Cosm-nutraceutical nanovesicles for acne treatment: Physicochemical characterization and exploratory clinical experimentation

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Abstract

The full exploration of the 'nutraceuticals' therapeutic potential in cosmetics has been hindered by their poor stratum corneum permeation. Therefore, the aim of the present study was to formulate a nutraceutical; quercetin, in novel vitamin C based nanovesicles (aspasomes), and to explore their beneficial effects in the treatment of acne. Aspasomes were characterized for their particle size, zeta potential, entrapment efficiency (EE%), 3-months storage stability, skin deposition/permeation, antioxidant potential, and morphology. Aspasomes antibacterial efficacy on *Propionibacterium acnes* using the zone of inhibition assay was also tested, whilst their safety on skin fibroblastic cells was assessed *in vitro* using 3T3 CCL92 cell lines. An exploratory clinical trial was conducted in acne patients, and the percentage reduction of inflammatory, non-inflammatory and total acne lesions was taken as the evaluation criterion. Results revealed that quercetin-loaded aspasomes displayed a desirable nanometer size (125-184 nm), negative charge with good storage stability, and high skin deposition reaching 40%. Aspasomes managed to preserve the antioxidant activity of quercetin, and exhibited a significantly higher antibacterial effect (15 ± 1.53 mm) against *Propionibacterium acnes* than quercetin alone (8.25 ± 2.08 mm), and were safe on skin fibroblastic cells. Upon clinical examination in 20 acne patients (14 females, 6 males), quercetin aspasomes exhibited reduction percentages of 77.9%, 11.8% and 55.3% for inflammatory lesions, comedones and total lesions respectively. This opens vast applications of the presented formulation in the treatment of other oxidative skin diseases, and delineates the nutraceuticals and nanoformulations prepared from natural materials as promising dermatological treatment modes.

Keywords: Vitamin C; Aspasomes; Acne; Quercetin; Nanoparticles; Antibacterial.

1. Introduction

From the plethora of recent research, many novel nanosystems with topical delivery benefits were discovered (**Bseiso et al., 2015; Abdelgawad et al., 2017; Fadel et al., 2017; Hatem et al., 2018a,b; Amer et al., 2019; El-Kayal et al., 2019; Elmowafy et al., 2019; Shaaban et al., 2019**), with the ability to improve the *ex-vivo* cutaneous drug deposition and permeation. An antioxidant nanovesicular system; aspasomes was first reported by **Gopinath et al., 2004**, but was only recently assessed for their dermatological application in the treatment of androgenic alopecia by **Hatem et al., 2018c**. The basic component of aspasomes responsible for the anti-oxidant effect is ascorbyl palmitate, which is the lipid soluble and more stable form of ascorbic acid (vitamin C) (**Gopinath et al.,2004; Saini et al., 2014**).

A dermatological disease which could benefit from the antioxidant properties of an aspasomal delivery system is acne (**Morris, 1954; Peirce et al., 1959**). Acne vulgaris is a chronic dermatological disease of the pilosebaceous gland (hair follicles associated with the oil glands) affecting people in their adolescence (**Strauss et al., 2007; Titus and Hodge, 2012**). It is marked by the presence of different forms of acne lesions, appearing as inflammatory pimples (whitehead & blackhead), papules, pustules, cysts, and nodules, and non-inflammatory lesions (comedones) (**Adityan et al., 2009; Fabbrocini et al., 2010; Najafi-Taher and Amani, 2017**). The main causative organism for acne is *Propionibacterium acnes* (*P. acnes*) residing in the sebaceous glands. Together with the excess sebum present in the skin, they result in the blockage of pores, and the greasy look of the skin in acne patients. The antioxidant nature of aspasomes mainly attributed to its content of ascorbyl palmitate would help to overcome the implicated action of reactive oxygen species and lipid peroxide in the pathogenesis and progression of acne (**Mills et al., 2016**).

Owing to the reported therapeutic power of nutraceuticals, an antioxidant molecule; quercetin was chosen in the current study as the therapeutic moiety of choice to be loaded within aspasomes, owing to its anti-inflammatory, anti-bacterial and anti-oxidant properties (Maciel et al., 2013). To further accentuate the cosmetic nature of the prepared aspasomes, several essential oils namely tea tree and neem oils were tested for inclusion in the aspasomal formulations, owing to their favorable contribution in acne treatment (Raman et al., 1995; Enshaieh et al., 2007; Ulbricht et al., 2011; Vijayan et al., 2013). The present manuscript represents the first attempt for the clinical use of aspasomes in acne treatment. The prepared aspasomes were characterized for their pharmaceutical properties such as size, charge, storage stability, skin deposition ability, antioxidant potential, in addition to their safety and their antibacterial activity against *P. acnes*. Furthermore, an exploratory clinical trial was conducted to assess their therapeutic potential in the treatment of acne.

2. Experimental Section

2.1. Materials

Quercetin, ascorbyl palmitate, cholesterol, diphenyl-1-picrylhydrazyl (DPPH) and dicetyl phosphate were all purchased from Sigma Aldrich, Germany. Tea tree oil was purchased from Alpha Pharmaceutical Co., Egypt. Neem oil was purchased from Chemajet chemical Co., Egypt. *Propionibacterium acnes* ATCC 6919, methanol, acetonitrile and water (HPLC analytical grade solvents), and sodium chloride were purchased from Fisher Scientific, UK. Disodium hydrogen phosphate, sodium chloride and potassium dihydrogen phosphate were purchased from El Nasr chemical Co., Egypt. Acetyl Uranyl (Uranyl acetate -2- hydrate) was purchased from Allied signal, Riedel- dehaen, Germany. Horse blood agar, 3T3 CCL92 skin fibroblasts, trypsin, penicillin, streptomycin, barium chloride dehydrate, sulfuric acid, hydrogen peroxide, neutral red, ethanol

HPLC analytical grade and chloroform were all purchased from Sigma Aldrich Co., UK. Dulbecco Modified Eagle's Medium (DMEM), and FBS (Fetal Bovine serum 10%) were 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 The Nile Co. for Pharmaceuticals and Chemical Industries, Egypt. Viva-spin tubes (PES membrane, MWCO 300 kDa) were purchased from Sartorius, Netherlands.

2.2. Methods

2.2.1. Preparation of quercetin aspasomes using the thin film hydration method

Aspasomes were prepared using the thin film hydration technique, in which 30, 56.6 and 13.5 mg of ascorbyl palmitate, cholesterol and dicetyl phosphate respectively, 10 mg quercetin and 10 µL of either tea tree oil, neem oil or both were used to formulate three different aspasomal vesicles, as shown in **Table 1**. The amounts were selected based on a preliminary study (data not shown).

