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### Formulation and Evaluation of Cubosomes as Skin Retentive System for Topical Delivery of Clotrimazole

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#### ABSTRACT

**Objectives:** Clotrimazole is a broad-spectrum antimycotic that widely used to treat fungal infections topically. However, poor water solubility of clotrimazole and low skin retention of its topical products present a hindrance for local availability and effectiveness of this drug. This study aimed to formulate and evaluate cubosomes as skin retentive system for topical delivery of clotrimazole. **Methods:** Six clotrimazole cubosomal dispersions (F1-F6) were prepared by emulsification of glyceryl monooleate (GMO) and poloxamer 407 at different compositions in water with and without polyvinyl alcohol (PVA). The dispersions were examined under polarizing microscope and transmission electron microscope (TEM) for confirming cubosomes formation. The four cubosomal dispersions (F3- F6) that found to be successfully developed were evaluated in terms of pH, particle size, zeta potential, rheological behavior, entrapment efficiency and *in vitro* drug release. The formulae F3 (without PVA) and F6 (with PVA) comprising the highest concentration of poloxamer in the disperse phase (15% w/w) provided the highest clotrimazole cumulative percentage release and subjected to comparative *ex vivo* skin retention and permeation studies with marketed clotrimazole cream. **Results:** Both F3 and F6 showed significantly lower clotrimazole permeation compared to the cream. Moreover, F6 achieved the highest skin retention for clotrimazole. The F6 was compared with the cream for its irritation potential in rats and also for antifungal activity in rats inducted with candida albicans. The investigations showed that F6 was well tolerated as the cream. Also, F6 showed superior antifungal activity compared to the available marketed cream. **Conclusions:** The developed F6 cubosomes is a promising dosage form for safe and effective topical delivery of clotrimazole.

**Keywords:** Clotrimazole; Cubosomes; Topical delivery; Skin retention; Poloxamer 407

#### INTRODUCTION

Clotrimazole is a broad-spectrum antimycotic drug used for treatment of Candida albicans and other fungal infections<sup>1</sup>. It is widely employed to treat fungal infections topically. The oral administration of this drug

is inconvenient because of its short half-life and large variations in bioavailability due to its low aqueous solubility (0.49 µg/ml)<sup>2</sup>. Also, gastrointestinal disorders, neurological reactions, elevation in liver enzymes, dysuria, and mental depression have been reported after oral administration of clotrimazole<sup>3</sup>.

The site of clotrimazole action is the stratum corneum where the pathogens reside. However, the specialized nature of the stratum corneum makes it an efficient barrier to drug molecules<sup>4</sup>. Therefore, stratum corneum penetration is the rate limiting step in percutaneous drug absorption<sup>4-5</sup>. Clotrimazole is usually formulated as a 1% cream, lotion, spray or solution for treatment of skin fungal infections. However, the commercially available topical products of clotrimazole have the limitations of low skin retention and deposition<sup>6-7</sup>. Since clotrimazole is poorly water-soluble, it requires a proper vehicle to improve its topical absorption<sup>8</sup>. Colloidal drug carriers such as microemulsions, vesicular carriers including liposomes, ethosomes and niosomes as well as both lipidic and polymeric particulate carrier systems were developed for topical delivery of clotrimazole, to ensure dermal penetration and targeting<sup>7,9-11</sup>.

Recently, it has been envisioned that cubosomes can act as an ideal drug delivery system for drugs with poor loading and permeability for the treatment of topical fungal infections<sup>12</sup>. Moreover, it has been reported that cubosomes can serve as a potential skin retentive system<sup>13-14</sup>.

Structurally, cubosomes are optically isotropic, viscous, self-assembled bicontinuous cubic liquid crystalline nanoparticles (10-500 nm); composed of lipid layers, separating non-intersecting water channels that provide unique properties of practical interest<sup>15</sup>. Cubosomes are biocompatible, bioadhesive, nontoxic, non-immunogenic and cost efficient drug delivery system. Unique structure of cubosomes can incorporate wide varieties of drugs (hydrophilic, lipophilic and amphiphilic) with high payload owing to their high internal surface area (400 m<sup>2</sup>/g)<sup>12, 16-17</sup>. Various investigations have also indicated the sustained and prolonged drug delivery properties of cubosomes<sup>14, 18</sup>. Moreover, similarities between the bicontinuous structures formed in human skin layers and those comprising cubic phases<sup>19</sup>, offering the promise of better skin retention and transport.

Therefore, in the present study, different formulations of clotrimazole loaded cubosomes were prepared and evaluated for their physicochemical properties and ability to deliver and retain clotrimazole in the skin.

## MATERIALS AND METHODS

### Materials

Clotrimazole was kindly donated as a gift by Al-Arabia Company (ADCO) (Egypt). Myverols 18-99 K, as a source of monoolein, was a gift from Kerry Ingredients & Flavours (Zwijndrecht, Netherlands). Poloxamer 407 (P407) and HPLC grade methanol, Poly vinyl alcohol (PVA) were purchased from Sigma-

Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Formic acid and Ammonia were purchased from Scharlau (Spain). Acetonitrile was purchased from JT beaker (USA). De-ionized water purchased from MEPACO-MEDIFOOD (Arab Company for Pharmaceutical and Medicinal plants) was used for all experiments. Sodium lauryl sulfate (SLS) was purchased from El-Nasr Pharmaceutical Company, Egypt. *Candida albicans* (ATCC®10231) was purchased from the American Type Culture Collection, USA. The YPD broth (Yeast extract 10 g/L, peptone 20 g/L, and dextrose 20 g/L) was purchased from Oxoid Limited, UK. Fetal bovine serum (FBS) was purchased from Biowest, France. Canesten® cream (clotrimazole 1% w/w, manufactured by Memphis Co. For Pharm. & Chem. Ind., Cairo, Egypt under license of Bayer Leverkusen, Germany) was purchased from a local pharmacy store. All other reagents were of analytical grade.

### Preparation of clotrimazole loaded cubosomal dispersions

Six cubosomal dispersions (F1-F6) were prepared by emulsification of lipid phase consisting of GMO and poloxamer407 in water with and without PVA<sup>20</sup>. The composition of the prepared cubosomal dispersions is shown in **Table 1**. The cubosomes were prepared by melting GMO and poloxamer 407 on a water bath kept at 60°C. Then, 350 mg of clotrimazole were dissolved in the molten mixture. De-ionized water containing 2.5 g PVA (F4, F5 and F6) that preheated at the same temperature was added to the molten mixture under mechanical stirring at 500 rpm. The final concentration of lipid in the dispersion was 5% (w/w) with respect to the final dispersion weight. The final clotrimazole concentration in cubosomal dispersion was 3.5 mg/g cubosomal dispersion. Dispersions were equilibrated for 24 hours magnetic stirring at room temperature and away from light. Then, the dispersions were subjected to probe sonication (UP50H Ultrasonic Processor, Hielscher ultrasound technology, Teltow, Germany) at 40°C for 4 min, then autoclaved at 121°C (Tomy autoclave, model: SS-235, Tomy Seiko Co. LTD., Tokyo, Japan) for 15 min plus an equilibration time of 5 min. Autoclaving was done to ensure transformation of any formed vesicular structures into particles of cubic structure<sup>21-23</sup>.

