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ORIGINAL RESEARCH



Evaluation of chamomile oil and nanoemulgels as a promising treatment option for atopic dermatitis induced in rats

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ABSTRACT

Background: Atopic dermatitis is a chronic inflammatory skin disease that remarkably affects the quality-of-life of patients. Chamomile oil is used to treat skin inflammations. We evaluated the efficacy of chamomile oil and nanoemulgel formulations as a natural alternative therapeutic option for atopic dermatitis.

Research design and methods: Formulations were developed comprising chamomile oil: olive oil (1:1), Tween 20/80 or Gelucire 44/14 as surfactant-cosurfactant mixtures, propylene glycol (10%w/w), water and hydroxypropyl methylcellulose (3%w/w). In-vitro physicochemical characterization, stability testing and in-vivo assessment of inflammatory biomarkers and histopathological examination of skin lesions were conducted in rats induced with atopic dermatitis.

Results: Nanoemulgels G₁ and X₁ which displayed the smallest particle size of 137.5 ± 2.04 and 207.1 ± 5.44 nm, good homogeneity and high zeta-potential values of -26.4 and -32.7 mV were selected as the optimized emulgel. Nanoemulgels were nonirritating of pH value 5.56, readily spreadable, and were physically stable following 10 heating-cooling cycles. Treatment with nanoemulgels showed a two-fold decrease in duration of skin healing and no spongiosis compared to chamomile oil. Levels of biomarkers were reduced after topical application of both nanoemulgels and chamomile oil.

Conclusion: Nanoemulgels are a potential cost effective, safe topical carrier system for chamomile in treating atopic dermatitis.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with remissions and exacerbations that affect individuals who genetically tend to overreact to external factors. It is commonly associated with other manifestations of atopy as allergic rhinitis or bronchial asthma. It has a significant impact on the quality-of-life of patients and their families [1,2]. The most common manifestations of AD are extreme skin dryness, itching, erythema, scaly patches, and thickened lichenified plaques with excoriation [3]. Extreme skin dryness is linked to activity change of $\Delta 6$ -desaturase, an enzyme catalyzing transformation of linolenic acid into γ -linolenic form. Patients affected with this disease exhibit a low level of essential fatty acids and disorder of lipid production in epidermis, which are of importance during formation and persistence of dermal changes. Moreover, AD patients suffer from frequent bacterial and viral skin infections [4].

The pathogenesis of the AD is inflammatory in origin, linked to activation of T cells. Antigen-specific T cells play an important role in the pathogenesis of AD at a cellular level [5], infiltrating AD skin lesions producing different cytokines. T helper 1 (Th1) cells secrete interleukin 2 (IL-2) and interferon

γ (IFN- γ), while T helper 2 (Th2) cells secrete interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 13 (IL-13). IL-4 is the major factor regulating immunoglobulin E (IgE) production by $\square$ cells. This is followed by the interaction of keratinocytes, endothelium cells, eosinophils, Langerhans cells, T cells, interleukins, and chemical mediators [4,6].

Interleukin-18 (IL-18) is a proinflammatory cytokine secreted by Kupffer cells of the liver and activated macrophages. IL-18 strongly enhances IFN- γ production by T cells, natural killer cell cytotoxicity, and T cell proliferation. The severity of AD highly correlates with the levels of serum Ig E and plasma IL-18 levels [7,8].

Nowadays aromatherapy is one of the increasingly used complementary therapies. Essential oils are major therapeutic agents used to treat several diseases, representing a major class of interest in drug research [9]. German chamomile (*Matricaria chamomilla*), belonging to the family Asteraceae, has been used as herbal medication since ancient times. Chamomile is widely used to treat skin inflammations and various bacterial skin infections, for its anti-inflammatory, antioxidant, mild astringent, and healing potential [10]. The pharmacological activity of

German chamomile is associated with its essential oil and flavonoid content. The principal anti-inflammatory and antispasmodic constituents of chamomile oil (CM) are matricin, chamazulene, α -bisabolol, and its oxides A and B [11].

Although chamomile is a very promising herb, research regarding its formulation and application in medicine is rare. CM is volatile in nature, with poor tissue permeability and degradation. Therefore, its beneficial therapeutic properties require a delivery approach that could improve its penetrative power and avoid its volatility. This might be achieved by delivering CM in a sustained manner by using a delivery carrier as nanoemulgel (EM). The EM is a nanoemulsion system intergraded into hydrogel matrix which affords favorable skin penetration properties [12]. In addition to being thixotropic and having a long shelf life, EM is greaseless, easily spreadable and removable. EM is emollient, non-staining, bio-friendly, with a pleasant appearance compared to pure CM. Moreover, nanoemulgels offer high patient acceptability, being noninvasive, and easily applicable [13–15]. Previous studies reported the easy incorporation and the enhanced skin permeability of lipophilic drugs in nanoemulgels. This was attributed to the fine distribution of nanoemulsion phase, resulting in a significant improvement of the drug therapeutic efficacy [16].

To the authors' knowledge, no studies concerning the formulation and evaluation of CM-loaded nanoemulgel have been published. In this light, the present study aimed to evaluate the efficacy of pure CM and its EM formulations as a natural alternative therapeutic option for AD. *In-vitro* physicochemical characterization and stability testing for EM was performed. *In-vivo* anti-inflammatory effect of the selected formulations was also evaluated by histopathological examination of AD skin lesions induced in rats and by assessing different inflammatory biomarkers related to AD.

2. Materials and methods

2.1. Chemicals and reagents

Chamomile and olive oils were purchased from Chemajet Chemical Company, Alexandria, Egypt. Propylene glycol and Hydroxypropyl methylcellulose (HPMC) 4000 cp were kindly gifted by Pharaonia Pharmaceuticals, Alexandria, Egypt. Gelucire 44/14 sample was a gift from Gattefosse, Lyon, France. Polysorbate 20 & 80 (Tween 20 & 80) were purchased from El-Nasr Pharmaceutical Chemicals Co. Capsaicin was purchased from Sigma Aldrich (CAT # 21,750), Enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturers' instructions: Boster, Picokine TM (CAT # EK0406), Aviva system biology (CAT # OKEH04042), Quantikine® ELISA (CAT # M2200), (antibodies-online.com CAT# ABIN455406), Hexabiogen (CAT # MBS703318) and Biodiagnostic (CAT #2533).

2.2. Preliminary screening of surfactants and co-surfactants for EM preparation

Concentrations of 300 mg of different surfactants (Tween 20, Tween 80, Span 20, Span 80, Brij 35 and Gelucire 44/14) were added to 300 mg of the oily phase (chamomile oil and olive oil, 1:1 ratio). The mixtures were gently heated at 50°C for

homogenization of the components. For each oil-surfactant mixture, 50 mg was then diluted with distilled water to 50 mL in a stoppered conical flask. The resulting mixture was observed visually for the relative turbidity and transmittance was assessed by UV-VIS spectrophotometer (Shimadzu-1700, Japan) at 638 nm, using double distilled water as blank [17].

