Original Article

Formulation and Characterization of Electrospun Nanofibers Loaded Fusidic Acid for Wound Dressing Technique

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Abstract

Electrospinning technology has been introduced as a new drug delivery system particularly when used for topical administration which shows excellent results in the treatment of various skin diseases. The combination of synthetic polymer (Polyvinyl alcohol) with biocompatible and biodegradable polymer (Chitosan) in a specific ratio to carry antibacterial agent (Fusidic acid) in the form of electrospun nanofibers to be used in wound dressing to treat different types of infections and ulcerations of the skin. The prepared electrospun nanofibers solution is tested for viscosity to assist in selection of suitable ratio of (PVA/CS) to load the antibacterial agent and produce nanofibers loaded fusidic acid. The evaluation of nanofibers is performed by studying the morphology of nanofibers, loading efficiency, swelling test, ex-vivo which carried by using Franz diffusion cell and in-vivo drug release profile by testing the nanofibers on rat to determine the percent of wound contraction and compare the average cumulative drug release with conventional dosage form to understand the bust mechanism from the surface of nanofibers and controlled drug release from the formula.

Keywords: Nanofibers, Electrospinning, Franz cell, in-vivo study

Introduction

Over the course of the last decade, wounds dressing and skin care have become worldwide interest in public health section because any serious infections and damages to this barrier may be dangerous and fatal if the complication is occurred; so the research and development in this area are necessary to achieve an efficient technique and new approaches to treat such conditions that related to skin infection in order to restore natural functions of the skin by improving the healing process which includes four stages; hemostasis, inflammation, proliferation and tissue remodeling [1].

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Dressing of wounds can be classified into four categories according to the severity and site of the injury; bioactive, passive, advanced and interactive dressing; the main role of wound dressing is to control the moisture content and stop bacterial invasion to the wound site [2].

Nowadays there are many biopolymers such as Chitosan and synthetic polymers such as polyvinyl alcohol are used to load antibacterial agent into the polymer solution to formulate as nanofibers of various properties according to the purpose of treatment and the time of administration [3].

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The main advantage of natural and synthetic polymer combination is to improve the action of each other by improving the mechanical strength, biocompatibility and biodegradability properties [4]. Studying the incorporation of natural with synthetic polymer such as PVA/Chitosan to prepare an electrospun nanofibers solution by co-axial, blend, emulsion and core-shield methods rely on therapeutic goal to treat primary and secondary burns, skin ulcers and wounds infection, through modified drug release [5]. In the current study the selection of suitable ratio of PVA/Chitosan to load antibacterial agent (fusidic acid) and produce nanofibers that used in the treatment of wound infections. Electrospun nanofibers loaded fusidic acid is used to form a barrier to stop bacterial invasion and provide moisture to the site of wound [6]; in addition the formulation of nanofibers based biopolymer improve the healing mechanism of the skin and enhance the antibacterial activity of fusidic acid [7].

Experiment

Materials

Chitosan (Deacetylated 80% of Chitin with moderate molecular weight of 88.000) is purchased from Anand Agro Care (India). Fusidic acid pure powder is purchased from Xa-Bc-Biotech (China), Polyvinyl Alcohol (Hydrolyzed 96% of average molecular weight 120,000) is purchased from Hainan Huarong (China) and acetic acid 0.1M is purchased from Drashti Chemicals (India).

Methodology

Characterization Study of Fusidic Acid

λmax Determination of Fusidic Acid

The solution of 0.1mg/mL fusidic acid in PBS pH 5.5 and methanol is prepared to determine λmax by using UV spectrophotometer at wavelength between 200nm-400nm [8].

Calibration Curve of Fusidic Acid

Fusidic acid calibration curve is determined by preparing a solution of 0.1mg/mL fusidic acid in Phosphate Buffer solution pH 5.5. Different concentrations of the solution are scanned for UV absorbance against concentration at λmax to obtain the calibration curve with mean value of ±S.D [8].

Determination of Melting Point of Fusidic Acid

Melting point is determined by using Stuart SMP20 device. Capillary tube method is used to obtain melting point of fusidic acid, the test is triplicated (n=3) with mean value of ±S.D [9].

Saturation Solubility of Fusidic Acid in Different Solvents

The study of fusidic acid solubility in different solvents is performed by using shake flask method, dissolving 1gm of fusidic acid in 10mL of DW and PBS pH5.5 in separated flasks to form a semi-saturated solution at 40ºC. Using 8µm filter paper and weighting the undissolved powder after excluding the weight of filter paper by using sensitive electronic balance (n=3) with mean value of ±S.D [10].

Electrospinning process

At the beginning setting the parameters of the device, filling the plastic syringe with 5mL of polymer solution, the syringe is fitted with a stainless steel needle of 3cm long 23G and 1mm of inner diameter [11]. The flow rate is controlled by using HPLC pump injection between 0.3-0.5mL/h. A high tension D.C voltage current of 16-30kv is applied. The distance between needle and collector is about 8cm. The nanofibers is deposited onto the collector and expelled after 1-hour of Electrospinning. The nanofibers are left to dry at room temperature for a few hours, to allow the evaporation of residual solvent [12].

Preparation Electrospun Nanofibers Polymer Solution

Preparation of PVA Solution

Solution of 10%PVA (96% hydrolyzed of molecular weight 120,000) is prepared by dissolving 10gm in 100mL DW with continues stirring at 80 ºC to obtain homogenous solution [13].

Preparation of Chitosan Solution

Solution of Chitosan powder (80% deacetylated Chitin of moderate molecular weight 88.000) is prepared by dissolving 2gm, 4gm, and 6gm in 100mL of 0.1M acetic acid with continues stirring for 2-hour at 80ºC to obtain 2%, 4% and 6% solution respectively [14].

