J. Integr. Sci. Technol. 2022, 10(1), 53-60 Article

Journal of Integrated
SCIENCE & TECHNOLOGY

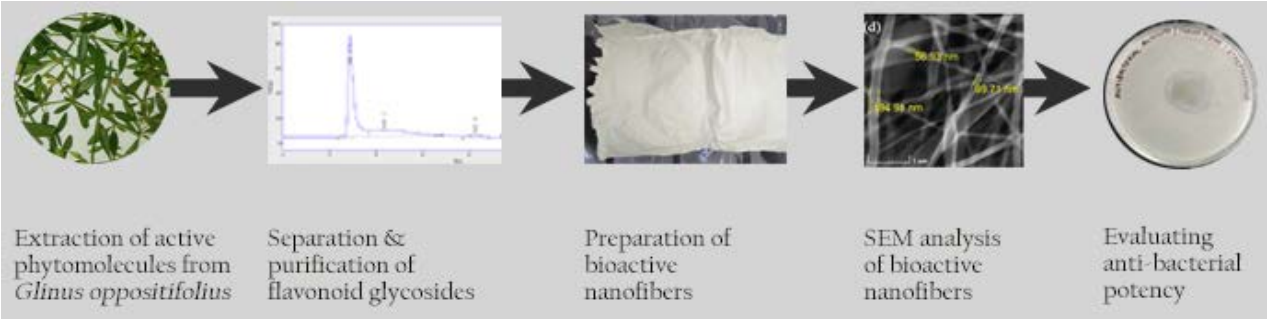
Novel electrospun nanofibers incorporated with flavonoid glycosides from *Glinus oppositifolius* (L.) Aug. DC. for antibacterial dressings

K. Suresh Kannan,[1] D. Kandavel,[2*] S. Balamurugan,[1] P. Rajalakshmi,[3] Selvakumar K[4]

[1]*Department of Botany, Thanthai Periyar Government Arts and Science College (Autonomous), Affiliated to Bharathidasan University, Tiruchirappalli - 620023, Tamil Nadu, India.* [2]*Department of Botany, Government Arts College for Men (Autonomous), Nandanam, Chennai - 600035. India.* [3]*Department of Zoology, Queen Mary's College, Chennai - 600004, Tamil Nadu, India.* [4]*Department of Applied Biosciences, BioLim Centre for Science & Technology, Chennai - 600023, Tamil Nadu, India*

Received on: 02-Jan-2022, Accepted and Published on: 04-Apr-2022

ABSTRACT



Electrospun nanofibers are non-woven fibers with diameters in nano size, produced from natural or synthetic materials or a combination of both using electrospinning apparatus. These nanofibers find usage in different fields, especially in medical research and the healthcare sector for their varied properties. Apart from the core polymers which are essential for nanofiber formation, several other bioactive ingredients are complexed in nanofiber production to add value to the end product. In this current research work, flavonoid glycosides from the medicinal plant *Glinus oppositifolius* were extracted, purified using column and high-performance liquid chromatographic methods and tested for their antibacterial efficiency. The bioactive compounds were then blended in the nanofiber formulation to produce nanofiber-based anti-bacterial dressings through the electrospinning technique. The nanofibers were tested through FTIR for analyzing the composition of the final product and further subjected to scanning electron microscopical analysis to study morphology and nano size. The nanofibers thus produced exhibited strong anti-bacterial activity against tested pathogens namely *K. pneumonia, P. vulgaris and Streptococcus sp*. The positive results showed that further research can be carried out to optimize the production and medical dressing applications of the nanofiber.

Keywords: Glinus oppositifolius, Electrospun nanofibers, Flavonoid glycosides, Antibacterial dressings

INTRODUCTION

Nanofibers are derived from both natural and synthetic materials and polymers of nanometer size. Nanofibers have high molecular weights and they belong to the size range of 1 to 1000 nm.[1] Nanomaterials including nanofibers, function as an alternative to many existing resources. They have several excellent properties, such as low cost and density and high energy and porosity. They also have a large surface area-to-volume ratio. Additionally, the nanofibers have the unique property of enhancing the functionality of the composite that has been incorporated with it. Over the past few years, this property of nanofibers has been capitalised on to produce promising applications in the biomedical field[2,3] as well as in materials sciences.[4–6] Nanofibers have been used in the biomedical field for drug delivery, tissue engineering applications, wound dressings[7] and in other applications like air filtration, microsensors, photocatalysis, etc.[4]

