect in liquid culture medium, the effects were not maintained when used as a coating. The reason for this observation is not clear, but it could be due to evaporation of volatile active compounds or complex surface reaction that resulted in inactivation the natural extracts after it was applied as coating. For pine bark extracts coatings, the three drying methods did not result in antibacterial effect after. Coat weight measured for pine bark coatings with MR-12 were similar, while MR-24 coated samples also had similar coat weight. It is entirely possible that higher coat weight of pine bark could have resulted in antibacterial effect when used as coatings, but this assertion would require further investigation.

4. CONCLUSION

In this study, antibacterial activity of two natural plant extracts were examined against gram-positive *S. aureus* and gram-negative *P. aeruginosa* after they had been applied as coatings on paper intend for food packaging application. Our results showed that natural extracts obtained from sea buckthorn leaves maintained their antibacterial activity when applied as coatings on paper suitable for packaging application. SB extract showed inhibition on the growth of *P. aeruginosa* but was inactive against *S. aureus* when applied as coatings on paper. On the other hand, the PB extract did not show any antibacterial effect against either of the tested bacteria as coatings, although the solvent extracts exhibited antibacterial effect. Efficacy of these natural extracts was compared to an antimicrobial blend that was effective against test bacteria only at higher coat weights, while SB extract was

effective both at low and high coat weights. Similar to penicillin, SB coatings showed antibacterial effect, but results are not comparable since different disc diameters were used. IR and oven drying of samples did not reduce the antibacterial efficacy of the extract that maintained antibacterial effect after being applied as coating.

We believe natural extracts would be used extensively in future applications as part of antimicrobial food packaging technologies, which promote better quality and improve shelf life of food. The coatings can be manufactured cost-effectively in a roll-to-roll process flow that allows large area application of antimicrobial surfaces in products ranging from antibacterial tissue paper to filters and packages.

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