Preparation of Electrospun Nanofibers Solution (PVA/Chitosan/FA)

The solution of 10% PVA is mixed with 2%, 4% and 6% Chitosan respectively; in ultra-sonication bath for 1-hour to obtain different ratios (8/2, 7/3 and 6/4 v/v) of polymer solution. The mixture is stored in refrigerator for 1-hour to ensure the consistency. Final step is the addition of 4% fusidic acid of polymer weight from 1% fusidic acid to the elctrospun polymer solution of different ratios to be ready for Electrospinning process [15].

Preparation of Electrospun Nanofibers Formulas

Fusidic acid solution of 4% of polymer weight is added to 10mL of (PVA/Chitosan) at different ratios to prepare 9-formula as show in the table 1.

Table 1: Formulation of PVA/Chitosan/F.A nanofibers solution

Formula	*PVA 10%(mL)*	*Chitosan 2%(mL)*	*Fusidic acid 4% (mL)*	*Total volume(mL)*
F1	8	2	3.5	13.5 mL
F2	7	3	3.2	13.2 mL
F3	6	4	2.8	12.8 mL
Formula	PVA 10%(mL)	Chitosan 4%(mL)	Fusidic acid 4% (mL)	Total volume(mL)
F4	8	2	3.6	13.6 mL
F5	7	3	3.4	13.4 mL
F6	6	4	3.2	13.2 mL
Formula	PVA 10%(mL)	Chitosan 6%(mL)	Fusidic acid 4% (mL)	Total volume(mL)
F7	8	2	3.8	13.8 mL
F8	7	3	3.6	13.6 mL
F9	6	4	3.5	mL

Optimization of Nanofibers Composition

Loading Efficiency of Fusidic Acid Nanofibers

The loading efficiency is determined by obtaining the amount actual drug to the theoretical amount that loaded into the formula by placing a piece of dried nanofibers in oven at 45ºC then immersed in 100mL of PBS pH5.5 for 8-hour; the amount of drug release is measured by using UV absorbance at 205nm, the test is performed three times (n=3) with a mean value of ±S.D [16]. According to the equation of percent loading efficiency:

Loading $efficiency$ (%) $=$ ($Drug\ loading$ / $Theoretical\ drug\ loading$)×100—[1]

Characterization of Electrospun Nanofibers Loaded Fusidic Acid

Fungilab S.A (Spain) is used to determine the viscosity of nanofibers solution. Functional groups are detected using FTIR spectrophotometer JASCO (Japan). Nanofibers diameter is analyzed by scanning electron microscope Thermo-Fisher Scientific (Japan), swelling percent, ex-vivo Franz diffusion cell and in-vivo study; are used to estimate the compatibility of the drug and excipient that used in the synthesis of nanofibers loaded fusidic acid of different ratios.

Viscosity Test

The determination of electrospun nanofibers solution viscosity is by using Fungilab viscometer. The principle of test is to select the suitable spindle size and speed at a constant temperature. The viscosity values are displaced on the screen in cPoise, the test is triplicated with mean value of ±S.D [17].

Scanning Electron Microscopy Analysis

Thermo Fisher Scientific Electron Microscopy China is used to evaluate the morphology and microstructure of the synthesized of electrospun nanofibers acceleration voltage of 10kV. Image analysis program ImageJ is used to determine the average nanofibers diameter by mean of ±S.D of about 100 fibers chosen randomly from each image sample [18].

Fourier-transform Infrared Spectroscopy (FTIR) analysis

Fourier-transform infrared spectroscopy JASCO (Japan) is used to obtain the functional groups of components by detecting the wavenumbers between 4000–400cm-1, using the KBr pellets of the classical technique [19].

Evaluation of Nanofibers Loaded Fusidic Acid

Swelling Test

The swelling percent is measured the amount of fluid being absorbed by the polymer; nanofibers piece of each prepared formula is immersed in PBS pH5.5 for 24-hour for different time interval (1, 3, 6 and 10 days) at 37 ºC. The swelling value is determined triplicate with mean value of ±S.D; using the following equation [20]:

$S = (Ww - Wd)/Wd \times 100$ ------ [2]

Ww is a function of immersion time; Wd is the dried weight of the samples

Ex-vivo Franz Diffusion Cell Drug Release and Release Kinetics

Franz diffusion cell apparatus is made by Thawabit Al-Masar for medical supplies (Iraq), the device is used to study the

drug release profile. Excised rat skin of 4.5cm diameter is place on the receptor compartment and a piece of 2×2cm of selected formulas is applied on the top of the excised rat skin then adjusting the donor compartment, the cell contains 20mL of PBS pH5.5 at 37ºC; the collected data is compared with the results from conventional dosage of fucidin 2% ointment Leo. The percent cumulative drug release during 7-hour is determined by using UV absorbance at 205nm. The test is triplicated with the mean value of ±S.D [21].

Release Kinetics

The obtained data of average cumulative drug release follow kinetics model to interpret the mechanism of release from the formula. The data may fit with zero order, first order, Hixson, Higuchi and Korse– Peppas [22].

Best Formula Selection

The selection of best formula is relied on the comparison of the detected results of average cumulative drug release from ex-vivo, in-vivo studies, loading efficiency and nanofibers diameter of nanofibers; which assist in the selection of best formula.

Stability Study

Short term stability test is performed for the selected nanofibers formula. Two pieces of nanofibers; batch 1 is kept inside sealed foil bag in refrigerator at 8ºC and batch 2 is placed in hot air oven at 40ºC without sealing; both are stored for one month. The stability test is using SEM imaging and short term drug release profile from the formula by using dissolution test. Short term stability assists in the evaluation of the structural morphology and the release behavior of the drug to confirm the activity and safety for medicinal use [23].

Statistical Analysis

The analyzed data are determined in triplicate (n=3) with mean value of ±S.D. according to one way analysis (ANOVA) at (P) level [24].

