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# PRODUCTION AND CHARACTERIZATION OF SPOROPOLLENIN REINFORCED ALGINATE-BASED COMPOSITE FILMS

**Bahar AKYUZ YILMAZ[1]***

[1]*Aksaray University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, 68100 Aksaray, Türkiye*

**Abstract:** Sporopollenin is a natural biomaterial that offers great potential for various applications due to its biocompatibility, biodegradability, non-toxicity, durability, and high thermal stability. In this study, sporopollenin was extracted from *Pinus nigra* pollen and incorporated into alginate-based films to utilize these advantageous properties. Sporopollenin samples were added to 100 mL of 1% alginate solution at different concentrations of 0, 5, 10, and 50 mg. Both sporopollenin-free alginate films and sporopollenin-enriched films were comprehensively characterized using FT-IR, TGA, SEM, and MTT analysis methods. The results revealed that sporopollenin obtained from *P. nigra* pollen was successfully incorporated into the alginate-based films. Furthermore, an increase in the amount of sporopollenin led to enhanced surface roughness. MTT test results also confirmed that the films were non-toxic. The aim of this work is to investigate the potential of sporopollenin as a functional additive in alginate films and to highlight its importance in developing sustainable and biocompatible biomaterials. These findings suggest that sporopollenin holds promise as an innovative biomaterial for various applications, such as food packaging.

**Keywords:** Alginate, Biodegradable, Composite, Cytotoxicity

***Corresponding author:** Aksaray University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, 68100 Aksaray, Türkiye
**E mail:** baharakyuzylmaz@gmail.com (B. AKYUZ YILMAZ)
Bahar AKYUZ YILMAZ https: //orcid.org/0000-0001-9760-9856

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## 1. Introduction

Recently, consumer demand for high-quality fruits and vegetables has been increasing day by day. This increasing demand has led to protecting foods with appropriate preservation techniques and extending their shelf-life by using various packaging. The use of synthetic and non-biodegradable packaging brings environmental problems. For this reason, scientists have turned to the research of edible films consisting of biopolymers or their composites obtained by using natural, edible, and biodegradable polymers for food preservation and shelf-life extension. These edible films and coatings create a semi-permeable safety barrier around vegetables and fruits, reducing the loss of quality attributes. Biopolymers such as starch, pectin, chitosan, carrageenan, alginate, xanthan gum, and gelatine are commonly used to produce these films (Martins et al., 2024).

Alginates are natural biopolymers found in the cell wall of brown seaweeds. It is structurally known as a linear copolymer containing (1 → 4)-linked β-d-mannuronate (M) and α-l-guluronate (G) residues (Dekamin et al., 2018). Alginates have attracted attention in many fields thanks to their biological properties, making them suitable for many applications in the medical, food, cosmetic, pharmaceutical, and surgical sectors (Tallis, 1950). It offers non-toxic properties such as thickening, stabilizing, suspending, film forming, gel forming, and emulsion stabilizing (Rahman et al., 2024). Thanks to its excellent film-forming ability and low cost, sodium alginate is widely applied in edible packaging. Although alginate edible films form strong films, they show poor resistance to water due to their hydrophilic nature (Pathak et al., 2008). Moreover, single alginate films have poor mechanical properties and low additional functionalities. It is more effective to improve the properties of alginate films when composite with other polymers or different inorganic, organic materials. Adding sporopollenin, known as the apple of the plant world, to alginate films can also improve film properties.

Sporopollenins are microcapsules that form the protective outer layer of plant spores and pollen grains and are obtained by removing the genetic materials, oils, and proteins of pollen with chemicals such as acid, base, and chloroform. They are cross-linked biopolymers that are mechanically robust, chemically inert, very high thermally stable, non-allergenic, and can remain intact in nature for thousands of years (Tønnesen and Karlsen, 2002). Sporopollenin microcapsules have attracted great interest as microencapsulation materials due to their environmentally friendly nature and uniform micron-scale size. Therefore, due to their mechanical and thermal durability, sporopollenin exine capsules have become a promising material for drug delivery, electronics, and food packaging applications (Alshehri et al., 2016; Martău

et al., 2019; Mohammed et al., 2021). Accordingly, composite materials reinforced with hollow sporopollenin microcapsules from different plant spores were developed (Mujtaba et al., 2022; Lingait et al., 2024).