Among these, nanofibers in wound dressings provide multiple benefits. Nanofibers with the base of natural polymers such as chitosan that have been synthesised using the electrospinning technology exhibit properties such as high surface-to-volume ratio, high microporosity and versatility. Thus, their structure is suitable

Corresponding Author Dr. D. Kandavel, Associate Professor, Department of Botany, Government Arts College for Men (Autonomous), Nandanam, Chennai - 600035. India
E-mail: kandavel1976@gmail.com

Cite as: *J. Integr. Sci. Technol., 2022, 10(1), 53-60.*

 ISSN: 2321-4635 http://pubs.iscience.in/jist

for the extracellular matrix, promoting cell growth, adhesion and proliferation. They can help maintain a moist healing environment due to their high permeability and rate of absorption of the exudate formed on the wound. Nanofiber wound dressings are also found to improve the antimicrobial properties of the dressings which helps in reducing the growth of the microbes at the site of wound healing. This antimicrobial ability has been exhibited naturally by certain polymers used in nanofiber production (E.g., chitosan) or through incorporating antimicrobial agents directly during nanofiber production (e.g. gentamicin).[8]

Antibacterial wound dressings play a crucial role in the wound healing process as they provide distinct advantages such as reducing the bioburden, reduce the risk of invasive infection, protecting against contamination and facilitating wound healing.[9] The application of antimicrobial nanofibers in wound dressing has exhibited better coverage and protection of the wound from infection.[10] Among different antimicrobial agents available, plant secondary metabolites were found better option for developing antimicrobial nanofibers due to their wide range of antibacterial, antifungal, antiviral, insecticidal, anti-inflammatory, biocompatibility and low toxicity properties.[11–14] Antibacterial cellulose nanofibers developed by Liakos *et al* through the incorporation of cinnamon, lemongrass and peppermint extracts were found to inhibit the growth of *Escherichia coli* and exhibited low cytotoxicity.[15] Extracts from *Lawsonia inermis L* containing lawsone (2-hydroxy-1, 4-naphthoquinone) have been reported to reduce bacterial growth and accelerate the proliferation of fibroblasts and tissue granulation.[10]

Glinus oppositifolius is one of the widely used traditional medicinal plants and is well known for its antiseptic and anti-dermatitis properties. *G. oppositifolius* contains various types of bioactive compounds comprising carbohydrates, alkaloids, tannins, flavonoids, saponins, steroids, etc.[16] Extensive studies have been carried out to provide scientific evidence for the antifungal, antibacterial, antiplasmodial, anthelmintic, antioxidant, and anti-inflammatory activity of *G. oppositifolius.* A study in 2020 by a group of researchers from Thailand demonstrated the antibacterial activity of *Glinus oppositifolius* against different bacterial species namely *Bacillus subtilis, B. cereus, Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *S. epidermidis.*[17] Martin-Puzon *et al* proved that the biochemical constituents of the *G. oppositifolius* were effective against antimicrobial resistant bacteria such as methicillin-resistant *Staphylococcus aureus,* vancomycin-resistant *Enterococcus*, multidrug-resistant *Escherichia coli* and *Pseudomonas aeruginosa.*[18] The current study focuses on isolating the antibacterial flavonoid glycosides from *G. oppositifolius* and incorporating them into nanofibers production using electrospinning and evaluating their efficiency in antibacterial wound dressing applications.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents used in the study were purchased from reliable manufacturers such as Sigma, Himedia and Merck, India.

Plant sample collection

G. oppositifolius (L.) leaf and stem parts were collected from Surapet, Chennai, Tamil Nadu, India.

Plant authentication

The plant specimen was authenticated as *G. oppositifolius* (PARC/2017/4765) by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, Institute of Herbal Science, Chennai - 600045, Tamil Nadu, India.

Extraction of active compounds using different solvents

The plant sample was air dried in clean laboratory conditions for 15 days. Dried plant materials were crushed into a fine powder using a grinder and sieved. The active compounds from the plant material were extracted using the maceration technique with methanol as the solvent. The dry plant powder and methanol were mixed in a ratio of 1:10. The mixture was stirred in a magnetic stirrer at 100 rpm at room temperature for 48 hours. The mixture was then centrifuged to obtain the supernatant which is stored at optimal refrigerated conditions for further experiments.[19]

Qualitative analysis of plant extract for selective phytochemicals

Test for glycosides:

About 2 ml of the extracts were hydrolyzed with 1 ml of concentrated glacial acetic acid, 1% ferric chloride and concentrated HCl. The test mixture was observed for the development of greenish blue colour which indicates the presence of glycosides.[20]