The lipidic ingredients were dissolved in organic solvent mixture of chloroform: methanol (2:1 v/v) (Nasr et al., 2008; Aref at al., 2017; Agiba et al., 2018). The solvent mixture was then allowed to evaporate at 40°C and 150 rpm (BUCHI Rotavapor R-114 WB B-480, USA) until the formation of a thin film of dry lipid. This was followed by portion-wise rehydration with 10 mL phosphate buffered saline (PBS, pH 7.4). The flask was rotated for 60 minutes at constant temperature allowing the hydration and maturation of the vesicles. The hydrated liquid aspasomal formulations were stored in glass vials at 4-8°C for further investigations.

2.2.2. Determination of the particle size and zeta potential of the aspasomal formulations

The particle size and zeta potential of the freshly prepared aspasomal formulations were determined using the Zeta-sizer (ZS90, Malvern Instruments, Ltd., Worcestershire, UK). Samples were appropriately diluted with filtered deionized water using a disposable cuvette at 25°C, detection angle 173°, following 2-minutes equilibration (**Bseiso et al., 2016; Mouez et al., 2016; Fadel et al., 2018**).

2.2.3. Determination of quercetin entrapment efficiency (EE%) in aspasomes

The entrapped amount of quercetin loaded in aspasomal formulations was determined by an ultra-filtration method using viva-spin centrifugal tubes, where the aspasomal formulations (2 mL) were transferred to the upper chamber of the viva-spin tubes and centrifuged at 4000 rpm at 25°C for 30 minutes (Bench Biofuge Centrifuge, Heraeus, Germany). An aliquot of the filtrate was then diluted using methanol (**Aref et al., 2017; Du et al., 2017**), and the amount of the un-entrapped (free) 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 x 250 mm, 5μm, USA), and the effluent was analyzed at 354 nm (**Kumari et al., 2010**).

The EE% was calculated according to equation 1:

$$EE\% = \frac{\text{Initial Quercetin amount} - \text{free unentrapped quercetin}}{\text{Initial quercetin}} \text{ X } 100 \qquad \text{(Equation 1)}$$

2.2.4. Physical stability assay of aspasomes

The aspasomal formulations were assessed for their physical stability, where samples were re-examined for changes occurring in the particle size, zeta potential and EE% after storage in the refrigerator at a temperature of 4-8°C for a period of three months. Samples were centrifuged first then assessed (**Nasr et al., 2008; Aref at al., 2017**).

2.2.5. *Ex-vivo* skin deposition of quercetin aspasomes

The *ex-vivo* skin deposition of quercetin selected aspasomal formulations was performed using the Franz diffusion apparatus (Variomag Telesystem, Germany) and analyzed by HPLC as described above. On the day of the experiment, the excised rat skin was defrosted, moistened with PBS of pH 7.4 one hour before usage, cut into small square pieces, and mounted between the receptor and the donor compartments of cells (1.77 cm^2) allowing the stratum corneum to face upwards (Singh et al., 2014; Bsieso et al., 2015; Nasr and Abdel-Hamid, 2016; Nasr et al., 2017). A specific amount of the aspasomes (0.1 mL) was placed onto the skin in the donor compartment, and the receptor compartment was filled with 7.5 mL PBS of pH 7.4 stirred at 150 rpm at 37°C (Gopinath et al., 2004; El Zaafarany et al., 2010). Five hundred microliter samples were withdrawn from the receptor compartments after 6 hours, diluted with methanol and analyzed for the permeated quercetin using HPLC as previously described. The skin samples were removed, washed five times with distilled water, then wiped with a clean tissue and dried by a clean filter paper to remove any excess aspasomal formulation, and finally placed in vials containing 10 mL methanol. Vials were then placed in the bath sonicator (Julabo, USR 3, Germany) for a period of one hour to allow extraction of the deposited quercetin. The amount of quercetin accumulated into the skin was expressed as a percentage of the total amount applied on the skin.

2.2.6. Diphenyl-1-picrylhydrazyl (DPPH) anti-oxidant assay of selected quercetin aspasomal formulations

The anti-oxidant activity of the selected quercetin aspasomal formulation was determined using the DPPH free radical scavenging assay. DPPH exhibits an obvious absorption maximum at 515 nm giving a characteristic purple color. The anti-oxidant potential is directly proportional to the disappearance of the purple color of the DPPH, and the formation of the yellow color of the

reduced DPPH, which depends on the number of hydrogen atoms donated by the anti-oxidant substance, and hence absorbed by the DPPH. Therefore, the anti-oxidant activity can be examined by following the decrease of UV absorption at 515 nm (Milton Roy, USA)(**Ashraf et al., 2018**). A 5 µL aliquot of quercetin, quercetin aspasomal formulations, and ascorbic acid control in methanol were added to 200 µL of DPPH methanolic solution of concentration 0.004% w/v. Absorbance readings of DPPH tested in 96 well plates were recorded immediately at 515 nm. The IC$_{50}$ values were estimated and the calculation of percentage inhibition of DPPH radical was calculated as previously described (**Ashraf et al., 2018**).

2.2.7. Morphological characterization of the selected quercetin aspasomal formulation using transmission electron microscope (TEM)

The selected aspasomal formulation was morphologically examined by the TEM (JEM-100S, Joel, Japan) after negative staining (**Nasr et al., 2013**). One drop of diluted sample was placed on a 200-mesh copper grid, then one drop of 1% uranyl acetate stain was added and allowed to dry and the excess fluid was removed before examination.

2.2.8. Assessment of the antibacterial potency of quercetin aspasomes

P. acnes was cultured under anaerobic conditions (a gas mixture of 80% nitrogen, 10% carbon dioxide and 10% hydrogen with no oxygen) at a temperature of 37°C (**Kishishita et al., 1980; Bojar and Holland, 2004; Perry and Lambert, 2006**). The bacteria were hydrated by brain heart infusion (BHI) and thoroughly mixed (**Tally et al., 1978; Kumar et al., 2007**), then they were inoculated onto a highly enriched blood agar prepared from Mueller-Hinton (MH) agar base and horse blood (**Dali et al., 2001**). The agar plates were placed into a jar where a gas tea bag and an anaerobic indicator were added to ensure that the conditions were free of oxygen (**Kumar et al., 2007**). The gas jar was then placed in the incubator and checked for bacterial growth on a two-

day basis for a period of 5-7 days. Following bacterial growth, a cotton swab of the bacteria was placed in PBS and vortexed several times allowing it to be the same color as 0.5 McFarland standard (equivalent to $1.5*10^8$ CFU/mL of micro-organisms) (**Wulansari et al., 2017**). Afterwards, a swab was taken from this solution to cover the blood agar plates which were then left to settle for a minute. Ten microliter samples of ethanol (as a control), quercetin ethanolic solution, and the selected aspasomal formulation were then added on to the agar plates (**Wulansari et al., 2017**). The plates were then covered and left in a well-sealed gas jar containing a tea bag, and an indicator to ensure that the conditions were free of oxygen. The jar was then placed in the incubator for 2 days after which the plates were removed, and the inhibition zones were measured in mm.

