

# Silk Fibroin Nanofibers Loaded with Hydroxytyrosol from Hydrolysis of Oleuropein in Olive Leaf Extract

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## **ABSTRACT**

*The purpose of this study was to prepare antimicrobial silk fibroin nanofibers from the aqueous formic acid solutions of silk fibroin and hydroxytyrosol with the in situ hydrolysis of oleuropein present in olive leaf extract using electrospinning method. With the use of aqueous formic acid solution of olive leaf extract and silk fibroin resulted in more uniform and beadless nanofibers. Morphological properties of electrospun nanofibers were investigated using Scanning Electron Microscope (SEM). The diameter of electrospun nanofibers ranged between 70 nm to 150 nm. The nanofiber diameter did not change much with increasing concentration of olive leaf extract added into silk fibroin solution to be used in electrospinning process. The increase in olive leaf extract concentration resulted in beadless and uniform nanofiber structures. The average diameter of the nanofibers prepared with fibroin solution having 10 % olive leaf extract was determined as  $85 \pm 10$  nm. Results revealing the formation of smoother and uniform nanofibers was attributed to the crosslinking effect of oleuropein and polyphenols present in olive leaf extract with certain functional groups in silk fibroin structure. Antibacterial properties of hydroxytyrosol loaded nanofibers against *Staphylococcus epidermidis* (Gram +) and *Escherichia coli* (Gram -) were confirmed with the clear inhibition zones observed in disc diffusion tests. Silk Fibroin nanofibers loaded with hydroxytyrosol may offer a new alternative biomaterial to be used in wound dressing or medical textile applications.*

## **KEYWORDS**

*Nanofibers, Silk fibroin, Electrospinning, Olive leaf extract, Antimicrobial*

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## **INTRODUCTION**

Nanofibers can be produced by various techniques such as drawing, template synthesis, phase separation, self-assembly and electrospinning. Among them, electrospinning is the most efficient and multifunctional method in order to produce nanofibers from polymer solutions. It allows us to prepare continuous fibres from both synthetic and natural polymers with a diameter ranging from micrometers to a few nanometers. By tuning the process parameters including applied voltage, hydrostatic pressure in the capillary, electric potential of the tip, the distance between the tip and the collection screen and feeding rate; solution properties including viscosity, conductivity, and surface tension; and ambient parameters including temperature, humidity, and air velocity in the electrospinning chamber, different conformation of nanofibers can be obtained [1, 2].

The electrospinning method can be used to prepare nanofibers with a wide varieties of biopolymers including collagen, chitin, chitosan, cellulose, silk fibroin, hyaluronic acid, and zein [3]. Besides, with the addition of some additives or natural compounds into the structure of nanofiber mats, unique and tailor made properties can be acquired [4]. In this manner, completely biodegradable, biocompatible and non-toxic functional nanofibers can be produced. The functional nanofibers can be used in many applications such as drug delivery, wound dressing, tissue engineering, sensor technology, filtration, energy storage, and reinforcement of composites [1].

Antimicrobial nanofibers can be obtained by adding some antimicrobial agents. There is an intense concern about antibacterial natural compounds. Especially, plant-derived components are commonly used for many industries. The olive tree is one of oldest cultivated trees. The leaves of this tree are used for centuries as a therapeutic agent and many researchers emphasize that there are a great deal of useful phenolic compounds in olive leaf [5]. Also, extract which is obtained from olive leaf destroys microorganism and free radicals that cause diseases and adverse effect on human health [5]. It was reported that main phenolic compounds present in olive leaves are oleuropein, hydroxytyrosols, rutin, verbaskosit, apigenin-7-glucoside, luteolin-7-glucoside, tyrosol, vanilic acid, diosmetin-7-glucoside, caffeic acid, luteolin, diosmetin, vanillin, and catechins [6, 7].

Although, oleuropein is the main phenolic component in olive leaf extract, the antioxidant capacity of pure oleuropein is limited. Olive leave extract having oleuropein as main constituent has enhanced antioxidant and antimicrobial activities after oleuropein is hydrolyzed into highly bioactive hydroxytyrosols [8-10].

