Pinosylvin-Based Polymers: Biodegradable Poly(Anhydride-Esters) for Extended Release of Antibacterial Pinosylvin

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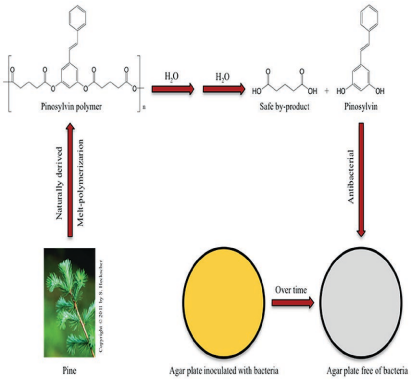
Pinosylvin is a natural stilbenoid known to exhibit antibacterial bioactivity against foodborne bacteria. In this work, pinosylvin is chemically incorporated into a poly(anhydride-ester) (PAE) backbone via melt-condensation polymerization, and characterized with respect to its physicochemical and thermal properties. In vitro release studies demonstrate that pinosylvin-based PAEs hydrolytically degrade over 40 d to release pinosylvin. Pseudo-first order kinetic experiments on model compounds, butyric anhydride and 3-butylstilbene ester, indicate that the anhydride linkages hydrolyze first, followed by the ester bonds to ultimately release pinosylvin. An antibacterial assay shows that the released pinosylvin exhibit bioactivity, while in vitro cytocompatibility studies demonstrate that the polymer is noncytotoxic toward fibroblasts. These preliminary findings suggest that the pinosylvin-based PAEs can serve as food preservatives in food packaging materials by safely providing antibacterial bioactivity over extended time periods.



1. Introduction

Pinosylvin is an analog of resveratrol and a natural stilbenoid present in *Pinus* species.[1,2] Owing to its intrinsic antimicrobial bioactivity, pinosylvin research has gained great interest. For instance, several studies have reported on pinosylvin antibacterial bioactivity against common foodborne pathogens such as Gram-positive (*Staphylococcus aureus, Listeria monocytogenes*) and Gram-negative (*Escherichia coli, Salmonella*) bacteria, even when the compound was physically admixed within food matrices of either vegetable or animal origin.[3–5] The inherent properties of pinosylvin and related stilbenoids have generated interest for extended release applications, from food protection to bioactive delivery carriers.

To achieve extended release of stilbenoids, researchers have physically encapsulated similar stilbenoids into polymer matrices. In one approach, chitosan microspheres were loaded with resveratrol, yielding however microspheres with low bioactive loadings (<10%) and a burst release of the bioactive (over 60% within 30 min).[6] In another approach, chemical modification of resveratrol into a triacetyl ester was attempted,[7] but the ester-protected resveratrol hydrolyzed completely within 1 h.[7] As resveratrol and pinosylvin are structurally similar, the physical and chemical modifications of pinosylvin, as described above, should result in similar outcomes. Consequently, novel polymer systems capable of achieving higher bioactive loading and providing extended release of pinosylvin as a food preservative into common food packaging materials are of interest; such targeted goals

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can be achieved by chemically incorporating pinosylvin into a poly(anhydride-ester) (PAE) backbone.

PAEs are predominantly surface-eroding polymers with minimal burst release, allowing for controlled and sustained release of bioactives in a near-zero order manner.[8,9] Additionally, PAEs can be prepared to achieve higher bioactive loadings (50%–100%),[10,11] and formulated into different geometries, hence offering the potential to satisfy diverse applications.[12,13]