In this study, sporopollenin particles obtained from *P. nigra* pollen were incorporated into alginate-based composite films, and their physicochemical properties were analyzed using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), and Thermogravimetric Analysis (TGA). In addition, the cytotoxicity of all films was investigated by MTT assay. The effects of sporopollenin added at different ratios to alginate-based films were evaluated.

## 2. Materials and Methods

### 2.1. Materials and chemicals

In this study, *Pinus nigra* pollen was collected from Aksaray University Campus, Aksaray, Türkiye. They were then left to dry at room temperature for 3 days. Then, to remove dust and other unwanted particles, they were first sieved through a sieve with a pore diameter of 10 µm and then through a sieve with a pore diameter of 100 µm to remove large particles. Pollen samples were stored in sealed tubes at -20°C for further experiments. The chemicals used for sporopollenin extraction; HCl, NaOH, Chloroform and Methanol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Glycerol, the plasticizing agent, was obtained from Sigma-Merck, USA. Distilled water was used in all experimental stages.

### 2.2. Isolation of Sporopollenin from *P. Nigra* Pollen

For the isolation of sporopollenin, the obtained *P. nigra* pollen was treated with acid for demineralization, base for deproteinization, and chloroform/methanol/water solution for depigmentation. Briefly, for demineralization, 10 g of *P. nigra* pollen was treated with 4 M HCl solution for 2 h at 50°C. Then, the samples were filtered with a Whatman filter paper with a pore size of 110 µm and then washed with distilled water to neutral pH. For deproteinization, the samples were incubated with 4M NaOH solution for 6 hours at 150°C using a magnetic stirrer with a heating system. The treated samples were then filtered using Whatman filter paper and washed extensively with distilled water to neutral pH. The demineralization and deproteinization processes were repeated 4 times to ensure complete removal of genetic and cellulosic materials in the pollen. Acid and base treated pollen samples were then kept in chloroform/methanol/water solution (4: 2: 1 v: v: v) for 1 h at room temperature. Finally, sporopollenin samples were washed thoroughly with distilled water and dried in an oven at 60°C for 48 hours. Figure 1a shows the isolation stages of sporopollenin schematically.

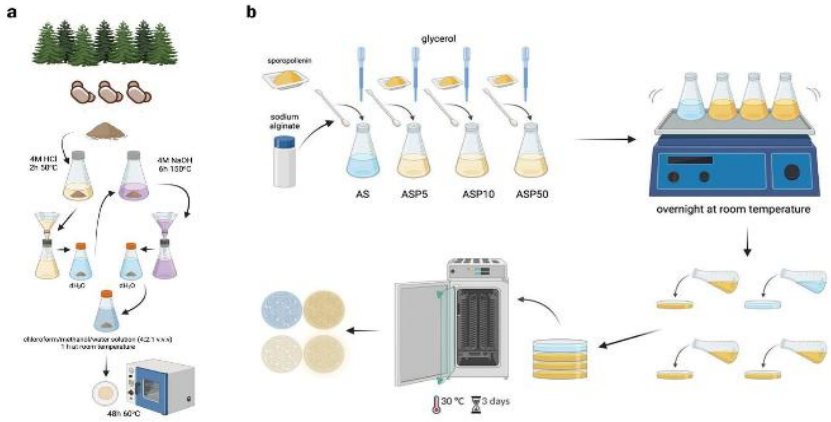


**Figure 1.** Schematic representation of the isolation steps of sporopollenin (a) and preparation of sporopollenin reinforced of alginate-based films (b).

### 2.3. Preparation of Films

Four different types of alginate-based films were prepared to investigate the effects of sporopollenin on film properties. Initially, a 1% (w/v) sodium alginate solution was prepared by dissolving sodium alginate in distilled water. For flexibility enhancement, 5 mg of glycerol was added as a plasticizer to each solution. The control film, containing only alginate and glycerol without sporopollenin, was labeled AS. To prepare the other film samples, sporopollenin was added to three alginate solutions at concentrations of 5 mg, 10 mg, and 50 mg, creating samples labeled ASP5, ASP10, and ASP50, respectively. The use of different concentrations was adapted from the method of previous study (Kaya et al., 2017). After adding glycerol and different amounts of sporopollenin, each solution was stirred overnight at room temperature using a magnetic stirrer at 100 rpm. This gentle stirring was done to ensure homogeneity and prevent fragmentation of the sporopollenin. The mixed solutions were then poured into plastic petri dishes and left to dry at 30 °C for three days. Figure 1b schematically shows the preparation of sporopollenin reinforced films. The original camera images of all films are shown in Figure 2. Film thickness was measured with a digital micrometer (Mitutoyo, China). For each film, measurements were taken at ten random points, and the average thickness was calculated.