In-Vivo Antimicrobial Activity

Animals study

The antimicrobial activity of topical nanofibers loaded fusidic acid is estimated by using scratch test on rats to study the effects on the healing process and comparing the results with the conventional dosage form fucidin 2% ointment Leo. Animal study has been performed in the University of Al-Farahidi pharmacology department and commencement in January 2022. Twelve laboratory rats weighing between 185-245gm are incubated under controlled conditions; humidity 50% and temperature 25°C; provided with rat food and water; the experiment is last for 24-day. Intraperitoneal injection of 85mg ketamine/kg and 15mg xylazine/kg of body weight about 0.13ml, within 5 minutes and lasts for about 2 hours. The chest and back side of the rat are shaved by using hair foam removal about 3×3cm area, the wound is induced through scratching on the external skin by surgical blade; and infected with 10µL suspension of Staphyloccocus aureus [25].

Experiment

Three groups, each group includes four rats (n=4); Group1 is treated with normal saline; Group2 with fucidin 2% ointment; Group3 with 2×2cm of selected nanofibers formula every 12-hour; the treatment is last for 8days (n=3). The assessment of wound area is expressed in percent reduction to the original size of the wound which determined by using percent wound contraction equation [25]:

$$\%\, Wound\ contraction\ = \\ (wound\ area\ on\ day\ 0 - wound\ area\ on\ day\ n)/ \\ wound\ area\ on\ day\ 0\ \times\ 100$$ ------- [3]

Results And Discussion

Characterization of Fusidic acid

Determination of λmax of Pure Fusidic Acid

Fusidic acid in PBS pH5.5 is scanned at 205nm UV; which agrees with the absorbance of fusidic acid in methanol using UV spectroscopy [26]. Figure 1 shows UV absorbance of fusidic acid.

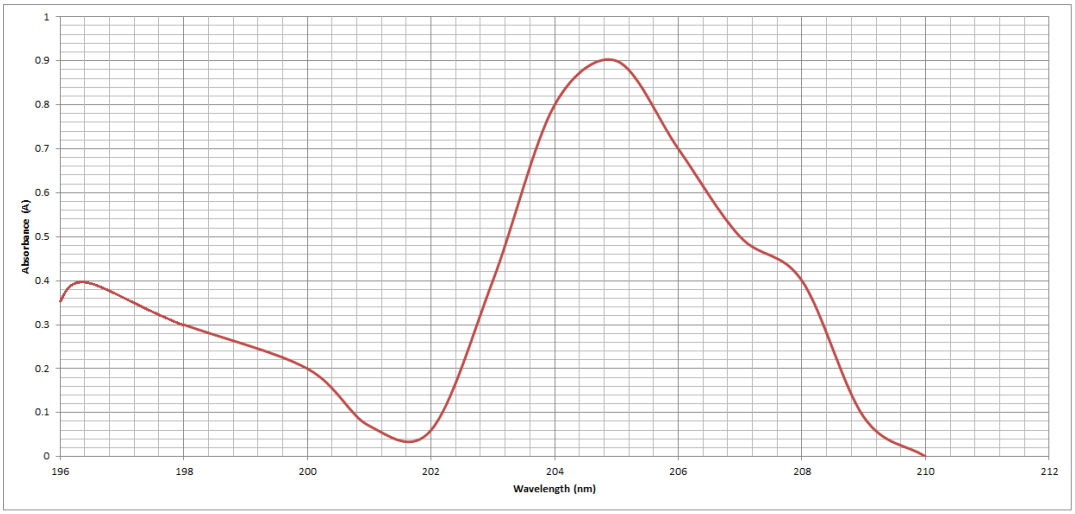

Fig.1: Fusidic acid UV absorbance in phosphate buffer solution pH5.5

Calibration Curve of Fusidic acid

The prepared fusidic acid in PBS pH5.5 solution is used to obtain calibration curve, at 205nm UV absorbance of different fusidic acid concentration which fits Beer-Lambert equation of straight line with correlation coefficient = 0.9981. Calibration curve is shown in figure 2 and table 2 illustrates the absorbance of fusidic acid.

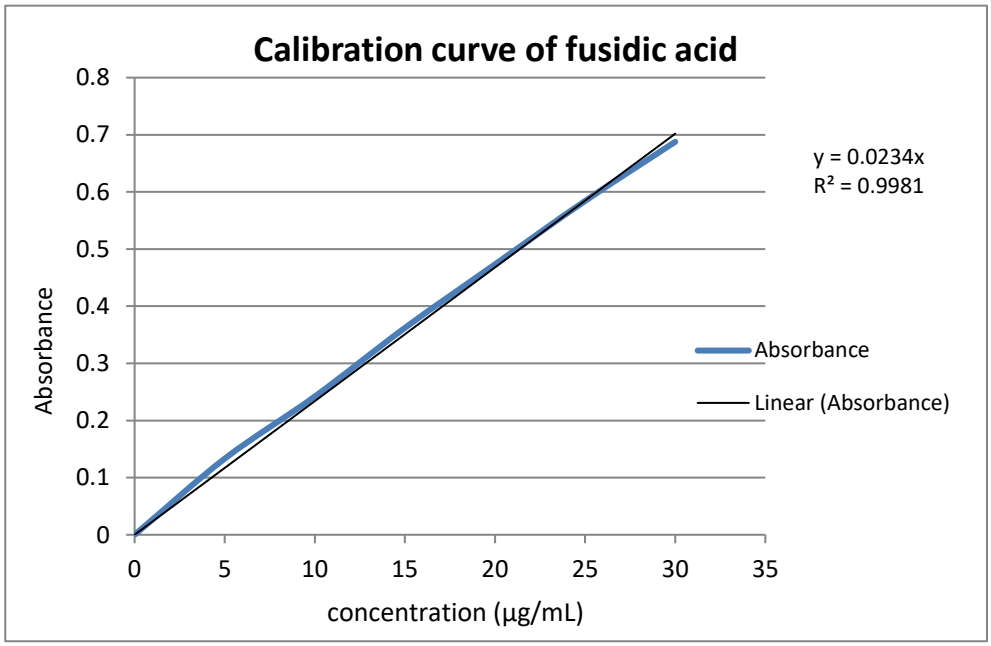

Fig.2: Fusidic acid calibration curve

Table 2: Calibration curve of fusidic acid phosphate buffer solution pH5.5

Concentration (µg/ml)	***5(µg/ml)***	***10(µg/ml)***	***15(µg/ml)***	***20(µg/ml)***	***25(µg/ml)***	***30(µg/ml)***
Absorbance	0.1332 ±0.04	0.2420 ±0.06	0.3621 ±0.03	0.4733 ±0.06	0.5842 ±0.05	0.6877 ±0.06

Melting point of Fusidic acid

The melting point of fusidic acid is detected at 193ºC; it agrees with reference of the pure fusidic acid data as mentioned in physical constant [27].