Test for polyphenols:

1 ml of extract is warmed and added with 2 ml of 5% ferric chloride ($FeCl_3$) and observed for green or blue colour formation.[20]

Test for flavonoids:

1 ml of the extract was taken and added with 1% sodium hydroxide solution. Yellow colour formation indicated the presence of flavonoids.[20]

Quantification of total flavonoids in the plant extract

250 µl of 5% $NaNO_2$ solution was added to 0.5 ml of the plant sample along with 150 µl of 10% $AlCl_3.H_2O$ solution and incubated at room temperature for 5 min. Following the incubation, 0.5 ml of 1M NaOH solution was dissolved, added and then the total volume was made up to 2.5 ml with distilled water and the absorbance was read at 510 nm.[21] Quercetin was used as standard for flavonoid quantification.

Separation of flavonoid glycosides using column chromatography

Flavonoid glycosides (FG) were separated and partially purified through the column chromatographic technique. 3 ml of the methanol extract loaded on a glass column packed with silica gel (100G, 60-120 mesh size). Aqueous methanol was used in the mobile phase. Totally seven fractions were collected and subjected to flavonoid estimation.[22]

Partial purification of flavonoid glycosides using High performance liquid chromatographic analysis

HPLC analysis of the partially purified flavonoid glycosides from fraction F3 was conducted using an Agilent HPLC instrument. The analysis was performed using a C18 column (reverse phase) of 250 mm in length; 4.5 mm in width and 5 micrometres of particle size. Acetonitrile and phosphoric acid were used as mobile phases

A and B respectively. Elution was performed under 5°C and at a rate of 0.5ml per minute. UV spectra analysis was performed and Quercetin is used as the standard flavonoid compound.[23]

Antibacterial analysis of isolated flavonoid glycosides

Flavonoid glycosides thus isolated and partially purified were further evaluated for anti-bacterial efficacy against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (MTCC 740). Mueller Hinton Agar plates were made and spread with the test organisms using the spread plate technique. Wells were made in the plate aseptically using a cork borer. Partially purified flavonoid glycoside (50 µl) was added to the test wells. Sterile deionised water and Gentamicin were used as negative and positive controls respectively. The plates were incubated in a bacteriological incubator at 37°C for 24 h in an upright position.[24] Following the incubation, the plates were observed for the zone of inhibition and the measurements were tabulated.

Polymer preparation and production of nanofibers using electrospinning

10% Poly Vinyl Alcohol (PVA) solution was made in distilled water. The solution was mixed in a hot plate *cum* magnetic stirrer for 120 min at 80°C with constant stirring. The PVA solution was then cooled and mixed with an aqueous solution of FG (1%). The PVA/FG solution was further subjected to stirring for 60 min. The PVA/FG solution was then loaded in a 5 ml syringe and attached with a syringe needle. The syringe was then fixed in the electrospinning equipment and the 15kV voltage was applied to produce the nanofibers. The electrospinning process was carried out at room temperature with a flow rate of 0.04 ml/h. The nanofibers thus produced were collected in a drum collector wrapped with clean and thick aluminium foil. The distance between the needle and the drum collector was maintained at 150 mm.[25]

FTIR analysis of flavonoid glycoside embedded nanofibers

The nanofiber embedded with flavonoid glycosides was powdered and subjected to Fourier Transform infrared (FTIR) spectroscopy using a Shimadzu FTIR spectrophotometer. The analysis was carried out in the region of 500 to 4000 cm^{-1}.[26]

SEM analysis of flavonoid glycoside embedded nanofibers

Electrospun Phyto-nanofibers were subjected to Scanning Electron Microscope (SEM) analysis for studying the surface morphology. Small piece of nanofiber in the size range 5mm x 5mm was taken and mounted in the stub made of aluminium and fixed using the adhesive tape made of carbon. Further, the stub is coated with a thin layer of gold and subjected to SEM analysis under different magnifications using TESCAN – VEGA3 scanning electron microscope.[27]

Antimicrobial analysis of electrospun nanofibers incorporated with flavonoid glycosides

Antibacterial activity ability of the electrospun Phyto-nanofibers was conducted using plate assay. Experiments were carried out against *Klebsiella pneumonia* (MTCC 7028), *Proteus vulgaris* (ATCC 8427) and *Streptococcus* sp. (MTCC 9724). The test cultures were plated on Mueller Hinton Agar. A square piece (1x1 cm) of electrospun nanofibers containing flavonoid glycosides was placed on the agar plate under aseptic conditions. Nanofiber without flavonoid was used as the negative control. The plates were then incubated in a bacteriological incubator in an upright position at 37°C for 24 h (Modified from Q. Liu *et al*., 2021). Following the incubation, observation was made for the zone of inhibition and the readings were tabulated.

