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Quercetin loaded cosm-nutraceutical electrospun composite nanofibers for acne alleviation: Preparation, characterization and experimental clinical appraisal

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Abstract

In the cosmeceutical field, it is essential to develop topical delivery systems which would allow drugs to create a depot and permeate within the skin. The aim of the present study was to develop composite nanofibers of polyvinyl alcohol/quercetin/essential oils using the electrospinning technique, and assess their efficiency in acne alleviation. Quercetin was chosen due to its anti-inflammatory, anti-oxidant, and antibacterial activities. Nanofibers were characterized for their morphology, *ex-vivo* deposition/permeation, physical/mechanical integrity, thermal properties, and chemical characteristics. In addition, the anti-bacterial efficacy was tested on *Propionibacterium acne (P. acne)*, and a cytotoxicity assay was carried out. Lastly, an experimental clinical trial was conducted on acne patients, where the percentage reduction of inflammatory, non-inflammatory and total acne lesions was taken as evaluation criterion. Results showed that quercetin was successfully loaded into the nanofibers which were homogenously dispersed. They showed a reasonable skin deposition percentage of 28.24% ± 0.012, a significantly higher antibacterial efficacy against *Propionibacterium acne* than quercetin alone, and were utterly safe on skin fibroblastic cells. Upon clinical examination on acne patients, the nanofibers showed 61.2%, 14.7%, and 52.9% reduction of inflammatory, comedonal, and total acne lesions respectively, suggesting a promising topical anti-acne delivery system.

Keywords: Acne, Electrospinning, Nanofibers, Polyvinyl alcohol, *Propionibacterium acne*, Quercetin.

1. Introduction

Acne is a chronic disease of the pilosebaceous gland (hair follicles associated with the oil glands) (**Strauss et al., 2007; Titus and Hodge, 2012**) affecting people in their adolescence with a percentage of 80% from the age of 11 to 30 years (**Ramanathan and Hebert, 2011; Thakur et al., 2013**). It is manifested by the appearance of different types of lesions, where they are either inflammatory, showing the whiteheads and blackheads, or non-inflammatory showing the comedones, or taking a more severe form of papules, pustules, cysts or nodules. The principle causative micro-organism responsible for pimples formation is *Propionibacterium acne (P. acne)* dwelling in the hair follicles. Together with the overabundance sebum present in the skin, the skin pores are obstructed, and hence give the oily appearance of the skin in acne patients. Due to the vast applications nanofibers (NFs) offer, they were widely used in many dermatological diseases, one of which is acne.

Nanotechnology has attracted considerable attention in the past decade as it accords in many medical and pharmaceutical fields. The fundamental goal of topical nano-delivery systems is to provide a localized action with an optimized clinical applicability and a good patient compliance. NFs were first founded back in 1934 by Formhals, yet they gained much interest lately due to their newly discovered advantages, specifically in topical and transdermal delivery (**Yu et al., 2009a**). NFs fulfill the requirement of providing high patient compliance as they are considered painless mat-like structures offering high surface area to volume ratio, and therefore allowing a large concentration of drugs to be loaded inside them. Moreover, NFs show high porosity, and hence they provide more drug penetration through the skin (**Hong et al., 2006; Yu et al., 2009a; Zargham et al., 2012**). Also, the release profile of the drugs loaded in NFs could be modified allowing sustained delivery. In addition, NFs are considered very promising cosmeceutical skin

care masks, due to their ability to be molded according to the skin's topography (**Goyal et al., 2016**).

NFs are normally prepared by different techniques, among which electrospinning showed a great potential in controlling the fiber diameters, size distribution and morphology. In electrospinning process, a polymer is used as the main matrix in which other materials are mixed resulting in a composite solution. Different parameters were reported to be responsible for the morphology and controlling the formation of uniform fibers of the formed NF mat, namely the solution flow rate, applied voltage, distance from the needle tip to the collector as well as solution concentration (**Ding et al., 2002; Jalili et al., 2005; Yu et al., 2009a; Zargham et al., 2012; Goyal et al., 2016**).

Several polymers were used as the main electrospinning matrix and were reported in forming uniform NFs such as poly caprolactone (PCl), polyvinyl alcohol (PVA) and many others among which PVA was chosen to incorporate different formulations due to its biodegradability, being biologically friendly, non-toxic and non-carcinogenic (**Arecchi et al., 2010; Jannesari et al., 2011**). It also offers many useful physical and chemical properties since it can enhance the mechanical properties of the NFs films produced (**Gaaz et al., 2015**), and it also exhibits high flexibility and a high swelling property in aqueous media (**Jannesari et al., 2011**). Furthermore, PVA shows high thermal stability (**Arecchi et al., 2010**), and high viscoelastic properties, while offering high chemical resistance (**Sasipriya et al., 2013**). Owing to all those properties, fiber formation using PVA became the most commonly reported technique, where (**Jannesari et al., 2011**) reported the beneficial use of PVA in the formation of wound dressings, due to its hydrophilicity, which hence, allows it to be washed off easily of the wounds surface.

Quercetin (QC), a natural flavonoid; has gained much interest lately due to its tremendous actions related to its anti-oxidant, anti-inflammatory, anti-cancerous, and anti-bacterial potentials (**Kelly, 2011; Chen-yu et al., 2012; Nam et al., 2016**). QC was reported to be promising in the treatment of acne, owing to the highly anti-inflammatory action it exerts on acne pimples (**Havsteen, 2002; Kelly, 2011**). It was reported to display effectiveness against *P. acne*; showing a bacteriostatic action and an improvement of different acne lesions (**Tan et al., 2011**). Moreover, it was reported by (**Chen-yu et al., 2012; Choi et al., 2012; Nam et al., 2016**) that QC inhibits the tumor necrosis factor alpha (TNF-α) production, which is a major cytokine, responsible for chronic inflammatory diseases and production of other inflammatory cytokines, chemokines or inflammatory mediators, hence shows an anti-inflammatory action. As for the direct application of quercetin on to the skin, it was not recommended to apply it as such due to its poor water solubility, instability, and poor epidermal permeation, which hence hinders its deposition, delivery, and efficiency. Therefore, it was loaded into nanofibers to allow more quercetin skin retention, deposition, and permeation into the epidermal layers. Different essential oils (EOs) namely tea tree oil, and neem oil were previously utilized as functional additives showing high acceptance rate among acne patients, where it was reported that both oils possess anti-inflammatory, anti-oxidant, and anti-bacterial potentials, which qualified them to act as perfect candidates for acne treatment (**Raman et al., 1995; Carson et al., 2006; Enshaieh et al., 2007; Vijayan et al., 2013; Hammer, 2014**), hence they were chosen in the current study to be loaded within the NFs of interest along with QC to be used clinically against acne patients. To the best of our knowledge, till the current date, only two papers reported the use of NFs in acne treatment with no clinical applicability (**Nada et al., 2016; Sangnim et al., 2018**).