2.2.9. Safety of quercetin aspasomes on skin fibroblasts

The study was carried on 3T3 CCL92 cells, also known as the skin fibroblasts 3T3-Swiss Albino mouse (ATCC® CCL92™) to test the topical safety of the selected aspasomal formulation, similar to what was reported with other authors (**Jírová et al., 2003; Calabro et al., 2008**). The experiment was carried out in an aseptic environment inside the tissue culture hood. The nutrient media used was Dulbecco modified eagle's media (DMEM), to which 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics were added (**Hockley and Baxter, 1986; Calabro et al., 2008**). The vial containing the cells was removed from the liquid nitrogen where it was stored, defrosted and thawed in the water bath (37°C), then it was sterilized before being placed in the hood. The desired volume was withdrawn and placed in three 75-mL flasks each containing previously prepared DMEM. Flasks were then placed in the incubator, where cells were checked after two days for viability, motility and confluency. After reaching half-confluency, they were incubated again for another two days. The cells were afterwards rinsed with 0.25% (w/v)

trypsin, then 3-mL trypsin were added to the flask, agitated gently, and incubated for 3-5 minutes to activate the cells and detach them. Afterwards, 7-mL nutrient medium were added to the flask to inactivate the trypsin, then all the 10 mL were placed in a centrifuge tube and centrifuged at a rate of 125 x g for 10 minutes to produce a pellet that was used for further sub-culturing. After reaching a cell count of 5 x 10^4 cells/mL, cells were placed in the wells. Samples tested were the selected aspasomal formulation, the quercetin solution, in addition to the negative control where nutrient media was added to cells, as well as the hydrogen peroxide which was used as a positive control. The plates were then covered and incubated at adequate conditions (37°C/5% CO2 in air atmosphere/humidified atmosphere) overnight in the incucyte (Thermo Fischer Scientific, USA). The neutral red dye solution at a concentration of 40 µg/ mL was prepared and incubated overnight in the same conditions as the cells. After 24 hours, plates were checked under the microscope (Olympus CX41-MET, UK) for their viability and morphological changes. Samples were then decanted, and nutrient media containing neutral red dye were added to the cells followed by incubation for another two hours. A de-staining solution was then added to the plates, and plates were then shaken in the microtiter plate shaker for 10 minutes, followed by extraction of the neutral red, and the optical density (OD) of the neutral red extract was measured at 540 nm using a spectrophotometer (EnSpire Alpha plate reader, Perkin Elmer, USA)(**Lasarow et al., 1992;Repetto et al., 2008; Ates et al., 2017**).

2.2.10. Clinical evaluation of quercetin aspasomes on acne patients

The clinical study included patients of both genders suffering from acne vulgaris, selected from the Dermatology Out-patient Clinic of Minia University Hospital, Al-Minya, Egypt. This study included patients suffering from mild or moderate acne vulgaris according to grading of **NilFroushzadeh et al., 2009**. The study included 20 patients suffering from acne vulgaris (6 males

and 14 females), their age ranged from 14 to 27 years, of acne lesions duration between 1 to 7 months. Inclusion criteria involved patients who did not receive any treatment for acne or oral contraceptive pills at least 3 months prior to the study. Exclusion criteria included pregnant and nursing patients or those who suffered from hyperandrogenism. The clinical trial has been carried out in accordance with "The Code of Ethics of the World Medical Association (Declaration of Helsinki). An informed consent was obtained from each patient or his guardians, and the study was approved by the Committee for Postgraduate Studies and Research of Minia University, and the Committee for Postgraduate Studies and Research of Ain Shams University (REC- ASU#31). Patients were instructed to apply a thin film of the selected quercetin aspasomal formulation (a volume of 100 µL corresponding to a quercetin dose of 100 µg) on the right side of the face once daily and a placebo formulation (Panthenol®) on the left side of the face once daily for 12 hours. Panthenol® was chosen as a negative control, since there were no topical products available in the market for quercetin. The treatment period continued up to 8 weeks, and patients were instructed to report any discomfort or irritation encountered during the study (**Nasr et al., 2018**). 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 total acne lesions by 2-blinded dermatologists. The percentage reduction was determined for each of the inflammatory, comedonal, and the total acne lesions where it was calculated by the following equation (**Barakat et al., 2017**):

$$\frac{Number\ of\ lesions\ before\ administration - Number\ of\ lesions\ after\ administration}{Number\ of\ lesions\ before\ administration} \times 100\% \quad (Equation\ 2)$$

2.2.11. Statistical analysis

Experiments were done in triplicate and 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 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.

3. Results and Discussion

3.1. Preparation of quercetin aspasomal formulations

Till current date, only few papers about aspasomes have been published. Aspasomes formation required the three basic ingredients; ascorbyl palmitate, cholesterol and dicetyl phosphate. Owing to the bilayer forming property of ascorbyl palmitate, it is able to form vesicles (aspasomes) which exhibit an anti-oxidant action as reported by **Gopinath et al., 2004**, with the aid of cholesterol and dicetyl phosphate. Some concerns were raised by **Meves et al., 2002** that ascorbyl palmitate might cause skin damage after exposure to UV radiation, however this study was conducted *in vitro* in keratinocytes, and was not verified further by *in vivo* or clinical studies.

The three ingredients (ascorbyl palmitate, cholesterol and dicteyl phosphate) are considered essential for the formation of stable vesicles, where cholesterol was reported to modify the membrane properties and increase its mechanical strength (**Kirby et al., 1980; Magarkar et al., 2014**). As reported by **Kirby et al., 1980**; **Lee et al., 2005**; **Magarkar et al., 2014**, cholesterol intercalates in the bilayer with its polar head, and due to its hydrophobic properties, it occupies the interior portion of lipid bilayers and is able to fill the gaps and restrict any membrane component movements. Furthermore, a study reported by **Owen et al., 2018** suggested that cholesterol is important as it protects the lipid bilayer from hydrolytic degradation, oxidative stress, and water injury. The third ingredient is the dicetyl phosphate which was included as a negative charge

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inducer to create vesicles with stronger interaction with the surface of the skin achieving better topical delivery (Chen et al., 2012), and creates an electrostatic repulsion between adjacent vesicles, hence delaying the formation of aggregates.

3.2. Determination of the particle size and zeta potential of the aspasomal formulations

As shown in **Table 1**, quercetin aspasomal formulations displayed a particle size range of 125-184 nm with no separated oil droplets, suggesting the successful intercalation of oils within the bilayer. F3 formulated with 20 µL total oil content displayed significantly lower particle size compared to F1 and F2 (P<0.05), which may be attributed to the double fluidizing ability of the oils on the lipidic ascorbyl palmitate (Herman and Herman, 2014). Zeta potential measurement showed that all formulations were negatively charged with no significant difference between them (P>0.05), which is attributed to the presence of the negative charge inducer dicetyl phosphate (Villasmil-Sánchez et al., 2010; Chibowski andSzczes, 2016).