2.3. Preparation of chamomile oil nanoemulgels (CM-EMs)

EMs were prepared using surfactants and co-surfactants selected on the basis of their % transmittance. The total amount of the oil phase in the prepared formulations composed of chamomile and olive oil were kept constant at 2% w/w in 1:1 ratio. The calculated quantities of oils, surfactant, co-surfactant, propylene glycol, and 1/3 the quantity of water were mixed and heated to 60°C for 2 minutes to obtain a clear mixture, then homogenized (Ultra Turrax T25, IKA, Staufen, Germany) at 10,000 rpm for 2 minutes to form the oil in water nanoemulsion. CM-EMs was then obtained by adding HPMC (3% w/w) and the remaining amount of water to the prepared nanoemulsion. The formulation was homogenized at 10,000 rpm for 5 minutes and then stored in the refrigerator (4°C) for further characterization. The detailed formulae are shown in Table 1.

2.4. Selection of the most stable formulations

The prepared CM-EMs formulations were inspected visually for color, homogeneity, and by centrifugation testing for phase separation. Centrifugation test was performed at 10,000 rpm in Eppendorf tubes using a high speed cooling centrifuge (Centurion Scientific Ltd, UK). The cooling centrifuge was operated at 0°C for 5 minutes. Formulations showing acceptable properties with no phase separation were selected for further characterization.

2.5. Characterization of the stable CM-EMs formulations

2.5.1. Droplet size analysis

The droplet size and polydispersity index (PDI) of the EMs were determined through dynamic light scattering at 25°C using Malvern Zetasizer (NanoZS, Malvern Instruments, Malvern, UK). Before measurement, the samples were diluted to a weak opalescence by filtered distilled water. Measurements were done using glass cuvettes with square apertures. The measurement position was 4.65 mm and the dispersant refractive index was 1.330. Each sample was measured in triplicates and the results were expressed as means $\pm$ SD.

Table 1. Composition of chamomile oil nanoemulgels.

Formulation composition (g)	Formulation code									
	T ₁	T ₂	T ₃	T ₄	G ₁	G ₂	G ₃	G ₄	X ₁	X ₂
Tween 80	1	1	2	2	1	1	2	2	1	2
Tween 20	1	2	1	2	0	0	0	0	0	0
Gelucire 44/14	0	0	0	0	1	2	1	2	0	0
HPMC	3	3	3	3	3	3	3	3	3	3
Water	34	33	33	32	34	33	33	32	35	34

*each formulation contains; chamomile oil (0.5 g), olive oil (0.5 g), propylene glycol (10 g)

2.5.2. Zeta potential

The zeta-potential (ZP) of the diluted EMs was determined using Zetasizer and the electrophoretic mobility determined at $25 \pm 0.5^\circ\text{C}$ and dispersant dielectric constant of 78.5 using a clear disposable zeta cell. The obtained electrophoretic mobility values were used to calculate the ZP using the software DTS version 4.1 (Malvern, England). All experiments were done in triplicates and expressed as means $\pm$ SD.

2.5.3. pH measurement

The pH of the EMs was measured in triplicates at 25°C using a calibrated pH meter (pH 211 Microprocessor pH meter, Hanna instruments, Romania). Samples were prepared by dilution of 0.2 gram of CM-EM to 10 mL with purified water before measurement [18].

2.5.4. Spreadability measurement

The spreadability test was conducted by pressing 0.2 gm of each prepared formulation between two glass slides and left for about 5 minutes until no more spreading occurred. Measurements were done in triplicates and the diameter of the formed circles were measured in cm and used as comparative values for spreadability [19–21]. Results were expressed as means $\pm$ SD.

2.6. Characterization of the selected CM-EMs formulae

2.6.1. Transmission electron microscope

The morphology of the selected EMs was observed using transmission electron microscope (TEM) (JEM-1400S plus, Joel, Tokyo, Japan) by negative staining method. One drop of the diluted EM sample was deposited on a carbon coated copper grid, then stained by a drop of uranyl acetate in ethanol (2% w/v). The excess liquid was removed and the grid was dried at room temperature then, the samples were observed using TEM [18].

2.6.2. Rheological studies

The viscosities of EMs were determined at 25°C using Brookfield viscometer (Brookfield digital viscometer, RV DV-II +Pro, Brookfield Engineering Laboratories, Middleborough, USA). A sample of the gel was placed in a suitable beaker and the instrument continuously sheared the material at various rates, using spindle 04. Measurement was done over a range of speeds: 8, 10, 15, 22, 40 rpm each for 60 seconds. All measurements were done in triplicates [22].

2.6.3. Stability tests

2.6.3.1. Freeze/thaw cycling. EMs were subjected to a total of ten complete cycles. Each cycle consisted of 24 hrs at 25°C followed by 24 hrs at -4°C . These cycles were important for determining the ability of the CM-EM to withstand thermal shock, as well as to evaluate their physical stability by determining droplet size and ZP measurements.

2.6.3.2. Aging test. The EMs were stored in well closed glass containers and kept in the refrigerator (4°C) for six months.

The changes in physical appearance, droplet size, and ZP were investigated.

2.7. In-vivo study

All experiments were performed in accordance to the Research Ethical Committee of the Faculty of Pharmacy and Drug Manufacturing, Pharos University in Alexandria, Alexandria, Egypt and comply with the National Research Council's guide for the care and use of laboratory animals.

Pregnant Sprague Dawley rats were housed for 2 or 3 days before parturition. Their newborns were weaned at postnatal week 3 according to Han *et al* study [23]. Thirty males only were separated and used. All animals were nurtured in a room maintained at a 12-h light/dark cycle with access to food and water.

2.7.1. Skin irritation study

A 0.5 gm of the two selected formulae was applied on a 5 cm^2 area of the shaved dorsal side of the rats daily for three consecutive days. The skin surface was monitored for any visible changes such as erythema and edema, after 24, 48, and 72 hrs of the formulation application. The mean erythema scores were recorded depending upon the degree of erythema as reported by Afzal Hussain *et al* [24].

2.7.2. Induction of dermatitis and treatment design

Neonatal rats at 7 weeks of age were shaved off their hair at the dorsal skin as previously described by Back *et al*. [25]. They were divided into five groups, five rats each. The negative control group (Group N) was injected by the vehicle (saline containing 10% Tween 80 and 10% ethanol), whereas the other four groups were injected subcutaneously with 50 mg/kg of capsaicin to induce dermatitis [26] at a volume of $10\text{ }\mu\text{L/g}$ body weight [27]. After development of cutaneous lesions, a group was left without treatment (Group P) and three groups received treatment with CM (Group CM) and the selected emulgels, (Group EM.X₁) and (Group EM.G₁). Topical treatment was applied in skin lesions two times daily started after appearance of ulcerative skin lesions and treatment continued till the lesions disappeared.