2. Experimental

2.1. Materials

Quercetin (QC) and polyvinyl alcohol (PVA) Mwt 125,000 were both purchased from Sigma Aldrich, Germany. Essential oils (tea tree oil, neem oil) were both purchased from Alpha Pharmaceutical Co., Cairo, Egypt. All analytical grade solvents were purchased from Fisher Scientific UK. Disodium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from El Nasr chemical Co., Cairo, Egypt. *Propionibacterium acne* (*P. acne*) ATCC 6919, horse blood agar, 3T3 CCL92 skin fibroblastic cells, trypsin, antibiotics (penicillin, streptomycin), barium chloride dehydrate, sulfuric acid, hydrogen peroxide, and neutral red, were all purchased from Sigma Aldrich Co., UK as previously mentioned in the work conducted by (**Amer et al., 2020**). Dulbecco modified eagle's medium (DMEM), and FBS (Fetal Bovine serum 10%) were both purchased from Gibco by life technologies, Thermo Fisher Scientific, USA. Mueller Hinton agar, brain heart infusion, and the anaerobic indicator were purchased from Thermo Scientific, Oxoid, Anaerogen, UK. Industrial methylated spirit 70% (IMS) was purchased from Atom Scientific, UK. Panthenol® cream was purchased from a local pharmacy (Sherryland Pharmacy) where it was manufactured by the Nile Co. for Pharmaceuticals and Chemical Industries, Egypt.

2.2. Methodology

2.2.1. Preparation of PVA/QC/EOs NFs

A horizontal home built electrospinner set up consisting of a programmable syringe pump (SK-500 I, China), a high voltage power supply (Gamma high-voltage power supply, USA) generating up to 30 KV, and a stationary ground copper plate collector covered with aluminum

foil was placed to collect the NFs (**Rho et al., 2006; Sasipriya et al., 2013; Gordon et al., 2015; Kegere et al., 2019** 14, 29-31).

A PVA solution of 10% concentration was prepared by dissolving PVA powder in distilled water where it was placed on a magnetic stirrer at a temperature of 80-100°C until complete dissolution. The solution was then left to cool down, and was loaded into a 10-mL syringe and mounted onto the pump, which was connected to the electrospinner through a silicon tube. A solution flow rate ranging between 0.2-4.5 mL/hour (h) was applied while being connected to the high applied voltage ranging between 15-30 kV. The NFs were collected on an aluminum foil grounded on the metallic collector located 8-17 cm away from the spinneret. The aforementioned electrospun NFs were termed **plain PVA nanofibers** (P1) as shown in **Table 1**, and were actually prepared as a control to settle on the optimum process parameters used for loading QC and different EOs as such within the NFs. QC and EOs were both added to the PVA in different concentrations, mixed, and electrospun, for reaching a synergistic effect in treating acne.

As shown in **Table 1, PVA/Quercetin/Essential Oils nanofibers** (**PVA/QC/EOs NFs**) were prepared, by mixing different amounts of QC and EOs dissolved in ethanol with the 10% PVA solution (v/v) and these solutions were fed into the syringe pump in the electrospinning instrument resulting in PVA/QC/EOs NFs. This ethanolic solution contained an amount of 1, 2, 5, and 10 mg QC (H1-4 respectively) in addition to different EOs concentrations. In all trials, the volume of the electrospun samples was fixed to 10-mL.

Table 1: Composition of different nanofibers formulations

Nanofibers code	Description	Composition of nanofibers			
		Amount of 10% PVA (mL)	Amount of QC (mg)	Amount of neem oil (µL)	Amount of tea tree oil (µL)
P1	Plain 10% PVA NFs	10.0	----------	----------	----------
H1	PVA/QC/EOs NFs 1	+	1.0	1.0	1.0
H2	PVA/QC/EOs NFs 2	+	2.0	2.0	2.0
H3	PVA/QC/EOs NFs 3	+	5.0	5.0	5.0
H4	PVA/QC/EOs NFs 4	+	10.0	10.0	10.0

* **+ denotes for the presence of the ingredient**
* **---------- denotes for the absence of the ingredient**

2.2.2. Characterization of the nanofibers

2.2.2.1. Selection by visual examination

Visual inspection was done to monitor the electrospinning process, where it was examined for the occurrence of dropping. Dropping occurred when the polymer dropped at the tip of the needle if it was not completely electrospun and was transferred to the aluminum foil placed on the collector as liquid droplets.

2.2.2.2. Scanning electron microscope (SEM)

The influence of the process parameters on the electrospun NFs morphological appearance was visualized by scanning electron microscope (LEO supra 55 field emission SEM, Germany). Samples were prepared by placing 1 cm^2 NFs on a double-sided carbon tape applied to a SEM stub. The aluminum foil was used to enhance the conductivity of the samples, and therefore improving the quality of images produced.

The average fiber diameter (AFD) of the NFs was determined from the selected SEM images by measuring the diameter of 85-100 NF7s chosen randomly at more than one magnification for each sample, by using **ImageJ analysis commercial software** (**Ding et al., 2002; Jia et al., 2007; Peresin et al., 2014; Gordon et al., 2015**). The results were expressed as AFD of 85-100 NFs and were illustrated graphically as histograms. In case of the beaded NFs mats, the AFD was measured in the beaded-free areas in the image.