3.3. Determination of quercetin EE% in aspasomes

As shown in **Table 1**, all formulations displayed high EE% for quercetin ranging from (96-100%). It was also shown that formulae F2 and F3 prepared using tea tree oil showed a significantly higher EE% for quercetin than F1 prepared using neem oil only (P<0.05), suggesting augmented solubilization of quercetin in tea tree oil, with consequent increase in its entrapment within the bilayers. The high EE% encountered for all formulations could be ascribed to the utilization of the thin film hydration technique which creates multilamellar vesicles capable of entrapping large amounts of hydrophobic drugs as quercetin (Sharma and Sharma, 1997; Morilla et al., 2002; Sinico et al., 2005). Furthermore, the high EE% may be attributed to the lipophilicity of the quercetin (log P= 1.82 ± 0.32) (Rothwell et al., 2005), enabling its subtle

incorporation within the hydrophobic ascorbyl palmitate bilayer membrane. Furthermore, the presence of cholesterol in the aspasomal composition increases the hydrophobicity of the bilayer, therefore prompting more drug entrapment within the bilayers and less drug leakage, thus allowing a high EE% (**Lee et al., 2005; Nasr et al., 2008; Briuglia et al., 2015**). Also, it was reported by **Moribe et al., 1999** and **Singh et al., 2014** that cholesterol increases the micro-viscosity of the bilayer, resulting in an increase in its stability, and a decrease in its permeability, and therefore causing higher drug loading, entrapment, and retention. Since formulations F2 and F3 displayed higher EE% than F1, they proceeded for further characterization experiments.

3.4. Physical stability of the selected aspasomal formulations

As observed in **Table 1**, Formulations F2 and F3 exhibited good stability after 3 months storage at 4°C, manifested by minor increase in the particle size, and a minor decrease in EE%. Moreover, no significant change in the zeta potential values occurred after storage (P>0.05), further confirming the stability of aspasomes. The stability study was conducted at refrigeration rather than ambient conditions since the former is the standard storage protocol for vesicular systems (**Nasr and Wahdan, 2019**), to avoid the hydrolysis of lipids.

3.5. *Ex-vivo* skin deposition of quercetin aspasomes

The *ex-vivo* deposition of the quercetin loaded aspasomes in rat skin was investigated. Both formulations were able to deposit quercetin in the skin (27.10%±0.018) and (40.93%±0.022) for formulations F2 and F3 respectively, as shown in **Figure 1**. This could be ascribed to the film forming nature of vesicles, resulting in an occlusive effect and a decrease in the trans-epidermal water loss, therefore allowing more deposition of the drug (**Jenning et al., 2000; Muller et al., 2007**). In addition, it was reported by **Lauchli et al., 2012** that neem oil exerts a semi-occlusive effect on the skin, causing a balanced moist environment, and hence increases deposition. The

significantly lower deposition obtained with F2 compared to F3 may be ascribed to the smaller particle size of the latter, which allowed better accumulation in the outer skin layers (**Puglia et al., 2006; Adib et al., 2016**).

The cumulative percentage permeated of quercetin to the receptor compartment was less than 1% in both formulations; suggesting the topical rather than the transdermal traits of the quercetin loaded aspasomes. This low permeation rate of quercetin could be ascribed to its hydrophobic nature, as well as the hydrophobic nature of aspasomes (**Tan et al., 2011; Jangde and Singh, 2016**). Lastly, the presence of cholesterol may have accounted for the low quercetin permeation rates of both formulations, since it was reported by **Nagarsenker and Londhe, 2003; Singh et al., 2014** and **Briuglia et al., 2015** that cholesterol limits the movement of the relatively mobile hydrocarbon chains, and hence decreases the vesicular membrane permeability, and consequently the drug's permeation.

3.6. Anti-oxidant assessment of quercetin aspasomes

As evident in **Figure 2**, both formulations F2, F3 as well as the quercetin solution displayed high DPPH scavenging percentages at different concentrations. The aspasomal formulation F2 displayed significantly higher IC_{50} value of 3.65 μg/mL compared to F3 which displayed IC_{50} value of 1.39 μg/mL (P<0.05). The IC_{50} represents the concentration of quercetin causing 50% inhibition of the DPPH free radical, and hence F3 was considered more efficient in the free radical scavenging ability compared to F2, and its action was comparable to quercetin control solution, which displayed an IC_{50} of 1.52 μg/mL (P>0.05), suggesting that F3 was able to preserve the antioxidant potential of quercetin. This came in accordance with **Sessa et al., 2012** and **Chen et al., 2015**. The better antioxidant activity of formulation F3 compared to F2 can be correlated to the smaller particle size of the latter, as reported by **Makanjuola, 2017**. Moreover, the presence

of neem oil which is known to contain many anti-oxidant molecules such as azadirachtin, ascorbic acid and a large number of phenolic compounds may have further contributed to its better free radical scavenging action (**Olabinri et al., 2009; Hossain et al., 2014**). Interestingly, the quercetin solution and aspasomal formulations displayed significantly lower IC_{50} values compared to the reference ascorbic acid, which showed an IC_{50} of 14.5 μg/mL, suggesting that quercetin was a more potent antioxidant due to the presence of many hydroxyl groups in its structure (**Rice-Evans et al., 1997; Pool et al., 2012**).

Based on the combined effect of particle size, EE%, physical stability, *ex-vivo* deposition and DPPH assays, F3 was chosen as the selected formulation for further characterization steps.

3.7. Morphological characterization of the selected aspasomal formulation

The quercetin aspasomal formulation F3 was visualized using TEM, which displayed the structure of well identified vesicles of sealed spherical core and coat nature, as shown in **Figure 3**.

3.8. Assessment of the antibacterial potential of the selected aspasomal formulation

The anti-bacterial effect was measured by calculating the average inhibition zone for quercetin and its aspasomal formulation F3, in which they displayed 8.25±2.08 mm and 15±1.53 mm respectively. The larger inhibition zone encountered with the latter suggests that the presence of tea tree oil, neem oil, ascorbyl palmitate and the other aspasomes forming ingredients had potentiated the anti-acne activity of the formulation. Tea tree oil was reported to exhibit antibacterial action on *P. acnes* (**Raman et al., 1995; Pazyar et al., 2013; Hammer, 2014; Wulansari et al., 2017**) owing to the presence of terpinen-4-ol (**Flores et al., 2011**). Neem oil was also reported to exhibit an anti-bacterial activity against *P. acnes* (**Alzohairy, 2016**) owing to its content of various anti-bacterial ingredients used in acne treatment (**Vijayan et al., 2013**).