RESULTS AND DISCUSSION

Extraction and qualitative analysis of selective phytocompounds

Fresh samples of *G. oppositifolius* (Figure 1) were collected and subjected to the study. The plant samples were subjected to a maceration-based extraction technique using methanol as the solvent. Methanol was reported to be a more suitable solvent for the extraction of phytochemical constituents from plants due to its high polar nature and hence used in the study.[29] The extracts were subjected to qualitative selective phytochemical analysis for phenols, glycosides and flavonoids. The study focused on flavonoids and hence the tests were limited to selective compounds. The extract has shown positive for all the tested compounds (Table 1). Flavonoids were reported to be existing in glycoside form in the *G. oppositifolius*.[30] Therefore, the positive results for the tested compounds confirm the presence of flavonoid glycosides in the methanolic extract.

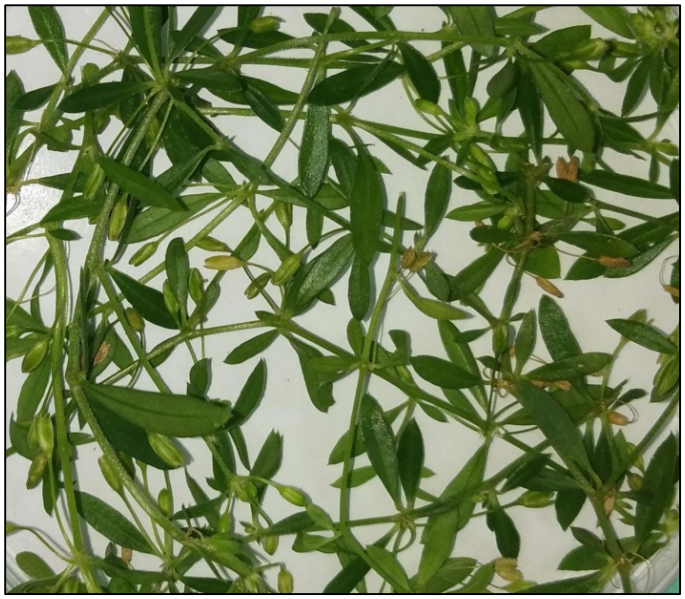


Figure 1: Leaf and stem parts of *G. oppositifolius*

Table 1: Selective qualitative analysis of methanolic extract of *G. oppositifolius*

Extract	Detection level
Phenols	+++
Glycosides	+++
Flavonoids	+++

+ Limited presence, ++ moderate presence, +++ strong presence,

Quantitative analysis of total flavonoid content in the methanolic extract

Further, the quantification of the total flavonoids present in the methanolic extract was made using an Aluminium chloride assay.

The assay works based on the complexation of the Aluminium and flavonoid and is one of the widely accepted spectrophotometric assays for quantifying flavonoids.[31] The total flavonoids were found to be 214.5± 2.53 mg Quercetin equivalent per gram of dry extract.

Isolation of flavonoid glycosides from methanolic extract

Silica gel column chromatography was used for the isolation of the flavonoid glycosides from the methanolic extract. 7 column fractions (F1 to F7) were obtained from methanolic extract of *G. oppositifolius* and the fractions were subjected to qualitative analysis and found to contain varying amounts of flavonoid in each fraction (Table 2). Among the 7 fractions, the F3 fraction was found to produce the strongest response for the flavonoid phytochemical test. There is no compound detection found in fraction 7.

Table 2: Total flavonoids content of methanol extracts of *G. oppositifolius*

Fraction	Qualitative observation value for the presence of flavonoids
F1	+
F2	++
F3	+++
F4	++
F5	+
F6	+
F7	-