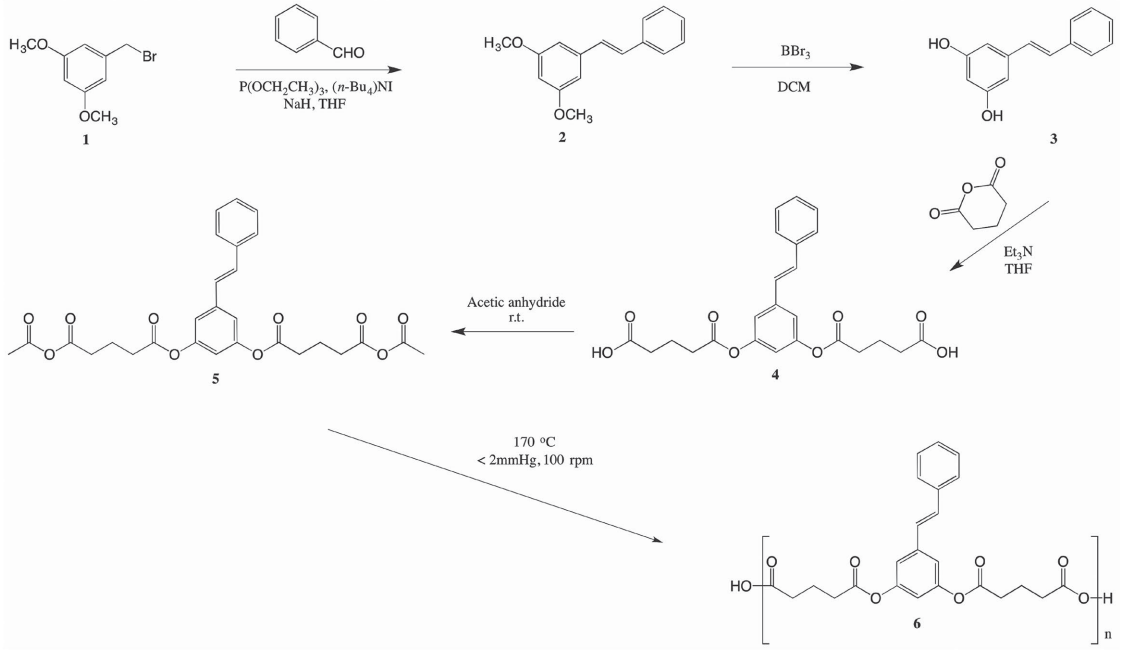
In this work, the synthesis of pinosylvin-based polymers via melt-condensation polymerization, followed by characterization of its physicochemical and thermal properties, is presented. In vitro degradation studies in phosphate buffered saline (PBS, pH 7.4) were performed over 40 d, and bioactive release was verified following polymer degradation. Pseudo-first order kinetic experiments were carried out on model compounds (butyric anhydride and 3-butylstilbene ester) to understand the hydrolytic degradation of pinosylvin-based PAEs. The polymer's cytocompatibility was assessed in vitro against fibroblast cells, and the release media confirmed antibacterial bioactivity via disc diffusion assay against *S. aureus* and *E. coli* bacteria.

2. Results and Discussion

2.1. Synthesis and Characterization

Pinosylvin was chemically incorporated in a PAE backbone, yielding 50 wt% loading as outlined in Scheme 1; pinosylvin synthesis was adapted from a methodology employed elsewhere.[14] Briefly, 3,5-dimethoxybenzyl bromide (**1**) was heated with triethyl phosphite in the presence of $(n\text{-Bu})_4$NI, then the intermediate reacted with benzaldehyde to yield 3,5-dimethoxystilbene (**2**) (Figure 1, **2A**), followed by demethylation to generate pinosylvin (**3**) (Figure 1, **3B**). The chemical composition of pinosylvin was also confirmed by infrared spectroscopy (IR) and mass spectrometry (MS). Ring-opening of glutaric anhydride linker molecules to generate pinosylvin diacid (**4**) was confirmed by the presence of the ester (C=O) and carboxylic acid (C=O) by IR spectroscopy (Figure S5, Supporting Information) and the relevant chemical shifts in the hydrogen nuclear magnetic resonance (^{1}H-NMR) spectra (Figure 1, **4C**).

Pinosylvin monomer (**5**) was formed by acetylation of diacid **4** in excess acetic anhydride, as confirmed by nuclear magnetic resonance (NMR) and IR spectroscopy. As pinosylvin monomer (**5**) had a moderate melting temperature (T_m = 155 °C) and a high decomposition temperature (T_d = 246 °C), melt-condensation polymerization was possible. Melt-condensation polymerization of activated monomer **5** at 170 °C produced polymer **6**, as confirmed via H-NMR (Figure 1, **6D**) and IR spectroscopy, which shows the presence of anhydride carbonyls and ester bonds, the preservation of the double bonds, and the disappearance of terminal carboxylic acids C=O (Figure S5, Supporting Information). Polymer **6** had a weight-average molecular weight of 61 kDa, a number-average molecular weight of 47 kDa, and a polydispersity index of 1.3. The polymer had a glass transition and decomposition temperatures of



Scheme 1. Synthesis of pinosylvin-based PAEs (**6**) from pinosylvin (**3**), an antimicrobial stilbenoid.