2.2.2.3. Drug loading analysis via HPLC

The drug loading percentage was evaluated via placing the electrospun nanofibers in a methanolic solution, where the amount of the quercetin was then analyzed using HPLC (Dionex Ultimate 3000, USA), utilizing a mobile phase acetonitrile:water (40:60) flowing at 1 mL/min through a C18 HPLC column (Thermo scientific, BDS Hypersil column, 4.6 × 250 mm, 5 µm, USA), and the effluent was analyzed at 354 nm (**Kumari et al., 2010**). The quercetin loading percentage was then calculated.

2.2.2.4. *Ex-vivo* deposition/permeation using Franz diffusion apparatus

The *ex-vivo* skin deposition and permeation of the selected QC NFs were evaluated using the Franz diffusion apparatus (Variomag Telesystem, Germany). As previously explained in prior work conducted by (**Amer et al., 2020**), and as reported by (**Bsieso et al., 2015; Nasr and Abdel-Hamid, 2016; Nasr et al., 2017**) the rat dorsal skin was prepared and mounted between the two compartments; the donor and the receptor compartment.

NFs were cut into 1 cm^2 square shaped mats and placed on to the mounted rat's skin, while the receptor compartment was filled with 7.5-mL PBS of pH 7.4 and stirred at 150 rpm at 37°C (**El Zaafarany et al., 2010**). A sample was withdrawn from the receptor compartments at different

time intervals of (0.25, 0.5, 1, 2, 4, and 6 hours (h)) where it was diluted with methanol and analyzed for the permeated QC.

The amount of QC deposited onto the skin was analyzed as previously reported by (**Amer et al., 2020**), where the amount of the accumulated QC into the skin was expressed as a percentage of the total amount applied on the skin using HPLC (Dionex Ultimate 3000 USA), following the specific method reported by (**Kumari et al., 2010**), where the mobile phase was acetonitrile:water (40:60 v/v), the injection volume was 20 µL, a flow rate of 1-mL per minute was used, and a chromatographic run time of 7 min per sample was attempted. Samples were injected into a C-18 HPLC column (Thermo scientific, C-18 BDS Hypersil column, 4.6x250mm, 5µm, USA) at 25°C, and the column effluent was analyzed at 354 nm (**Kumari et al., 2010**).

2.2.2.5. Fourier transform infrared (FT-IR) spectroscopy

Experiments were performed by the Fourier transform infrared spectroscopy (FT-IR, Nicolet 380, Thermo Electron Scientific Instruments, LLC Madison, USA) in the wave number region of 4000-400 cm^{-1} to examine the characteristic peaks of the best NFs mat, in addition to each individual component in order to compare the spectra of those samples, detect any chemical interaction occurring, and to make sure that the drug was fully incorporated within the prepared NFs. For the solid NFs, a 1 cm^2 of the NFs was placed in front of the IR beam directly. As for both EOs, they were directly placed in-front of the IR beam as liquids. QC and PVA powders as such were first mixed with KBr pellets and pressed on a hydraulic press (15T manual press machine for powder hydraulic pressing, China) to form a disc followed by examination of the spectra (**Zhong et al., 2010; Dhandayuthapani et al., 2011; Thipkaew et al., 2017**).

2.2.2.6. Differential scanning colorimetry (DSC)

The thermal behavior of the single pure components (PVA and QC) was examined by the DSC (Simaltaneous Thermal Analyser, Perkin Elmer diamond, model STA 6000, USA). Moreover, the selected NFs formulation was also examined by the DSC at a rate of 10ºC/min under constant dry nitrogen where they were heated from 25 to 350°C, on which samples of approximately 0.2-mg were sealed in a standard aluminum pan, and thermograms were obtained (**Taepaiboon et al., 2006; Peresin et al., 2014; Gordon et al., 2015**).

2.2.2.7. Physical integrity (water-retention test)

The physical integrity of the NFs was tested by immersing the selected NFs mat in distilled water. Square webs of 1 cm^2 of the NFs were cut and submerged in distilled water for a period ranging from 24 h to 1 month at room temperature. The NFs were checked visually after 24 h, 48 h, and after 1 month. The strength and physical integrity were checked every 8 h visually for the first two time periods (24 h and 48 h) to make sure that the NFs did not dissolve and were still intact, after which they were checked in one month (**Peresin et al., 2014**).

2.2.2.8. Assessment of the antibacterial potency of PVA/QC/EOs NFs

As described in previous work carried out by (**Amer et al., 2020**), *P. acne* was cultured under anaerobic conditions. Afterwards, samples were added on to the agar plates where ethanol, QC ethanolic solution and the selected NFs were tested individually. Ethanol which was used as a control, and QC ethanolic solution were added by a volume of 10 µL while the selected NFs were cut into square pieces of 1 cm x 1 cm and placed directly onto the plates (**Wulansari et al., 2017**). The plates were left in the incubator for two days, after which the inhibition zones were measured in mm.

2.2.2.9. Cell compatibility assay of PVA/QC/EOs NFs on skin fibroblasts

The safety of the selected NFs formulations was assessed via a cell compatibility assay carried on 3T3-Swiss Albino mouse (ATCC® CCL92™). As illustrated in previous work conducted by **Amer et al., 2020**, the experiment was carried out in sterile conditions, inside the tissue culture hood (Thermo Fisher Scientific, MSC-Advantage, Class II Biological safety cabinet, Waltham, MA 02454, USA).

The cells were prepared and sub-cultured till it reaches a cell count of 5 x 10^4 cells/mL, where different samples were then added (**Repetto et al., 2008**). The selected NFs formulation, the QC solution, in addition to the nutrient medium as well as the hydrogen peroxide were all added onto the cells and tested for their safety on the report of the methodology previously conducted by **Amer et al., 2020**.

2.2.2.10. Clinical assessment of PVA/QC/EOs NFs on acne patients

The clinical study included patients of both genders suffering from mild to moderate acne vulgaris according to grading of (**NilFroushzadeh et al., 2009**). Patients were selected from the Dermatology Out-patient Clinic of Minia University Hospital, Al-Minya, Egypt. The study included 20 patients with acne vulgaris (2 males and 18 females). Their age ranged from 14 to 28 years (average 18.6 ± 3.26 years). Regarding the duration of acne lesions, it ranged from 2 to 60 months (average 25.1 ± 15.8). On the report of the selection criteria carried out by (**Amer et al., 2020**), the inclusion criteria included patients who did not receive any anti-acne treatment, or oral contraceptives three months prior to the clinical study, while the exclusion criteria included pregnant, nursing mothers, and patients who suffered from hyperandrogenism. An informed

consent was taken from each patient or his guardians, and the study was approved by the research ethics committee for experimental and clinical studies of Ain Shams University **(REC- ASU#31)**.