Saturation Solubility Study of Fusidic Acid

The solubility of fusidic acid is determined in DW (11±0.3mg/mL) and in PBS pH5.5 (38±0.2mg/mL). Fusidic acid is more soluble in slightly acid media and suitable for topical preparation. Table 3 shows the solubility data of fusidic acid in different solvent [28].

Table 3: The saturation solubility of fusidic acid in DW and PBS pH 5.5

Method	***Fusidic acid in solvent (mg/ml)***	***Result ±S.D***
Solubility	FA/PBS pH5.5	38±0.2mg/1ml
	FA/DW	11±0.3mg/1ml

Fusidic Acid Nanofibers Formulation

Solution of 1% fusidic acid in phosphate buffer solution pH5.5 is used to prepare 4% fusidic acid of polymer weight according to the ratio of polymer solution (8/2), (7/3) and (6/4) 10% PVA/ Chitosan 2%, the required volume is equivalent to 3.5mL, 3.16mL and 2.83mL of 1% fusidic acid solution respectively; and the same calculation to the remaining ratio [29].

Optimization Study of Fusidic Acid Nanofibers

Loading Efficiency of Fusidic Acid Nanofibers

The percent of loading efficiency represents the actual amount to the theoretical amount of the drug' it is important to understand the factors affect the accuracy of the experiment and therapeutic effectiveness of formula that contains a particular amount of drug. The collected data is ranged from 90.26% to 99.1% (F9-F1 respectively) due to the interaction between the molecules and the extent of drug solubility in each formula which is crucial in selection of best formula [30]. The percent of loading efficiency is shows in figure 3.

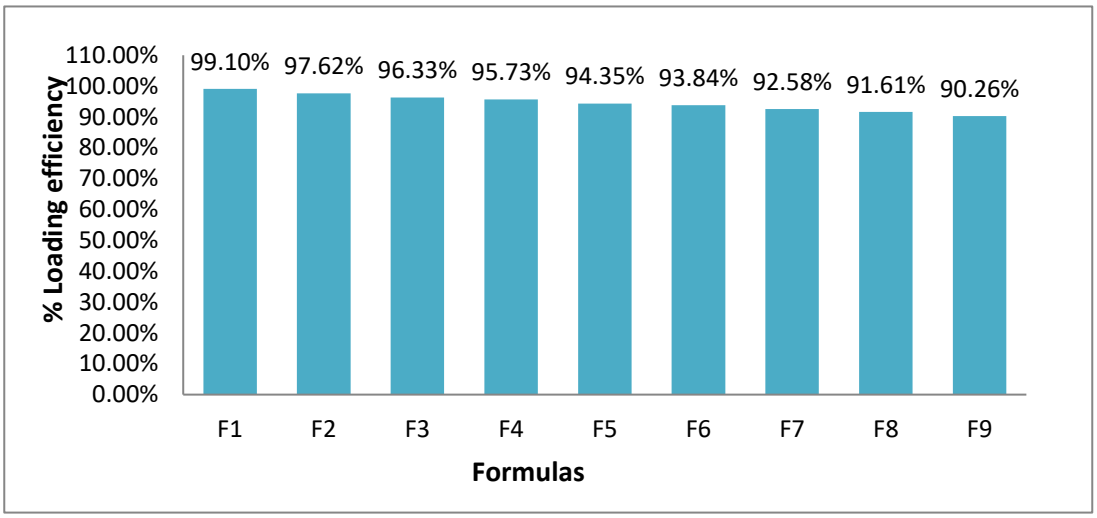

Fig. 3: The percent loading efficiency of fusidic acid

Characterizations of Nanofibers

Viscosity of The Polymer Solution Loaded Fusidic Acid

The viscosity data of polymer solution loaded fusidic acid is tested triplicate with the mean value of ±S.D and displaced in cPoise as shown in table 4. The recorded data shows that the viscosity of the solution increases with increasing Chitosan concentration due to the formation of H-bonds between the polymers molecules and thereby affects the flow rate of the polymer solution from the nozzle and nanofibers diameter during the process of Electrospinning; because of small quantity of fusidic acid in formula, so there is no significant effect on the viscosity of the solution [31].

Table 4: Viscosity values of nanofibers solution using Fungilab viscometer

Formula	*Viscosity cPoise (±SD)*
F1	1132±4
F2	1202±2
F3	1239±4
F4	1441±3
F5	1463±2
F6	1495±5
F7	1579±3
F8	1591±4
F9	1634±5

Morphology and Structural Analysis of Nanofibers

The morphological study of nanofibers is performed by SEM to determine the diameter of the fibers, the main factor affecting the morphology is polymer ratio (PVA/Chitosan), when the concentration of Chitosan increases, reduces the diameter of nanofibers due to improving of charge density of the droplet surface [32], but it may decrease the wettability of the polymer and drug release from the formula because of increasing the rigidity of the fibers. The data of average diameter of formulas is displaced in table 5. The SEM image of uniform nanofibers formula is shown in figure 4 for F1 with defined nanofibers structure and arrangement. Morphology and topology studies are important to interpret the effect of ratio variation on the nanofibers diameter; figure 5 illustrates the acceptable average nanofibers diameter for F1, F2 and F3. So to obtain optimum nanofibers size for wound dressings, the diameter should be within the average range for nanofibers between 361±45 to 487±85nm that used in wound dressing [33].