Silk is a polyamino acid based protein which is produced by silkworms in order to protect themselves during their metamorphosis. For centuries, humans have harvested silk cocoons so as to produce textile manufacturing. Silk has a great deal of characteristic properties like luster, moisture absorbance and strength [11]. Silk fibroin is a good candidate in many biotechnological applications, for instance; medical textile, drug delivery and tissue scaffolding [11]. The characterization of biomaterials made from silk fibroin nanofibers have been the focus of many studies in the literature [12-14]. In the literature, the adsorption/desorption behaviour of oleuropein on different types of silk fibroin matrices including silk fibroin microfibers, regenerated silk fibroin, and silk fibroin nanofibers were also investigated [15]. There are only a few studies about the incorporation of natural compounds into silk fibroin nanofibers in the literature [16, 17]. In several studies, nanofibers loaded with natural compounds have been prepared by electrospinning polymer blend solutions including natural compounds [18, 19].

To the best of our knowledge, there is no article about the preparation of antimicrobial electrospun silk fibroin nanofibers from the aqueous formic acid solutions of silk fibroin and hydroxytyrosol as a result the in situ hydrolysis of oleuropein present in olive leaf extract.

In this study, first the aqueous formic acid solutions of silk fibroin and olive leaf extract containing oleuropein were prepared. During the preparation of these solutions in situ acid hydrolysis of oleuropein resulted in the formation of hydroxytyrosol with relatively higher antimicrobial property. Then silk fibroin nanofibers having antimicrobial properties were obtained by electrospinning of these prepared solutions [20].

## EXPERIMENTAL

### Materials and Methods

The olive leaf extracts were obtained using the olive leaves (*Olea europaea*) collected from the olive trees in Urla-İzmir. Analytical grade ethanol purchased from Merck, Germany was used in all extraction experiments. Raw silk fibroin fibres (SF) were obtained from Bursa Institute for silkworm Research (Bursa, Turkey).

For the removal of sericin, sodium carbonate (Aldrich, Germany) was used. Calcium chloride-2-hydrate (Riedel-de Haën, Germany) was used for the preparation of aqueous silk fibroin solution. Dialysis tubing (MW Cut-off: 12-14 kDa, Sigma, USA) was used to prepare purified aqueous silk fibroin solutions. Formic acid (98+ % purity) was purchased from Merck (Germany). HPLC grade acetonitrile (Sigma-Aldrich, Germany) and HPLC grade acetic acid (Merck, Germany) were used for the mobile phase of High Performance Liquid Chromatography (HPLC) analyses. Ultra-pure water was used for all experiments.

#### *Preparation of crude olive leaf extracts*

The collected olive leaves were first washed and then dried at 35 °C in an oven (Memmert UFP 800TS) for 3 days. The dried olive leaves were ground to prepare powder to be used for extraction experiments. Extraction was performed in 70 % aqueous ethanol solution with solid-liquid ratio of 1:20, at 180 rpm at room temperature in a bench top orbital shaker (Thermo MaxQ-4000) for 5 hours. Aqueous ethanolic extract was first filtered and then subjected to evaporation using rotary evaporator (Heidolph laborata 4001) to remove the ethanol under vacuum at 35 °C. Remaining aqueous phase of extract was centrifuged at 4000 rpm for 5 min to remove solid residues. The liquid aqueous extracts were first frozen and then lyophilized using Telstar cryodos-50 freeze drier for 3 days. After lyophilization, dry crude extracts were obtained. The crude olive leaf extracts were stored in glass bottles in a dark, cool, dry place for further use in experiments [15, 20].