### HPLC assay of clotrimazole

Samples were analyzed for clotrimazole concentration by using a validated HPLC assay<sup>24</sup> with slight modifications. The HPLC system consisted of a LC-UV/PDA Shimadzu (Japan) chromatographic system. The used column was a C18 (hypersil BDS, 250 x 4.6 mm, 5 µm) and the column temperature was maintained at 40 °C. A mobile phase consisting of

**Table 1. Composition of Clotrimazole loaded cubosomes**

Formulations	Dispersed phase		Stabilizer PVA (% w/w )
	Lipid Glyceryl monooleate (% w/w)	Surfactant Poloxamer 407 (% w/w)	
F1	4.75	0.25	0
F2	4.5	0.5	0
F3	4.25	0.75	0
F4	4.75	0.25	2.5
F5	4.5	0.5	2.5
F6	4.25	0.75	2.5

**N.B:**

Clotrimazole was used in concentration of 0.35% w/w

Dispersed phase (5% w/w) with respect to the total weight of the dispersion

mixture of 0.1% v/v formic acid solution adjusted to pH 3 with ammonia / acetonitrile (45:55, v/v) was used in an isocratic elution mode. The flow rate was 1ml/min. Samples were injected using a manual injector at injection volume of 20 µl. Data acquisition and integration were carried out using LabSolutions software integrator. The detection wavelength was 210 nm using Photo Diode Array detector.

**Characterization of the prepared clotrimazole loaded cubosomal dispersions**

**Visual inspection**

All dispersions (F1-F6) are examined visually for color, homogeneity, presence of macroscopic aggregates, and deposition of material on the surface of the glass vial at the air-dispersion-glass contact line (referred to as “ring formation”)<sup>25</sup>.

**Structural examination**

*Structural examination under Polarizing Microscope*

To confirm the formation of isotropic cubic phase and exclude other liquid crystalline systems, a drop of the dispersions (F1-F6) was placed between a cover slip and a glass slide and then examined under the polarizing microscope (Ray Wild, model: Fluoroscope Polar, Ray Wild Limited Company, and Germany). All dispersions (F1-F6) were observed under plane polarizing light (PPL) illumination technique. As well, crossed nicols (XN) illumination technique was used for examination of the absence of birefringence.

*Structural examination under Transmission Electron Microscope*

Cubosomal structure of the dispersions was examined using a transmission electron microscope

(JEOLJEM-2100, Japan). A droplet of cubosomal dispersion was placed on a carbon-coated copper grid, and the excess fluid was removed by filter paper. The samples were stained with sodium phospho-tungstate solution (1% w/v) then viewed.

**Examination of the interaction of clotrimazole with cubosomes forming excipients**

The possibility of interaction between clotrimazole with cubosomes forming excipients was examined using Differential Scanning Calorimetry (DSC) and liquid chromatography mass spectrometry (LC-MS) scan.

Since cubic gel is more easily to be handled for these examination techniques than the liquid dispersion form of cubosomes, clotrimazole loaded cubic gel was prepared as follows:

On a thermostatically controlled water bath, GMO (4.25 g) and poloxamer 407 (0.75g) were melted at 60°C. Then 350 mg of clotrimazole was dissolved in GMO/poloxamer molten mixture. PVA (2.5 g) was dissolved in 2 mL of deionized water previously heated at 60 °C and then added drop wise to the molten mixture. The whole mixture was vortex mixed at room temperature to obtain a homogenous state then equilibrated at room temperature for 48 h to obtain the cubic gel.

*Differential Scanning Calorimetry (DSC) Analysis*

The DSC thermograms of pure clotrimazole, GMO, poloxamer 407, PVA as well as clotrimazole loaded cubic gel and blank cubic gel, were recorded using a thermal analysis system (DSC-50, Shimadzu, Japan). Samples of (4-5 mg) were placed in an aluminum pan and heated at a constant rate of 10 °C/min to a temperature of 400 °C under a nitrogen atmosphere with flow rate 25ml/min.

#### Liquid chromatography-tandem mass spectrometry (LC-MS/MS) scan

The LC-MS/MS scan was carried out for 5  $\mu$ l samples of clotrimazole loaded cubosomal dispersion (F6) diluted with methanol using a reported (LC-MS/MS) assay method<sup>26</sup> with some modifications. Mass spectrometric analysis (LC-MS/MS) was carried out in positive Heated Electrospray ionization (HESI) mode at Spray voltage [V] of 3600, using a TSQ Quantum Access MAX triple quadrupole system (Bruker Daltonik GmbH, Bremen, Germany) with Accela 1200 LC- 10 AD pump (Thermo Scientific, California, USA). The chromatographic separations were carried on a Hypersill gold column C18 (Phenomenex 50  $\times$  20 mm, 2.1  $\mu$ m), preceded by a security guard cartridge Gemini C18 (Phenomenex 4  $\times$  3mm, 5  $\mu$ m). A mobile phase of acetonitrile (mobile phase A): 0.2% aqueous formic acid solution (mobile phase B) (90:10 v/v) was conducted in an isocratic elution mode at a flow rate of 0.2 ml/min under ambient temperature. Data was processed using Thermo Scientific Xcalibur 2.1 software. The *m/z* values of clotrimazole and/or the major degradation products were recorded and compared with the values reported in literature.

#### Determination of Particle size and zeta potential

The particle size, polydispersity index (PDI) and zeta potential of cubosomal dispersions (F3-F6) were determined using Zeta sizer Nano-series (Nano ZS, Malvern, Worcestershire, UK). Samples of (50–fold) aqueous dilution were sonicated for 5 min before measurement. All samples were measured at  $25 \pm 0.5$  °C in triplicates and results were represented as mean value  $\pm$  standard deviation.

#### pH Measurements

The pH of the cubosomes (F3-F6) was measured by direct immersion of the electrode of a pH meter (HI 2211 PH/ORP Meter HANNA instrument, UK) in the dispersion at room temperature.

#### Rheological behavior examination

The rheological behavior of dispersions (F3-F6) was determined using Brookfield DV-III Ultra programmable cone and plate rheometer, equipped with spindle CPE 40 (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). The rpm was increased linearly from 20 to 100 units at  $25 \pm 0.5$  °C, with 15 seconds interval between each two successive speeds. The rheograms of the dispersions were plotted; the Y-axis was taken to represent the shear rate ( $\text{sec}^{-1}$ ) and X-Axis was taken to represent shear stress ( $\text{dyne/cm}^2$ ).

#### Determination of clotrimazole content in cubosomal dispersions

One milliliter of clotrimazole loaded cubosomal dispersion was dissolved in 25 ml of HPLC grade methanol in volumetric flask and shaken very well to obtain clear solution<sup>27-28</sup>. Concentrations of clotrimazole in methanol solution were determined using HPLC at wavelength 210 nm<sup>24</sup>. The drug content was calculated using the following equation:

$$\text{Drug content} = (\text{Actual yield} / \text{Theoretical yield}) \times 100 \quad \text{Eq.1}$$

#### Clotrimazole entrapment efficiency (EE %) in cubosomes

Clotrimazole entrapped in cubosomes was determined by ultrafiltration centrifugation<sup>29</sup>. Five gram of freshly prepared clotrimazole loaded cubosomal dispersions (F3-F6) was diluted to 10 mL with deionized water, then 3 mL of the diluted samples was placed in centrifuge tubes (Amicon Ultra 3000 MWCO, Millipore, USA) and centrifuged at 4°C and 4000 rpm for 15 min using cooling centrifuge (Sigma 3-30K, Sigma-Aldrich). Free clotrimazole contained in filtrate was measured after appropriate dilution with methanol using a validated HPLC assay<sup>24</sup>.

