Anais da Academia Brasileira de Ciências
ISSN: 0001-3765
aabc@abc.org.br
Academia Brasileira de Ciências
Brasil

UBAID, MUHAMMAD; ILYAS, SADAF; MIR, SADULLAH; KHAN, ABIDA K.; RASHID, REHANA; KHAN, MUHAMMAD Z.U.; KANWAL, ZAINAB G.; NAWAZ, AHMAD; SHAH, AMNA; MURTAZA, GHULAM

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Anais da Academia Brasileira de Ciências, vol. 88, núm. 4, octubre-diciembre, 2016, pp. 2303-2317
Academia Brasileira de Ciências
Rio de Janeiro, Brasil

Anais da Academia Brasileira de Ciências (2016) 88(4): 2303-2317
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201620160162
www.scielo.br/aabc

Formulation and *in vitro* evaluation of carbopol 934-based modified clotrimazole gel for topical application

MUHAMMAD UBAID[1], SADAF ILYAS[1], SADULLAH MIR[2], ABIDA K. KHAN[2], REHANA RASHID[2], MUHAMMAD Z.U. KHAN[1], ZAINAB G. KANWAL[1], AHMAD NAWAZ[1], AMNA SHAH[1] and GHULAM MURTAZA[1]

[1]Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan
[2]Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan

Manuscript received on March 30, 2016; accepted for publication on June 18, 2016

ABSTRACT

The aim of present study was to enhance topical permeation of clotrimazole gel preparation by using various permeability enhancers such as coconut oil, pistachio oil and sodium lauryl sulphate (SLS). Clotrimazole gel preparations were prepared and optimized by using three factor, five level central composite design. A second-order polynomial equation was generated in order to estimate the effect of independent variables i.e. coconut oil (X_1), pistachio oil (X_2) and sodium lauryl sulphate (X_3) at various dependent variables i.e. flux (Y_1), lag time (Y_2), diffusion coefficient (Y_3), permeability coefficient (Y_4), and input rate (Y_5) of clotrimazole gel formulations. *Ex vivo* skin permeation study was performed through rat skin by using modified Franz diffusion cell system. Optimized formulation F8 exhibited highest flux 2.17 µg/cm^2/min, permeability coefficient 0.0019 cm/min and input rate 1.543 µg/cm^2/min, along with moderate lag time 77.27 min and diffusion coefficient 0.063 cm^2/min, which is further supported by anti-fungal activity that exhibited more prominent zone of inhibition against *Candida albicans*, *Aspergillus niger* and *Mucor*. Thus, it can be concluded that permeation of clotrimazole gel was enhanced by various combination of coconut oil, pistachio oil and sodium lauryl sulphate but optimized formulation F8 containing 0.4 ml pistachio oil, 0.8 ml coconut oil and 0.04 g of SLS exhibited more pronounced and promising effect through rat skin.

Key words: Central composite design, clotrimazole, permeability enhancer, topical gel.

INTRODUCTION

Topical drug delivery system (TDDS) is a type of dosage form which distribute an adequate amount of drug across the skin (Allen et al. 2004). TDDS gives a greater chance of success of drug delivery over traditional methods like use of injectable and oral formulations. It augments the patient acceptability bypassing first pass metabolism. The load that is commonly placed by oral route on the digestive tract and liver can be minimized by using TDDS. It also decreases the untoward effects of a drug arised due to overdose of medicament (Aggarwal and Dhawan 2009). Moreover, TDDS offers sustained delivery of the drug by providing constant and steady administration of drug with short biological half-life. By eliminating pulsed entry of the drug into systemic circulation it may also decrease the adverse effects. It is not possible to use topical route