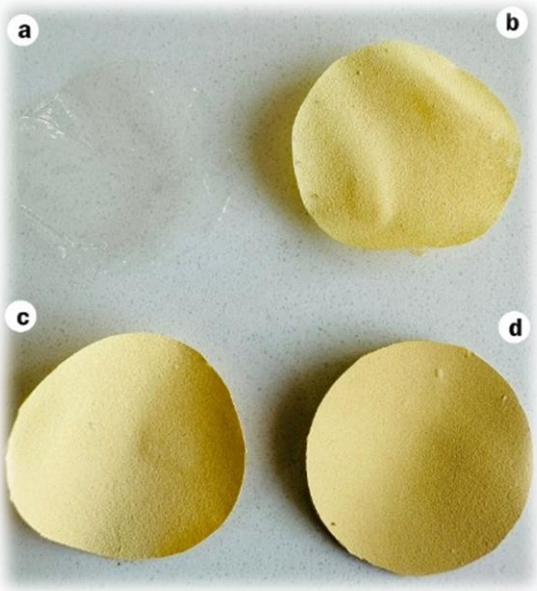


**Figure 2.** Original camera images of alginate-based control film (a), and sporopollenin-alginate composite films; ASP5 (b), ASP10 (c), ASP50 (d).

### 2.4. Physicochemical Characterization

#### 2.4.1. Fourier transform infrared spectroscopy (FTIR)

Infrared spectra of sodium alginate-based films (AS, ASP5, ASP10, and ASP50) reinforced with sporopollenin obtained from *P. nigra* pollen were recorded in the wavelength range of 4000-400 $cm^{-1}$ using Perkin Elmer Spectrum Two FT-IR Spectrometer.

#### 2.4.2. Thermogravimetric analysis (TGA)

The thermal stability of AS, ASP5, ASP10 and ASP50 film samples was determined by EXSTAR S11 7300 thermogravimetric analyzer. The samples were tested by heating at a constant temperature of 10 ˚C $min^{-1}$ from 30 ˚C to 700 ˚C under a nitrogen atmosphere.

#### 2.4.3. Scanning electron microscopy (SEM)

The surface morphologies of *P. nigra* pollen, sporopollenin isolated from pollen samples, and all film samples were analyzed with FEI Quanta FEG 250 Scanning Electron Microscopy device at different magnifications between 500X-50.000X. With the help of these images, the surface morphologies of the samples were investigated.

### 2.5. MTT Assay

Within the scope of the study, biocompatibility assessments of biomaterials with physicochemical characterizations were conducted using healthy mouse fibroblast cells (L929) (HUKUK, Sap Institute). For the relevant analysis, cells were briefly cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a 5% $CO_2$ atmosphere. When cells reached 70% confluency, they were trypsinized and passaged at a 1: 4 dilution twice a week. Cell viability was monitored with trypan blue during procedures, and experiments proceeded only if the viability level was above 90%. Suspended cells obtained after passaging were prepared for the MTT assay by adjusting the cell density to 3 x $10^4$ cells/mL per well and were seeded into 96-well culture plates. The culture plates were incubated at 37°C in a 5% $CO_2$ atmosphere for 24 hours. After 24 hours, pre-sterilized 2x2 mm samples were added to each well, and the cultures were further incubated under the previously mentioned conditions for incubation periods of 24, 48, and 72 hours without any medium change. For biocompatibility assessments, control cells were maintained in growth medium only, without any treatment, for the same durations. The cytotoxicity of the materials on cells was evaluated using the standard MTT assay. After the incubation period, the materials were removed from the wells, and 10 µL of MTT solution (final concentration 0.5 mg/mL) was added to all test and control wells. The plates were then incubated in the dark at 37°C for 3 hours. Next, the reaction mixture was removed from the wells, and 100 µL of DMSO solution was added to dissolve the formazan crystals. The plates were left for 5 minutes to stabilize the color, and the absorbance was measured at 492 nm using a ChroMate® ELISA reader. The viability of untreated cells without any material was considered 100%. The cell viability percentage (%) was calculated using the following equation 1:

$$\%\, Viability\ = \frac{(Abs_{sample})}{(Abs_{control})} x100 \qquad (1)$$

### 2.6. Statistical Analysis

Data analysis was performed using version 5 of GraphPad Prism software (GraphPad Software®). Data is presented as the mean ± standard error of the mean (SEM). Statistical differences were evaluated by one-way ANOVA followed by Tukey's post-hoc test (95% confidence interval). p-values of $P<0.05$ were considered statistically significant.