+ Limited presence, ++ moderate presence, +++ strong presence, - absence

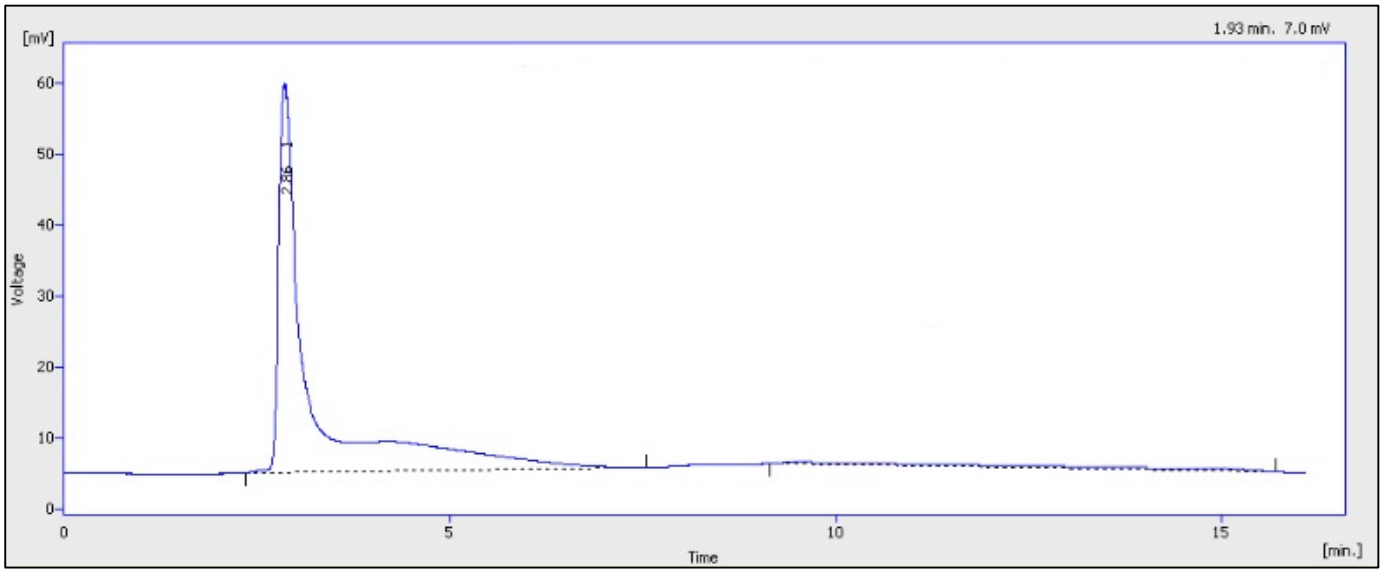


Figure 2: HPLC Chromatogram of Quercetin standard

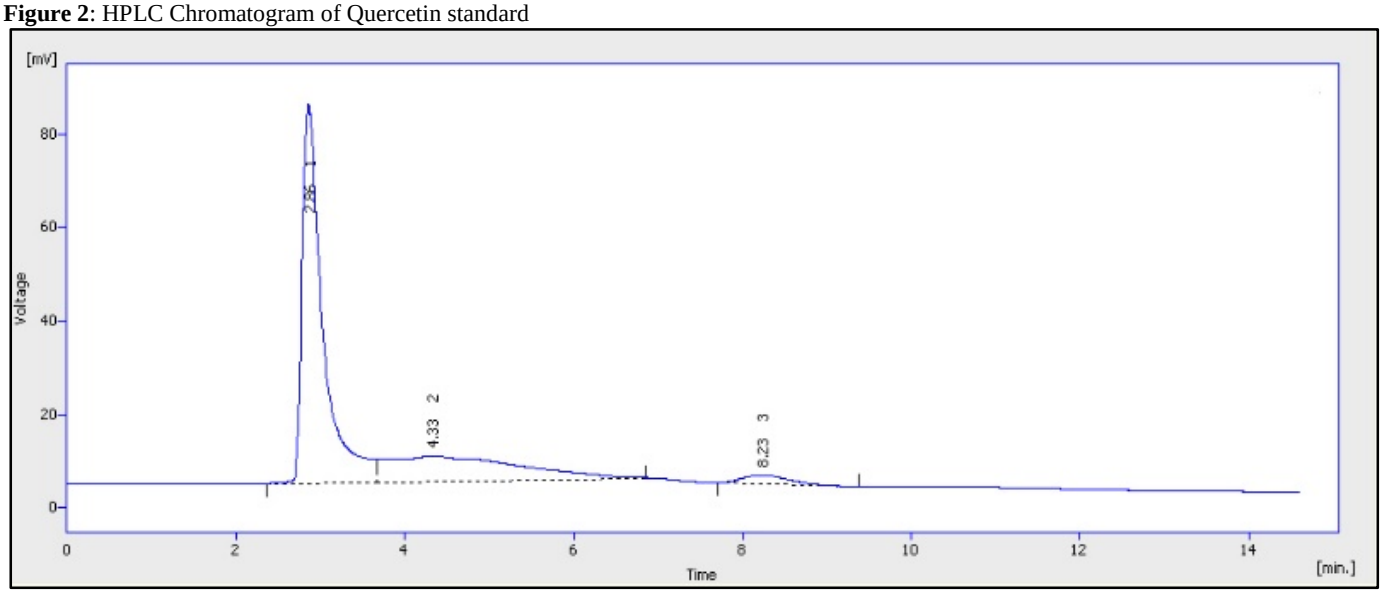


Figure 3: HPLC Chromatogram of chromatographic fraction 3

Partial purification of flavonoid glycosides using high-performance liquid chromatography