Correspondence to: Ghulam Murtaza
E-mail: gmdogar356@gmail.com

of administration for all types of drugs. The major hindrance to permeability of the drug through topical route is stratum corneum. A variety of efforts have been made to boost the flux and enhance the penetration of the drug through skin. The use of penetration enhancers is one of those efforts. A penetration enhancer is a substance that is used to promote the delivery of an active pharmaceutical ingredient across the stratum corneum (Sloan and Wasdo 2006). The accelerants or sorption promoters are the chemical substances that dwindle the barrier function of the skin for time being and increase the drug permeability. Penetration enhancers may be chemical, physical or formulation based enhancer (Rao et al. 2016). The most employed approach in penetration enhancement is the utility of chemical penetration enhancers, they interfere with well-arranged lipid layer of stratum corneum (Walker and Smith 1996). It is an ideal property of penetration enhancers to abridge reversibly the barrier property of the stratum corneum without causing any harm to viable cells (Barry 1983, Williams and Barry 2012). Penetration enhancers produce their action either by interrupting highly sequenced lipid structure of stratum corneum or by interacting with intercellular proteins. Another mode of action is improvement in the partition co-efficient of the drug which acts as co-enhancer into the stratum corneum (Barry 1983). Coconut oil, pistachio oil and SLS are chemical penetration enhancers that increase the skin permeability by certain alteration in the lipid layer of stratum corneum and by perturbation of intercellular lipids and protein domain integrity (Hussain et al. 2012).

Gels are biphasic swollen networks occupying both the cohesive characteristics of solids, and the diffusive transport properties of liquids. In contrast to ointments and creams, gels often grant immediate release of active pharmaceutical ingredient, regardless of the water solubility of the drug. They have limited risk of inflammation and unwanted reaction and are remarkably biocompatible. Gels are easy to apply on the skin and there is no need to remove it. Gels for skin application possess various agreeable characters. They are thixotropic and non-greasy having emollient action. Gels are readily spreadable that can be conveniently wiped out upon washing since the gels are washable with water (Helal et al. 2012).

Carbopol is a synthetic polymer made of carbomers. Carbomer polymers are cross linked together and form a microgel structure that is useful in dermatological applications. As these polymers are anionic in nature so neutralization in necessary for microgel structure that's why organic amines like tri ethanolamine are used for such purpose (Mohammad et al. 2004).

Coconut oil also known as edible oil riches in short and medium chain fatty acid along with 92% saturated fatty acid and 45- 56 % lauric acid (Gopala 2010), while on other side pistachio oil contain high concentration of unsaturation fatty acid (53%) along with oleic acid(51%) and linoleic acid(30%) (Tehrani et al. 2013). Sodium lauryl sulphate, an-ionic surfactant, possesses skin permeation enhancing effects by enhancing fluidity of epidermis lipid (Jocelyne et al. 2000).

Conventionally, mycosis have been classified into two major classes: systemic fungal infection and superficial mycosis. On the basis of mycosis antifungal agents are also divided into two classes accordingly i.e. systemic and oral drugs. They are also grouped on the basis of their chemical structure. Classes on the basis of chemical structure include polyene antifungals and azole antifungals. Other groups include allylamine antifungals and echinocandin antifungals (Helal et al. 2012). Complex flora resides on oral and vaginal mucosae which makes it susceptible to various infections. *Candida albican*, a widespread fungus exists commonly at various body parts like skin, mouth, throat, vagina and recum and causes mycosis especially in immune compromised patients (Elisabetta et al. 2013).

Clotrimazole (CTZ) belongs to imidazole family of antimycotic agents and has lipophilic characteristics with a partition coefficient of log $K_{ow} > 4$. The estimated half-life of clotrimazole is more than 60 days

(Corcoran et al. 2014) along with broad-spectrum activity. Clotrimazole has a molecular formula of $C_{22}H_{17}N_2Cl$ with a molecular weight of 344.8. The melting point of clotrimazole is between 141-145°C (Florey 1992). Clotrimazole has very low vapour pressure i.e. $3.31.10^{-07}$ Pa (Iannelli et al. 2011). The water solubility of clotrimazole is 3-8 mg/L at 23°C (Buchanan 2007).