3.9. Safety of quercetin aspasomes on skin fibroblasts

The neutral red assay is based on the ability of the viable cells to incorporate and bind the cationic neutral red dye after being treated with the test substance, by actively transporting the dye into the lysosomes (Repetto et al., 2008). When the viable cells are lysed, they release the dye, where the amount of the released dye is equivalent to the number of viable cells, and it can be measured spectrophotometrically at 540 nm.

As shown in **Figure 4**, cells receiving no treatment displayed 100% viability, while those receiving hydrogen peroxide as a positive control displayed significant death (26% viability). The quercetin solution, and the aspasomal formulation F3 displayed high viability for the cells of 93.73± 3.14, and 92.47± 3.99 respectively (P<0.05). This may be attributed to the herbal nature of quercetin, and the use of highly safe ingredients in the preparation of the aspasomal formulation. The individual components of the formulations were all reported safe for topical use; Andersen, 1999; Lanigan, 1999 and Khan et al., 2016 reported that ascorbyl palmitate was considered safe, non-irritating for the skin, and showed no signs of sensitization in clinical studies. Cholesterol was also confirmed to be a non-toxic and a non-irritant ingredient when applied on the skin, where it is used as an emulsifier in the cosmetic products and considered safe to be used in skin products. As for the dicetyl phosphate, Ueoka and Moraes, 2018 reported its use as a surfactant and as an emulsifier as well in the cosmeceutical industry. Moreover, Pazyar et al., 2013; Hammer, 2014 and Rajkowska et al., 2014 reported that tea tree oil was considered non-toxic and safe to be applied on the skin. As for the neem oil, Aneesa and Gayathri, 2016 reported its use as an anti-inflammatory, anti-allergic and an anti-bacterial oil when applied externally, and hence is considered safe when applied on to the skin. Quercetin itself was also reported to be used topically for wound healing and guarding against skin oxidative damage, which ensured its topical safety

(**Martin, 1996; Kumar et al., 2017**). All this shows that both the quercetin solution, and the aspasomal formulation F3 are considered highly safe on the cellular level, hence nominating aspasomes for clinical testing.

3.10. Clinical evaluation of quercetin aspasomes on acne patients

After 2 months of applying the quercetin aspasomal formulation F3 on the acne lesions, the percentage reduction for the inflammatory lesions, the comedones, and the total acne lesions was calculated. As can be seen in **Tables 2** and **3** and **Figures 5** and **6**, the aspasomal formulation F3 applied on acne lesions of the right side of the face displayed reduction percentages of 77.9%, 11.8% and 55.3% for inflammatory lesions, comedones and total lesions respectively. There was a significant decrease in the count of inflammatory and total acne lesions ($P< 0.05$) with no significant decrease in the count of comedones ($P> 0.05$) compared to the left side receiving topical Panthenol® cream, which displayed reduction percentages of 6.7%, 19.1% and 10.1% for inflammatory lesions, comedones and total lesions respectively. Aspasomes were more powerful on inflammatory lesions than on comedones, as evident from the significant higher percentage reduction for the former compared to the latter ($P<0.05$). Moreover, no local irritation, burning or dermatitis was observed during the treatment period (8 weeks) as reported by the patients treated with the aspasomal formulation F3. Patients reported that the formulation was devoid of greasiness and was light to apply. Moreover, they reported that it had retentive properties on the skin and was sufficiently acceptable.

Based on the aforementioned results, quercetin loaded aspasomes proved to be a promising nano-vesicular system for the treatment of different acne lesions. Their anti-inflammatory action as manifested by the significant reduction of the total lesion count (mainly the inflammatory lesions) could be ascribed to the use of tea tree oil, which exhibits anti-inflammatory properties as

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reported by **Hart et al., 2000; Pazyar et al., 2013**. Furthermore, **Yadav and Rao, 2012; Aneesa and Gayathri, 2016** also reported that neem oil shows anti-inflammatory properties, which hence allows it to work better on inflammatory papules, rather than comedones. In addition, the presence of quercetin which also acts as an anti-inflammatory molecule majorly contributes to the anti-inflammatory properties of F3 (**Kumar et al., 2017**). Moreover, **Telang, 2013** reported that vitamin C was able to inhibit NF-kB, exerting an anti-inflammatory action, and hence, allowing the enhancement of the anti-inflammatory properties of F3 on the inflammatory papules rather than the comedones. Lastly, the amphipathic nature of the aspasomal vesicles in addition to their nanometer range allowed for their good skin interaction, and promotion of their therapeutic action (**Hatem et al., 2018c**).

4. Conclusion

As seen from all presented results, the preparation of vitamin C-based nano-vesicular system loaded with quercetin was proven to be successful in the treatment of acne vulgaris. This paves the way for the emergence of whole line of natural nanocosmeceutical delivery systems that can be successfully used in the treatment of other topical dermatological diseases.