The fraction F3 which was found to contain a relatively high quantity of flavonoids were subjected to further purification using high-performance liquid chromatography. Quercetin was used as the standard reference compound. The chromatogram of F3 was found to produce a peak at 286 nm, similar to the reference compound. The purified fraction of F3 was obtained and used in further study.

Antibacterial activity of flavonoids glycosides

The purified flavonoid glycosides were tested for their efficacy in controlling the growth of pathogenic bacteria using a gel diffusion assay.[32] Flavonoid glycosides isolated from different plants were found to exhibit antimicrobial activity.[33,34] Flavonoid glycosides of *G. oppositifolius* are found to inhibit the growth of both the test organisms *E.coli* and *S. aureus.* The zone of inhibition was measured to be 24 and 25 mm respectively. Flavonoid glycosides were reported to exhibit bactericidal activity through disruption of the cytoplasmic membrane of the organism[33] and also through essential enzyme inhibition in the cell wall[35] and nucleic acid synthesis[36] etc.

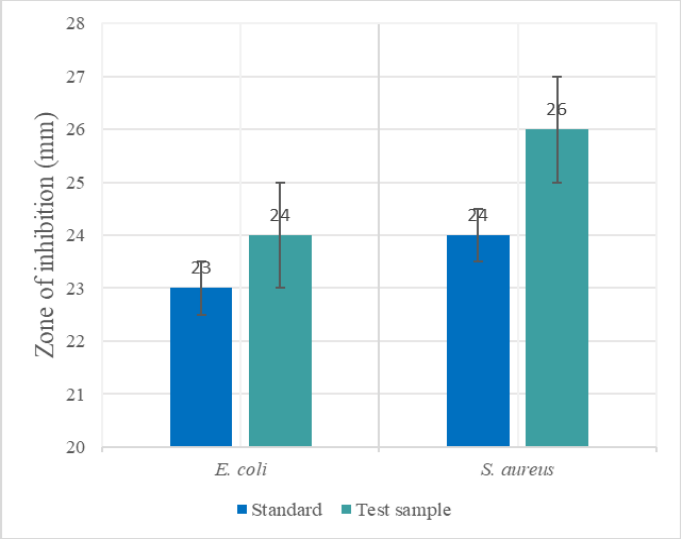


Figure 4: Zone of inhibition by flavonoid glycosides (Standard = Gentamycin)

Electrospun antibacterial nanofiber dressings

The flavonoid glycosides which show promising ability in controlling the growth of pathogenic bacteria were incorporated along with PVA polymer and the composition was used to produce the nanofiber sheet using electrospinning.[37] PVA is a biodegradable and biocompatible polymer and has been reported for applications in wound dressing material development due to its easy processable nature and mechanical strength.[38] Simplicity and versatile nature of the electrospinning mechanism help to create nanofibers with unique structures and properties to be used as dressing material.[8] We have produced a very thin sheet of dull white PVA/FG nanofiber, which we have further subjected to characterization and application studies.

Figure 5: PVA/FG nanofiber sheet produced through electrospinning

FTIR spectral analysis of PVA/FG nanofibers

FTIR test analysis has been performed to identify and confirm the presence of essential compounds that were used in nanofiber production. Figure 6 indicates the functional groups and bonding related to the PVA and FG components of the nanofibers. The O-H bond stretching identified in the 3200 to 3550 represents the alcohol. CH2 bending related to PVA was observed with the peak at 1436 and further stretching of C—O could be visualized with a peak at 1217[39]. The stretching found in the fingerprint region between 700 to 1800 represents the aromatic ring stretching and C-H stretching corresponding to the flavonoid glycoside components.[40]