Table 5: Average nanofibers diameter

Formula	*Average NF Diameter (nm) ±S.D.*
F1	420±40nm
F2	380±52nm
F3	322±31nm
F4	325± 25nm
F5	302±11nm
F6	284±26nm
F7	290±35nm
F8	278±30nm
F9	246±22nm

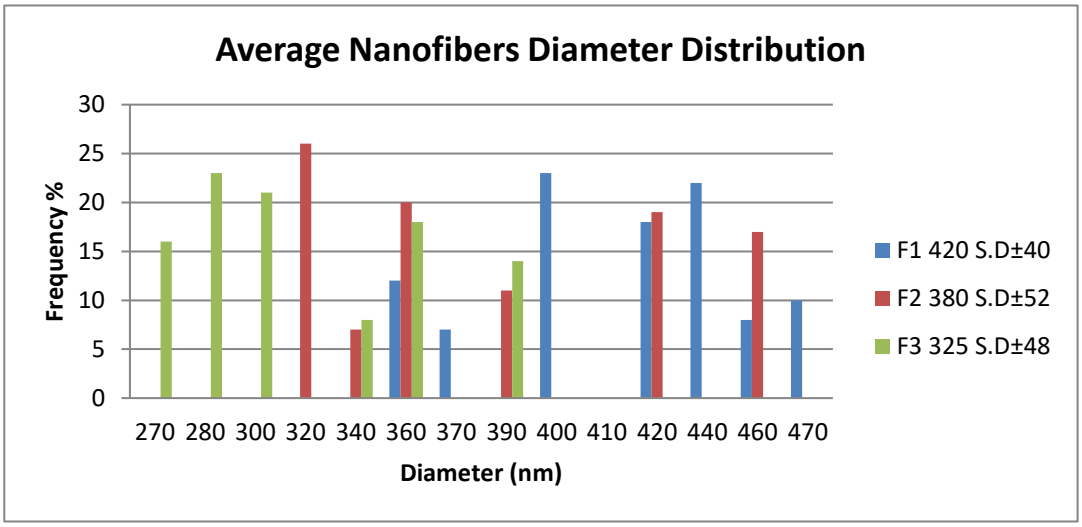

Fig.4: Average diameter distribution of nanofibers for F1, F2 and F3

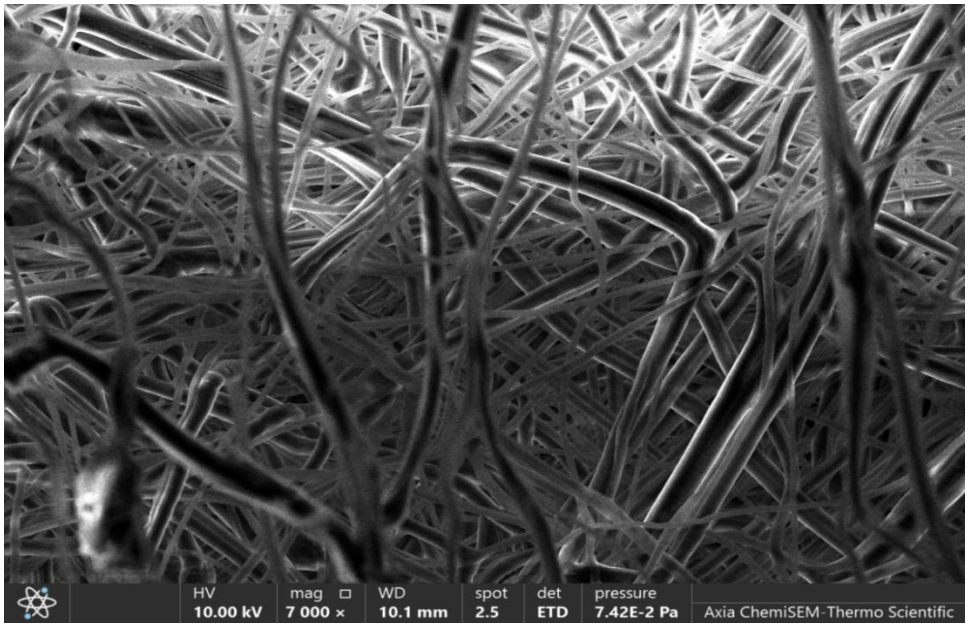

Fig.5: SEM imaged of obtained F1 (PVA/Chitosan/FA) nanofibers

Fourier-transform infrared spectroscopy (FTIR) analysis

FT-IR spectroscopy is used to interpret the compatibility of chemical groups in PVA, Chitosan, fusidic acid, mixture of them and nanofibers mat The interaction between functional groups influences the characteristics of final products; in addition to the effects of electrostatic and conductivity of the polymer solution [34].

For fusidic acid at the lower wave numbers there is some vibrations related to the presence of planar double bond deformation of C=C at 609cm-1, C=C–C at 748cm-1 and 752cm-1, C–H at 852 and 913cm-1 vibration of C-H bond, C=C–H at 975cm-1, C–H bonds outside the plane at 781.12 cm-1. C-H methyl group at 1443-1379cm-1; an intense stretching at 1686-1748cm -1, C-H at 2949cm-1 it's also essential for activity, finally there is a strong stretching vibration at 3446cm-1 that correlated with the O-H bond which is responsible for the interaction with the polymers [35]. Chitosan characteristic peaks between 844 to 1151cm−1 saccharide structure, at 1346 and 1480cm−1 presence of amide I and II peaks respectively, at 1655cm-1 N-acetylation of amide I mode, peaks at 2345, 1122 and 1072 cm−1 for C-O bond and peak at 2921cm-1 of C-H. The FTIR for PVA peaks at 3568cm−1 O-H bond, band at 2923cm−1 of the C-H of alkyl groups and peaks at 2345, 1654 and 1458cm−1 of C=O and C-O from acetate groups [36]. The interpretation of PVA/Chitosan/FA nanofibers peaks is shown in figure 5; at 1542 and 1560cm−1 the deformation of NH3+ groups and peaks of COOH dimers, lower amount of hydroxyl group O-

H at the peaks 3568-3588cm-1 [37]. The blend shows good compatibility result that may explain the stability of the nanofibers mat in PBS pH5.5 solution.