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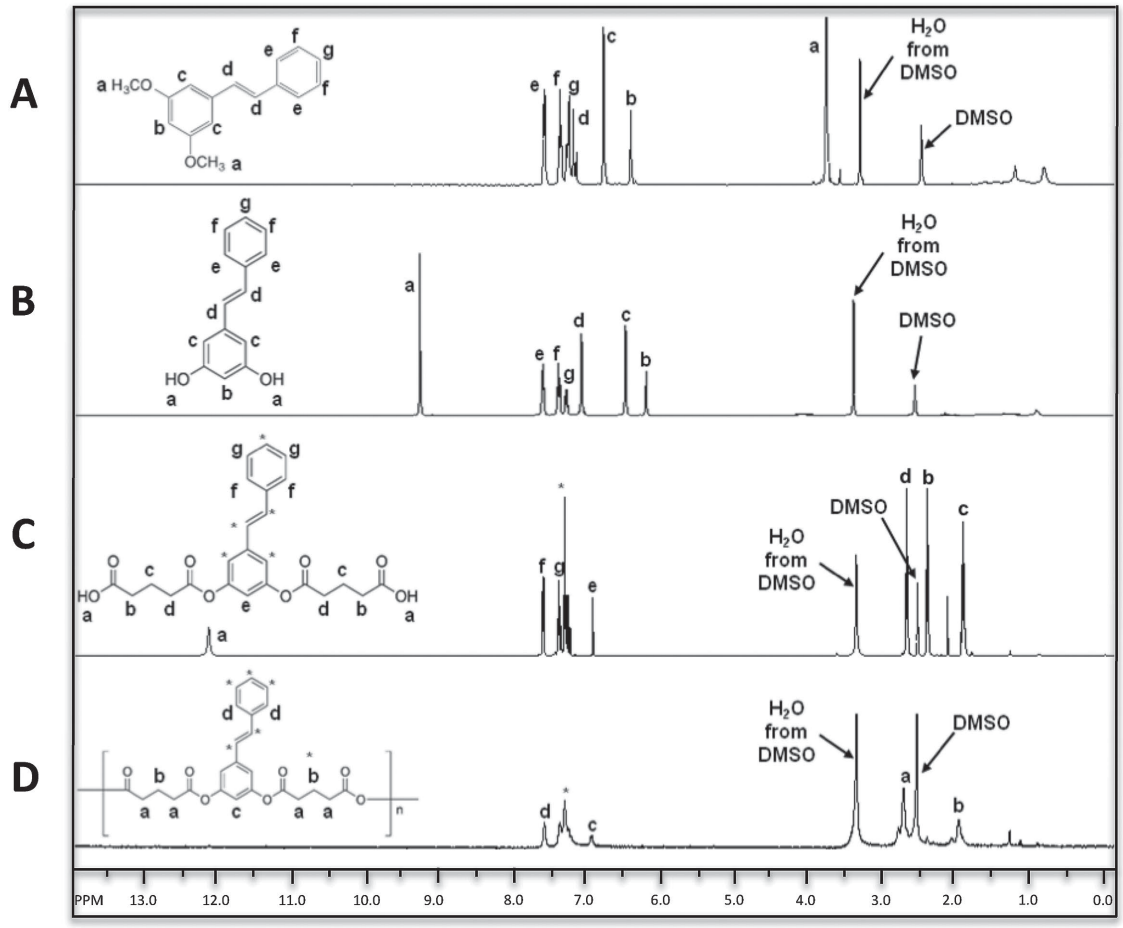

Figure 1. ^{1}H-NMR spectra highlighting key chemical shifts of A) 3,5-dimethoxybenzyl bromide (**2**); B) pinosylvin (**3**); C) pinosylvin diacid (**4**), and D) pinosylvin-based PAEs (**6**).