## 3. Results and Discussion

### 3.1. Transparency and Thickness

Stereo microscopy images of all films are presented in Figure 2. The surface of the AS control film has a uniform and smooth morphology due to the absence of sporopollenin. With the addition of sporopollenin, significant roughness was observed on the surface. It is also observed that the surface roughness becomes more pronounced as the concentration of sporopollenin in the alginate matrix increases. The thickness of the films was measured as 0.109 ± 0.0145 mm, 0.053 ± 0.0017 mm, 0.0537 ± 0.0006 mm, and 0.086 ± 0.0047 mm for AS, ASP5, ASP10, and ASP50, respectively. These results show that there is a significant increase in the thickness of the films with an increasing amount of sporopollenin.

### 3.2. FTIR

FT-IR spectra of AS, ASP5, ASP10, and ASP50 films were recorded (Figure 3). The broad peak observed at 3279.17

$cm^{-1}$ in the spectrum of the alginate-based control film (AS) was attributed to -OH groups forming intermolecular hydrogen bonds. Two peaks at 2929.5 and 2107.8 $cm^{-1}$ were associated with aliphatic C-H stretching vibrations. The sharp peak at 1600.2 $cm^{-1}$ indicated the presence of sodium ions attached to the carboxylate ($COO^-$) group in the structure of sodium alginate, and the peak around 1407.3 $cm^{-1}$ indicated aliphatic C-H bending vibrations. The peak at 1025.6 $cm^{-1}$ indicated C-O-C sugar ring groups and showed that alginate has a polysaccharide structure. These spectra show the structural and chemical properties of the AS film. The 3239.59 $cm^{-1}$ peak in the spectrum of the 0.05% sporopollenin-alginate composite film (ASP5) is attributed to intermolecular OH bonding. The 2924.34 $cm^{-1}$ peak is produced by aliphatic C-H stretching vibrations. 1599.12 $cm^{-1}$ peak is aliphatic C=C stretching vibrations. 1406.7 $cm^{-1}$ are aliphatic C-H bends. 1025.91 $cm^{-1}$ peak is produced by C-O-C group (Figure 3). The FTIR spectrum reveals the characteristic functional groups and structural features of sporopollenin. The broad OH bond peak at 3400.1 $cm^{-1}$ indicates the presence of hydroxyl groups present in the structure of sporopollenin (Tønnesen and Karlsen, 2002). The peak observed at 1678.5 $cm^{-1}$ indicates the presence of a carbonyl (C=O) group. The aromatic C=C stretching band at 1516.2 $cm^{-1}$ is one of the features that supports the durable, chemically and biologically degradation-resistant structure of sporopollenin. The presence of aromatic rings can provide structural stability and rigidity to sporopollenin (Kaya et al., 2017; Martău et al., 2019).

Some changes were observed when comparing the AS spectrum with the FT-IR spectra of ASP5, ASP10, and ASP50 alginate sporopollenin films. In general, peaks at higher wavelengths were observed as the sporopollenin ratio increased and because of intermolecular interactions. As the amount of sporopollenin increased, the OH bond shifted to 3324.92 $cm^{-1}$ and gave a broader peak. The intensity of aliphatic C-H stretching vibration peaks increased due to the increase in aliphatic C-H bonds because of the combination of sporopollenin with sodium alginate. It was observed that the C-O-C group shifted from 1025 $cm^{-1}$ in the control to 1032 $cm^{-1}$ in the presence of sporopollenin (ASP50). The results obtained from FT-IR analysis demonstrated the successful incorporation of *P. nigra* sporopollenin into alginate films.