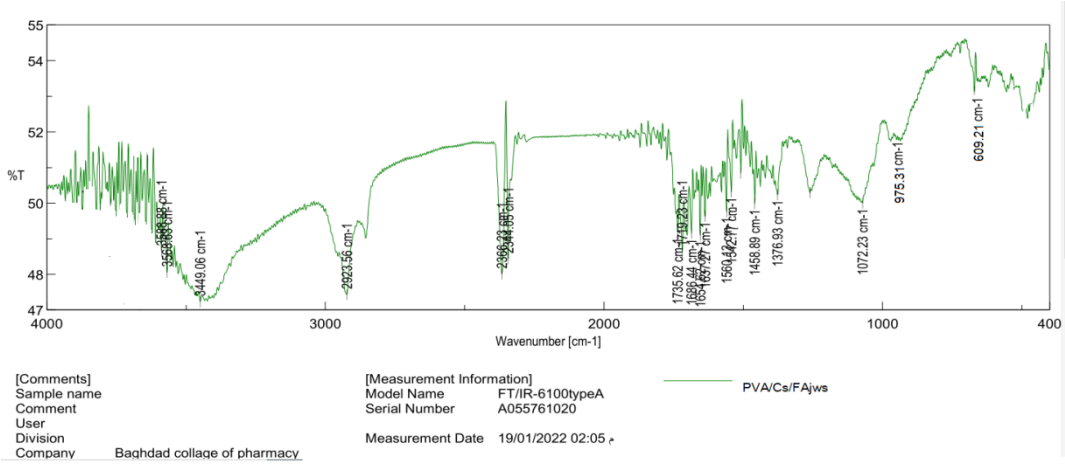

Fig.5: Fourier transform infrared peaks of nanofibers mixture

Evaluation of fusidic acid nanofibers

Swelling Test

The swelling percent of the selected formulas from SEM analysis represent the nanofibers that fall within the acceptable diameter range F1, F2 and F3. It is obtained by measuring the amount of fluid absorbed during day 1, 3, 6 and 10; the data is shown in figure 6; which reflect the effects of Chitosan on swelling of the polymer matrix, with increasing the amount of Chitosan the swelling percent decreases due to the formation of strong intermolecular forces between them, the small amount of fusidic acid does not affect H-bond formation between polymers [38]. The average weights for F1, F2, and F3 are 0.041, 0.051 and 0.061gm in mean value 0.001±S.D respectively before absorbing the buffer solution.

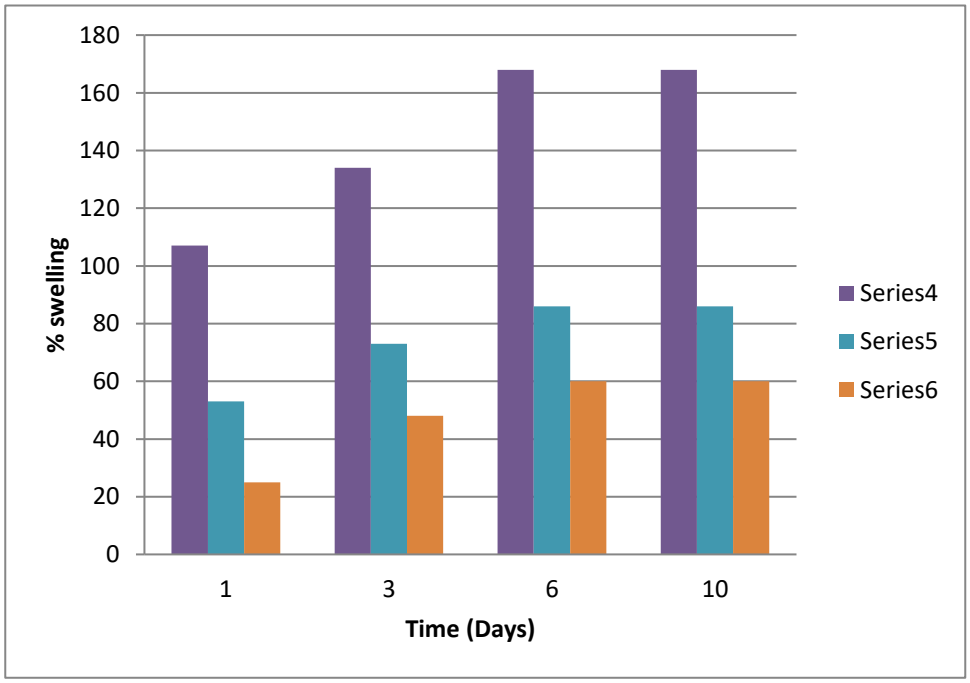

Fig. 6: Percent swelling of F1, F2 and F3 over 10-day (Series1,2,3) respectively

Ex-vivo Franz Diffusion Cell Drug Release and Release Kinetics

The drug release study is to predict the capability of nanofibers to enhance fusidic acid release from the nanofibers through the skin when compares with the conventional dosage form fucidin 2% ointment [39]. The average cumulative drug release during 7-hour is obtained by UV absorbance at 205nm as shown in figure 7; the maximum drug release according to the ranking 93.83% > 84.18%> 78.52% > 72.17% ; F1, F2, Fucidin and F3 respectively with mean value of ±S.D. The average percent of drug release in F1 and F2 appears to be higher than the ointment and F3. Obviously the solubility and drug release from the formula are mainly affected by the polymer ratio and the degree of swelling of the polymer matrix that control the release behavior.