50 and 190 °C respectively, exhibited no T_m, and exhibited a contact angle (CA) value of 84°.

2.2. Relative Rate of Hydrolysis: Kinetic Experiments

Pseudo-first order kinetic experiments were carried on two small molecules representing the PAE structure, namely butyric anhydride (**12**) and 3-butylstilbene ester (**11**), in order to better comprehend hydrolytic degradation of pinosylvin PAEs. Hydrolysis rate constants of the model compounds are shown in Table 1 and determined using the following formula[15]

Table 1. Hydrolysis rate constants of model compounds, butyric anhydride (**12**), and 3-butylstilbene ester (**11**), representing degradable linkages of pinosylvin PAEs (**6**, Scheme 1).

Compound	k [L mol^{-1} s^{-1}]
Butyric anhydride	$(30.6 \pm 2.24) \times 10^{-6}$
3-Butylstilbene ester	$(56.7 \pm 5.01) \times 10^{-7}$

$$k = k_{observed} / [water] \quad (1)$$

where k is the reaction rate constant, $k_{observed}$ the pseudo-first order rate constant, and [*water*] the molar concentration of water.

In the case of 3-butylstilbene ester (**11**), hydrolysis rate constants for 2.5%, 5%, and 10% 1,4-dioxane/PBS media were determined using Equation (1). Then, hydrolysis rate constants of the ester (**11**) for 0% 1,4-dioxane/PBS were extrapolated from the values obtained for 2.5%, 5%, and 10% 1,4-dioxane/PBS media (Figure S7, Supporting Information). As the percentage of 1,4-dioxane increased, the rate constants decreased: this effect is likely due to 1,4-dioxane dispersing water molecules and preventing water from interacting via hydrogen bonding with charged species in the transition state. Therefore, the activation energy of the transition state increased relative to that of water alone, and rate of ester hydrolysis consequently slowed. This observation

corresponds to a water-catalyzed hydrolysis, because for acid- or base-catalyzed hydrolysis reactions, addition of 1,4-dioxane would increase the hydrolysis rate constants.[16]

At 37 °C, butyric anhydride (**12**) manifested a faster hydrolysis rate constant [$k = (30.6 \pm 2.24) \times 10^{-6}$ L mol^{-1} s^{-1}] than 3-butylstilbene ester (**11**) [$k = (56.7 \pm 5.01) \times 10^{-7}$ L mol^{-1} s^{-1}]. Numerous studies in the literature have investigated the hydrolysis of similar anhydrides and esters, but they were performed under either acid- or base-catalyzed conditions, at room or higher temperatures (>>37 °C).[17–19] Therefore, because the hydrolysis conditions and parameters are extremely different, no direct comparisons can be made between the hydrolysis rate constants obtained in the literature and the ones from this study. Nonetheless, the experimental data obtained from this work correlate with the fact that the anhydride bonds, being more labile, are more susceptible to hydrolysis compared to ester bonds.[20,21]

2.3. Hydrolytic Degradation of Pinosylvin-Based PAEs

Bioactive release from polymer discs was monitored in vitro by high-performance liquid chromatography on polymer discs at physiological conditions (37 °C, pH 7.4). Polymer degradation through hydrolysis of anhydride and ester bonds is a primary factor for controlled release of the stilbenoid. To evaluate the hydrolysis of pinosylvin PAEs (**6**), pseudo-first order kinetic experiments were performed on butyric anhydride (**12**) and 3-butylstilbene ester (**11**) model compounds. Based on the kinetic analysis on these small molecules, butyric anhydride (**12**) underwent faster hydrolysis [$k = (30.6 \pm 2.24) \times 10^{-6}$ L mol^{-1} s^{-1}] than 3-butylstilbene ester (**11**) [$k = (56.7 \pm 5.01) \times 10^{-7}$ L mol^{-1} s^{-1}]. These findings suggest the anhydride bonds of pinosylvin PAEs (**6**), being more hydrolytically labile,[20,21] hydrolyze first to yield pinosylvin diacid (**4**), followed by hydrolysis of the ester bonds to generate pinosylvin (**3**).