2.7.3. Evaluation of cutaneous lesions

The severity of dermatitis was measured according to the scoring system previously described by Mihara and coworkers [28].

2.7.4. Sampling of the tissues and blood

Rats were ether anesthetized, euthanized by cervical dislocation, and decapitated. Skin samples were taken in the middle of the back of the neck of all rats. Samples were taken for group N, P and after disappearance of cutaneous lesions for the treated groups (CM, EM.X₁, and EM.G₁). Skin samples were immediately washed in phosphate buffered saline (PBS), then fixed in 10% neutral buffered formalin solution and prepared for histological examination. Blood was simultaneously collected in tubes containing Ethylene diamine tetra acetic acid (EDTA), from the posterior vena cava through a laparotomy incision. Samples were centrifuged and plasma was collected and stored at -80°C until they were used in ELISA procedures.

2.7.5. Histopathology of skin tissues

Skin samples were trimmed, subjected to serial passages of ethyl alcohol, cleared in xylene, embedded in paraffin and then sectioned using a microtome at 5 μ m slices (Microtome, Leica RM2155, Leica Inc, Nussloch, Germany). The sections were stained by hematoxylin and eosin (H&E) dye. Histological examination was done under light microscope (Olympus Electron Microscope, Olympus, Japan).

2.7.6. Enzyme-linked immunosorbent assay (ELISA)

The skin lesions were homogenized on ice five times with ice-cold PBS at a pH of 7.4. Skin homogenates were then centrifuged (15,000 rpm, 4°C for 10 min). Supernatants were aliquoted into Eppendorf tubes and stored at –80°C until they were used in ELISA measurement. Skin homogenates were used to assess the level of IL-4, IL-18, IL-22, IgE, eosinophil cationic protein (ECP) and nitrous oxide (NO) in all studied groups, while plasma samples were used to assess the levels of IL-22 and NO. ELISA kits were used according to the manufacturers' instructions.

2.8. Statistical analysis

Data were reported as mean $\pm$ standard deviation (SD) and compared using one-way ANOVA analysis followed by Tukey post hoc test and independent T-test for data; $p < 0.01$, 0.05 was considered significant. Statistical analyses were performed using the software package Prism® 7 (GraphPad Software, Inc., CA, USA).

3. Results

Different nonionic surfactants were screened for their emulsification efficacy based on the percentage transmittance. Among the screened surfactants; Tween 80, Tween 20 and Gelucire 44/14 were selected (Table 1), where they revealed a % transmittance of 38, 17.3, and 12.9% respectively. Propylene glycol also showed 18.9% transmittance. It was used in a concentration of 10% w/w in all formulations as a co-surfactant and humectant.

Based on preliminary trials (data not shown), HPMC in concentration of 3% w/w was found to be suitable for gelling the nanoemulsion because of its desirable consistency, feel and ease of topical spreadability.

3.1. Physical examination

All prepared CM-EMs formulations were bluish in color, viscous, washable, creamy preparations with a smooth and homogeneous appearance. Although one of instability problems of an emulsion in general is creaming, formulation as

an emulgel is expected to minimize this problem due to the increased viscosity. In this context, centrifugation at a high speed can be employed to indicate the potential of destabilization process and to reflect the strength of interfacial film [29].

After centrifugation test under high speed (10,000 rpm) for 5 min, only four formulae showed physical stability with no phase separation. These formulations namely were T₁ (Tween 80: Tween 20; 1:1), G₁ (Tween 80: Gelucire 44/14; 1:1), X₁ (Tween 80; 1% w/w), and X₂ (Tween 80; 2%w/w) and were subjected to further characterization.

3.2. Particle size, polydispersity index and zeta potential

Particle size (PS) analysis was performed to confirm the manufacture and uniform distribution of nanosized emulsion droplets. All CM-EMs formulations were found to be in the nano-size ranges from 137 to 414 nm. The PDI values were found to be low (0.187–0.39), indicating narrow distribution of droplet size within the formulations. All formulations exhibited relatively high ZP ranging from –20.5 to –32.7 mV indicating good stability of the formulated EMs and supporting the findings of centrifugal test (Table 2).

pH measurement was essential to ensure the nonirritating nature of the topical emulgel when applied to the skin. The pH values of the CM-EMs ranged from 5.41 to 5.72, which is considered acceptable to avoid the risk of irritation upon application (Table 2).

3.3. Spreadability

Spreadability has a crucial role for an emulgel to meet the ideal qualities. As shown in Table 2, the values of the spreadability indicated that the CM-EMs were easily spreadable by small amount of shear. The order of increasing spreadability was as follows G₁ > X₁ > X₂ > T₁.

The selection of CM-EMs for *in-vivo* studying was based on globule size, zeta potential, and spreadability. Therefore, the two formulae; G₁ and X₁ which showed the smallest particle size (137.5 $\pm$ 2.04 nm and 207.1 $\pm$ 5.44 nm respectively) with high ZP values; –26.4 $\pm$ 5.8 mv (G₁) and –32.7 $\pm$ 6.14 mv (X₁) were selected. Moreover, G₁ & X₁ showed the highest spreadability values of 6.45 and 4.38 cm, respectively.

3.4. TEM

TEM images as shown in Figure 1(a,b) revealed that the droplets of diluted nanoemulgels were spherical in shape, discrete and in the nanometer size range.

Table 2. Characterization properties of the stable chamomile nanoemulgels.

	T ₁	G ₁	X ₁	X ₂
Particle size (nm)	290 $\pm$ 2.60	137.5 $\pm$ 2.04	207.1 $\pm$ 5.44	414.1 $\pm$ 0.65
Polydispersity index	0.187 $\pm$ 0.003	0.337 $\pm$ 0.044	0.390 $\pm$ 0.035	0.363 $\pm$ 0.007
Zeta potential (mV)	–30.6 $\pm$ 6.34	–26.4 $\pm$ 5.8	–32.7 $\pm$ 6.14	–20.5 $\pm$ 10.6
pH	5.63 $\pm$ 0.01	5.41 $\pm$ 0.02	5.72 $\pm$ 0.01	5.59 $\pm$ 0.03
Spreadability (cm)	2.60 $\pm$ 0.0	6.45 $\pm$ 0.06	4.38 $\pm$ 0.05	3.08 $\pm$ 0.05

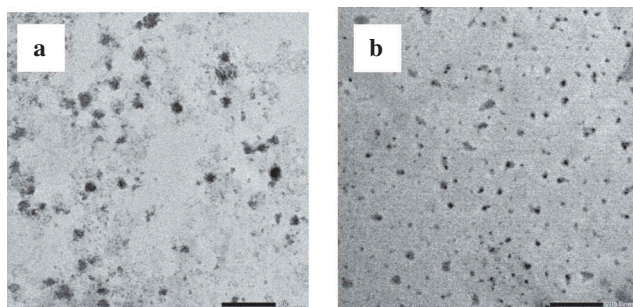


Figure 1. TEM images of the optimized emulgels; (a) EM.G₁ and (b) EM.X₁.