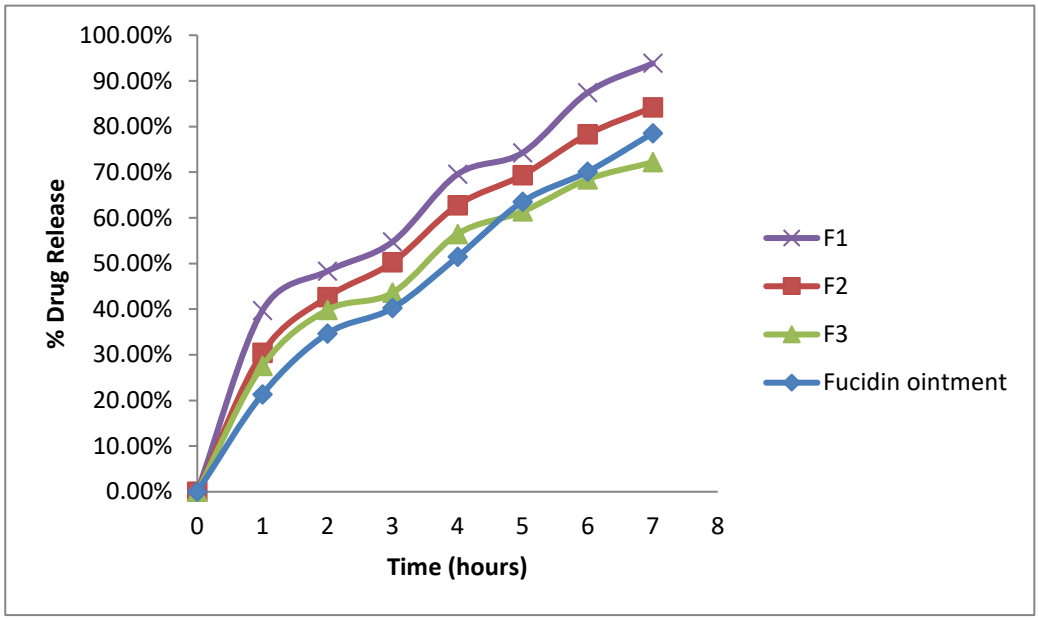

Fig.7: Average percent cumulative drug release of F1, F2, F3 and Fucidin nanofibers using Franz diffusion cell

Release kinetics

The data of average cumulative drug release fits with Higuchi kinetic model of regression coefficient (R2) = 0.986 and slope (m) = 0.348. The obtained data are plotted as average percent cumulative drug release against square root of time (Mt/M0 = kt0.5) [40].

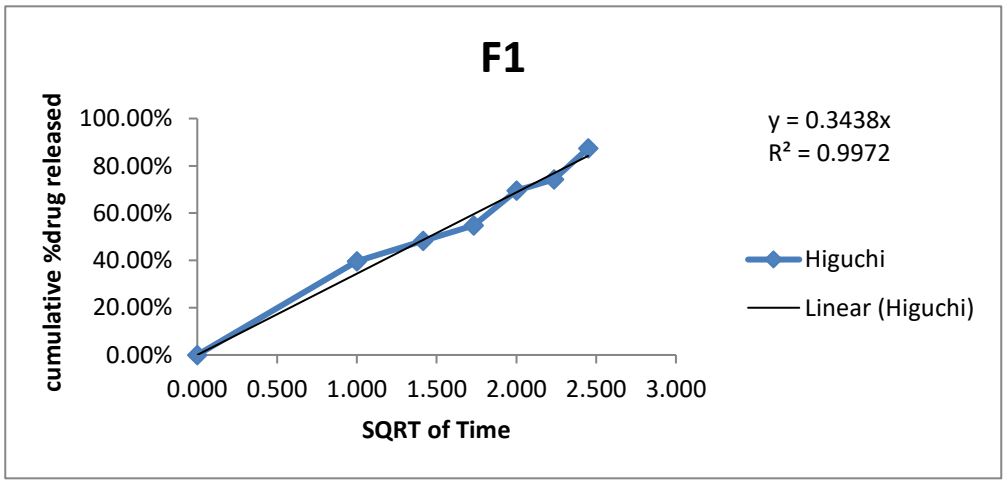

Fig.8: Higuchi kinetic model fitting with drug release from nanofibers

Best formula selection

The selection of best formula is based on the data of percent loading efficiency, viscosity, swelling percent, morphology by SEM and ex-vivo drug release study. All the data show F1 has the highest values among the remaining nanofibers formulas, thus it is selected as best formula.

Stability Test

Short term stability study is to detect the factors influencing the drug release form nanofibers under predetermined condition for 1-month; the results show that, the average percent of cumulative drug release from batch 1 (92.47%) has little difference with original tested formula (F1) in ex-vivo study (93.83%); while batch 2 shows a decrease in the initial burst release from nanofibers and at the end of 7-hour (29.83%) and (90.25%) respectively due to the loss of water molecules from the matrix and delay the wettability and drug release with time [41].

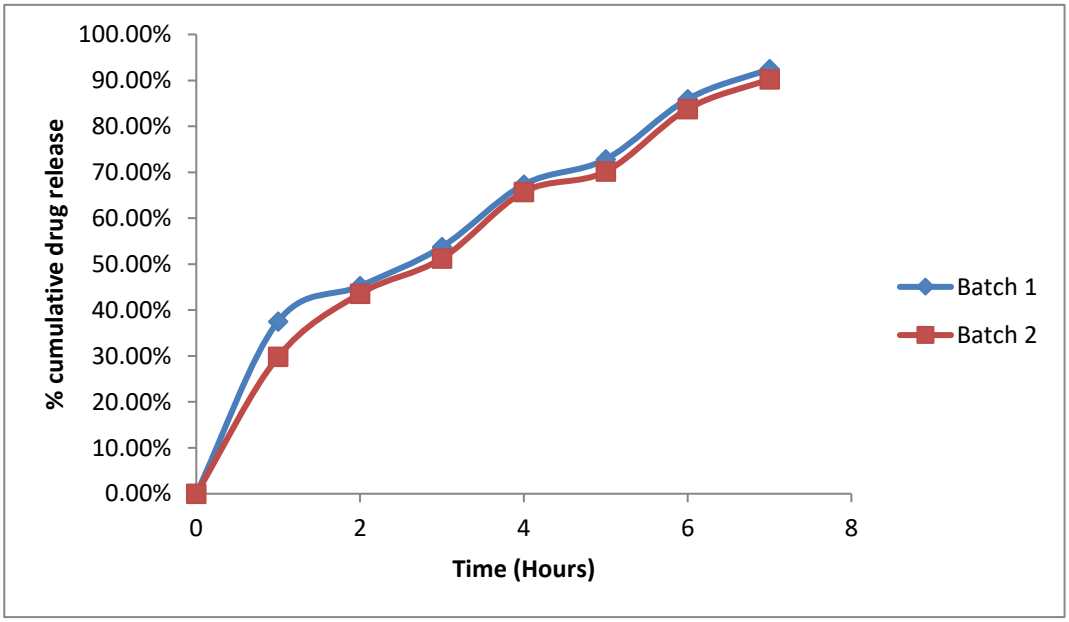

Fig.9: Short term stability of F1 under two conditions (Batch1 and Batch2)