As shown in Figure S8 (Supporting Information), a minor amount (≈5%) of pinosylvin (**3**) is quickly released

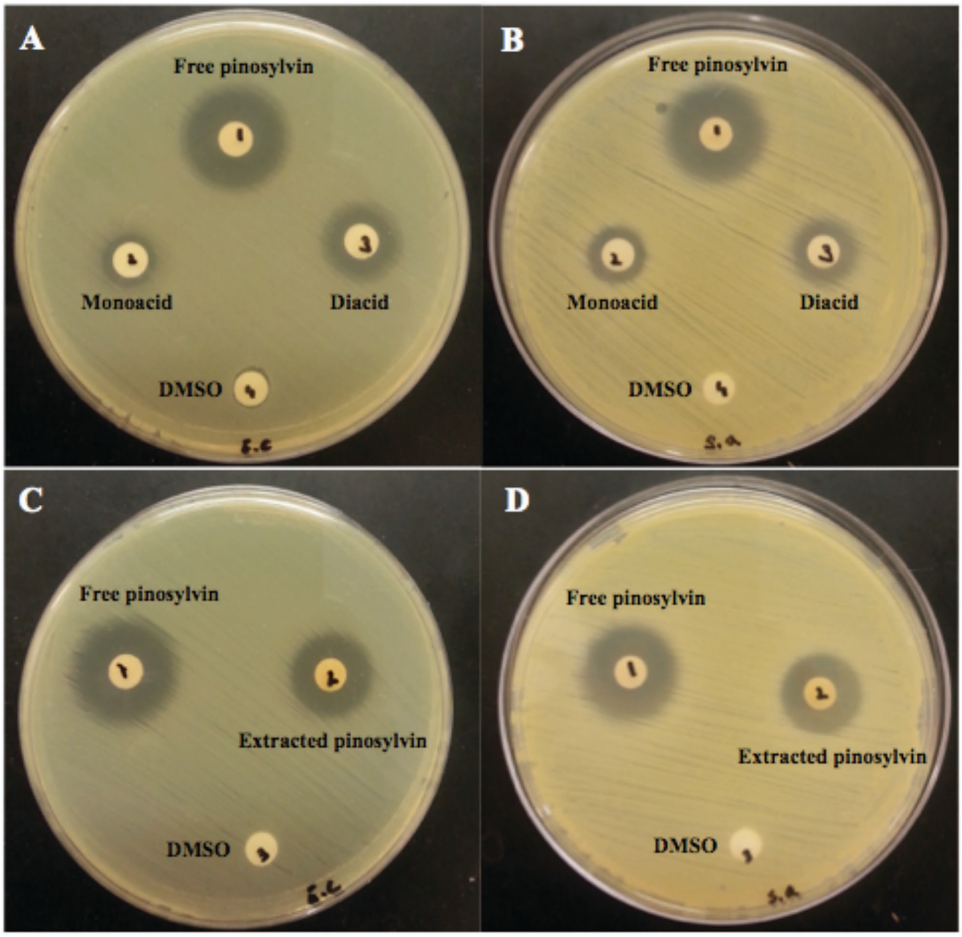

Figure 2. Disc diffusion assay results for A,C) *E. coli* and B,D) *S. aureus* showing zones of inhibition (Z_H) for free pinosylvin (Z_H = 17.5 mm), extracted pinosylvin (**3**, Z_H = 17.0 mm), monoacid (**7**, Z_H = 10.0 mm), and diacid (**4**, Z_H = 11.0 mm) at 10 mg mL^{-1} in DMSO.

into the degradation media of polymer **6**. After the first 2 d, polymer **6** exhibited a sustained release of pinosylvin (**3**). The polymer is relatively slow degrading, releasing ≈10% of pinosylvin (**3**) in 40 d. This slow-degrading behavior of **6** is likely due to the relatively high hydrophobicity (CA 84°), and **6** is estimated to release 100% of pinosylvin (**3**) in 28 months, on extrapolation (Figure S8, Supporting Information). At the end of study, a mass balance was performed (96% mass accounted for) following analysis of bioactive in residual polymer **6**.