#### *HPLC analysis of prepared olive leaf extract*

HPLC analyses were performed using the method described earlier in the literature [21]. The HPLC Equipment (Hewlett-Packard Series HP 1100) installed with LiChrospher® RP-18 analytical column (250 mm × 4 mm i.e.; with a particle size of 5 µm) thermostated at 30 °C was used. A diode array detector was used to monitor the absorbance changes at 280 nm chosen as stationary phase. The mobile phase flow rate for chromatographic analysis was 1 ml min<sup>-1</sup>. Briefly, the mobile phases were: (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95 % (A) and 5 % (B) to 75 % (A) and 25 % (B) during 20 min; it changed to 50 % (A) and 50 % (B) in 20 min (40 min, total time); in 10 min it changed to 20 % (A) and 80 % (B) (50 min, total time), after re-equilibration in 10 min (60 min, total time) to initial composition. Followed by HPLC analysis, concentration and abundance of oleuropein in samples were determined based on calibration curve of oleuropein standard (purity ≥ 90 %, Extrasynthese, Genay Cedex, France) [15, 20].

#### *Preparation of silk fibroin aqueous solution and regenerated silk fibroin (foam)*

For the degumming (removal of sericin) the raw silk was boiled in aqueous solution of 0.05 % sodium carbonate (50 times v/w) for 30 min. This boiling process was repeated three times. The degummed silk was washed with distilled water and dried at ambient conditions. In order to prepare aqueous silk fibroin solution 1.2 g degummed silk was dissolved in 20 (v/w) CaCl<sub>2</sub>/distilled water/ethanol (molar ratio 1:8:2) by stirring at 78 °C for 2 hours and then dialyzed at 4-8 °C for three days to remove neutral salts [22].

Aqueous silk fibroin solution obtained as the dialysate was filtered and then freeze dried for 5 days in order to obtain completely dried material in the form of foam as described in Figure 1 [15, 20].

#### *Electrospinning of solutions including silk fibroin and olive leaf extract*

Electrospinning setup (Figure 2) consist of High Voltage Power Supply, iseg T1CP 300 and Syringe pump, Newera NE1000. The Syringes and needles used during fabrication of nanofibers were purchased from medical suppliers. Regenerated silk fibroin foams were dissolved in formic acid along with olive leaf extract [15, 20].

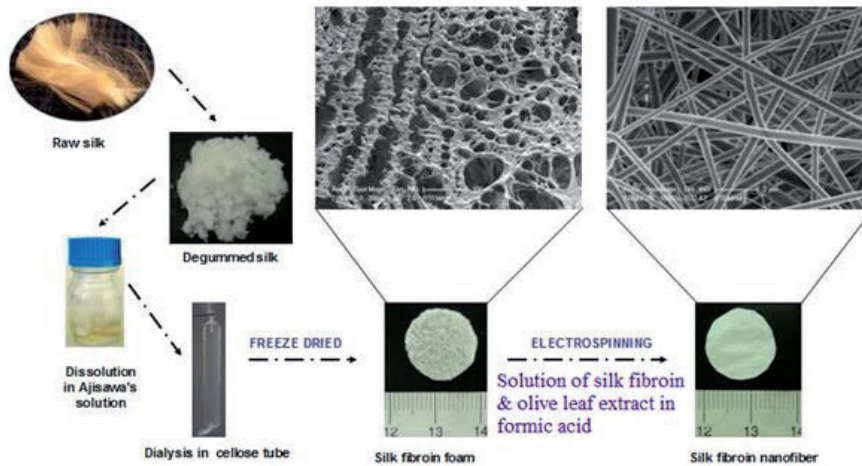


Figure 1. Process of preparation silk fibroin nanofibers from aqueous formic acid solution of regenerated silk fibroin (foam) and olive leaf extract

The SF solution with a concentration 80 mg/ml was prepared and olive leaf extract was added into this solution. Phenolic compounds present in the solution of olive leaf extract in formic acid were analysed with HPLC (Agilent Technologies 1100series).

Electrospinning was performed with a nozzle having a diameter of 0.8 mm. The nozzle tip was maintained at a high electric potential for electrospinning and mounted in the parallel plate geometry. A constant volume flow rate was maintained using a syringe pump. The voltage was kept at 20 kV and the distance between the syringe needle and the grounded collection plate was 10 cm. The electrospun nanofibers with and without olive leaf extract were collected on a collection plate covered with aluminium foil. Morphological properties of nanofibers were investigated using Phillips XL-30S FEG Scanning Electron Microscope (SEM). The samples were coated by gold sputtering in an argon atmosphere before SEM analysis. The Image measurement and visualization software was used to determine the average diameter of fibres based on the randomly chosen nanofibers from SEM images.