Statistical Analysis

One way ANOVA analysis, using F distribution df (2, 15) (right tailed) shows P-value equals 0.000488515, [P(x ≤ F)=0.999511]. It means the chance of type1 error (rejecting a correct H0) is small: 0.0004885 (0.049%). The smaller the P-value the stronger it supports H1 [42].

In-vivo Antimicrobial Activity (Animal Study)

The obtained data of percent wound contraction on excised rat skin during 8-day show that treated with saline, fucidin 2% and fusidic acid nanofibers F1; statistically F1 is the higher percent after 8-day treatment as shown in figure 10 [43], the percent of wound contraction by using nanofibers gives rapid result and the skin healing mechanism shows improvement in shorter time. Nanofibers application on wound appears in the images of figure 11 clearly the healing of skin during 8-day of treatment [44].

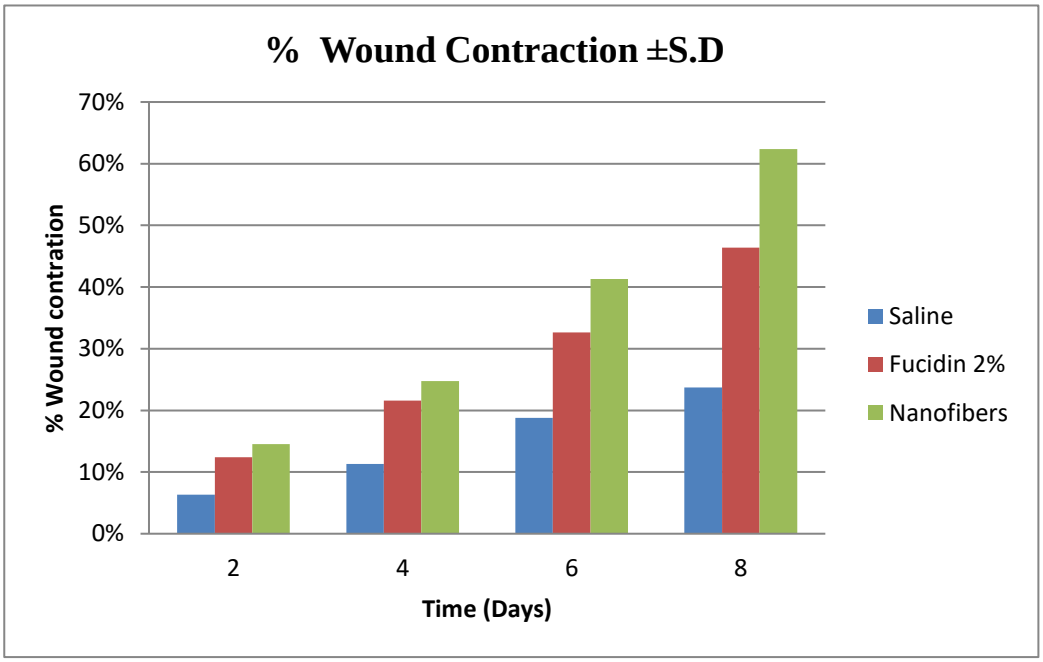

Fig.10: Percent of wound contraction in three treated groups for 8-day

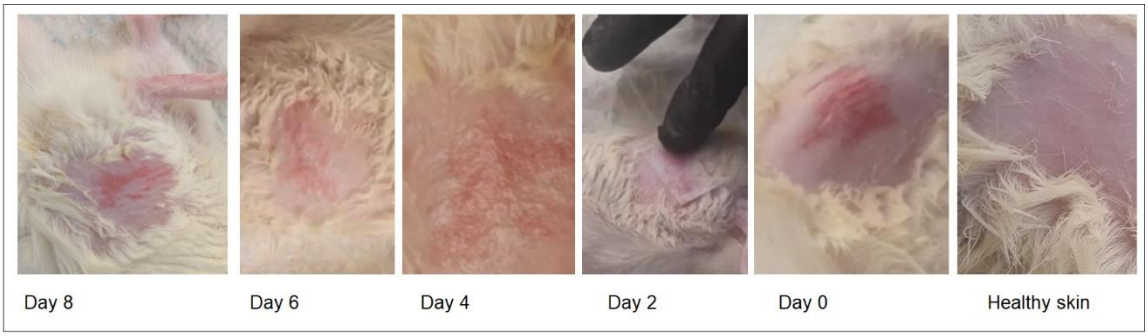

Fig.11: Wound healing during 8-day treatment with nanofibers F1

Conclusion

The electrospun nanofibers loaded fusidic acid is prepared by Electrospinning process. The average nanofibers diameter is changed due to the effect of PVA/Chitosan ratio, with increasing the Chitosan concentration, reduces the diameter. Loading efficiency reflects the uniformity of nanofibers and perfect distribution of fusidic acid on the nanofibers surface. F1 shows initial burst drug release within first 2-hour; in addition the percent of wound contraction is faster during 8-day treatment of excised rat skin. Finally short term stability study demonstrates the influence of elevated temperature on the morphology and drug release profile of nanofibers to provide optimum conditions for the storage to ensure the activity and safety for medicinal use.

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