Patients were instructed to apply the selected QC-loaded NFs patch of dimensions 1 cm x 1 cm on the right side of the face and placebo formulation (Panthenol®) on the left side of the face once daily. The marketed product Panthenol® was chosen as the negative control in our clinical study, since there were no topical quercetin and/or essential oils marketed cream products to use as a control. Also, Panthenol® showed an anti-acne action as reported by (**Fabbrocini and Panariello, 2016**), hence was considered suitable to be used in comparing the clinical efficacy. Patients were informed to wet the nanofibers patch with one drop of water before applying it on to the face. The nanofibers patch was left onto the face for a period of 8 hours/day for a period of 8 weeks. Patients were photographed every 2 weeks and evaluated clinically after 8 weeks on both sides of the face through the counting of comedones, inflammatory and the total acne lesions by 2-blinded dermatologists and the percentage reduction was accordingly calculated. Percentage reduction was calculated for each type of lesion as previously mentioned by (**Barakat et al., 2017; Amer et al., 2020**), where the number of pimples after administration are subtracted from the number of pimples before administration and divided by the latter. This was done for each type of lesion. A comparison between the percentage reductions was made between both; the anti-acne nanofibers formulation, and panthenol in all different types of acne lesions.

2.2.2.11. Statistical analysis

All the formulations were prepared in triplicate (except for measuring the NFs size where the results were calculated as the average of 85-100 NFs). Results were expressed as mean ± standard deviation (S.D). A comparison using one-way analysis of variance (ANOVA) was carried

out, followed by Tukey Kramer post-test test or paired t-test using Graphpad®Instat software. A difference between means was considered significant if the P value was less than or equals to 0.05. Wilcoxon rank-sum test was used to compare the percent reduction in acne count between acne patients exposed to the formulation and those exposed to placebo. The test was used to test the null hypothesis of equal population medians. A P-value of ≤0.05 was considered to be statistically significant.

3. Results and Discussion

3.1. Nanofibers preparation by electrospinning

The optimal electrospinning parameters for the plain 10% PVA solution that resulted in uniform bead free NFs were determined to be: solution flow rate of 3 mL/h, applied voltage of 20 KV and 10 cm distance from the spinneret tip to the plate collector. Parameters other than the aforementioned ones exhibited undesirable NF traits. It was observed that upon increasing the solution flow rate from 0.2 to 3 mL/h, the dropping percentage decreased, where at 3 mL/h a stable electrospinning process was achieved with a significantly lesser degree of falling droplets. At lower solution flow rates, the solution was ejected very slowly resulting in either inhibition of the electrospinning process with no formation of NFs or the occurrence of a very thin layer of NFs with significant dropping and beading. This came in accordance with (**Rodoplu and Mutlu, 2012; Eleyas et al., 2017**) who reported that low solution flow rates were not considered desirable due to the incidence of dropping and the formation of beads. On the other hand, increasing the solution flow rates higher than 3 mL/h (4 and 4.5 mL/h) again resulted in significant dropping and beading (to be demonstrated in the SEM section), which could be attributed to the large volume of the liquid drawn from the needle tip that required long time to dry, with no complete formation of NFs. Moreover, it was reported by (**Athira et al., 2014**) that high solution flow rates influenced

the jet velocity and the material transfer rate, causing beaded fibers to form. In addition, the residual undried liquid may cause merging of the fibers, forming an unwanted web instead of a smooth fiber mat. Also, this high solution flow rate may allow the solution to be electro-sprayed instead of being electrospun, hence forming nanoparticles instead of NFs (**Buchko et al., 1999; Thompson et al., 2007; Eleyas et al., 2017**). It was also evident that the distance from the spinneret tip to the collector played an important role in the morphology and the uniformity of the NFs, where very short and very long distances were considered unfavorable, hence it had to be controlled (**Ki et al., 2005**). It was observed that very short distances as 8 cm caused a lot of dropping and beading with the formation of minute fraction of fibers, since it prevents the fibers from having enough time to solidify before reaching the collector, and hence remaining as liquid droplets (**Yuan et al., 2004**). In addition, at distances as long as 17 cm, droplets and beads in large numbers were also formed, similar to what was reported by (**Ki et al., 2005; Athira et al., 2014**), which could be ascribed to the capillary instability developed at longer distances as reported by (**Thompson et al., 2007**), hence allowing the liquid to drop and cause significant beading. Finally, regarding the effect of applied voltage on the electrospinning process, it was observed that changing the voltage from 15 to 30 KV had a little effect on the NFs morphology in terms of dropping and beading, and hence the optimum plain NFs were electrospun at 20 KV, which was a common voltage used for electrospinning PVA NFs (**Lee and Lyoo, 2010; Lyoo et al., 2010**).

Regarding PVA/QC/EOs NFs (H1-H4), different amounts of QC and EOs were incorporated into the 10% PVA solution (1, 2, 5, 10 mg or μL for QC and EOs respectively). Both NFs formulations H1 (1 mg and 1 μL for QC and EOs respectively) and H2 (2 mg and 2 μL for QC and EOs respectively) displayed smooth, uniform and consistent NFs, with optimum properties attained at spinneret tip to collector distance of 15 cm, a solution flow rate of 1 mL/h and applied

voltage of 25 KV, since the optimum electrospinning conditions for P1 were not suitable for the NFs formation. On the other hand, formula H3 produced a large amount of NFs, however a lot of dropping, and beads formation occurred, and this might be attributed to the high amount of the incorporated polyphenolic QC, which caused an increase in the viscosity of the solution by cross-linking with PVA, leading to partial occlusion of the syringe and difficulty of ejection, similar to what was reported by (**Ramakrishna et al., 2006; Martins et al 2008**). Finally, H4 failed to produce any NFs, due to the discontinuation of the electrospinning process because of the highly viscous solution produced on adding large amount of QC (10 mg), causing possible cross-linking with PVA.