The amount of entrapped clotrimazole was obtained by subtracting the amount of free drug from the total drug incorporated in 5g cubosomal dispersion. The entrapment efficiency (EE %) was calculated using the following equation (Eq.2):

$$\text{Entrapment Efficiency (EE\%)} = \frac{\text{Total amount of drug fed initially} - \text{Amount of drug in the filtrate}}{\text{Total amount of drug fed initially}} \times 100$$

#### In-Vitro Release Study

The *in-vitro* release of clotrimazole from the cubosomal dispersions (F3-F6) was performed. Prior to testing, a piece of cellulose membrane (Molecular weight cut off 12,000–14,000 Da, Spectra/Pro, Spectrum Laboratories, Inc., USA) was soaked in deionized water for about 24 h. Then, the membrane was fixed in position using rubber band to cover one end of a top-cut plastic syringe acting as a dialysis tube of 1.9 cm internal diameter. An accurately weighed 3 g of the test preparation (equivalent to 10.5 mg clotrimazole) was placed in the designed release assembly. The tube enclosing the test sample was then attached to the shaft of a dissolution apparatus I (Hanson Research, California, USA). The dialysis tube was carefully adjusted to a position so that the membrane just touched the surface of the release medium. A volume of 50 ml Sorenson's phosphate buffer (pH 7.4) containing 1% sodium laurylsulfate (SLS) was used as release medium<sup>7, 30</sup>. The temperature was maintained at  $37^\circ\text{C} \pm 0.5$  °C and the stirring speed was adjusted to 100 rpm. Two ml aliquots of the release medium were withdrawn at 1, 2, 3, 5 and 8 h time intervals, and replaced with 2

ml fresh medium to maintain the volume. The samples were filtered with micropore 0.22  $\mu\text{m}$  syringe filter and analyzed for clotrimazole content using HPLC. The mean cumulative amount of clotrimazole released ( $n=3$ ,  $\pm$  SD) was plotted as a function of time.

#### Release kinetics and mechanisms

The *in-vitro* release data obtained were subjected to analysis for fitting to various kinetics models (zero order, first order and Higuchi) as follows:

Zero order rate equation:

$$Q_t = Q_0 + K_0t \quad \text{Eq.3}$$

Where  $Q_t$  is the amount of drug released at time  $t$ ,  $Q_0$  is initial amount of drug, and  $K_0$  is zero order release rate constant.

First order rate equation:

$$\log Q = \log Q_0 - K_1t / 2.303 \quad \text{Eq.4}$$

Where  $Q$  is the remaining amount of drug at time  $t$ ,  $Q_0$  is the initial amount of drug,  $K_1$  is first order release rate constant, and  $t$  is time.

Higuchi's model:

$$Q = K_H t^{1/2} \quad \text{Eq.5}$$

Where  $Q$  is the amount of drug released in time  $t$  per unit area at time  $t$ ,  $K_H$  is Higuchi diffusion rate constant.

The best-fitted model was selected based on the regression coefficient ( $R^2$ ) value.

To find out the mechanism of drug release, the *in vitro* release data of all cubosomal dispersions (F3-F6) were fitted to Korsmeyer and Pepp as equation<sup>31</sup>.

$$Mt / M_\infty = Kt^n \quad \text{Eq.6}$$

Where  $M / M_\infty$  is a fraction of drug release at time  $t$ ,  $K$  is the release rate constant, and  $n$  is the release exponent. The value of exponent ( $n$ ) indicates the mechanism of drug release.

#### Ex-vivo skin permeation and skin retention study

The protocol to carry out the *Ex-vivo* skin permeation and retention studies was approved by the Animal Ethics Committee of Faculty of Pharmacy, Helwan University, No. 009A2018. Guidelines of the ethics committee were followed throughout this study. The animals were kept under standard laboratory conditions till the experiment was performed, with free access to standard laboratory food and water, at a constant room temperature of 25°C and a natural day/night cycle. Euthanasia of the animals and disposal of killed animals was done according to the lab regulation.

Full -thickness skin was obtained from the dorsal region of male Wistar albino rats weighing from 150 to 200 g. The dorsal skin was excised after hair removal with a depilatory cream. Subcutaneous fat and other extraneous tissues were trimmed then the skin was rinsed with isotonic phosphate buffer saline (PBS,

pH 7.4) and visually inspected for integrity to ensure the absence of any imperfections.

#### Skin permeation

The excised rat skin was fixed in position to top-cut plastic syringe representing a dialyzing tube of 1.9 cm internal diameter providing an effective permeation area of 2.84  $\text{cm}^2$ . The skin was placed with its stratum corneum facing upward (inside of the tube) and dermal side downward (facing the permeation medium). The position-fixed skin was made watertight by a rubber band. The tested preparations (F3, F6 and commercial clotrimazole cream) were accurately weighed in the plastic tube at an amount equivalent to 10 mg of drug. Then the tube enclosing the test sample was attached to the shaft of a dissolution apparatus I (Hanson Research, California, USA). The permeation medium was 30 ml (Sorenson's phosphate buffer pH 7.4 containing 1% SLS). The dermal side of the skin was just touching the surface of permeation medium. The temperature of the experiment was kept at 37°C and stirring rate was adjusted to 50 rpm. A 2 ml aliquot was periodically withdrawn at time intervals (3, 6, 9, 12 and 24 h) and was replaced immediately with fresh permeation medium. All samples – in triplicates – were filtered through a 0.22  $\mu\text{m}$  pore size syringe filter and analyzed by HPLC described before. The cumulative amount of drug permeated per unit area rat skin was calculated and plotted versus time. The flux ( $\text{mg}/\text{cm}^2/\text{h}$ ) was calculated as the slope of the plot.

#### Skin retention study

After 24 h of skin permeation study, skin retention of clotrimazole was measured. The effective diffusion area of the skin was carefully separated and washed many times with de-ionized water to remove excess formulation remaining on the skin surface then was cut into small pieces using a pair of scissors, weighed into capped glass vials and vortexed with 3ml methanol and left soaked for 24 h. The samples were subjected to 3 sonication cycles of 30 min each in an ultrasound bath (Power sonic 410, Hwa-Shin instrument Company, South Korea) followed by probe sonication for 5 min using UP50H Ultrasonic Processor, Hielscher ultrasound technology, Teltow, Germany. The resulted mixtures were filtered using micropore 0.22  $\mu\text{m}$  syringe filter. One milliliter from the filtered solutions was diluted with 1 ml Sorenson's phosphate buffer (pH 7.4) containing 1% SLS then re-filtered and clotrimazole was then quantified by HPLC. The study was performed in triplicates.

#### Skin irritation study

This study was carried out to detect the irritation potential of the selected cubosomal formula (F6) after topical application in comparison with

commercial clotrimazole cream. The study was performed using fifteen male albino rats (150 - 200 g). The study proposal was approved by the Animal Ethics Committee of Faculty of Pharmacy, Helwan University, No. 009A2018. Animals were divided into four groups with five animals each as follows:

Group 1 was not subjected to any treatment, group 2 is the positive control group where formalin was used as a standard irritant<sup>32-33</sup>, group 3 included the rats treated with the selected cubosomal dispersion (F6) and group 4 included the rats treated with commercial clotrimazole cream.