Clotrimazole is rapidly metabolized in liver and excreted after 48 hour of (Sawyer 1974). Fungicidal action is achieved by the blockage of fungal sterol 14α-demethylase, a microsomal cytochrome P450-dependent enzyme (Tian et al. 2006) as a result of which structural and functional deterioration occurs in the cytoplasmic membrane. Therefore the basic mechanism for fungicidal effect of clotrimazole is wrecking of the cell membrane, which ultimately leads to the efflux of intracellular phosphorus entities. This efflux leads to disintegration of cellular nucleic acids along with the discharge of potassium. Whenever the fungal strains are exposed to the drug, they are immediately killed by the fast and extensive onset of fungicidal action. The inhibition of fungal growth by clotrimazole is a time-dependent and concentration-dependent phenomenon. However, not only the fungal cytochrome P-450 is suppressed, the interference with mammalian cytochrome P-450 has also been reported (Köfeler et al. 2000).

So, the aim of present study was to prepare an optimize carbopol based clotrimazole gel for topical application along with the study of various permeability enhancers like coconut oil, pistachio oil and sodium lauryl sulphate on the permeation of drug through rat skin layer.

MATERIALS AND METHODS

MATERIALS

Clotrimazole pure drug was purchased from Bayer Pharmacuticals (Karachi, Pakistan). Carbopol 934 was a generous gift from Saffron Pharma (Faisalabad, Pakistan). Pistachio oil and coconut oil were gifted from Mushtaq Herbal Pharma (Multan, Pakistan). Sodium lauryl sulphate and ethanol was purchased from (Sigma-Aldrich, USA). Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and sodium dihydrogen phosphate were purchased from (Fluka, Germany) while sodium hydroxide was purchased from Shama Laboratory Chemical Works (Lahore, Pakistan). All the chemicals including penetration enhancers were of analytical grade and double distilled water was used throughout the study.

METHODS

Solubility Study

Solubility of clotrimazole was determined in different solvents e.g. distilled water, ethanol, methanol, chloroform and diethyl ether. Excess quantity of clotrimazole was dissolved with each of above solvent in thermostat (37 ± 2˚C) for 48 hours. Samples were taken from each solvent system, filtered, and the concentration of clotrimazole in each solution was determined after dilution by using UV spectrophotometry (UV-VIS Spectrophotometer O.R.I 3000, Germany) at 254 nm using ethanolic buffer (pH 7.4) as blank.

Formulation Design

For the optimization of clotrimazole gel, a computerized optimization technique, named as central composite design was employed, that involved three independent factors i.e. coconut oil, pistachio oil and sodium lauryl sulphate as permeability enhancers (Table I).

TABLE I
Coded levels and values of the independent variables.

Variable		Coded Levels				
		-α	-1	0	+1	+α
X_1	Coconut oil	0.26	0.40	0.6	0.80	0.94
X_2	Pistachio oil	0.13	0.20	0.3	0.40	0.047
X_3	SLS	0.020	0.25	0.032	0.038	0.043

According to central composite design (CCD), the optimization technique involves four basic and important stages. First is the performance of experiments that are designed statistically for the experimental plan. Then is to explain the experimental data, generation of mathematical models and to emphasize on the data of ANOVA. Next is to evaluate the efficiency of the model that controlled directly with diagnostic plots. Finally the prediction of responses along with verification of model is accomplished (Box and Draper 1987).

In this study, Response Surface methodology (RSM) in combination with the factorial experimental design of CCD was employed as shown in equation 1 to optimize the process. RSM is an efficient method to illustrate the effect of various parameters that affect the responses. By altering the parameters simultaneously and carrying out a limited number of experiments, the effect of variables upon response can de elaborated.

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

In above equation, Y denotes dependent variable (Response), $b_0$is intercept, b_1 to b_{33} are regression coefficients which are calculated from response Y while X_1, $X_2$and X_3 are coded values of factors used in gel preparation.

The experimental results were analyzed using design expert version 8.0.4, and the regression model was proposed. Coconut oil, pistachio oil and sodium lauryl sulphate were selected as independent parameters. Moreover, Y is the estimated response and X_1, X_2 and $X_3$are coded variables for coconut oil, pistachio oil and sodium lauryl sulphate respectivelly. Accordingly, 20 experiments that covers the full design of factors were performed as shown in Table II.