3.2. Scanning Electron Microscope (SEM)

The morphology of the prepared NFs and their average fiber diameter (AFD) were inspected using SEM, to delineate whether the NFs were homogenous or not, and to inspect the possibility of beading. Regarding the plain PVA NFs (P1) electrospun at a distance of 10 cm from the spinneret tip to the collector, a solution flow rate of 3 mL/h and an applied voltage of 20 KV, the fibers appeared as thin round rods, with the smooth surface arranged in a network shape, showing no beads. The AFD of 100 fibers was measured and ranged from 198.66-295.51 nm, and histograms displayed size uniformity as shown in **Figure 1**. **Figure 2** represent SEM images of P1 electrospun at solution flow rate or spinner tip to collector distance conditions other than the optimal respectively, where the solution flow rate played an important role in the morphology of the NFs, as shown in the SEM images, in which very low and very high flow rates were considered unfavorable due to the dropping and the formation of beads. Furthermore, very short and very long distances were both considered undesirable, due to the presence of a vast number of beads, as shown in the SEM images.

NFs H1 and H2 containing small amounts of QC and EOs, displayed smooth and uniform fibers, as shown in **Figures 3(A), (C)** respectively, with respective AFD of 354.95 nm and 313.08 nm. Meanwhile, PVA/QC/EOs NFs H3 containing larger amount of QC and EOs (5-mg and 5-µL respectively) displayed significant beading, as shown in **Figure 3(E)**, where the yellow arrows demonstrate the beads present.

Based on all aforementioned data, the NFs formulation H2 was chosen for further characterization, owing to its desirable appearance and properties, as well as its higher content of QC compared to H1.

3.3. Drug loading analysis via HPLC

The drug loading percentage was examined using the HPLC, where the amount of loaded quercetin was calculated to be 96% ± 0.006, which was considered advantageous in our topical NFs formulation. This may be due to using a polymer as PVA with a high concentration of 10%, which allows a high crystallinity, and therefore a more closely packed nanofibers network system. Hence, entrapping a high amount of quercetin (**Hassan and Peppas, 2000; Gajra et al., 2014**).

This percentage was then used in the skin deposition/permeation experiment for calculating the skin deposition percentage.

3.4. Ex-*vivo* deposition/permeation using Franz diffusion apparatus

Upon conduction of *ex vivo* deposition of the NFs formulation H2, it showed a reasonable skin deposition for QC of 28.24% ± 0.012. The high skin deposition % obtained may be attributed to the fact that the NF mat provides a high surface area to volume ratio (**Shin et al., 2001; Park et al., 2004; Taepaiboon et al., 2006; Fathi-Azarbayjani et al., 2010; Levengood et al., 2017**), which allows drugs to be deposited onto the skin at a high percent. In addition, the microporous structure offered by the NFs allows a higher adhesion and attachment on to the skin cells, and

hence increasing the contact time with the skin, and therefore, allowing more deposition (**Bhattarai et al., 2004; Ma et al., 2005; Jia et al., 2007; Fathi-Azarbayjani et al., 2010**). Furthermore, PVA exhibits a rubbery adhesive nature with spreadable properties, allowing it to show high viscoelastic properties, leading to enhancement of drug diffusion and hence deposition (**Zhu and Qian, 2007; Lu and Fassihi, 2015**). Moreover, as reported by (**Lauchli et al., 2012; Amer et al., 2020**) neem oil enhanced skin deposition, where it exerted a semi-occlusive effect onto the skin, hence, causing a balanced moist environment and therefore allowing for a better quercetin deposition. Also, essential oils specifically tea tree oil had shown an enhanced percutaneous absorption/penetration bypassing the stratum corneum using different mechanisms of action (**Herman & Herman, 2014**). Less than 1% of QC was permeated into the receptor compartment over a period of 6 h. This low permeation rate is considered beneficial in the topical delivery of QC, therefore the action would be localized onto acne pimples. This low permeation rate may be attributed to the usage of PVA as the polymer, where a high concentration of PVA (10%) was used to form the NFs, and hence a higher crystallinity, and a denser PVA network system is formed, which allowed the QC to be entrapped into the NF mats, decreasing its release (**Morimoto et al., 1989; Hassan and Peppas, 2000; Gajra et al., 2014**). Moreover, this low permeation rate may be ascribed to the hydrophobicity of QC, and hence the NF mat could not transport it into the hydrophilic dermis, resulting in low permeation rate (**Tan et al., 2011**).

3.5. Fourier transform infrared (FT-IR) spectroscopy

The FTIR spectra of all the pure ingredients, and the selected NFs formulation H2 were examined, and the charts are presented in **Supplementary 1**. As shown in **Supplementary 1(A) (B)**, tea tree and neem oils displayed similar FT-IR charts respectively, owing to the fact that they are both EOs. The tea tree oil showed a hydroxyl group peak at 3469 cm^{-1}, which is similar to that

of the neem oil formed at 3470 cm^{-1}. Also, a strong sharp band at 3008 cm^{-1} seen in both the tea tree, and the neem oils could be assigned to the presence of C-H groups of the aromatic compound (**Elzey et al., 2016**), while the absorption peak at 1655 cm^{-1} present in both EOs accounts for the presence of C=C bond. The region of 1163-1098 cm^{-1} in the tea tree oil, and 1163-1099 cm^{-1} in the neem oil could be ascribed to the presence of C-O bonds (**Tanwar et al., 2013**). Lastly for the tea tree oil, the absorption band at 1239 cm^{-1} confirmed the presence of C-O bond. Similarly, for the neem oil, the strong peak at 2851 cm^{-1} indicated the presence of C-H bonds (**Tanwar et al., 2013**), while the absorption peak at 1743 cm^{-1} is a sign of having C=O bond (**Tanwar et al., 2013; Elzey et al., 2016**).