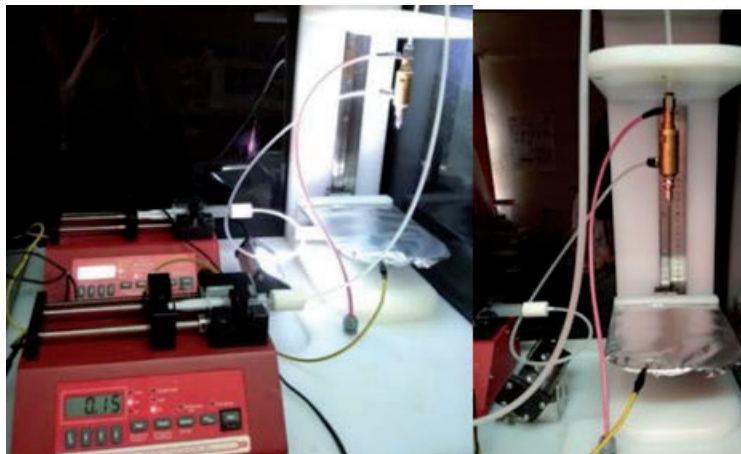


Figure 2. The photo of electrospinning experimental set-up used to prepare silk fibroin nanofibers from aqueous formic acid solution of regenerated silk fibroin (foam) and olive leaf extract

#### Antibacterial tests

Sterile cultures were prepared daily in 8 ml broth by transferring one loop of stock bacteria (*Staphylococcus epidermidis* (Gram +) and *Escherichia coli* (Gram -) which are kept in - 80 °C. These cultures incubated for

18 hours and subcultures were obtained by transferring 80  $\mu$ l from this 18 hour-incubated cultures to fresh broth (8 ml). Experiments were performed with this daily prepared subcultures which are standardized for inoculation on agar surface corresponding to certain numbers of CFU/ml. Log phases of growth curves were taken into account to reach approximate inoculation numbers also the standardized inoculums were confirmed by measuring OD values. In this study, six hours subcultures were taken in all experiments so as to use the same number of bacteria ( $6 \times 10^7$  CFU/ml). 100  $\mu$ l of bacteria culture (from 6 hours subculture) were inoculated onto agar surface. Inoculated culture was dispersed by streaking the sterile swab over the entire sterile agar surface by rotating the plate 60° each time to ensure the inoculum uniformly spread. The inoculated plates were allowed to sit for 5-10 minutes to let the broth absorb into agar. Silk fibroin nanofiber discs were placed on these petri dishes. Vancomycin (VA), Gentamicin (CN) and Penicillin (P) discs were used as a (+) control antibiotics. Plates were incubated for 24 hours at 37 °C. After 24 hours the inhibition zones were observed [15].

## RESULTS AND DISCUSSION

### *Content of olive leaf extract treated with formic acid*

The HPLC chromatogram of olive leaf extract dissolved in aqueous solution having no formic acid can be seen in Figure 3. As seen from the chromatogram, in the twenty-second minute oleuropein was detected as the major compound among other phenolics. Hydroxytyrosol and its derivatives along with other phenolic acids are detected between 0-12 minutes interval in the chromatogram.