Acknowledgement

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Figure legends
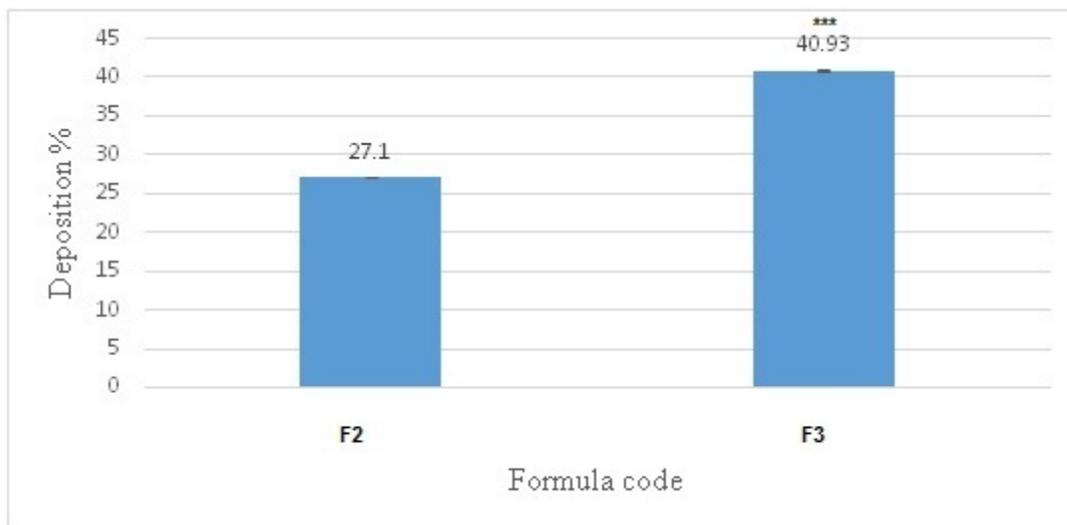
Figure 1: Skin deposition percentage of quercetin from formulations F2 and F3, displaying significantly higher deposition for the latter (*** P<0.0001).
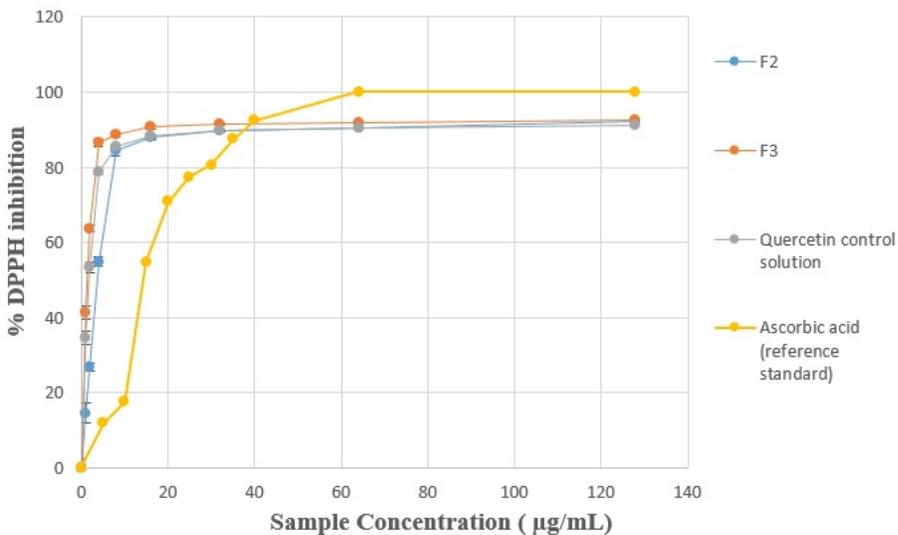
Figure 2: DPPH free radical scavenging effects of quercetin vesicular formulations F2 and F3 compared to quercetin control solution and ascorbic acid reference (Mean±S.D), showing the ability of formulation F3 to preserve the antioxidant potential of quercetin.
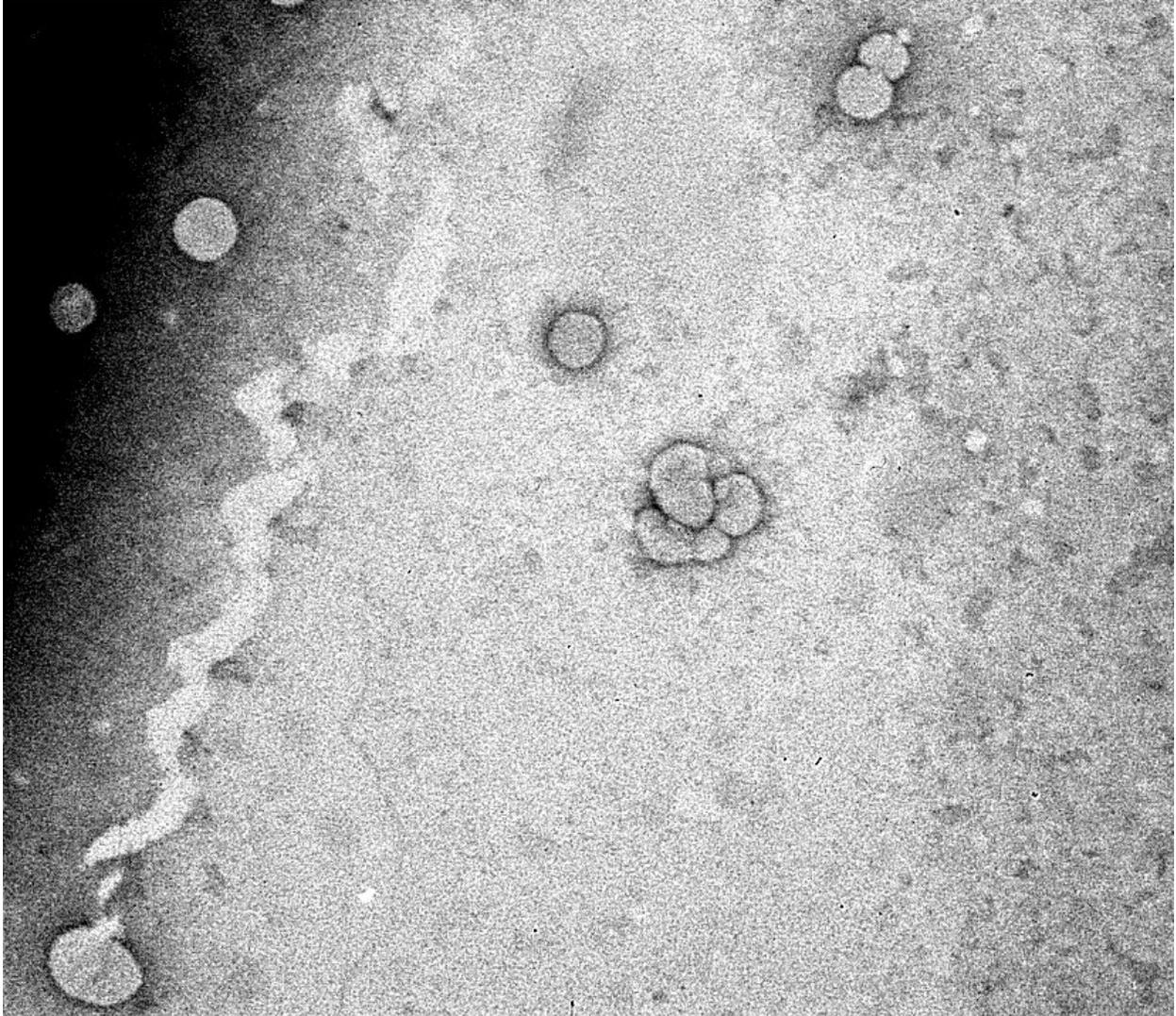
500 nm