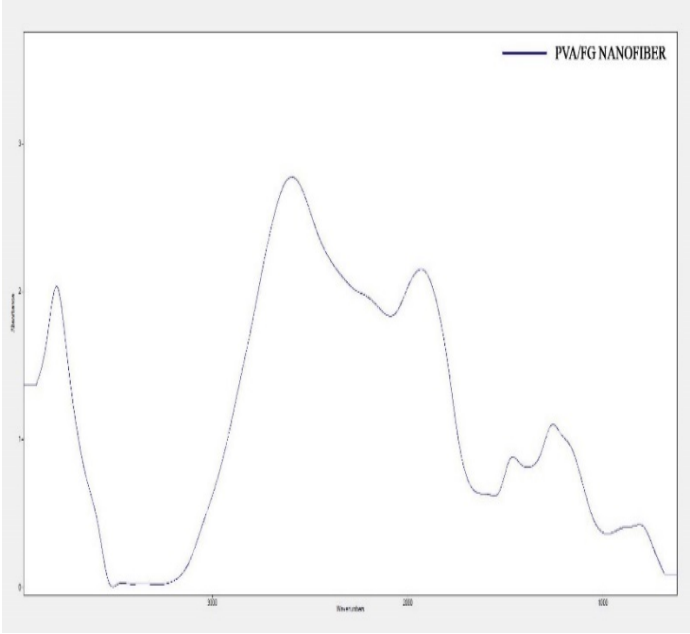


Figure 6: FTIR spectrum of PVA/FG nanofibers

Scanning Electron Microscopical analysis of PVA/FG nanofibers

SEM analysis of the PVA/FG nanofibers was carried out using standard procedure and the images were represented in Figure 7. Analysis was carried out under different magnification from 50 µm to 1 µm. Thin and branched nanofibers were observed under 10 µm. The size of the nanofibers ranged from 50 nm to 200 nm. At this lesser nano size range, PVA nanofibers can contain a high surface area to volume ratio and thereby can accommodate more flavonoid glycoside active molecules and can also assist in controlled release of the flavonoid over the wound healing for protecting the wound site from bacterial infections.[41]