3.5. Viscosity

Viscosity measurement of the selected formulations indicated that, both emulgels (G₁ and X₁) exhibited pseudo-plastic rheology, as evidenced by shear thinning and a decrease in viscosity with increased angular velocity (Figure 2). The apparent viscosity values at 22 s⁻¹ and 25°C for the two CM-EMs were 4282 cp for formula X₁ (emulgel with 1%Tween 80) and 1400 cp for formula G₁ (emulgel with combination of Tween 80 and Gelucire 44/14; in 1:1 ratio).

3.6. Stability testing

The two selected formulae G₁ and X₁ were subjected to stress storage conditions of 10 freeze/thaw cycles with temperature ranging from -4°C to 25°C. No visual sign of phase separation was shown after 10 cycles, indicating the stability of the prepared EMs under such condition, which could likely be

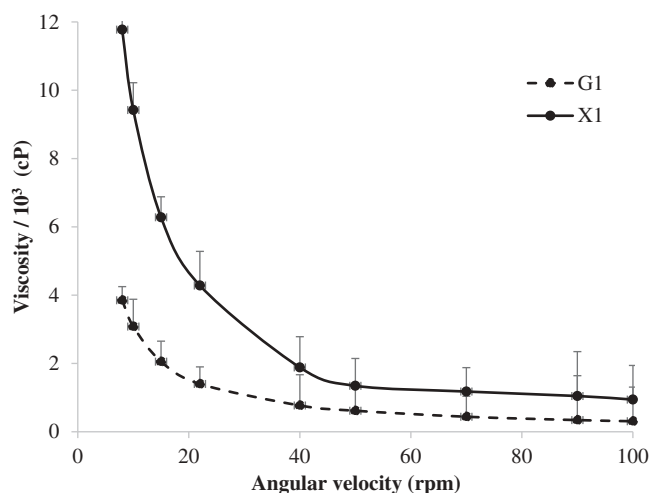


Figure 2. Rheogram of the optimized emulgels; G₁ and X₁ at 25°C.

encountered in real life. Moreover, the results of PS, PDI, and ZP measurements of these two formulae (Table 3) after being exposed to this stress condition did not show any significant differences ($p > 0.05$, Paired Student *T*-test) from the zero time which confirm their stability.

The two optimized formulations G₁ and X₁ showed also aging stability at refrigerated condition (4°C) during the study period of 6 months, with reference to the organoleptic properties, color, odor, consistency, and no signs of phase separation were observed. In addition to the basis of measuring size and ZP during the study period (Table 3 and Figure 3), no significant changes ($p > 0.05$, Paired Student *T*-test) were also revealed.

3.7. Skin irritation test

The results demonstrated, no visual alteration in the skin or development of apparent signs of skin irritation (erythema and edema), indicating that the developed EM is safe and nonirritant. It is worth mentioning that, the dose used for topical application was 0.5 gm of the emulgel (equivalent to 5 mg CM)/5 cm² skin lesion. This amount of gel was the least sufficient to cover the lesions with a uniform layer and allowed the oil nanodroplets to diffuse through skin without significant loss during application.

3.8. Histopathology of skin tissues

AD was induced in rats by subcutaneous injection of capsaicin. Erythema, excoriation and scarring were observed on the skin as major characteristics of dermatitis. Rats were subjected to different topical application of therapies (CM, EM.G₁, and EM.X₁) on the skin lesions to explore an ideal treatment. The severity of lesions was reduced after topical applications of all therapies. The duration of the clinical disappearance of skin lesions in AD induced rats varied from 10 days with the application of pure CM to only 5 days treatment using emulgel formulae X₁ or G₁ (Figure 4).

Figure 5 represents the histopathological changes in skin tissues stained with H & E. Histopathological results revealed that skin tissue of the normal control group (N) showed normal epidermis thickness with normal prominent skin appendages in the dermis. Dermatitis in the positive control rats (P) showed many pathological changes in both epidermis and dermis layers such as spongiosis (dermal edema), acanthosis (thickening of epidermis), hyperkeratosis, and parakeratosis. Treatment of AD-induced rats by emulgels formulae X₁, G₁ or CM showed improvement in skin lesions compared with the positive control rats. Rats treated by CM showed presence of spongiosis in the

Table 3. Change in particle size, PDI and zeta potential of the two selected emulgels after six months storage at refrigerate conditions (4 ± 2°C) and after 10 freeze/thaw cycles (-4°C to 25°C).

Storage time (month)	PS (nm) ± SD		PDI ± SD		ZP (mV) ± SD	
	G ₁	X ₁	G ₁	X ₁	G ₁	X ₁
0	137.5 ± 2.04	207.1 ± 5.44	0.337 ± 0.044	0.390 ± 0.035	-26.4 ± 5.8	-32.7 ± 6.14
2	140.5 ± 4.04	212.4 ± 3.44	0.471 ± 0.024	0.367 ± 0.025	-35.7 ± 7.2	-34.8 ± 6.30
4	147.3 ± 1.04	230.4 ± 2.45	0.350 ± 0.043	0.422 ± 0.015	-32.8 ± 2.8	-28.8 ± 5.24
6	158.4 ± 0.35	255.9 ± 2.18	0.170 ± 0.014	0.330 ± 0.030	-30.2 ± 4.6	-22.6 ± 4.49
After 10 freeze/thaw cycles	194.6 ± 4.57	205.2 ± 5.86	0.473 ± 0.079	0.359 ± 0.068	-28.3 ± 4.38	-35.2 ± 4.66