Rats' hair on dorsal area was removed one day prior to application of formulations<sup>34</sup> using a depilatory cream. Formalin solution and doses of the commercial clotrimazole cream and cubosomal dispersion equivalent to 10 mg drug were applied with uniform spreading on the shaved rats' skin once daily for three consecutive days.

#### *Erythematous scoring system and visual observation*

The skin was checked for any sensitivity reaction or visible difference, such as erythema after the application of formulations throughout the three successive days of the study.

The mean erythematous scores were recorded (ranging from 0 to 4) depending on the degree of erythema as follows:

No erythema = 0, slight erythema (barely-light pink) = 1, moderate erythema (dark pink) = 2, moderate-to-severe erythema (light red) = 3 and severe erythema (extreme redness) = 4<sup>35-37</sup>.

#### *Histopathological examinations*

After three days, rats were euthanized and the defined part of the shaved skin were excised and fixed in 10 % formal- saline solution for 24 h. The skin was washed with tap water then serial dilutions of alcohols (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared through sectioning at 4 µm thickness by a microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained with hematoxylin and eosin stains for histopathological examination under the light electric microscope<sup>38</sup>.

#### *Microbiological studies*

##### *Preparation of the microorganism*

*Candida albicans* (ATCC®10231) was grown overnight in YPD broth (Yeast extract 10 g/L, peptone 20 g/L, and dextrose 20 g/L) containing 10% fetal bovine serum (FBS) for 14–18 h with vigorous shaking at 30°C. Next day, the grown culture was subcultured

into fresh YPD broth containing 10% FBS and was further shaken at 37°C for 2–3 h. This step converted *Candida* to the virulent pseudohyphae form which has the ability to establish cutaneous infection<sup>39</sup>.

##### *Preparation of the animal*

Eighteen Male albino rats (150-200 g) were used in the experiment. All animal experimentation procedures were approved by the Animal Ethics Committee of Faculty of Pharmacy, Helwan University, No. 009A2018. Animals were housed under standard laboratory conditions as described above. An area of approximately 2 cm<sup>2</sup> was shaved using a depilatory cream in the back of each rat 24 h before getting infected.

##### *Induction of *Candida albicans* cutaneous infection*

*Candida albicans* suspension (1.1 X10<sup>6</sup> cfu/ml) was gently rubbed onto the shaved skin area with help of sterile cotton-tipped swab until no more visible fluid is observed<sup>40</sup>. This induction was done once daily for a period of consecutive 14 days. Only 18 rats out of 24 had been subjected to infection induction.

##### *Treatment of the infection.*

Animals were divided into three groups each containing six rats. The first group (positive control) was inducted with cutaneous candidiasis and not treated, the second group inducted with cutaneous candidiasis and treated with the selected formula (F6) while the third group inducted with cutaneous candidiasis and treated with commercial clotrimazole cream. Both group 2 and 3 were treated by equivalent doses of 10 mg drug once daily for seven consecutive days started after 15 days from the induction (24 h after infection signs had been developed on the fourteenth day of induction). The skin of rats not subjected to infection induction was used as negative control.

Rats of groups (2 and 3) were assessed visually and with photographs daily at application site for presence of nodules, ulceration, erythema, and crusting. Score of disease was recorded (ranging from 0 to 4) on the basis of the presence of the four clinical features (nodules, ulceration, erythema, and crusting). Rats were considered to be healed when the four disease parameters are absent<sup>39</sup>.

##### *Histopathological Examination*

After seven days of treatment, rats were euthanized and the defined part of the shaved skin were excised and fixed in 10 % formal- saline solution for 24 h for histopathological examination. The skin was processed according to the method described earlier reported by<sup>38</sup>.

### Statistical analysis

The data obtained throughout this study were analyzed by unpaired samples Student's t - test using Microsoft Office 2007, Excel package. A statistically significant difference was considered at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Visual inspection

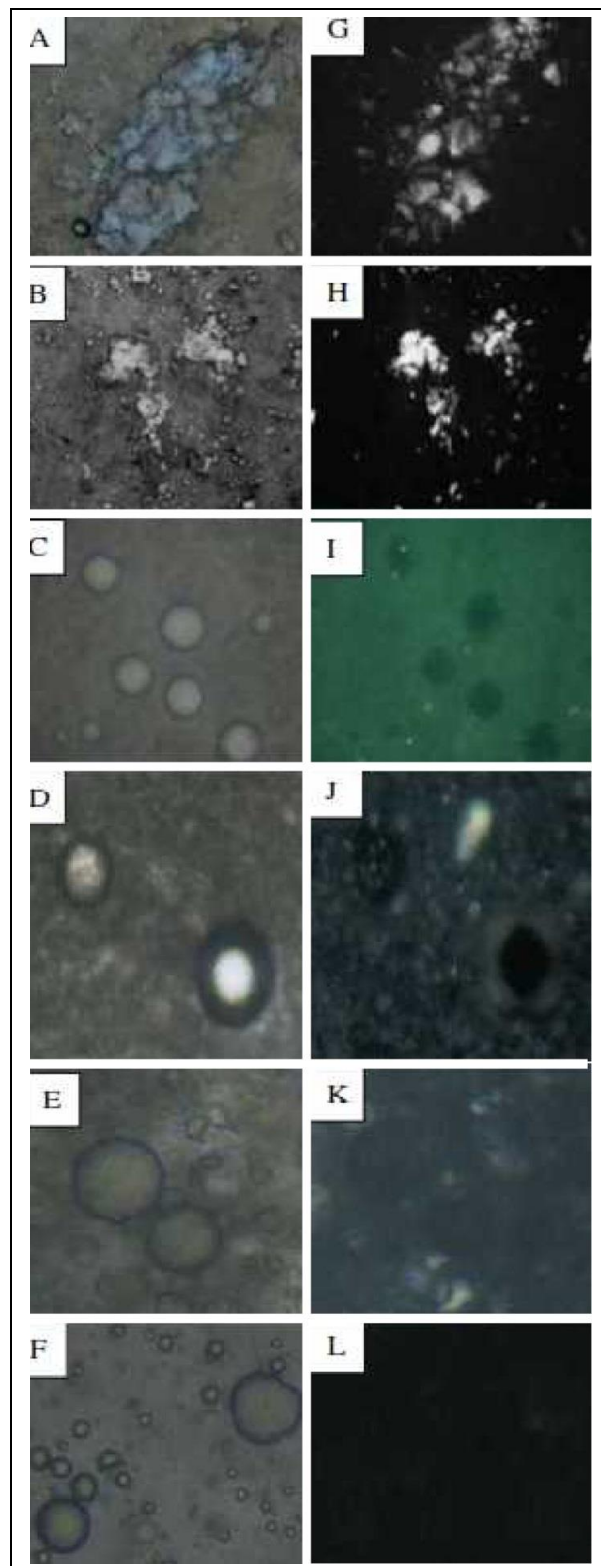
All the prepared cubosomal dispersions were physically stable with homogeneous milky white appearance except F1 and F2 showed ring formation and macroscopic aggregates. These observations agreed with those reported by<sup>22</sup>. The authors found that the dispersions with poloxamer concentrations less than 12 % w/w of total disperse phase were physically unstable with macroscopic aggregates. Although F4 and F5 contained poloxamer concentration of 5% and 10 % (w/w), respectively, these formulae were homogenous with no macroscopic aggregates due to presence of PVA that stabilizes the cubosomal dispersions. PVA was recognized as an efficient stabilizer acting as a protective agent by being adsorbed at the lipid/aqueous interface; hence, lowers the fusion of the particles and decreases the aggregate formation<sup>27, 41</sup>.