On the basis of RSM results, polynomial regression modelling on the responses of corresponding coded values of process variables was performed. The co-efficient of determination (R^2 and adj-R^2) were used to express the quality of polynomial model.

Preparation of Gels

Hydroalcoholic gels have earned great popularity owing to their ability to provide effective dispersion performance. This study involved the use of direct method for preparing hydroalcoholic gels. For the preparation of gels, polymer (Carbopol 934) was dispersed gently into known quantity of water with constant stirring by using magnetic stirrer (Clifton-Germany), so that there was no lump in the dispersion. Then the alcoholic solution of clotrimazole was added to the mixture with constant stirring, , so homogeneous dispersion was obtained. To it, the known quantities of permeability enhancers were added (Tables I and II). In order to adjust the pH of gel formulation, triethnolamine was used (Chaudhary et al. 2013).

TABLE II
Central Composite Design Experiments and Composition of Clotrimazole gels.

Formulation Code	Drug (mg)	Polymer (Carbopol) (g)	Ethanol (ml)	Water (ml)	Enhancers			Coded levels		
					Coconut oil	*Pistachio oil*	(SLS)	X_1	X_2	X_3
F1	20	0.7	1	20	0.4	0.2	0.025	-1	-1	-1
F2	20	0.7	1	20	0.8	0.2	0.025	1	-1	-1
F3	20	0.7	1	20	0.4	0.4	0.025	-1	1	-1
F4	20	0.7	1	20	0.8	0.4	0.025	1	1	-1
F5	20	0.7	1	20	0.4	0.2	0.04	-1	-1	1
F6	20	0.7	1	20	0.8	0.2	0.04	1	-1	1
F7	20	0.7	1	20	0.4	0.4	0.04	-1	1	1
F8	20	0.7	1	20	0.8	0.4	0.04	1	1	1
F9	20	0.7	1	20	0.25	0.3	0.035	-1.68	0	0
F10	20	0.7	1	20	0.92	0.3	0.035	1.68	0	0
F11	20	0.7	1	20	0.6	0.15	0.035	0	-1.68	0
F12	20	0.7	1	20	0.6	0.45	0.035	0	1.68	0
F13	20	0.7	1	20	0.6	0.3	0.025	0	0	-1.68
F14	20	0.7	1	20	0.6	0.3	0.041	0	0	1.68
F15	20	0.7	1	20	0.6	0.3	0.035	0	0	0
F16	20	0.7	1	20	0.6	0.3	0.035	0	0	0
F17	20	0.7	1	20	0.6	0.3	0.035	0	0	0
F18	20	0.7	1	20	0.6	0.3	0.035	0	0	0
F19	20	0.7	1	20	0.6	0.3	0.035	0	0	0
F20	20	0.7	1	20	0.6	0.3	0.035	0	0	0

CHARACTERIZATION OF CLOTRIMAZOLE GEL

Homogeneity

All developed clotrimazole gel formulations were characterized for homogeneity assessment. This was done by visual inspection of gel after the settlement of gel in suitable containers. Gels were analyzed for their appearance and existence of any clog (Nawaz et al. 2013).

pH evaluation

The pH of the gel was recorded using digital pH meter (Beckman, Germany). It was done by bringing the gel in direct contact with pH meter. After this, pH meter was allowed to equilibrate, then pH is recorded. All the experiments were performed triplicate (Chaudhary et al. 2013).

Skin irritation test

This test was performed on human volunteers. Twenty volunteers were chosen for single formulation and study was performed after taking their informed consent. It was performed by applying gel on an area of 2 square inch to the back of hand. Then the examination for the presence of lesion or irritation was done (Shivhare et al. 2009, Rajinikanth and Mishra 2008).

Viscosity

Brookfield viscometer (model DV-1+, USA) was used to measure the viscosity of clotrimazole gel. For this purpose, spindle No 04 was used. Viscosity was recorded by rotating spindle at 10 rpm.