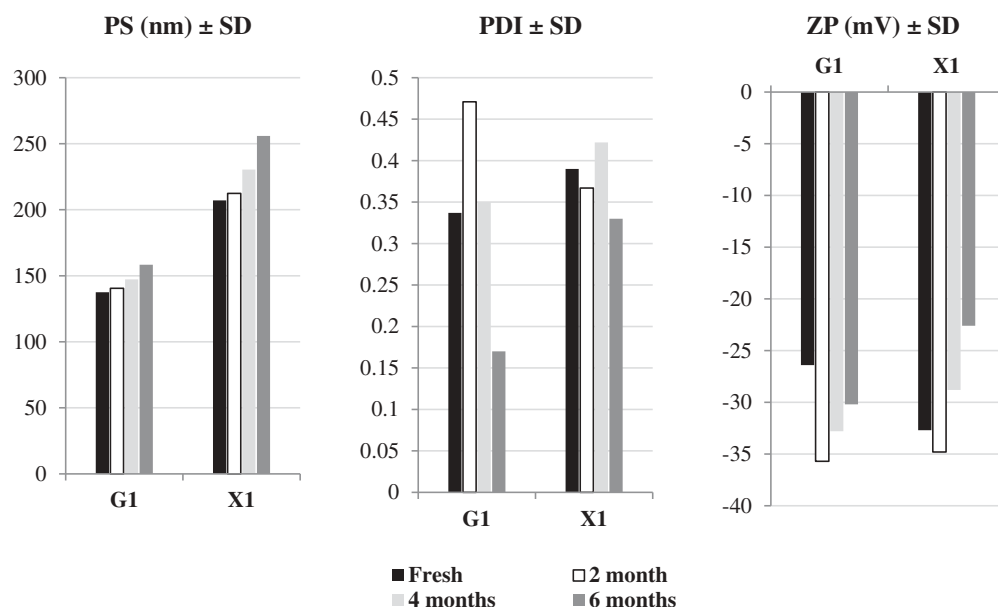


Figure 3. Effect of storage time at 4°C on particle size, polydispersity index and zeta potential of the two selected emulgel formulae G₁ and X₁.

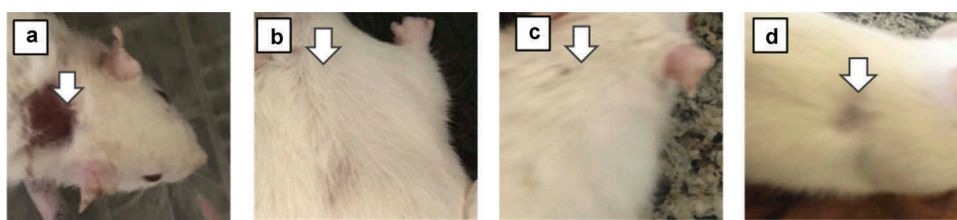


Figure 4. Representative photographs in different animal groups; showing (a) induced AD in rats, (b,c) lesion healing after 5 days treatment with EM.G₁ and EM.X₁, respectively and (d) lesion healing after 10 days treatment with CM.

dermis and thin epidermal layer. While, those treated by EM.X₁ and EM.G₁ showed prominent skin appendages that mimic the normal skin and no spongiosis was observed.

3.9. Enzyme-linked immunosorbent assay (ELISA)

Different inflammatory markers were assessed in both the rats' skin homogenate (IL-4, IL-18, IL-22, IgE, ECP, and NO) and plasma (IL-22 and NO) (Figure 6). In the skin homogenates, the different markers were significantly elevated in the positive controls compared to the negative controls ($p < 0.01$, 0.05). After treatment with CM and CM-EMs, the levels of IL-4 and IL-22 significantly decreased ($p < 0.01$, 0.05) compared to the positive controls (Figure 6(a,b)). However, in case of IL-18 level, the decrease was significant ($p < 0.05$) only in the group treated by formula G₁ (Figure 6(c)). Estimation of IL-22 in plasma coincided with the skin homogenate results. Plasma level of IL-22 (Figure 6(b)) was significantly increased in the positive controls compared to the negative controls ($p < 0.01$). Treatment with pure CM or formula G₁ showed significant decrease in plasma level of IL-22 ($p < 0.01$), whereas treatment with formula X₁ did not reveal a significant change in plasma IL-22 levels compared to the positive control rats.

Overproduction of IgE antibodies is a characteristic feature of AD. IgE level in skin homogenate was significantly

increased in the positive controls compared to the negative control rats ($p < 0.01$). Moreover, total IgE levels were significantly reduced ($p < 0.05$) after the application of topical therapies (Figure 6(d)). Oxidative stress including NO has been implicated in AD. Compared to the negative controls, the positive control showed a significantly higher level of NO ($p < 0.01$) in both skin homogenate and plasma. The elevated level of NO was significantly attenuated after the application of topical treatments ($p < 0.05$) (Figure 6(e)). The positive controls of the AD model rat demonstrated significantly high levels of ECP in skin homogenate ($p < 0.01$). However, the level of ECP was significantly reduced ($p < 0.05$) in rats of different treated groups compared to the positive control group (Figure 6(f)).

4. Discussion

AD is a multifactorial chronic inflammatory skin disorder that is a result of complex genetic, epigenetic, environmental and immunological interactions. It is characterized by both the epidermal barrier dysfunction [30] reduced antimicrobial peptide release, genetic polymorphisms, and diminished recruitment of polymorphonuclear cells, dendritic cells and natural killer cells as well as chronic Th2 cells' inflammation within the skin [30]. The increase of proteolytic enzymes with the