As also shown in **Supplementary 1(C)**, PVA powder alone showed many characteristic absorbance peaks, where a broad peak was observed at 3529 cm^{-1} corresponding to the stretching inter-molecular and intra-molecular hydrogen bonds caused by hydroxyl groups (**Fathi-Azarbayjani et al., 2010; Sasipriya et al., 2013; Peresin et al., 2014; Shukry et al., 2014**). The two absorption peaks at 1709 cm^{-1} and 1661 cm^{-1} could be assigned to the presence of the C-O and C=O carbonyl stretching bonds respectively, resulting from the residual acetyl content (2%) present in the PVA matrix (**Sasipriya et al., 2013**), while the absorption peak detected at 1436 cm^{-1} represents the bending of the C-H, O-H, and CH_2 groups (**Jia et al., 2007; Peresin et al., 2014**), and it may also be attributed to the C-H stretching (**Fathi-Azarbayjani et al., 2010**).

As also shown in **Supplementary 1(D)**, the QC powder showed a strong band at 3383 cm^{-1} which is indicative of the presence of hydroxyl groups (O-H) (**Koga et al., 2014; Hurai et al., 2014**). Another sharp strong intensity peak at 2818 cm^{-1} can be assigned to the C-H bonds, and its stretching. The peaks at 1654 cm^{-1} and 1600 cm^{-1} are attributed to the presence of the C=O bond. The two peaks at 1563 cm^{-1} and 1514 cm^{-1} indicate that the compound (QC) is aromatic, and finally

absorption peaks in the region of 1432-1372 cm^{-1} indicate the presence of strong C-H groups, while the peaks in the region of 1161-1257 cm^{-1} correspond to the presence of C-O bonds (**Heneczkowski et al., 2001**).

Lastly, for the selected NFs formulation H2, the broadening shown in **Supplementary 1(E)** at 3473 cm^{-1} might be related to one of two reasons; it may be related in principle to superimposition, in which the PVA peaks observed in **Supplementary 1(C)** at 3529 cm^{-1} might have superimposed most of the peaks of the tea tree oil, neem oil, and the QC, observed in **Supplementary 1(A), (B), and (D)** respectively in the range of 3383-3470 cm^{-1}. Alternatively, it may be attributed to a possible chemical interaction between the PVA, and any of the other components present in the mat. It is worth mentioning that in this system of QC, EOs and PVA, there is a large number of hydroxyl groups, which will certainly affect the intensity of the peaks, and the formation of the intra-molecular and inter-molecular hydrogen bonds, and hence all this may result in a slight shift/broadening of the peaks, where (**Li et al., 2014**) reported that hydrogen bonds between QC itself can occur within the NF core due to the presence of a huge number of hydroxyl groups.

3.6. Differential scanning colorimetry (DSC)

DSC analysis was carried out to check the changes occurring to QC upon loading it into the NFs. As presented in **Figure 4(A)**, the DSC thermogram of the PVA powder showed a melting range from about 200 to 230 °C 42 owing to the melting of its crystalline domains (**Mohsin et al., 2011**). As shown in **Figure 4(B)**, QC DSC thermogram showed a sharp endothermic peak at 323 °C which corresponds to its melting point, as reported in previous studies (**Vijaya Sri et al., 2007; Wu et al., 2008; Zheng and Chow, 2009**). However, as shown in **Figure 4(C)**, the DSC

thermograms of our selected NFs formulation H2 showed the disappearance of the melting peak of QC, suggesting the complete drug encapsulation in the NFs. This came in accordance with several reports on the amorphization to occur during the electrospinning process of NFs (**Zhou et al., 2008; Yu et al., 2009b, Gonçalves et al., 2017**).

3.7. Physical integrity/Water retention test for the NFs

The physical integrity test determines the rigidity and the firmness of the NFs, where inspection of the NFs was carried out by immersing the electrospun 2-mg QC best formula NFs formulation (H2) in water at room temperature, and visually inspecting it over a period of 24 h,48 h, and one month (**Peresin et al., 2014**). The NFs formulation H2 remained intact, compact, and retained their shape without dissolution or disintegration, hence displaying good physical and mechanical integrity. This is considered advantageous for topical delivery, as was reported previously (**Ueda et al., 2009; Patel et al., 2012; Peresin et al., 2014**) that films should be smooth, flexible, of high physical and mechanical integrity, and can withstand high mechanical pressure, in order to adhere consistently on to the skin surface and allow topical drug delivery. Lastly, it is worth to mention that this rigidity may be due to the cross-linking of the QC to the PVA polymeric molecules via hydrogen bonding as previously mentioned, or due to the inter-molecular and intra-molecular hydrogen bonds occurring between any of the nanofiber mat components as the tea tree oil, neem oil, PVA or quercetin due to the presence of a vast amount of hydroxyl groups, which hence allowed a high physical integrity, and stability.

3.8. The anti-bacterial assay using the disc diffusion method

The anti-bacterial efficiency was measured by calculating the average inhibition zone for each sample in mm. As shown in **Figure 5**, the NFs formulation H2 showed significantly higher antibacterial activity (average zone of inhibition of 18 ± 0.01 mm) compared to QC (8.25 ± 2.08 mm) ($P<0.05$) where this finding showed that NFs formulation H2 encountered an increase in the percentage inhibition compared to the quercetin of 118% (1.18 fold). The larger inhibition zone encountered with the NFs suggests that the presence of tea tree oil, neem oil, together with the quercetin and the other nanofibers forming ingredient (PVA) had potentiated the anti-acne activity of the formulation. As reported by (**Lv et al., 2017**), the essential oils, were added to quercetin to improve its solubility, stability, and skin permeation, hence a better anti-bacterial action. Also, this large inhibition zone could be ascribed to their structure as NFs mats, similar to previous reports (**Bottino et al., 2013; Albuquerque et al., 2014**), that NFs are good candidates for the treatment of the bacterial infections, since they are characterized by high surface area to volume ratio and hence they were considered highly permeable and highly porous, resulting in more intimate interaction with the bacteria, causing its death (**Kurtz and Schiffman, 2018**). In addition, it was reported by **Goyal et al., 2016**, that the interconnected structure of the NFs allows a controlled interaction with the bacteria and other micro-organisms causing their death. Furthermore, it was reported that cellulose NFs electrospun with the cationic polymer poly (diallyldimethylammonium chloride) (pDADMAC), transformed the NF mats into contact killing materials for *E. Coli* (**Rieger et al., 2016**). All this may have contributed to the marked prominent anti-bacterial activity produced by the NFs formulation H2.