Spreadability

To measure the spreadability of the gels wooden block and glass slide apparatus was used. Approximately, 2 g of developed clotrimazole gel was placed in the pan. The time taken by upper slide to separate completely from the fixed slides was noted (Hussain et al. 2012). The gel spreadability was evaluated through following equation:

$$S = ML/T \quad (2)$$

Where S = spreadability, M = weight tied to upper slide, L = length of glass slide and T = time taken by the slide to separate from.

Drug content

A measured amount of formulated gel was taken and dissolved in 100 ml of ethanolic phosphate buffer of pH 7.4. Similarly, solution of marketed clotrimazole cream was also prepared. Mechanical shaker was used to shake the gel solution continuously for 2 hours. The solution thus prepared was filtered and analyzed spectrophotometrically (Spectronic, Genesys 5) at 254 nm using ethanolic buffer (pH 7.4) as blank (Sera and Ramana 2006).

EX VIVO SKIN PERMEATION STUDIES

Preparation of Standard Curve

A standard calibration curve was used to calculate the concentration of the dug penetrated through rat skin. To construct the calibration curve, stock solution was prepared by dissolving 10 mg of clotrimazole in 100 ml of ethanolic phosphate buffer pH 7.4. The mixture was placed in a sonicator to ensure solubilization of the drug. Then the dilutions of 10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml were made and analyzed spectrophotometrically by using UV spectrophotometer (Beckman, Germany) (Nawaz et al. 2013).

Preparation of Rat Skin

The Sprague-dawle rats were received from animal facility of COMSATS Institute of Information and Technology Abbottabad (Ref. no. PHM-0033-FC/M-4). This animal study was conducted in accordance with international guidelines for animal use in lab experiments after getting approval from departmental ethical committee. The rats were 5-8 weeks of age and with average weight of 150 g – 200 g. The rats were provided with food and water *ad libitum*. The rats were sacrificed by cervical dislocation. Skin was excised from the rat and hairs were removed by means of hair clipper (Chaudhary et al. 2013).

Permeation Studies

Modified Franz diffusion cell system was used to carry out the *ex vivo* permeation studies. Appropriate sized trimmed rat skin was mounted on Franz cell diffusion system in such a way that the stratum corneum

faces the donor compartment while dermis faces the receiver cell. In receptor compartment of Franz cell ethanolic phosphate buffer [i.e. ethanol: phosphate buffer pH 7.4 (40:60)] was filled while in donor compartment, 2 g of gel was loaded. This assembly was placed in a water bath, which was placed on magnetic stirrer. Magnetic beads were placed inside the receiver compartment to ensure the constant stirring. Drug permeability studies were conducted for 6 h. The temperature was maintained at 37 ± 0.05˚C. The samples were withdrawn from the receptor cell at regular time intervals of time and were analyzed spectrophotometically (Beckman, Germany) (Pankaj et al. 2013).

Antifungal Activity

Antifungal activity was evaluated by using agar well method against *Candida albicans, Aspergillus Niger* and *Mucor*. Microbial inoculums of tested organisms (0.5% v/v) were spread over the nutrient-agar media in the separate petri dishes for each fungal strain. After solidifying the agar plate, well of 10 mm in diameter were made. The gel formulations were added to the wells by means of sterile syringe. Then the zone of inhibition of each well was visualized after the incubation period of 24 hrs. The radius of zone of inhibition was calculated and compared to the zone of inhibition of control formulation (Akhtar et al. 2012).

Stability studies

The stability studies for optimized formulation were conducted in the accelerated conditions as per guidelines of International Conference on Harmonization (ICH). Well closed container was used for the storage of optimized gel formulation. The gel formulations were stored at 40˚C and 75% relative humidity for 90 days. Samples were drawn at aforethought time interval of 30 days, 60 days and 90 days. The gel formulation was evaluated for their physical properties including appearance, color, and presence of clogs, consistency and phase separation. Gel was also evaluated for chemical parameters like change in pH and drug content (Chaudhary et al. 2013).