### Structural examination

#### Structural examination under Polarizing Microscope

Polarizing microscopy can offer the morphology of liquid crystalline based on the optical birefringence phenomena of liquid crystalline. Anisotropic molecular arrangement of non-cubic liquid crystalline (layered and hexagonal) formed a bright birefringence in the cross polarized light photograph<sup>42</sup>.

The molecular arrangement of cubic liquid crystalline is non-birefringence<sup>43</sup>. **Figure 1** shows photographs of drug loaded dispersions observed without cross polarizer (**Figure 1.A, 1.B, 1.C, 1.D, 1.E, 1.F**) and with cross polarizer (**Figure 1.G, 1.H, 1.I, 1.J, 1.K, 1.L**). Upon examination under cross polarized microscope, the majority of the examined field in case of F1 and F2 showed aggregates of anisotropic nature and low yield of isotropic cubic structures (**Figure 1.G and 1.H**, respectively). All the other formulae (F3-F6) were isotropic which reveals the cubic nature of these dispersions. However, the formulae F3 and F6 showed only cubic isotropic crystals without aggregates (**Figure 1.I and 1.L**, respectively) while F4 and F5 showed few discrete of anisotropic non - aggregated structures in addition to the main cubic structures (**Figure 1.J and 1.K**, respectively).



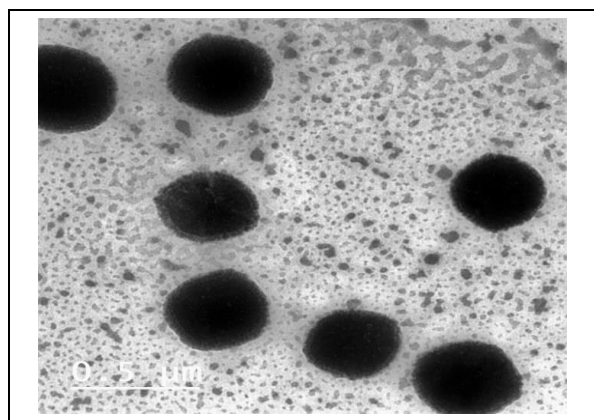
**Figure 1.** Photographs of drug loaded dispersions observed without cross polarizer: (A) F1, (C) F2, (E) F3, (G) F4, (I) F5, (K) F6 and with cross polarizer: (B) F1, (D) F2, (F) F3, (H) F4, (J) F5, (L) F6 .

Table 2. The characterized parameters of clotrimazole loaded cubosomes

Formula Code	pH	Particle size (nm)	PDI	Zeta potential (mV)	Drug content (%)	EE%	Viscosity (cp)
F3	6.96±0.02	184.10±7.92	0.30±0.03	16.30±0.14	106.00±0.05	97.50±0.03	2.36±0.22
F4	7.07±0.02	114.00±1.41	0.36±0.01	14.15±0.21	104.00±0.00	98.00±0.05	5.80±0.18
F5	6.96±0.01	174.30±0.14	0.34±0.05	15.60±0.28	105.00±0.02	98.80±0.54	7.86±1.10
F6	6.95±0.01	180.70±2.40	0.30±0.04	22.90±0.14	100.00±0.02	97.00±0.01	9.50±0.15

### Structural examination under Transmission Electron Microscope

Negative stain transmission electron microscope photograph **Figure 2** revealed that cubic nano-structures that dispersed as individual particles were indeed formed.



**Figure 2.** Transmission electron microscope photograph of clotrimazole loaded cubosomal dispersion (F6) stained with sodium phospho-tungstate solution (1% w/v).

### Examination of the interaction of clotrimazole with cubosomes forming excipients

#### Differential Scanning Calorimetry (DSC) Analysis

Differential Scanning Calorimetry (DSC) is a powerful technique in evaluating the physicochemical drug- excipient interactions and also in detecting polymorphic modifications. **Figure 3** shows the DSC thermograms of clotrimazole, GMO, poloxamer407, PVA, blank cubosomal gel and clotrimazole-loaded cubosomal gel. The DSC thermogram of clotrimazole exhibited a single sharp characteristic, endothermic peak at about 145 °C, corresponding to its melting point which was in agreement with that reported previously<sup>44</sup>. Such sharp peak indicates the crystalline state of the drug. However, the thermogram of

clotrimazole loaded cubic gel revealed the disappearance of the drug melting peak and the appearance of endothermic peak at 71 °C. The disappearance of the drug melting peak indicating that the drug was molecularly encapsulated in the bicontinuous structures in non-crystalline form which was in agreement with that reported previously<sup>45-46</sup>. The appearance of endothermic peak at 71 °C in the thermograms of drug loaded cubic gel as well as blank cubic gel might be due to the phase transition from cubic to reversed hexagonal phase; such transition is known to occur at 71°C<sup>47-49</sup>. This finding can be also considered as further confirmation of formation of cubic phase in addition to polarized microscopy and TEM.

#### Liquid chromatography-tandem mass spectrometry (LC-MS/MS) scan

LC-MS/MS is a Complementary method for detecting drug-excipient incompatibility<sup>50</sup>. It can be used to conduct qualitative analysis for components and gives structural information through scanning positive or negative ions and comparing the peaks of the resulted fragments with standard literatures<sup>51</sup>.

The LC-MS/MS spectrum of clotrimazole loaded cubosomal dispersion (F6) diluted with methanol (data not shown) displayed [M+H]<sup>+</sup> ion peak (m/z 485.87) and fragmentation pattern that was very similar to what reported in literature for clotrimazole<sup>52</sup>. This result confirmed the drug stability in cubosomes.

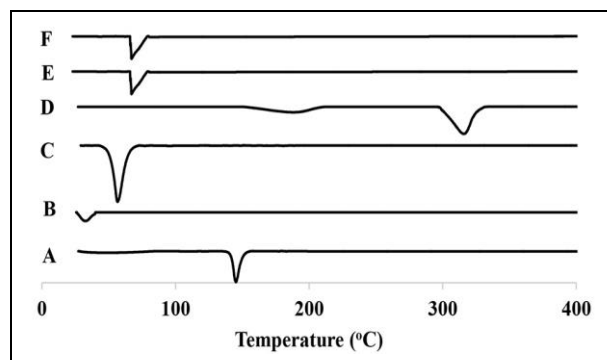
#### Characterization of the prepared clotrimazole loaded cubosomal dispersions