In addition, tea tree oil was reported to exhibit antibacterial properties by destruction of the bacterial cell wall (**Carson et al., 2002**), especially on *P. acne* (**Raman et al., 1995; Pazyar et**

al., 2013; Hammer, 2014; Wulansari et al., 2017) owing to the presence of terpinen-4-ol which shows a high anti-bacterial activity (**Flores et al., 2011**). In addition, neem oil was also reported to exhibit an anti-bacterial activity against *P. acne* (**Alzohairy et al., 2016**) owing to its content of various anti-bacterial ingredients, and hence, was recommended for treatment of many skin diseases such as acne (**Chandel et al., 2012; Vijayan et al., 2013; Singh et al., 2014**).

3.9. Cytocompatibility study of PVA/QC/EOs NFs on 3T3 CCL92 skin cells

As shown in **Table 2**, cells receiving no treatment showed 100% viability, while those receiving hydrogen peroxide as a positive control displayed significant death (26.10% ± 4.65% viability). The QC solution, and the NFs formulation H2 (1 cm2) displayed high viability percentages of 93.73% ± 3.14%, and 98.26% ± 4.82% respectively with no significant differences between the viability percentages among the two formulations ($P<0.05$). This may be ascribed to the natural origin of QC, and the use of highly safe ingredients in NFs formulation H2. The individual components of NFs formulation H2 were all reported safe for topical use in which tea tree oil was considered non-irritating and safe to be applied on the skin (**Carson et al., 2007; Prajapati, 2007; Pazyar et al., 2013; Amer et al., 2020**). The neem oil was also reported previously to be non-toxic and innocuous when applied on to the skin (**Aneesa and Gayathri, 2016; Amer et al., 2020**). QC itself was also reported as safe to be used topically where it plays an important role in wound healing and protecting against skin oxidative damage (**Kumar et al., 2017; Amer et al., 2020**). Regarding PVA which was used to fabricate the NFs mat, it was also reported to be biodegradable and biocompatible (**Muppalaneni and Omidian, 2013**). Cells treated with the NFs formulation H2 appeared under the microscope as differentiated elongated fibroblastic strands similar to what was reported previously (**Pohl and Christophers, 1979; Fisher et al., 2015**).

Table 2: The average viability percentages of the cells when treated with different formulations and controls (n=3)

Sample #	Sample composition	Average viability % (Mean± S.D)
1	Media only (Negative control)	100± 1.25
2	Hydrogen peroxide (Positive control)	26.10± 4.65
3	Quercetin solution	93.73± 3.14
4	NFs formulation H2	98.26± 4.82

3.10. Clinical assessment of PVA/QC/EOs NFs on acne patients

As previously mentioned in work conducted by (**Amer et al., 2020**), after 2 months of topical administration of the NFs formulation H2, the percentage reduction for each different type of lesion was calculated.

Table 3: The percentage reduction for the inflammatory lesions, comedones, and the total lesions for patients after application of nanofibers formulation H2 on the right side of the face

Patient number	Inf.B	Inf.A	% reduction*	Com.B	Com.A	% reduction*	Tot.B	Tot.A	% reduction*
1	25	10	60.0	0	0	0.0	25	10	60.0
2	11	4	63.6	2	2	0.0	13	6	53.8
3	11	3	72.7	1	1	0.0	12	4	66.7
4	16	5	68.8	5	3	40.0	21	8	61.9
5	22	8	63.6	4	3	25.0	26	11	57.7
6	12	2	83.3	3	3	0.0	15	5	66.7
7	16	6	62.5	2	1	50.0	18	7	61.1
8	20	8	60.0	4	4	0.0	24	12	50.0
9	14	7	50.0	0	0	0.0	14	7	50.0
10	12	4	66.7	5	4	20.0	17	8	52.9
11	13	4	69.2	8	7	12.5	21	11	47.6
12	15	5	66.7	1	1	0.0	16	6	62.5
13	25	13	48.0	4	3	25.0	29	16	44.8
14	24	7	70.8	2	2	0.0	26	9	65.4

15	17	6	64.7	8	7	12.5	25	13	48.0
16	10	4	60.0	4	3	25.0	14	7	50.0
17	12	6	50.0	9	8	11.1	21	14	33.3
18	11	5	54.5	3	2	33.3	14	7	50.0
19	15	8	46.7	5	5	0.0	20	13	35.0
20	12	7	41.7	5	3	40.0	17	10	41.2
	Average % reduction		61.2± 10.2	Average % reduction		14.7± 16.5	Average % reduction		52.9± 9.9

* Percent reduction was calculated by subtracting the number of lesions after administration A from the number of lesions before administration B and dividing by the latter
Inf.: Inflammatory lesions
Com.: Comedones
Tot.: Total number of inflammatory lesions and comedones

As could be seen in **Table 3, 4, Figure 6**, and the box and whisker plot, the count of inflammatory and total acne lesions was significantly decreased ($P<0.05$) compared to the left side of the face receiving Panthenol® cream, while the count of comedones was not affected in all patients ($P>0.05$) compared to the left side receiving Panthenol® cream. The NFs formulation displayed reduction percentages of 61.2% ± 10.2, 14.7% ± 16.5, and 52.9% ± 9.9 for inflammatory lesions, comedones and total lesions respectively, in comparison with 12.5% ± 15.2, 22.5% ± 19.9 and 15.3% ± 10.7 for Panthenol® cream. No signs of local irritation, burning sensation or dermatitis were recorded during the treatment period of almost 8 weeks.