2.4. Antibacterial Disc Diffusion Assay

To confirm the benign effects of polymerization processes on pinosylvin's (**3**) antibacterial bioactivity, a disc diffusion assay was carried out against Gram-positive *S. aureus* and Gram-negative *E. coli* foodborne bacteria.[22] The concentration of each substance (free pinosylvin, extracted pinosylvin (**3**), monoacid (**7**), and diacid (**4**)) in dimethyl sulfoxide (DMSO) was greater than the minimum inhibitory concentration (MIC) for pinosylvin (**3**) (250 μg mL^{-1}) to evaluate inhibition zones on the inoculated agar plates.[3] From the data collected (Figure 2), both free and extracted pinosylvin (**3**) diffused from the discs and exhibited similar zones of inhibition against both bacterial strains. Pinosylvin monoacid (**7**) and diacid (**4**) also prevented bacterial growth, with diacid (**4**) showing slightly larger zone of inhibition. This difference may be due to the greater aqueous solubility of **4** (2.24 mg mL^{-1}) compared to **7** (0.80 mg mL^{-1}), therefore allowing diacid (**4**) to diffuse more readily through the hydrophilic agar plate. Furthermore, release studies have shown that diacid (**4**) breaks down readily into monoacid (**7**) and subsequently pinosylvin (**3**) within the timeframe the disc diffusion assay was performed (100% pinosylvin release in 18 h). This observation, coupled with diacid's aqueous solubility and larger diffusion area, would explain the greater zone of inhibition for **4**. Overall, this assay demonstrates that polymerization processes did not affect pinosylvin's (**3**) antibacterial bioactivity against two of the most common foodborne pathogens.

2.5. In Vitro Cytocompatibility Studies

All polymers were cytocompatible at 0.5, 0.1, and 0.05 μg mL^{-1} over 72 h (Figure S3, Supporting Information). No significant difference in cell viability was found between the polymer groups and the media control. However, polymers at 3, 1.5, and 1 μg mL^{-1} were cytotoxic toward fibroblasts. Since pinosylvin's (**3**) antibacterial MIC against common Gram-positive and Gram-negative bacteria is 250 μg mL^{-1},[3] pinosylvin polymers (**6**) will be toxic at such high concentrations in vivo (500 μg mL^{-1} of polymer **6** needed for 250 μg mL^{-1} therapeutic effect of pinosylvin (**3**)). Therefore, pinosylvin polymers (**6**) are recommended for external applications, such as preservatives in food packaging materials.

3. Conclusions

Sustained release of pinosylvin (**3**) may provide prolonged antibacterial effects against common foodborne bacteria when released from polymers in a sustained fashion. Attempts in the literature for achieving extended release of pinosylvin (**3**) and similar stilbenoids by the use of polymers as delivery vehicles have been unsuccessful.[6,7] As an alternative, the chemical incorporation of pinosylvin (**3**) into a PAE backbone for the extended release of the bioactive is investigated in this work. Physicochemical and thermal testing revealed successful synthesis while in vitro hydrolytic degradation of polymer (**6**) confirmed sustained bioactive release of pinosylvin (**3**) over 40 d. To understand the chemical degradation of pinosylvin-based PAEs (**6**), pseudo-first order kinetic experiments performed on butyric anhydride (**12**) and 3-butylstilbene ester (**11**) model compounds revealed faster hydrolysis for the anhydride [$k = (30.6 \pm 2.24) \times 10^{-6}$ L mol^{-1} s^{-1}] compared to the ester [$k = (56.7 \pm 5.01) \times 10^{-7}$ L mol^{-1} s^{-1}]. Pinosylvin (**3**) released from polymer **6** retained its antibacterial biological activity as observed via a disc diffusion assay. In vitro cytocompatibility studies demonstrated that polymer **6** is cytocompatible up to 0.5 μg mL^{-1}, which concentration falls well below pinosylvin's MIC of 250 μg mL^{-1}. As a result, pinosylvin PAEs (**6**) constitute a promising technology to be employed, as part of future work, for external applications, such as food additives into common food packaging materials for food preservation and food safety.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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