##### Determination of Particle size and zeta potential

The mean particle size, poly dispersity index (PDI) and zeta potential values of the prepared cubosomes (F3- F6) are shown in **Table 2**. The particle size analysis showed that the average particle size values of all these formulae were within the nano range (114 ±1.14 nm – 184.10 ±7.92 nm). The narrow particle size distribution of the cubosomes nanoparticles was



indicated by the low polydispersity index values that ranged from  $0.30 \pm 0.03$  to  $0.36 \pm 0.01$ . Zeta potential measurements showed that all clotrimazole-loaded cubosomes nanoparticles carried a negative charge. The negative charge might be due to presence of the free fatty acid (oleic acid) in GMO as well as GMO and PVA hydroxyl groups that are anchored on the lipid / water interface<sup>53-54</sup>. The negative charge of these nanoparticles could facilitate their permeation through the skin via channels created by the repulsive forces between negatively charged skin lipids and nanoparticles<sup>55</sup>. The mean zeta potential values of the dispersions were found to be slightly low and ranged from  $-14.15 \pm 0.212$  mV to  $-22.9 \pm 0.14$  mV. This might be attributed to the partial neutralization of the negative charge of cubosomes by the positive charge of clotrimazole as the pH values of all the prepared dispersions were very near to the drug pKa. However, these measured zeta potential values can be acceptable to provide sufficient electric repulsion to prevent particles aggregation. It was previously reported that potential values of (8 mV -21 mV) are considered to be advisable for the stabilization of nanoparticles, since very little or no agglomeration takes place<sup>56</sup>. Moreover, presence of poloxamer 407 provides additional stability mechanism by creating steric repulsion between cubic particles due to its polymeric bulky structure<sup>57-58</sup>



**Figure 3.** The DSC thermograms of **A)** Clotrimazole, **B)** GMO, **C)** Poloxamer 407, **D)** PVA, **E)** Blank cubosomal gel and **F)** Clotrimazole-loaded cubosomal gel.

#### pH Measurements

The results of pH measurements are shown in **Table 2**. The pH values of the prepared clotrimazole-loaded cubosomes were in the range of 6.95 to 7.07 which is within the physiologically acceptable range for topical products<sup>59</sup>.

#### Rheology

All the investigated formulae (F3-F6) exhibited Newtonian flow behavior with viscosity values ranged from  $2.36 \pm 0.22$  cp to  $9.5 \pm 0.15$  cp as shown in

**Table 2**. Such low viscosity values can increase drug penetration into skin<sup>46</sup>. Also, it can be noticed from comparing the viscosity values of formulae (F4, F5 & F6) that increasing the content of poloxamer 407 in the dispersion led to increasing viscosity. This might be due to formation of more solid-like liquid crystal materials<sup>46</sup>. In addition, it was observed that the presence of PVA had a potential effect on increasing the viscosity of the dispersions (F4-F6) in comparison with (F3) that not contain PVA.

#### Determination of clotrimazole content in cubosomal dispersions

As shown in **Table 2** cubosome dispersions (F3-F6) had drug content ranging from  $100.00 \pm 0.02$  % to  $106.00 \pm 0.05$  %.

#### Clotrimazole entrapment efficiency (EE %) in cubosomes

The EE% for the prepared clotrimazole-loaded cubosomes ranged between  $97.00 \pm 0.01$  % and  $98.80 \pm 0.54$  %, as shown in **Table 2**. Clotrimazole was successfully entrapped into the cubosomes which reveals the ability of cubosomes to be a promising delivery system for lipid soluble drugs.

#### In-Vitro Release Study

As illustrated in **Figure 4**, the cumulative percent clotrimazole released after 8 h was  $29.68 \pm 0.51$  %,  $8.00 \pm 0.15$  %,  $15.00 \pm 0.09$  % and  $42.87 \pm 0.73$  % for F3, F4, F5 and F6, respectively. The investigated formulae (F3-F6) showed controlled drug release profiles demonstrating that GMO provides release-retarding properties. This slow drug release might be related to the firm entrapment of the lipophilic clotrimazole by GMO and the slow diffusion of the drug from the inner water channels in the cubic phases<sup>49</sup>.

It can be observed from the *in-vitro* drug release study that F3 and F6 showed the highest drug release within 8h and thus were selected for further investigations. Both of these cubosomal dispersions contained highest weight ratio (~1: 5.67) of poloxamer to GMO. Thus the high drug release from F3 and F6 could be explained on the basis that poloxamer 407, has the ability to solubilize clotrimazole in the aqueous release medium facilitating the drug release from the cubosomes nanoparticles. These results agreed with those reported by Salah et al.<sup>46</sup>.

#### Release kinetics and mechanisms

The kinetic analysis of the release data showed that all formulae (F3-F6) followed zero order release kinetics as indicated from the highest regression coefficient  $R^2$  (data not shown). The formulae had mean  $t_{50}$  values of  $15.85 \pm 0.10$  h,  $44.45 \pm 0.39$  h,

32.13 ± 0.58 h and 17.12 ± 0.84 h for F3, F4, F5 and F6, respectively.

The release mechanisms of clotrimazole from the prepared cubosomal dispersions (F3–F6) were studied by applying the Korsmeyer - Peppas model. The dispersions F3 and F5 exhibited (n) values more than 0.45 and less than 0.89 indicating non-Fickian anomalous transport i.e. the rate of clotrimazole release from the cubosomes F3 and F5 was governed by both drug diffusion and polymer relaxation (erosion). In addition, dispersion F4 showed (n) value more than 0.89, indicating super case II (polymer relaxation) while F6 showed (n) value less than 0.45 demonstrating Fickian release mechanism.

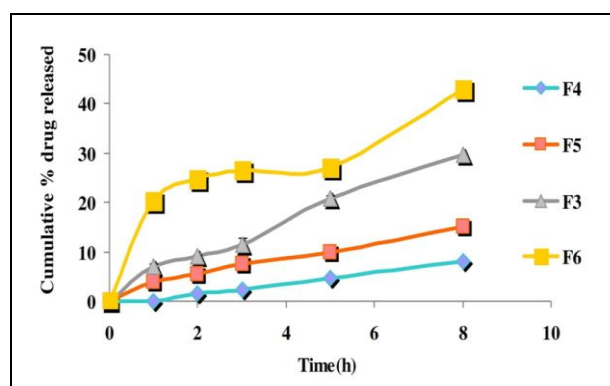


Figure 4. Release profiles of clotrimazole from different cubosomes dispersions.

### Ex-vivo skin permeation and skin retention study

#### Skin permeation

This study aimed for investigating the permeation of clotrimazole from the selected cubosomal formulae (F3 and F6) through rat skin in comparison with commercially available clotrimazole cream. The results illustrated in Figure 5 showed that the flux of clotrimazole from F3, F6 and cream was  $0.25 \pm 0.02 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ ,  $1.16 \pm 0.3 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ , and  $17.348 \pm 2.6 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ , respectively. Both F3 and F6 exhibited significantly ( $p < 0.05$ ) lower flux than the cream. Furthermore, the flux of clotrimazole from F3 and F6 was not significantly different ( $p > 0.05$ ). These results agreed with those previously reported by Peng et al.; the authors found that GMO – based cubosomes reduced the skin permeation of capsaicin through rats skin within 24 h as compared to both phytantriol– based cubosomes and the conventional capsaicin cream<sup>14</sup>.

#### Skin retention study

A significantly ( $P < 0.01$ ) higher retention of drug in the skin 24 hours post application was obtained

for the tested formulae (F3 and F6) than the conventional clotrimazole cream (Figure 6).

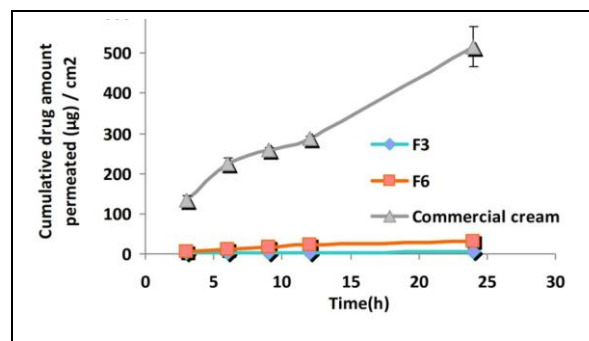


Figure 5. Permeation profiles of clotrimazole from cubosomal dispersions (F3, F6) and conventional cream.