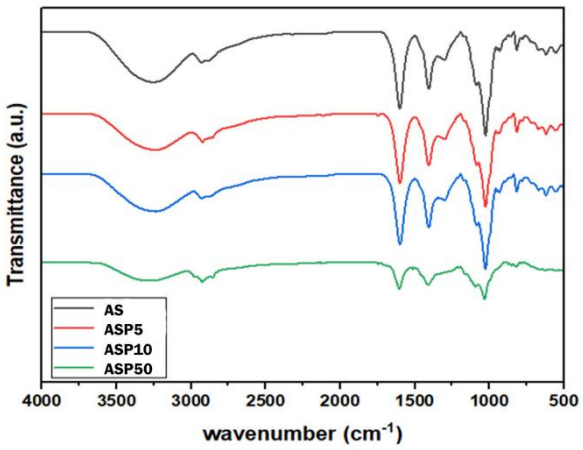


**Figure 3.** FT-IR spectra of AS and sporopollenin-alginate composite films (ASP5, ASP10, and ASP50).

### 3.3. TGA

The thermograms of all films are presented in Figure 4. For the AS film, only three mass loss stages were observed, whereas for ASP5, ASP10, and ASP50 films, the mass loss occurred in three distinct stages. In all films, the first mass loss corresponds to the evaporation of adsorbed water (Tavassoli-Kafrani et al., 2016). The second mass loss is attributed to the decomposition of glycerol (nearly 200 °C), as reported in the literature. The third mass loss is associated with the degradation of the polymeric structure of alginate (between 335-480 °C) (Chiappe et al., 2017). The third mass loss, observed between 370-550°C (DTGmax, °C), is attributed to the degradation of the sporopollenin structure. The mass loss during the third stage increased progressively with the increasing sporopollenin content in the films. The degradation temperatures of both alginate and sporopollenin were close, giving a single broad degradation step. These findings suggest that sporopollenin is successfully incorporated into the alginate matrix.

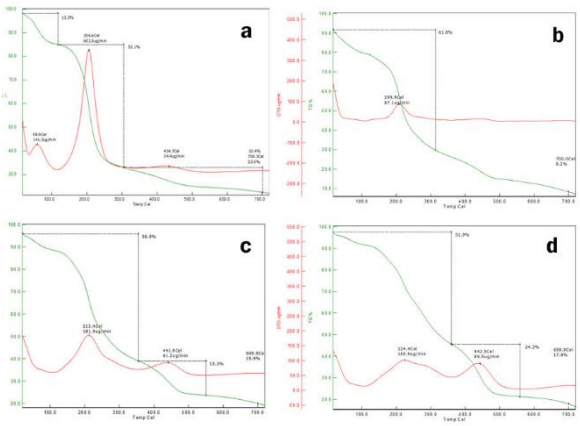


**Figure 4.** Thermograms of a) Control film; AS and sporopollenin-alginate composite films; b) ASP5, c) ASP10, and d) ASP50 films.

### 3.4. SEM

Scanning electron micrographs of dry *P. nigra* pollen, sporopollenin microcapsules are presented in Figure 5. As observed in Figure 5, the structure of sporopollenin is approximately 20-25 µm. In the extraction of sporopollenin, the genetic material and the inner part of the pollen were removed and sporopollenin microcapsules were obtained. As a result of heavy chemical treatments, no breakage of sporopollenin structures was observed and the pore openings formed are approximately 0.7-1 µm.

Figure 6 shows SEM images of alginate control film (AS) and alginate-based composite films to which sporopollenin was added at different ratios (ASP5, ASP10, and ASP50). It was observed that the AS film had a smooth structure (Figure 6a), while the roughness of the films increased with increasing sporopollenin concentration (Figures 6b-d). The homogeneous distribution of sodium alginate and glycerol to form a film matrix and the presence of H bonds between them are the main reasons for the smooth structure of the AS film. According to the SEM images, sporopollenin is trapped in the film, but in homogeneously distributed. Increasing the sporopollenin concentration increased the roughness by disrupting the integrity of the film matrix. Moreover, the breakage of H bonds between alginate and glycerol and the formation of new interactions with sporopollenin caused the film to become more brittle. In conclusion, SEM analysis shows that sporopollenin was successfully integrated into the alginate film.