TEM Mag = 60000x

Figure 3: TEM micrograph of F3 at a magnification of 60000X displaying the spherical nature of the aspasomal vesicles.
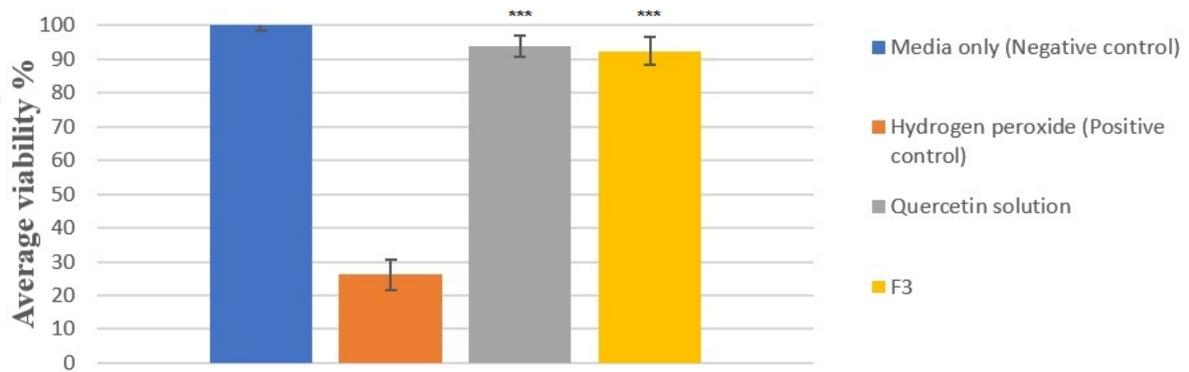
Figure 4: The average viability percentages of the cells when treated with different samples (the selected formulation F3 in comparison to the media as negative control, hydrogen peroxide as positive control and the quercetin solution (*** significantly higher viability from positive control (P<0.0001), and insignificantly different from each other P>0.05) .
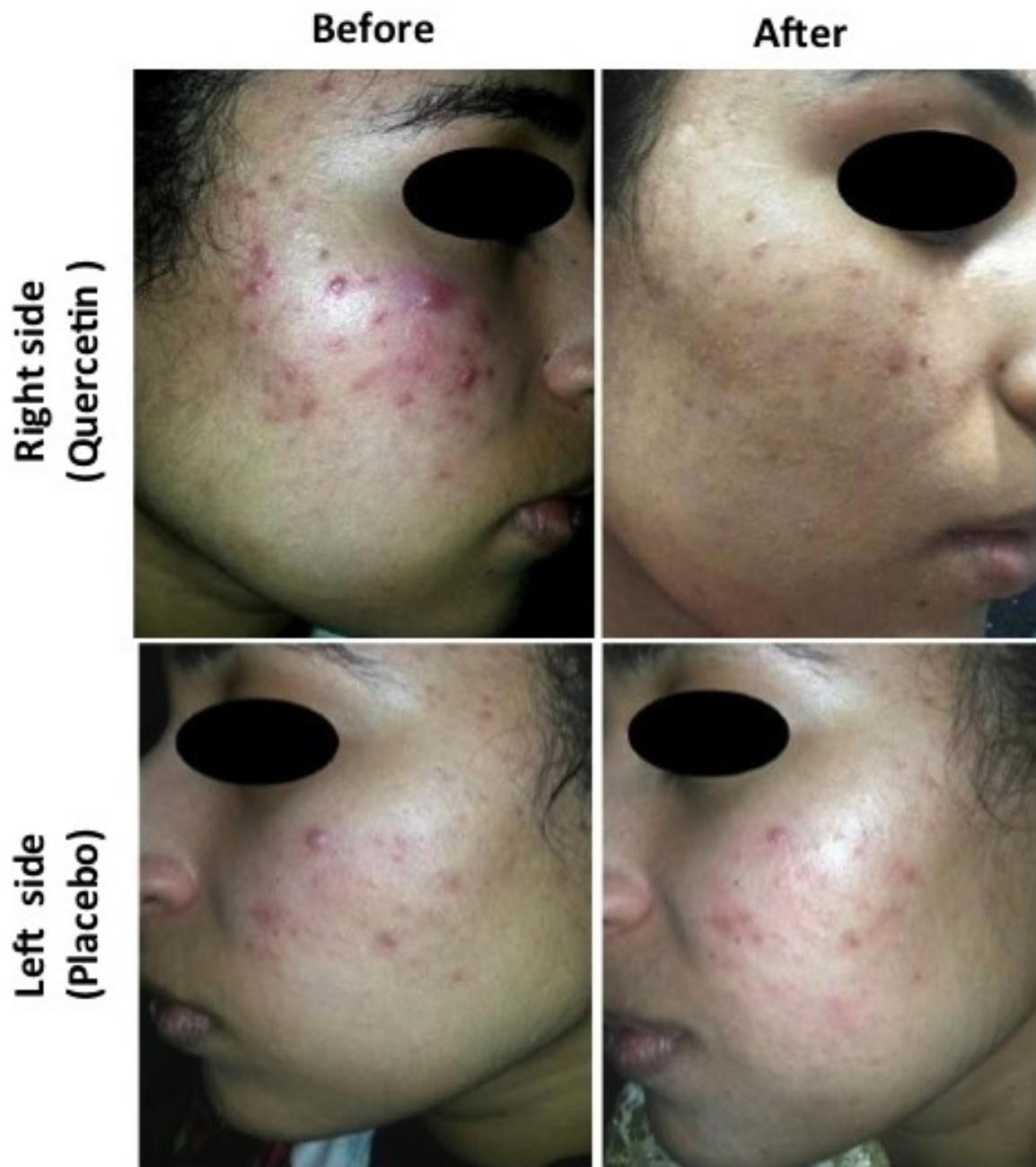
Figure 5: Representative patient receiving aspasomal formulation F3 on the right side and Panthenol® cream on the left side, showing better anti-acne effect of the former manifested by better reduction in the number of acne lesions.
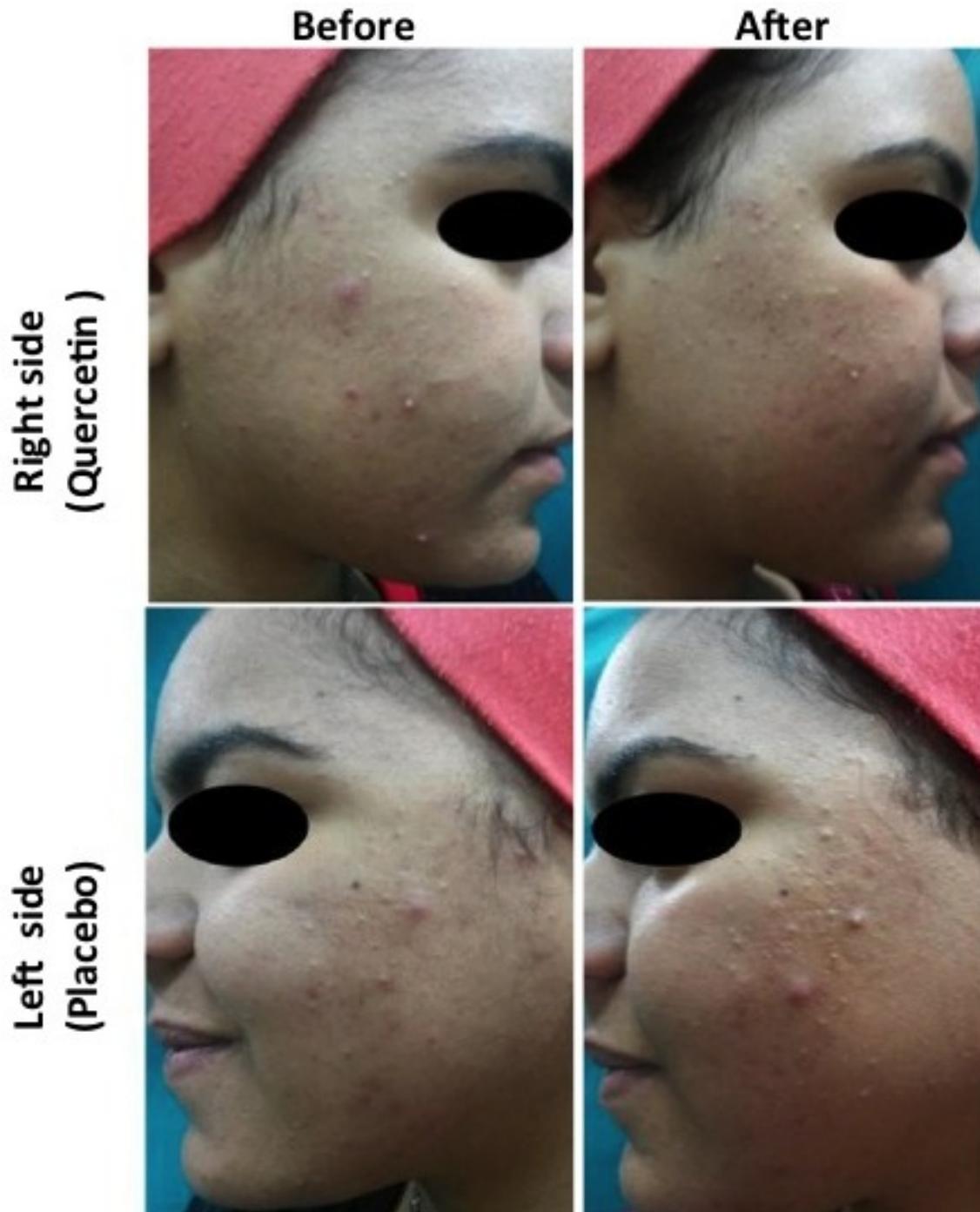
Figure 6: Representative patient receiving aspasomal formulation F3 on the right side and Panthenol® cream on the left side, showing better anti-acne effect of the former manifested by better reduction in the number of acne lesions.