The cubosomal formulae F3 and F6 provided 2.87 and 5.87 fold increase in skin retention, respectively, compared to the conventional cream. Thus, based on the results of *ex vivo* skin permeation and skin retention studies, the clotrimazole loaded cubosomes (F3 and F6) were found not only promoted drug retention in the skin, but also reduced the drug entering into the blood circulation. This might be due to that the penetration of a drug loaded on cubosomes through the skin involved two phases. The first phase characterized by fast drug penetration followed by phase of slower sustained drug penetration<sup>46</sup>. The fast penetration through the skin may be due to the ability of the cubosomes to penetrate into the skin between corneocytes through the paracellular route<sup>60</sup>. While the decrease in the drug penetration by time may be due to the ability of cubosomes nanoparticles to form a depot in the lipid part of the stratum corneum<sup>61</sup>. This may be due to the similarity between the structure of the nanoparticle cubic phase and the structure of the stratum corneum<sup>62</sup>.

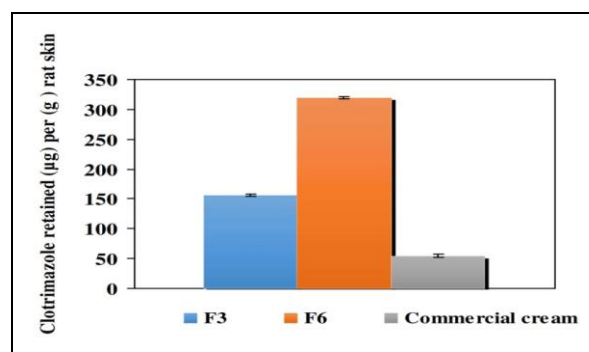
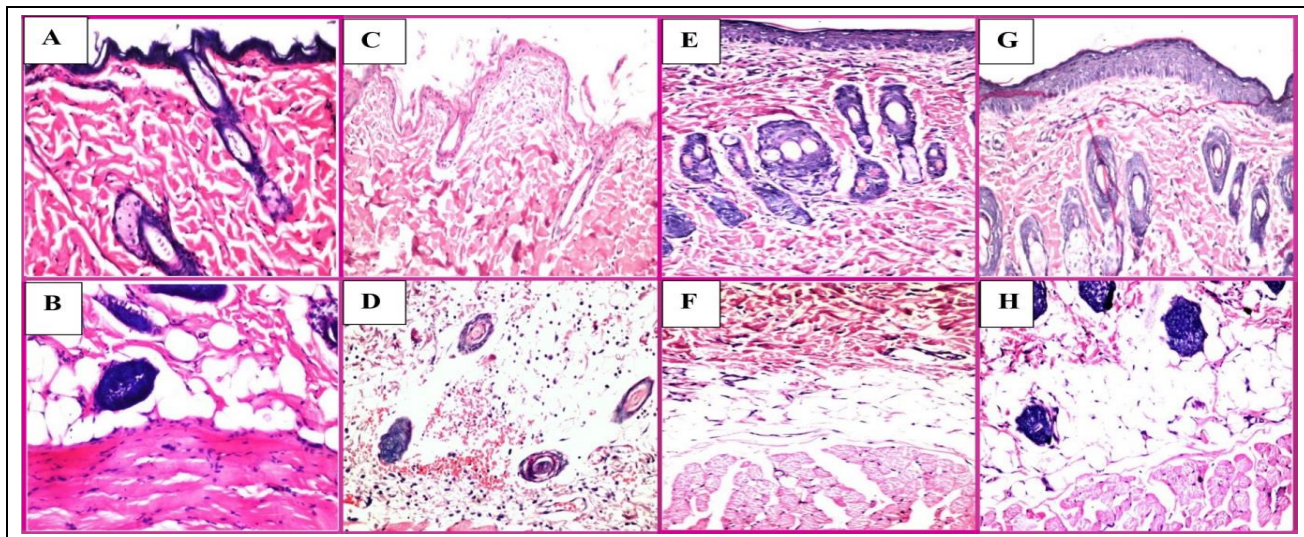
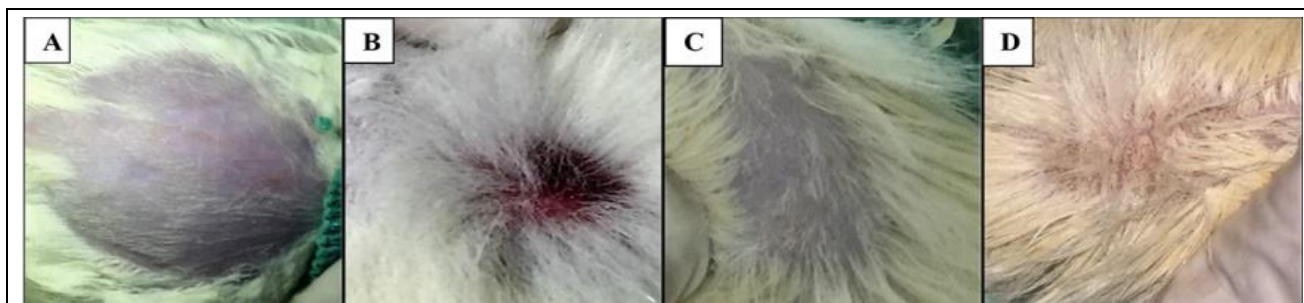


Figure 6. Skin retention of clotrimazole cubosomal dispersions (F3, F6) and conventional cream.



**Figure 7.** Histopathological examination of Skin of **A)** rat in group (1) showing normal histological structure of the epidermis and dermis, **B)** rat in group (1) normal histological structure of the subcutaneous tissue, **C)** rat in group (2) showing sloughing of the keratin and superficial cell layers of the prickles cells in the epidermis, **D)** Skin of rat in group (2) showing inflammatory cells infiltration and focal haemorrhage in subcutaneous and adipose tissues, **E)** Skin of rat in group (3) showing normal histological structure of epidermis and dermal cells, hair follicles and sebaceous gland, **F)** Skin of rat in group (3) showing normal histological structure of subcutaneous adipose tissue. **G)** Skin of rat in group (4) showing normal histological structure of the epidermis and dermis, **H)** Skin of rat in group (4) showing normal histological structure of subcutaneous adipose tissue.