Table 4: The percentage reduction for the inflammatory lesions, comedones, and the total lesions for patients after application of Panthenol® cream as placebo on the left side of the face

Patient number	Inf.B	Inf.A	% reduction*	Com.B	Com.A	% reduction*	Tot.B	Tot.A	% reduction*
1	30	20	33.3	6	6	0.0	36	26	27.8
2	11	10	9.1	3	2	33.3	14	12	14.3
3	8	8	0.0	2	1	50.0	10	9	10.0

4	14	7	50.0	6	5	16.7	20	12	40.0
5	23	19	17.4	5	2	60.0	28	21	25.0
6	15	13	13.3	4	4	0.0	19	17	10.5
7	10	8	20.0	7	6	14.3	17	14	17.6
8	10	12	-20.0	5	4	20.0	15	16	-6.7
9	15	11	26.7	8	6	25.0	23	17	26.1
10	11	12	-9.1	5	4	20.0	16	16	0.0
11	13	12	7.7	3	3	0.0	16	15	6.25
12	10	10	0.0	6	4	33.3	16	14	12.5
13	21	17	19.0	10	9	10.0	31	26	16.1
14	20	16	20.0	3	3	0.0	23	19	17.4
15	16	14	12.5	7	6	14.3	23	20	13.0
16	13	12	7.7	10	10	0.0	23	22	4.3
17	12	12	0.0	11	8	27.3	23	20	13.0
18	14	13	7.1	5	4	20.0	19	17	10.5
19	19	16	15.8	5	3	40.0	24	19	20.8
20	15	12	20.0	3	1	66.7	18	13	27.8
	Average % reduction		12.5± 15.2	Average % reduction		22.5± 19.9	Average % reduction		15.3± 10.7

* Percent reduction was calculated by subtracting the number of lesions after administration A from the number of lesions before administration B and dividing by the latter
Inf.: Inflammatory lesions
Com.: Comedones
Tot.: Total number of inflammatory lesions and comedones

The NFs formulation H2 exhibited anti-inflammatory properties which was affirmed by the significant reduction of the total lesions count (mainly the inflammatory lesions). This could be attributed to the use of several anti-inflammatory ingredients, namely the tea tree oil (**Carson et al., 2007; Pazyar et al., 2013; Amer et al., 2020**), and hence allowing the enhancement of the anti-inflammatory properties of H2 on the inflammatory papules rather than the comedones. Also, the neem oil which exhibits anti-inflammatory properties owing to the fact of having many anti-

inflammatory active ingredients such as azadirachtin may have contributed in the anti-inflammatory action (**Yadav and Rao, 2012; Aneesa and Gayathri, 2016; Amer et al., 2020**). In addition, the presence of QC acting as an anti-inflammatory molecule majorly contributes to the anti-inflammatory properties of H2 (**Sinha et al., 2014; Li et al., 2016; Kumar et al., 2017**). All of the aforementioned have contributed to the success of the QC loaded NFs as an anti-acne agent.

In addition, the polymeric NFs were proven effective in the clinical treatment of acne due to the countless favorable topical properties they offer, owing to their high surface area to volume ratio and high porosity which may therefore allow the drug to diffuse and penetrate more through the patch directly onto the skin, therefore allowing a pronounced effect (**Hong et al., 2006; Jia et al., 2007; Yu et al., 2009a; Zargham et al., 2012**).

It was reported by Tang et al., 2021 that different electrospun nanofibers had shown a significant decrease in the acne lesions size, a decrease in the hyperpigmentation occurring due to acne scars, and a significant decrease in TEWL when compared to placebo, and this was due to the physical properties the nanofibers possess, and the anti-oxidative and anti-bacterial compatibilities of the NFs loaded ingredients, while our NFs formulation H2 had shown a significant decrease in the count of inflammatory acne lesions and total acne lesions, which is considered comparable to the previously mentioned NFs, owing to the usage of anti-bacterial, and anti-inflammatory ingredients, and the nanofibers drug delivery system itself.

4. Conclusion

As can be observed from the above results, the QC loaded NFs successfully prepared via the electrospinning technique were proven to be effective in the treatment of acne, where they showed high skin deposition, high physical and mechanical integrity, a satisfactory anti-bacterial

efficacy, high cell viability, and propitious clinical results. Therefore, the NFs prepared in the present study can be considered as promising materials for encapsulation of different drugs used in the treatment of many skin diseases. All this accounts for the development of a new line of natural nano-cosmeceutical conveyance frameworks that can be effectively utilized in the treatment of other dermatological illnesses.

Credit author statement

The manuscript has been read and approved by all the authors.

S. S. Amer, M. Nasr and O. A. Sammour conceived the idea. S. S. Amer, prepared the nanofibers under the guidance of W. Mamdouh. S. S. Amer, A. ElShaer, E. Polycarpou, R. T.A. Abdel-Aziz carried out the in-vitro experiments. S. S. Amer prepared the manuscript. W. Mamdouh, M. Nasr and O. A. Sammour reviewed, and approved the work.

Declaration of competing interest/ Financial and competing interest disclosure

The contribution represents original work that has not been previously published or simultaneously submitted for publication elsewhere.

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Figure Legends

Figure 1: Scanning electron microscope images (A, C, E) for the plain NFs formulation (P1) electrospun at a spinneret tip to collector distance 10 cm, solution flow rate 3 mL/h and applied voltage 20 KV, and their histograms (B, D).

Figure 2: Scanning electron microscope images for the plain NFs formulation (P1) showing the effect of increasing the flow rate on the morphology of fibers, where (A) 0.5 mL/h, (B) 3 mL/h, and (C) 4.5 mL/h. Figure 2 (D, E) Scanning electron microscope images of the plain NFs formulation (P1) showing the effect of increasing the distance from 8 cm as in (D) to 17 cm (E) on their morphology. Beads were indicated by a yellow arrow in images (A), (C), (D), and (E), indicating conditions other than the optimal.

Figure 3(A): Scanning electron microscope image for the PVA/QC/EOs NFs formulation (H1), and its histogram (B) showing an AFD of 354.95 nm. Figure 3(C): Scanning electron microscope image for the PVA/QC/EOs NFs formulation (H2), and its histogram (D) showing an AFD of 313.08 nm. Electrospinning best parameters for H1 and H2: Tip to collector distance 15 cm, solution flow rate 1.0 mL/h, applied voltage 25 KV. Figure 3(E): Scanning electron microscope image for the PVA/QC/EOs NFs formulation (H3) showing a significant number of beads as indicated in yellow.

Figure 4: Differential scanning colorimetry thermograms of (A) PVA powder (B) quercetin powder, and (C) selected NFs formulation H2.

Figure 5: Photograph for the anti-bacterial assay plates representing the *P. acne* inhibition zones when PVA/QC/EOs NFs formulation (H2) was placed on the right side of the plate (#15), and quercetin, on the left side of the plate (#16).

Figure 6: Representative patients receiving NFs formulation H2 (quercetin patches) on the right side, and Panthenol® cream (placebo), on the left side.