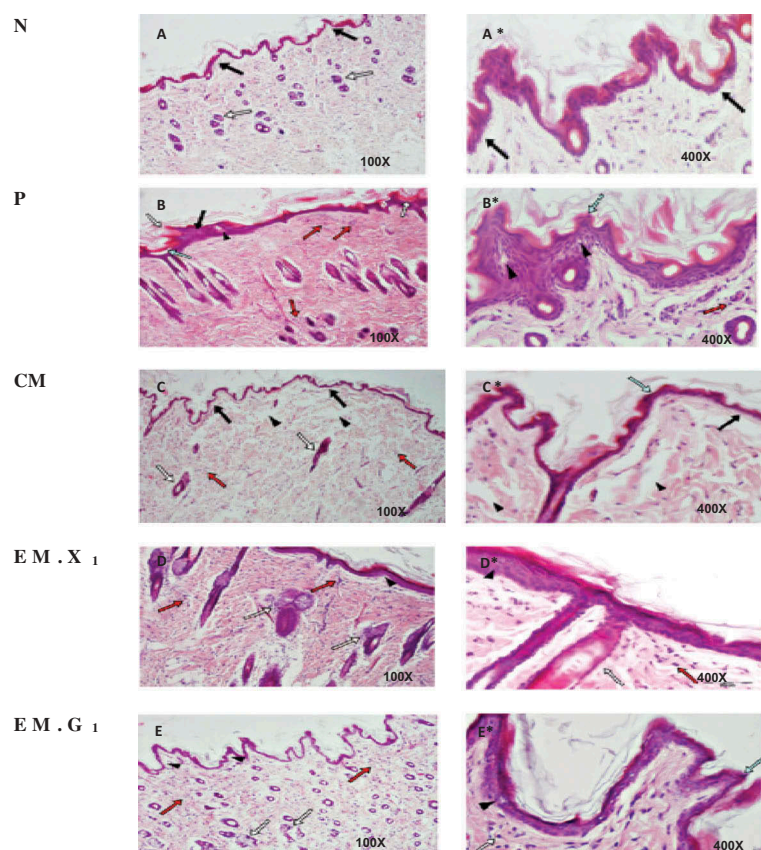


Figure 5. Representative histology photomicrograph of skin tissues of negative control group (N), positive control group (P), chamomile oil treated group (CM) and emulgels treated groups (EM.X₁ & EM.G₁). H&E stain; (A–E) magnification 100X; (A*– E*) magnification 400X. (a) Normal epidermis thickness (black arrow), dermis shows prominent skin appendages (white arrow). (A*) epidermis with normal thickness (black arrow head). (b) Epidermal spongiosis; intraepidermal edema (black arrow head), acanthosis; thickening of the epidermis in some areas (black arrow), hyperkeratosis; thickening of stratum corneum (white arrow) and parakeratosis; abnormal stratum corneum, in which surface keratinized cells of the skin retain nuclei (blue arrow); mononuclear cellular infiltration in the dermis (red arrow). (B*) Epidermal spongiosis (black arrow head) and parakeratosis (blue arrow); mononuclear cellular infiltration in the dermis (red arrow). (c) Thin epidermis (black arrow). Dermis shows few skin appendages (white arrow), mononuclear cellular infiltration (red arrow) and dermal edema separating collagen fibers (black arrow). (C*) Thin epidermis (black arrow) and parakeratosis (blue arrow); dermal edema separating collagen fibers (black arrow head). (d) Mild epidermal spongiosis (black arrow head). No acanthosis, hyperkeratosis nor parakeratosis was noticed. Dermis shows mononuclear cellular infiltration (red arrow) and prominent skin appendages (white arrow). (D*) Mild epidermal spongiosis (black arrow head). Dermis shows mononuclear cellular infiltration (red arrow) and prominent skin appendages (white arrow). (e) Mild epidermal spongiosis (black arrow head), no acanthosis nor hyperkeratosis was noticed. Dermis shows mononuclear cellular infiltration (red arrow) and increased skin appendages (white arrow). (E*) Mild epidermal spongiosis (black arrow head) and parakeratosis (blue arrow). Dermis shows mononuclear cellular infiltration (white arrow).

decrease of ceramide in the skin are genetic defects that lead to trans-epidermal water loss, resulting in skin dryness, and pruritus. This barrier breakdown creates a portal of entry for pathogens, allergens, and toxins causing inflammation and/or infection [31,32].

As the quality of life of patients with AD is seriously affected; it is mandatory to seek reasonable treatment options. Unfortunately, there is no complete cure for AD; current treatment aims to control the symptoms, and prevent exacerbations and flare ups to improvement of the patient's quality of life. Symptomatic control comprises proper skin care, daily use of emollients, anti-inflammatory treatment, in addition to avoiding contact with provocative allergens and irritants [33,34]. Anti-inflammatory therapy include topical corticosteroids (TCSs) and/or topical calcineurin inhibitors depending on the disease activity (periods of exacerbation and remission, location of skin lesion, exposure to irritants) However, treatment failure with current drugs and available treatment options could pose clinical problems that have a significant economic impact as loss

of working days [33,35]. Moreover, long life treatment with such anti-inflammatory drugs may lead in most cases to noncompliance and discontinuation of therapy as a result of the disturbing side effects especially when applying TCSs alone. Cutaneous side effects of TCSs include skin atrophy, striae, purpura, telangiectasia and hypertrichosis. They can cause, aggravate or mask skin infections such as impetigo, dermatophytosis, herpes simplex, and malassezia folliculitis and molluscum contagiosum [36].

New treatments are under development, where the majority focus on targeting the altered immune response in AD. Recent studies revealed that suppressing the inflammatory cytokines, restoring skin barrier function and thus inhibiting skin infections represent the main targets for management of AD [33,37].

Safe novel approaches toward the application of natural therapies using chamomile and olive oil have been evaluated in this study as an attempt for its application as a natural, biofriendly alternative to the current drug therapies. Olive oil is well known to possess antimicrobial activity and emollient