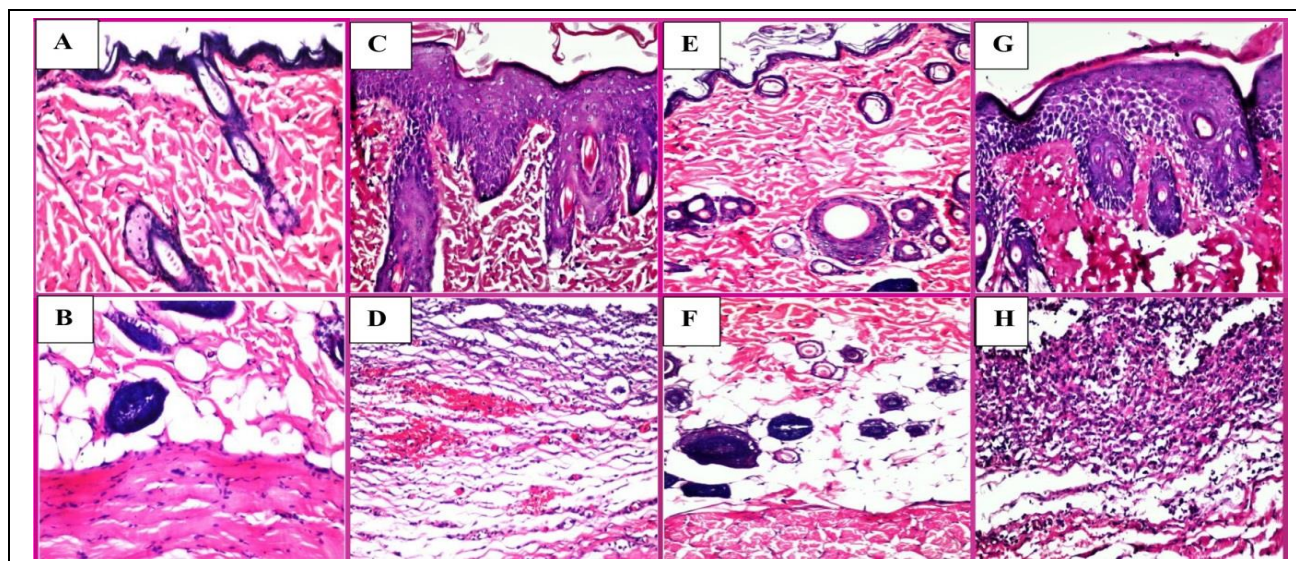
**Figure 8.** The images of skin surface morphology of **A)** Untreated uninfected rat, **B)** Untreated infected rat in group (1), **C)** F6 treated rat in group (2) after 7 days treatment, **D)** Commercial cream treated rat in group (3) after 7 days treatment.

However, significantly ( $P < 0.01$ ) higher drug retention in the skin was observed for F6 compared to F3. This might be due to the faster release observed for the drug from F6 compared to F3. Moreover, the hydrophilicity of PVA might enhance the cross and retention of F6 cubosomes in viable epidermis which is hydrophilic in nature. This can act as driving force for further cross and accumulation of F6 cubosomes in skin layers. Thus, F6 was selected for further investigations.

#### Skin irritation study.

Since cubosomes dispersions were composed of lipid, surfactant and stabilizer; hence, it was particularly important to consider the potential skin

irritation resulting from topical application. The test was carried out for commercial clotrimazole cream and cubosomal dispersion (F6) and positive group treated by formalin as a standard irritant<sup>32-33</sup>. After application of the tested preparations, the skin was observed once daily for any visible change for successive three days. Following the three days, the observations indicated that the positive control group developed severe erythema of average score  $3.8 \pm 0.45$  while the marketed clotrimazole cream and the cubosomal dispersion F6 did not show any sign of skin reaction. Thus, it can be assured that cubosomal dispersion F6 does not cause any skin irritation and can be well tolerated for topical application.



**Figure 9.** Histopathological examination of rat skin of: **A)** Uninfected rat showing the normal histological structure of the epidermis and dermis, **B)** Uninfected rat showing the normal histological structure of the subcutaneous tissue, **C)** Rat in group (1) showing acanthosis of the prickle cell layer of the epidermis, **D)** Rat in group (1) showing inflammatory cells infiltration and haemorrhage in subcutaneous tissue, **E)** Rat in group (2) showing normal histological structure in the epidermis and dermis, **F)** Rat in group (2) showing normal histological structure of subcutaneous tissue, **G)** Rat in group (3) showing acanthosis in the epidermis and hyalinosis in the dermis, **H)** Rat in group (3) showing suppuration in the subcutaneous tissue with pus formation.

Skin specimens were histopathologically examined after 3 days of the experiment to confirm the clinical assessment of skin sensitivity study. The images of skin tissues subjected to application of formalin, cream and cubosomes F6 are shown in **Figure 7** Group 1 showed normal histological structure of the epidermis, dermis, sebaceous glands and hair follicles as well as the underlying subcutaneous adipose tissue and the muscular layer (**Figure 7.A, 7.B**, respectively). The histopathological examination of the skin of rats included in the formalin treated group (group 2) indicated that the keratin layer and the underlying superficial layer of the prickle cells were sloughed (**Figure 7.C**). Also, inflammatory cells infiltration and focal hemorrhages in the subcutaneous tissue occurred (**Figure 7.D**). However, it was observed that the skin structure of the two groups treated with F6 (**Figure 7.E, 7.F**) and the cream (**Figure 7.G, 7.H**) was clear and integral without inflammatory reaction or infiltration of the inflammatory cells which showed very similar characteristics as that of normal skin. These results matched the visually inspected scores and confirmed that both F6 and cream formulations had no irritation effect on the skin.

#### Microbiological studies

The images of skin surface morphology of the involved groups versus the normal skin of uninfected rats after 7 days treatment were shown in **Figure 8**.

A significant ( $p < 0.01$ ) reduction in the scores of symptoms of the evaluated skin fungal infections was observed after 2 days treatment with F6 cubosomes (group 2) and the commercial cream (group 3) compared to the untreated positive control (group 1). However, the group that treated with F6 cubosomes showed significant reduction in the scores after 3 days treatment compared to the cream treated group. Moreover, after 7 days treatment, all rats treated with F6 cubosomes were completely treated and the affected skin appeared to have normal morphology with re-growth of baby hairs indicating that the skin recovered its normal state. However, only 1 rat out of 6 in the cream treated group was completely cured after 7 days while the other 5 rats showed average symptoms score of  $2 \pm 0.82$ .

The histopathological examination of the skin of the four groups after seven days of the experiment was shown in **Figure 9**, the non inducted rat skin showed normal histological structure of the epidermis and dermis **Figure 9.A**. Also, sebaceous glands and hair follicles as well as the underlying subcutaneous adipose tissue and the muscular layer showed normal histological structure **Figure 9.B**. The untreated inducted rat skin (group 1) showed acanthosis (hypertrophy or thickening) in the prickle cell layer of the epidermis in focal manner associated with hyalinosis in the underlying areolar connective tissue of the dermis **Figure 9.C**. Focal massive inflammatory

cells infiltration and pus cells with haemorrhage were detected in the subcutaneous tissue **Figure 9.D**. The induced rat skin treated with the selected cubosomal dispersion F6 (group 2) showed no histological alteration in the epidermis, dermis and subcutaneous tissue (**Figure 9.E, 9.F**). The induced rat skin treated with the commercial clotrimazole cream (group 3) showed acanthosis in the prickle cell layer of the epidermis in association with suppuration in the subcutaneous tissue with pus formation (**Figure 9.G, 9.H**). Thus, the results proved the more effectiveness of the selected formula F6 in treatment of topical fungal infections.

## CONCLUSION

The (F6) cubosomal dispersion consisting of 350 mg clotrimazole, 4.25 g GMO, 0.75g poloxamer 407, 2.5 g PVA and water up to 100 g can be considered as a promising effective and safe dosage form for topical delivery of clotrimazole because of its significantly higher drug retention in skin compared to the commercial cream. However, clinical studies should be conducted on humans with skin fungal infections to prove the effectiveness of this formula over the available marketed products.

## Conflict of interest

Authors declared that there is no conflict of interest

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