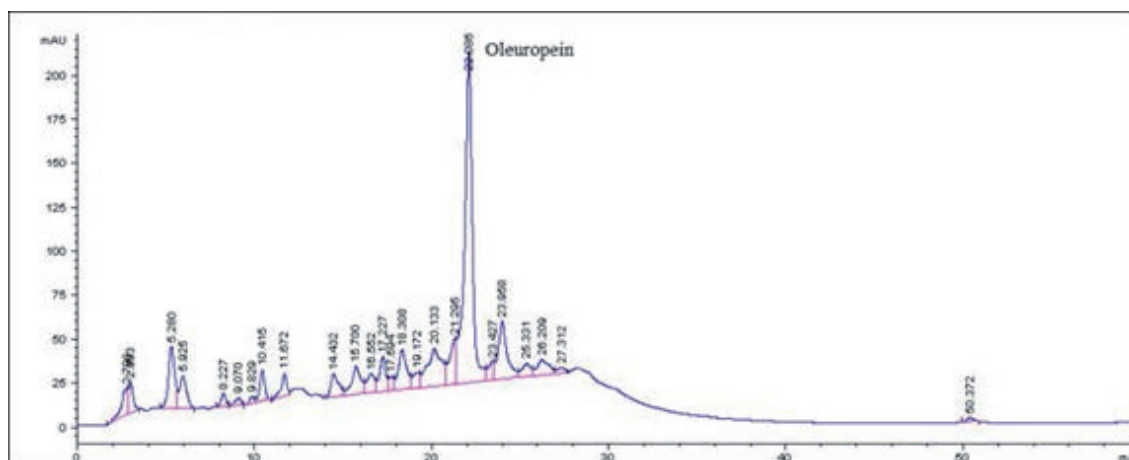


Figure 3. HPLC chromatogram of olive leaf extract dissolved in aqueous solution having no formic acid

HPLC chromatograms of olive leaf extracts dissolved in aqueous solutions having different amounts of formic acid (0 %, 25 %, 50 % and 100 %) are also given in Figure 4. As seen from the chromatograms the amount of oleuropein decreased with increasing formic acid content of the solutions. This indicated either degradation or the acid hydrolysis of oleuropein and formation of hydroxytyrosol/tyrosol derivatives. With increasing formic acid content, the solution became more acidic as expected. This acidic environment caused the hydrolysis of the oleuropein into hydroxytyrosol and its derivatives. The higher the acidity of the solution with increasing formic acid content, the higher amounts of hydroxytyrosol were formed with the hydrolysis of oleuropein.



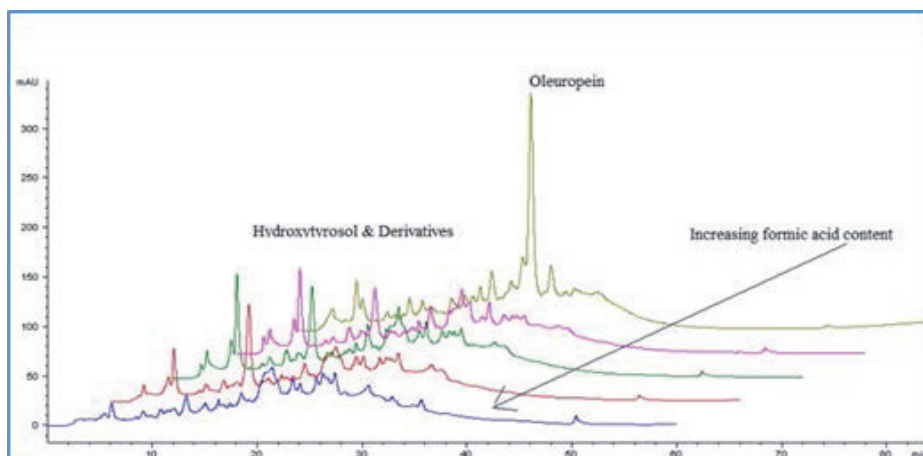


Figure 4. HPLC chromatograms of olive leaf extracts dissolved in aqueous solutions having different amounts of formic acid (increasing formic acid content: 0 %, 25 %, 50 % and 100 % formic acid)

Our results are in accordance with the results of studies reporting the acid and enzymatic hydrolysis of oleuropein to form hydroxytyrosol in the literature [9, 10]. The hydrolysis of oleuropein into hydroxytyrosol and its derivatives helped the enhancement of the antibacterial activity of the olive leaf extract having oleuropein as main constituent. Antimicrobial activity of oleuropein and hydroxytyrosol were also reported in the literature [23, 24]. Hydroxytyrosol has higher antimicrobial activity compared with that of oleuropein. Since nanofibers were fabricated via electrospinning of solutions including silk fibroin and olive leaf extract in formic acid, the prepared silk fibroin nanofibers contained the highly antimicrobial hydroxytyrosol due to the *in situ* hydrolysis of oleuropein during electrospinning process.