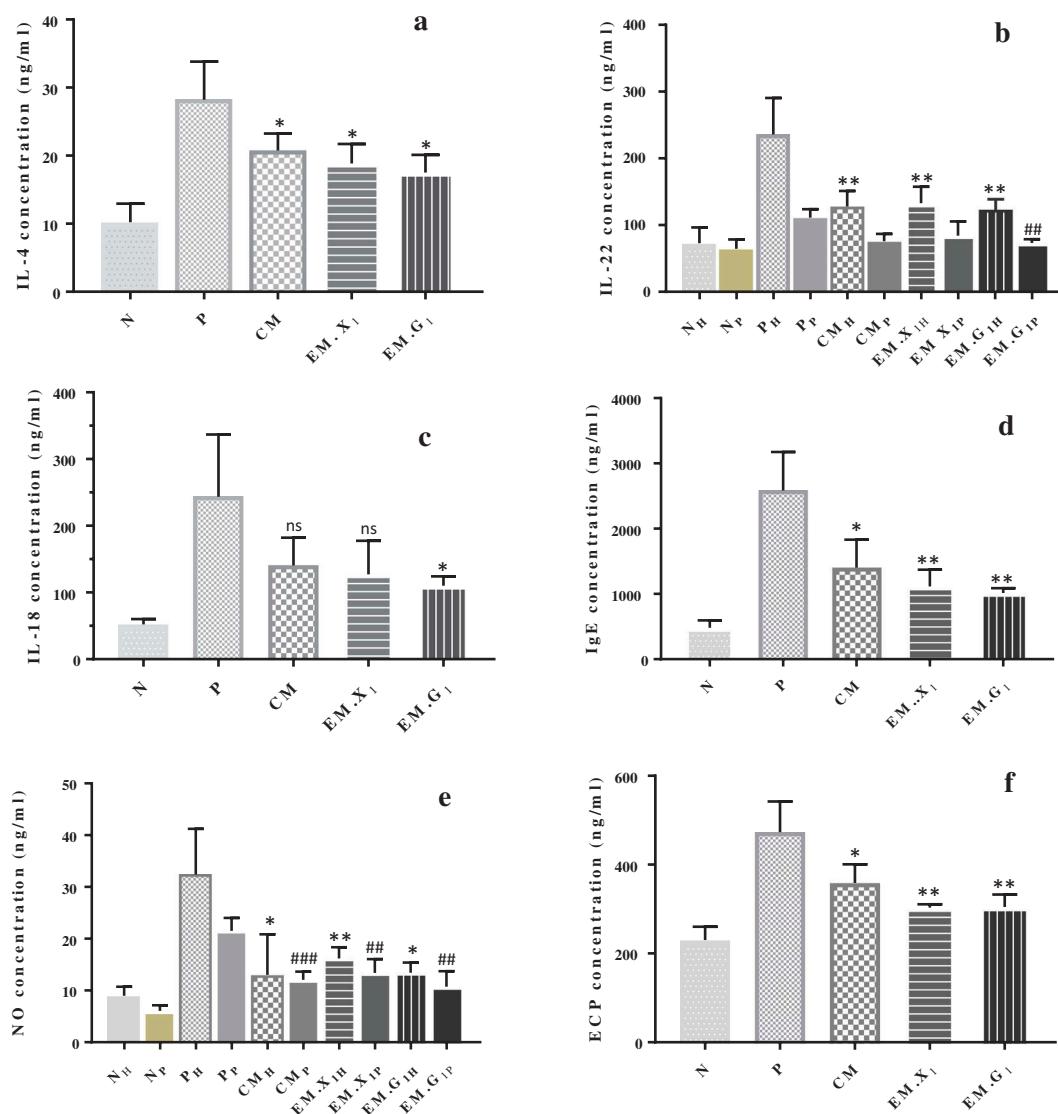


Figure 6. Levels of different biomarkers (IL-4, IL-22, IL-18, Ig E, NO, ECP) in skin homogenate (a–f), and biomarkers (IL-22, NO) in plasma (b,e) after induction of AD by s.c. injection of capsaicin in rats. Abbreviations; N: negative control, P: positive control, CM: treated with chamomile oil, EM.X₁: treated with emulgel X₁, EM.G₁: treated with emulgel G₁, H: skin homogenate & P: Plasma (n = 5 ± SD). (Values with different superscripts; *in skin homogenate, # in plasma, are significantly different, where; * *p* < 0.05, ** *p* < 0.001, *** *p* < 0.0001, # *p* < 0.05, ## *p* < 0.001, ### *p* < 0.0001, ns; non-significant).

property [38–40]. In addition, it has been reported that, oleic acid (an unsaturated fatty acid constituent of olive oil) revealed skin permeation enhancement [41,42]. Therefore, olive oil (0.5g/50g emulgel) was added in all formulations to acquire its reported beneficial properties.

The volatility of CM and its instability due to the oxidation of its components constrains its therapeutic efficiency. Therefore, oil encapsulation in a nanocarrier might improve the physical stability and its protection against volatilization during storage. EM formulations is the only nanocarrier systems which shows ease of application compared to pure oil in dermatological cases [13].

4.1. CM-EMs preparation

In emulsion preparation, selection of a suitable surfactant which will emulsify the oil with water is considered a critical factor. The most stable emulsion systems usually consist of

blends of lipophilic and hydrophilic emulsifiers which align alongside each other through hydrogen bonding imparting more rigidity and strength to the film at the interface of oil droplets [43]. The safety profile of surfactants and co-surfactants is an important criteria in formulating the emulgels which cannot be ignored [21].

Surfactants used in this work were Tween 20, Tween 80 and Gelucire 44/14. All these surfactants are reported as pharmaceutically acceptable and categorized as generally regarded as safe 'GRAS.' It is worth mentioning that the Hydrophilic-lipophilic balance (HLB) value of the selected surfactant or surfactant blend was found to be in the range of 14–15, which fulfills the requirement of minimum HLB value for the formulation of a stable o/w nanoemulsion [24]. Propylene glycol was added in the formulations as a co-surfactant. In addition it has been reported to act as a humectant, increase the spreadability property and consequently enhance the esthetic properties regarding to skin feel of the final product [44,45]. The pH

measurement has confirmed the nonirritating nature of the nanoemulgels.

4.2. Spreadability and viscosity

Spreadability denotes the area on which the emulgel spreads easily upon application to the skin. Also, viscosity is a crucial factor since it greatly affects the spreadability and the overall elegance of the formulations. The shear thinning behavior observed of the emulgels (G_1 and X_1) is a favorable property since it should be thin during topical application and thicken otherwise [44]. This pseudoplasticity could be a result from the colloidal network structure that aligns itself in the direction of shear, thereby decreasing the viscosity as the shear rate increases [17]. Both emulgels (G_1 and X_1) contained the same surfactant Tween 80. EM- G_1 had an additional surfactant; Gelucire 44/14 which showed less viscosity and hence a higher spreadability value of 1.5 fold. This observation may be due to interaction of Gelucire with the gelling agent HPMC leading to decreased viscosity, possibly by solubilizing the hydrophobic chains of HPMC affecting its hydration and conformation in water, consequently resulting in a viscosity decrease [46].

4.3. Stability tests

Stability tests were based on PS and ZP determination after exposure to different thermodynamic stability stress conditions. The two selected emulgel formulae G_1 and X_1 were subjected to different thermodynamic stability stress tests by using centrifugation, 10 freeze/thaw cycles test, in addition to aging at refrigerated condition (4°C) for 6 months. The two optimized formulations G_1 and X_1 showed good stability in all tests. The stability of the selected EMs could be due to several factors; firstly the homogenization conditions (speed and duration) were adequate to disperse the oily phase as fine droplets (in the nanometer range), where the small droplet size obtained could reduce the creaming movement of droplets preventing the breakage of the formulations during testing [47]. Secondly, EMs stability could be due to the development of a surface charge over the nanoemulsion droplets (as seen in the high ZP results). This surface charge was maintained and resulted in repulsive electrical forces among approaching oil droplets and thus hindered coalescence [22]. Finally, stability could be attributed to the high viscosity of the EMs constraining the Brownian movement [17]. Our results were in agreement with Yang *et al* [48] and Srivastava *et al* [49] studies.

4.4. In-vivo testing

Although all the materials used for preparation of EMs fell under the GRAS category, concentrations of all materials is a very critical issue for these formulations. Using a large amount of surfactants is usually irritant to the skin. Therefore, skin irritation test was performed and results indicated that the proposed SAA concentration in the developed EMs is safe and nonirritant.

In the present study, AD was induced by the subcutaneous injection of capsaicin. Based on reported data, this model

relies on a simple procedure, in addition, the manifestation of AD-like symptoms is consistent compared to other models [26,27,50,51]. Within 3 to 4 days, several histological changes were recorded in the rat's skin. These results agree with the results of Ji and Xiao-Kang [52] who illustrated that, these histopathological changes are mainly observed in the acute stage of patients suffering from AD [53]. Topical application of CM alone showed improved skin lesions with the presence of spongiosis within the dermis of AD-induced rat. While the application of (X_1 , G_1) formulations ameliorated AD lesions by diminishing edema (spongiosis), parakeratosis, inflammatory cells, scaling, and dryness.