Table 1: Characterization of the prepared quercetin aspasomal formulations

Formula code*	Oil	Particle size (nm) Mean ±S.D. (freshly prepared)	Particle size (nm) Mean ±S.D. (after storage)	Zeta potential (mV) Mean± S.D. (freshly prepared)	Zeta potential (mV) Mean± S.D. (after storage)	EE % Mean ± S.D. (freshly prepared) and content (mg/g of formulation)	EE % Mean ± S.D. (after storage) and content (mg/g of formulation)
F1	10 µL neem oil	184±7	N.D.	-100±13	N.D.	96±0.1 (0.96 mg/g of formulation)	N.D.
F2	10 µL tea tree oil	182±5	206±3	-87±7	-84±4	100±0.01 (1 mg/g of formulation)	93±0.02 (0.93 mg/g of formulation)
F3	10 µL neem oil +10 µL tea tree oil	125±3	142±2	-80±2	-77±2	100±0.01 (1 mg/g of formulation)	91±0.01 (0.91 mg/g of formulation)

* All formulae were rehydrated with 10 ml phosphate buffer (PB pH 7.4), and contained 10 mg quercetin

Table 2: The percentage reduction for the inflammatory lesions, comedones, and the total lesions for patients after application of aspasomal formulation F3 on the right side of the face

Patient number									

Patient number	Inf.B	Inf.A	% reduction*	Com.B	Com.A	% reduction*	Tot.B	Tot.A	% reduction*
1	13	3	76.9	11	10	9.1	24	13	45.8
2	17	2	88.2	8	7	12.5	25	9	64.0
3	15	3	80.0	2	2	0.0	17	5	70.6
4	13	2	84.6	9	8	11.1	22	10	54.5
5	14	2	85.7	11	9	18.2	25	11	56.0
6	8	1	87.5	4	4	0.0	12	5	58.3
7	9	1	88.9	10	9	10.0	19	10	47.4
8	12	3	75.0	7	7	0.0	19	10	47.4
9	10	3	70.0	6	4	33.3	16	7	56.3
10	11	2	81.8	8	6	25.0	19	8	57.9
11	13	2	84.6	4	3	25.0	17	5	70.6
12	14	2	85.7	7	7	0.0	21	9	57.1
13	17	2	88.2	9	9	0.0	26	11	57.7
14	11	4	63.6	2	2	0.0	13	6	53.8
15	12	2	83.3	8	7	12.5	20	9	55.0
16	13	6	53.8	11	11	0.0	24	17	29.2
17	11	5	54.5	10	8	20.0	21	13	38.1
18	12	3	75.0	5	5	0.0	17	8	52.9
19	15	2	86.7	5	2	60.0	20	4	80.0
20	14	5	64.3	3	3	0.0	17	8	52.9
	Average % reduction		77.9 ± 11.1	Average % reduction		11.8 ± 15.4	Average % reduction		55.3± 11.2

* 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

Table 3: 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

Left side									
Patient number	Inf.B	Inf.A	% reduction*	Com.B	Com.A	% reduction*	Tot.B	Tot.A	% reduction*
1	14	13	7.1	10	9	10.0	24	22	8.3
2	18	17	5.6	7	6	14.3	25	23	8.0
3	16	17	-6.3	3	3	0.0	19	20	-5.3
4	15	14	6.7	7	6	14.3	22	20	9.1
5	14	13	7.1	10	8	20.0	24	21	12.5
6	9	9	0.0	4	4	0.0	13	13	0.0
7	11	9	18.2	11	9	18.2	22	18	18.2
8	13	11	15.4	8	7	12.5	21	18	14.3
9	11	11	0.0	7	6	14.3	18	17	5.6
10	13	12	7.7	7	5	28.6	20	17	15.0
11	12	11	8.3	3	2	33.3	15	13	13.3
12	12	10	16.7	8	5	37.5	20	15	25.0
13	15	14	6.7	10	9	10.0	25	23	8.0
14	10	10	0.0	3	3	0.0	13	13	0.0
15	15	13	13.3	7	6	14.3	22	19	13.6
16	14	11	21.4	10	10	0.0	24	21	12.5
17	11	11	0.0	11	8	27.3	22	19	13.6
18	14	14	0.0	5	4	20.0	19	18	5.3
19	17	16	5.9	5	3	40.0	22	19	13.6
20	14	14	0.0	3	1	66.7	17	15	11.8
	Average % reduction		6.7± 7.3	Average % reduction		19.1± 16.5	Average % reduction		10.1± 6.8

* 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

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