#### *Electrospinning of solutions including silk fibroin and olive leaf extract*

The diameter of electrospun nanofibers ranged between 70 nm to 150 nm. The nanofiber diameter did not change much with increasing concentration of olive leaf extract added into silk fibroin solution to be used in electrospinning process. The increase in olive leaf extract concentration resulted in beadless and uniform nanofiber structures (Figure 5). The average diameter of the nanofibers prepared with fibroin solution having 10 % olive leaf extract was determined as  $85 \pm 10$  nm. Results revealing the formation of smoother and uniform nanofibers could be attributed to the crosslinking effect of oleuropein and polyphenols present in olive leaf extract with certain functional groups in silk fibroin structure.

In the presence of  $\beta$ -glucosidase aglycones produced from Iridoid glycosides are responsible for the denaturation of proteins and being a crosslinking agent. Poly  $\alpha$ ,  $\beta$  - unsaturated aldehyde is produced by deglycosidation and oxidation of oleuropein. Oleuropein was also used as crosslinking agent in collagenic films [25]. In another study, oleuropein in OLE has been introduced as a natural, non-toxic cross linker for electrospun zein fibres. Homogeneous fibre morphology detected with alterations in bond structure in FTIR was attributed to the effects of crosslinking effect of oleuropein [3].

To the best of our knowledge, this is the first study to present OLE as a crosslinking agent in zein fibres which are commonly used in tissue engineering applications. In the light of these findings it can be proposed that OLE has a potential to be used as a cross linker in zein fibres as well as providing functionality attributed to its high antioxidant capacity and antimicrobial property.

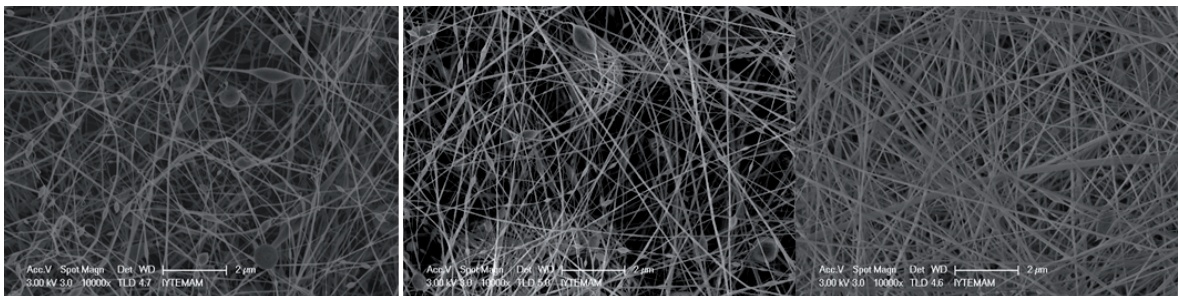


Figure 5. SEM images of prepared silk fibroin (SF) nanofibers without and with different olive leaf extract (OLE) by weight %; Left: 0 % OLE; Middle: 5 % OLE; Right: 10 % OLE. Magnification for all images: 10000x

### Antibacterial tests

In Figure 6 positive (+) and negative (-) controls are presented. The inhibition zones are seen belong to Gentamicin (labelled as G), Vancomycin (labelled as V) and Penicillin (labelled as P), respectively. Silk fibroin nanofiber discs (labelled as C) without olive leaf extract was used as the control.