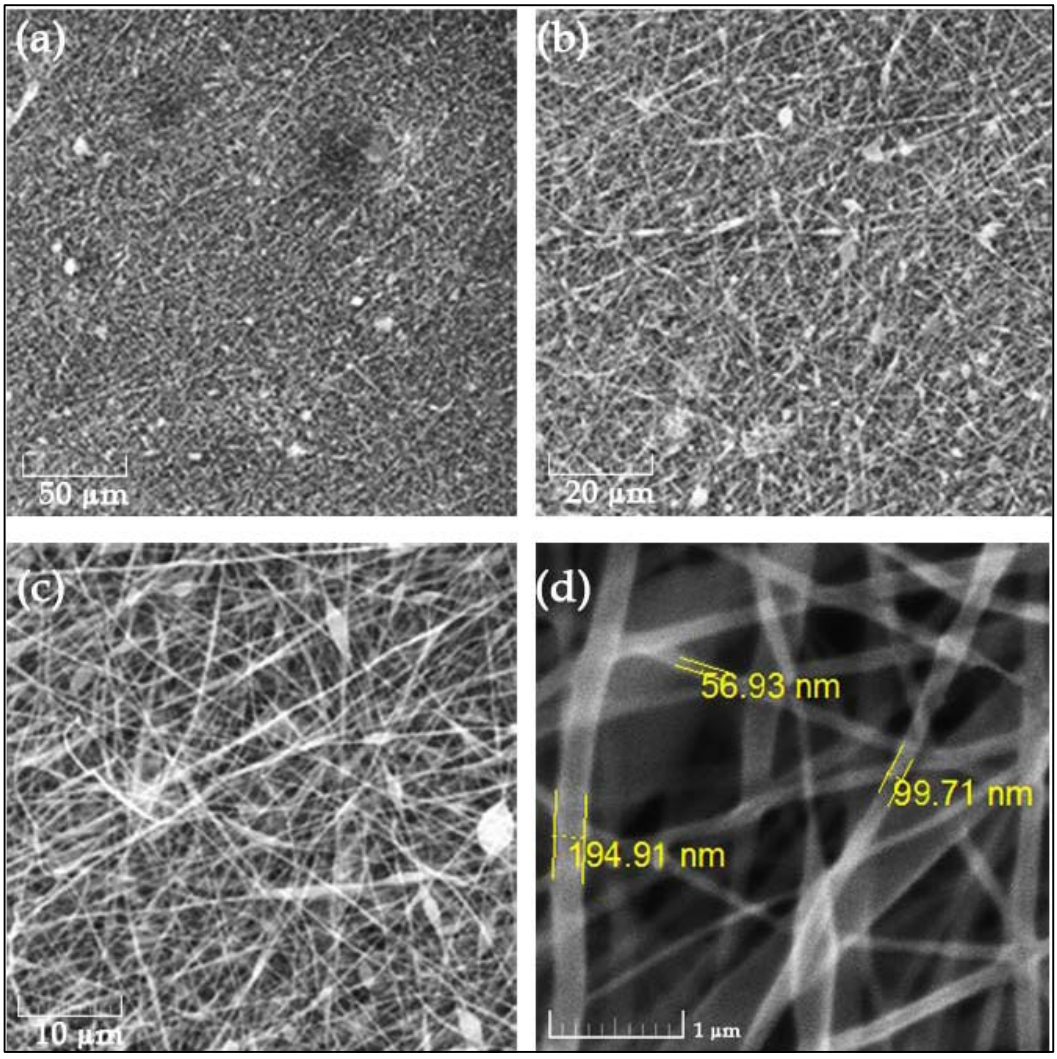


Figure 7: SEM images of PVA/FG nanofibers

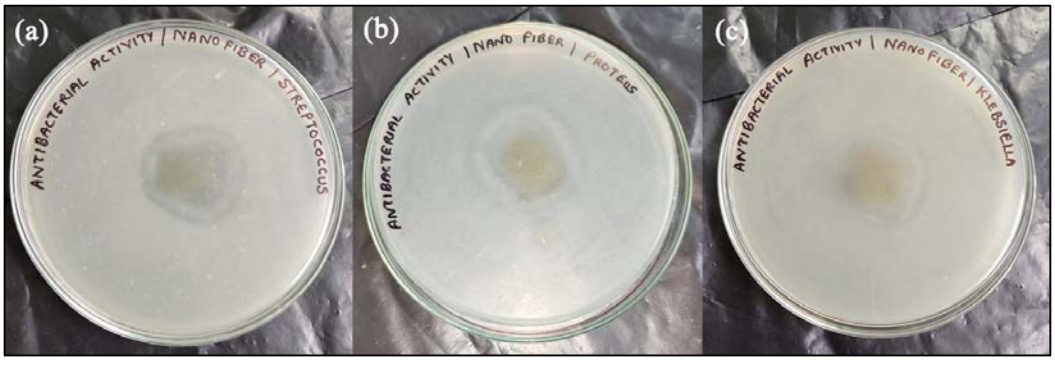


Figure 8: Antibacterial activity of PVA/FG nano fibers. (a) *S. pneumonia; (b) P. vulgaris;* (c) *K. pneumonia*

Antibacterial analysis of PVA/FG nanofibers

PVA/FG nanofibers were developed in the study to employ it as bioactive wound dressing material to control bacterial growth in wound infection. Antibacterial analysis of the PVA/FG nanofibers was carried out using three different bacterial pathogens namely *K. pneumonia, P. vulgaris* and *Streptococcus sp*. PVA/FG nanofibers have shown a clear zone of inhibition around the material which confirms the anti-bacterial activity exhibited by the PVA/FG nanofiber dressing material. Previous research studies have shown that nanofibers made with the composition of Poly Lactic-co-Glycolic acid (PLGA) and *Aloe vera* extract have shown very good antibacterial efficiency against tested pathogens, *Staphylococcus aureus* and *Staphylococcus epidermidis.*[42] Miguel *et al* have developed polyethylene oxide-Chitosan-Aloe vera nanofibers for wound healing applications and also studied the antibacterial properties. They have reported strong efficiency of the aloe vera extract microbes, *S. aureus* and *E. coli.*[43] PVA/Chitosan nanofibers made by Kegere *et al*, by blending the polymers with extract of *Biden pilosa* have shown antibacterial properties

against *E. coli* and 51.7% of *S. aureus*.[44] In accordance with the existing research results (Figure 4 & 8), the currently developed PVA/FG nanofibres have also shown promising results against tested pathogens.

CONCLUSIONS

Nanofibers are nanoscale entities that have wonderful applications in dynamic sectors. The current research work involved the production of nanofibers blended with bioactive flavonoid glycosides isolated from *G. oppositifolius*. Nanofibers are usually involved in wound dressing purposes and the incorporation of the flavonoid glycosides in the nanofiber extends its application with anti-bacterial potency. Such plant-based value-added nanofiber can act as cost-effective and less toxic wound dressing material that can be a good alternative to existing materials. Further research should be carried out to optimise the composition and characterization of this flavonoid glycoside enriched nanofiber-based wound dressing material.

CONFLICT OF INTEREST

The authors declare no conflicts of interest relevant to this article.

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