Inflammatory cytokine pathway has a central role in the pathogenesis of AD where the cytokine mediators diminish the outmost layer of the epidermis that functions as skin barrier [53–55]. IL-18 is a potent pro-inflammatory cytokine involved in chronic inflammatory conditions such as AD. It is produced by skin resident dendritic cells and keratinocytes. IL-18 with IL-12 induces the Th1 response, resulting in the production of IFN- γ , while IL-18 without IL-12 can stimulate Th2 responses in allergic inflammations. In this study, IL-18 in skin homogenate was significantly decreased only in the rat group treated by formula G_1 . IL-18 contributes to CD4⁺ T cell-dependent Ig E production that directly activates mast cells, which release chymase that may accelerate the inflammatory responses in AD lesions. IL-18 also stimulates natural killer T cells that are the major source of IL-4. IL-4 increases production of Ig E, skin inflammation, and aggravates the skin barrier defect in AD [8,56].

In this work, IL-22 was significantly increased in the AD skin of rats after the subcutaneous injection of capsaicin. AD lesion was significantly improved after 5 and 10 days of treatment with formulae X_1 , G_1 and CM, respectively as the inflammatory cytokines IL-4, IL-18 and IL-22 levels in skin homogenates and in plasma were significantly decreased. This decrease was also accompanied by signs of AD lesions healing and restoring the normal skin histostructure. Immune dysregulation of Th2 (IL-4) and Th22 (IL-22) are the key features in the pathogenesis of AD [57]. Studies have recorded the presence and activation of Th22/IL-22 in both acute and chronic skin lesions in patients with AD, as well as positive correlation of IL-22 with the AD disease activity [58–61]. IL-22 belongs to the IL-10 cytokine family and it signals through IL-22 receptor 1 (IL-22R1), which is only expressed in tissue cells. In the skin, IL-22 acts on keratinocytes through binding to IL-22R expressed on these cells [37,62].

Our study revealed high levels of IgE and ECP in skin homogenate of the rats' model, where these elevations were mitigated after topical application of formula X_1 , G_1 , and CM. ECP is the main basic protein found in the secondary granules of eosinophils [63]. Eosinophilia and Ig E are strongly correlated and related to AD. Increased level of IgE sensitizes mast cell which stimulates the production of histamine and inflammatory cytokines leading to itching and edema [64]. Plasma ECP concentrations have also been linked to AD activity thus correlating with the symptoms, (lichenification, erythema, papules, pruritus, and excoriations) [65].

We demonstrated an increased level of NO in both plasma and skin homogenates in AD-induced rats that were then reduced after topical treatment with formula X_1 , G_1 , and CM. It was suggested that the increased level of oxidative stress

contributes in the pathogenesis of AD by upregulation of proinflammatory cytokine genes and induction of skin lipid oxidation which damages skin structure that appears as spongiosis/edema. Moreover, inflammation activates several enzymes leading to generation of oxidative stress. Understanding the relation between oxidative stress and inflammation and their role in pathogenesis of AD will encourage studies to find effective treatment. Thus preventing oxidative stress by antioxidants, suppressing proinflammatory cytokines by anti-inflammatory agents and maintaining intact skin barrier are promising approaches in AD therapy [52].

We assumed that the developed CM-EMs formulae would be beneficial for increasing the oil retention time and penetration at the application area. Notably, the amount of oil permeated from the emulgels in an *ex-vivo* permeation study (data not shown) was negligible. This could be attributed to the entrapment and retention of the oil within the skin layers. This assumption was supported by our promising results that reflected the anti-inflammatory effects of CM, revealed from both the histopathological examination and the assessment of biomarkers of induced AD in rats. *In-vivo* evaluation showed that, the skin lesions disappeared after treatment with both, the oil and the prepared emulgels. Yet this assumption needs further examination for confirmation.

However, the duration of treatment was remarkably reduced to only 5 days following the application of both formulae (G_1 & X_1), compared to 10 days following pure oil application. Furthermore, treatment using EM formulae (X_1 or G_1) did not show spongiosis which was observed in CM treatment.

PS and surface charge of the drug carrier system are important parameters in dermal application. The nano size of the oil droplets in the prepared emulgel formulation would enhance the embedment of the oil into the stratum corneum as previously reported [22]. In addition, the presence of surfactants/cosurfactant blend in the composition of the prepared emulgels would appreciate the permeation potential of the oil and reduce the barrier effect of the stratum corneum, thus, enhance the oil diffusion in the skin layers.

It was also found that, low viscosity results in better permeation as indicated by the superiority of the low viscosity formula EM. G_1 compared to EM. X_1 in treating AD. EM. G_1 resulted in a reduced IL-18 in skin homogenate and plasma IL-22 compared with the positive control.

However, a further study is planned to ensure the superiority of this nanoemulgel formulation compared to conventional moisturizers for AD treatment.

5. Conclusion

Novel insights in alternative AD therapy are required to overcome the disadvantages of conventional topical therapies. The use of natural herbal remedies is a promising approach for future therapies. The present study was conducted to explore an effective treatment of AD which is a recurrent chronic disease and exerts major problem worldwide. The developed chamomile emulgels showed nanometer size of oil droplets with relatively high negative surface charge supporting their physical stability under stress conditions. Topical application of CM-EMs

contributed to speed skin lesions disappearance two fold higher than pure oil, in addition to improvement of histological progress of the skin, where treatment by the two formulae X_1 or G_1 did not show spongiosis as observed in chamomile treatment. All inflammatory biomarkers were significantly attenuated after topical application of oil and emulgels. Formula G_1 was found superior in treating AD as it reduced IL-18 in skin homogenate and plasma IL-22 compared with the positive control. IL-18 is a reliable biomarker for the diagnosis of AD. However, from this study, IL-22 could be considered as a potential surrogate biomarker for the diagnosis of AD.

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Author contributions

Ragwa M. Farid, Noha S.El-Salamouni and Mai M. Ali contributed to the conception and design of the in-vitro pharmaceutical part of the work. Noha S.El-Salamouni and Mai M. Ali performed the practical in-vitro pharmaceutical experiments. Gihan A. El-Batouti, Sherien A. Abd El-Hady, and Lamia S. Kandil contributed to the conception and design of the in-vivo study. Lamia S. Kandil helped in performing the in-vivo practical work with Sherien A. Abd El-Hady. Ragwa M. Farid, Gihan A. El-Batouti, and Sherien A. Abd El-Hady contributed in writing the paper and interpretation of the data. All authors revised the paper critically for intellectual content. All authors approved the version to be published. All authors agree to be accountable for all aspects of the work.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer Disclosures

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