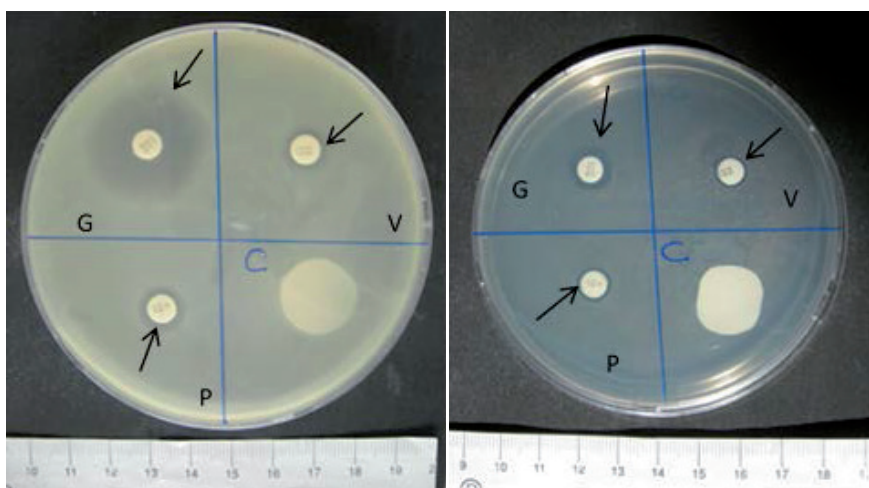


Figure 6. The pictures of inhibition zones observed in antibacterial disc diffusion tests for antibiotics, Gentamicin (labelled as G), Vancomycin (labelled as V) and Penicillin (labelled as P) against *E.coli* (petri dish on the left); *S. Epidermidis* (petri dish on the right). Silk fibroin nanofiber discs (labelled as C) without olive leaf extract. Arrows show the observed clear inhibition zones

As shown with arrows in Figure 6 significant inhibition zones were observed for all antibiotics. However, no inhibition zones were observed for the silk fibroin nanofiber discs (labelled as C) without olive leaf extract. The picture given on the left in Figure 6 shows the results of disc diffusion tests for silk fibroin discs with OLE against *E.coli*. The picture given on the right in Figure 7 shows the results of disc diffusion tests for silk fibroin discs with OLE against *S. Epidermidis*.

In Figure 7 discs labelled as A and C are silk fibroin nanofiber mats prepared with solutions having 40 mg/ml and 80 mg/ml olive leaf extract, respectively. The inhibition zones shown with arrows are clearly observed around the nanofiber discs. These inhibition zones indicated the antibacterial properties as a result of the hydroxytyrosol formed by the hydrolysis of oleuropein present in olive leaf extract in acidic environment.

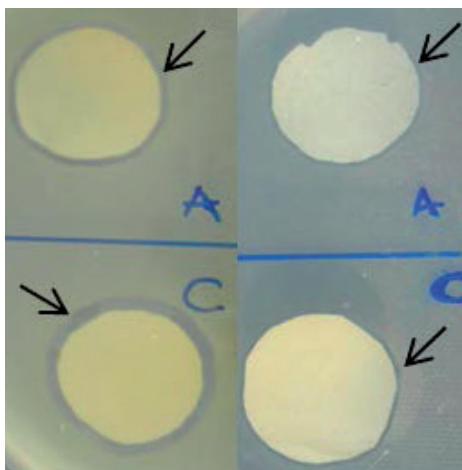


Figure 7. Results of antibacterial disc diffusion tests of nanofiber discs prepared from silk fibroin and olive leaf extract in formic acid: Left: *E.coli*; Right: *S. Epidermidis*. Arrows show the observed inhibition zones

## CONCLUSION

In this study, aqueous formic acid solutions of silk fibroin and olive leaf extract containing oleuropein were successfully prepared. In these solutions acidic environment caused the hydrolysis of oleuropein into hydroxytyrosol having relatively higher antimicrobial property. Hydrolysis was confirmed with help of chromatograms obtained from HPLC analyses. Silk fibroin nanofibers having antimicrobial properties were successfully obtained by electrospinning of these prepared solutions having hydroxytyrosol. Antimicrobial properties of these electrospun silk fibroin discs were confirmed with the results from disc diffusion tests against *E.coli* and *S. Epidermidis*.

Silk Fibroin nanofibers loaded with Hydroxytyrosol from the hydrolysis of oleuropein in OLE may offer a new alternative biomaterial to be used in wound dressing or medical textile applications.

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