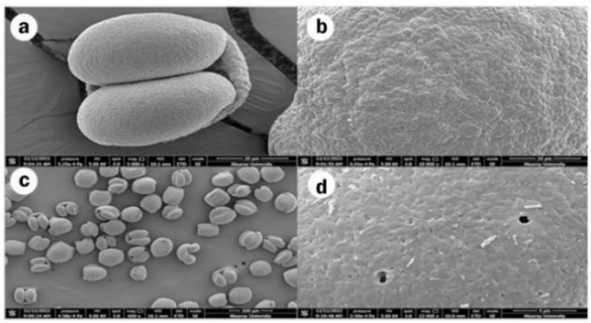


**Figure 5.** SEM images of *P. nigra* pollen (a-b), sporopollenin microcapsules (c-d).

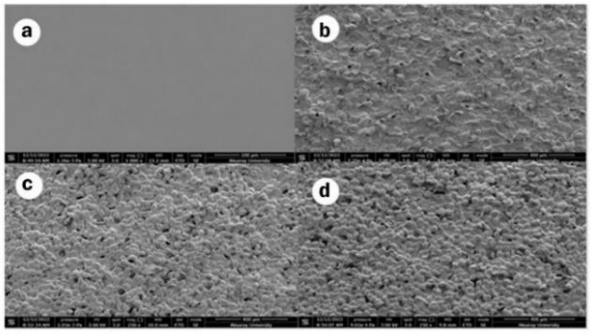


**Figure 6.** SEM images of a) AS, b) ASP5, c) ASP10, and d) ASP50 films.

### 3.5. MTT Assay

The main purpose of the MTT assay is to measure cell viability in connection with the activity of mitochondrial dehydrogenase enzymes. In living cells, ongoing mitochondrial activity reduces the MTT salt, forming formazan crystals. When these formazan crystals dissolve in solvents such as DMSO, they produce a purple color with intensity proportional to cell viability. The resulting coloration is then evaluated spectrophotometrically to obtain the MTT assay results.

MTT assays were performed at 24, 48, and 72 hours for the relevant biomaterials (Figure 7). As a control group, cells without any material, containing only DMEM culture medium, were selected. The percentage cell viabilities of the film samples compared to the control group at 24 h, 48 h, and 72 hours are shown in Figure 7. The cell viability results (MTT results) indicate that all 2x2 mm diameter samples did not exhibit cytotoxic effects on L929 cells at 24, 48, and 72 hours (values > ~70%). Additionally, at 72 hours, ASP50 showed a significant level of proliferation compared to samples ASP5 and ASP10 (**; $P<0.01$). These findings are consistent with previous studies reporting the biocompatibility of sporopollenin-based materials. Dyab et al. (2018) demonstrated that sporopollenin microcapsules preserved cell viability and maintained excellent cytocompatibility even after antibiotic encapsulation. Similarly, Akyuz Yilmaz et al. (2021) showed that chitosan–fungal spore blend films did not adversely affect cell viability. Together with our results, these studies reinforce the cytocompatible nature of sporopollenin composites and highlight their strong potential for safe biomedical applications.

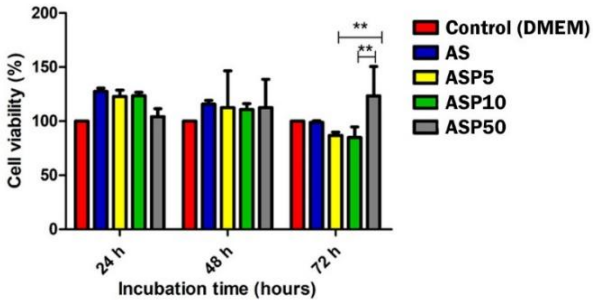


**Figure 7.** Cell viability assays of L929 cells after cultured on sporopollenin-alginate composite films for 24 h and 72 h (Bar represent mean of cell viability ± SEM; n = 3 statistical difference is showed as **$p < 0.05$).

## 4. Conclusion

In this study, sporopollenin grains isolated from *P. nigra* were used in the design of alginate-based composite films. It has been reported that mixing sporopollenin with alginate improves the thermal stability and acid-base resistance of the alginate film. Sporopollenin reinforced alginate-based composite films can be used in application areas such as material science and food

industry. Sporopollenin used in the study was obtained from *P. nigra* pollen. It is thought that microcapsules of sporopollenin obtained by removing the genetic material can be used as an ideal biomaterial in different application areas. Especially for the design of food coating, drug delivery and biomedical materials.

**Author Contributions**

The percentages of the author' contributions are presented below. The author reviewed and approved the final version of the manuscript.

| | B.A.Y. |
|---|---|
| C | 100 |
| D | 100 |
| S | 100 |
| DCP | 100 |
| DAI | 100 |
| L | 100 |
| W | 100 |
| CR | 100 |
| SR | 100 |
| PM | 100 |
| FA | 100 |

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= Submission and revision, PM= project management, FA= funding acquisition.

**Conflict of Interest**

The author declared that there is no conflict of interest.

**Ethical Consideration**

Ethics committee approval was not required for this study because there was no study on animals or humans.

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