PERMEATION KINETIC ANALYSIS

Fick's law of diffusion was used in order to know the permeation rate of drug by believing that diffusion is involved in drug permeation through rat skin.

$$J = A.\ dm/dt \tag{3}$$

As we know that flux is proportional to concentration gradient so,

$$J = -D.\ dc/dx \tag{4}$$

Where J denotes permeation rate of drug through membrane, dc/dx is concentration gradient while D is the diffusion coefficient of permeant.

Lag time was calculated from X intercept of the slope at steady state concentration while Steady state flux was calculated from given equation.

$$J_{ss} = K_p C_o \tag{5}$$

Where K_p is permeability coefficient and C_o is drug concentration remains constant in vehicle.

And the permeability coefficient that is derived from the steady state flux is

$$K_p = J/C_o \quad (6)$$

Formulation responses like flux, lag time, diffusion coefficient, permeability coefficient and input rate were treated by design expert software version 8. The best fit model was selected based adjusted multiple correlation coefficients (adjusted R^2). One-way ANOVA with $p < 0.05$ was performed to know the significance of the model.

RESULTS AND DISCUSSION

PHYSICAL CHARACTERIZATION OF GEL FORMULATIONS

All clotrimazole gel preparations were evaluated for their physical characterization like pH, homogeneity, spreadability, viscosity, drug content, toxicity and anti-fungal activity as shown in Table III. The drug contents of the formulation was in the range of 0.99 - 0.91 mg/g of gel which indicates content uniformity. The pH of all developed formulations were in range of 5.0 ± 0.1 to 5.9 ± 0.2, which fall in normal pH range of the skin, while viscosity of all gel formulations were in between 16696 ± 2.0 to 69870 ± 2.0 cp. All tested formulations was safe and did not develop any skin irritation, lesion or inflammation along with spreadability values ranges ranges 5.2 ± 0.2 to 6.7 ± 0.1 (g.cm/s).

TABLE III
Characterization of Clotrimazole gel Formulations.

F code	Homogeneity	pH	Skin irritation	Viscosity (cp)	Spreadability (g.cm/s)	Drug content (mg/gm)	Anti-fungal activity Zone of inhibition (mm)		
							Mucor	*A. niger*	*C. albican*
F1	++	5.9 ± 0.1	Nil	17045 ± 2.5	5.2 ± 0.2	0.99	19	8	7
F2	++	5.7 ± 0.3	Nil	69870 ± 2.0	5.9 ± 0.2	0.91	20	7	8
F3	+++	5.3 ± 0.3	Nil	16870 ±3.0	6.0 ± 0.2	0.95	18	7	8
F4	++	5.8 ± 0.2	Nil	16696 ± 3.0	5.9 ± 0.1	0.98	18	8	8
F5	++	5.4 ± 0.2	Nil	17354 ± 1.1	6.1 ± 0.2	0.90	19	7	8
F6	++	5.5 ± 0.3	Nil	17167 ± 1.0	5.8 ± 0.2	0.92	19	7	7
F7	++	5.7 ± 0.1	Nil	17279 ± 2.5	5.4 ± 0.1	0.94	23	10	8
F8	+++	5.8 ± 0.3	Nil	17489 ± 2.0	5.5 ± 0.2	0.99	27	11	13
F9	+++	5.3 ± 0.2	Nil	69870 ± 2.0	6.3 ± 0.2	0.97	19	8	7
F10	++	5.8 ± 0.1	Nil	16870 ± 2.0	5.8 ± 0.1	0.99	24	9	9
F11	+++	5.5 ± 0.3	Nil	16696 ± 2.0	6.5 ± 0.1	0.96	20	8	7
F12	++	5.9 ± 0.2	Nil	17354 ± 2.0	5.5 ± 0.2	0.90	19	8	8
F13	++	5.0 ± 0.1	Nil	69870 ± 2.0	5.7 ± 0.1	0.93	18	8	8
F14	++	5.2 ± 0.3	Nil	17487 ± 2.0	6.7 ± 0.1	0.99	20	8	8
F15	+++	5.1 ± 0.1	Nil	16871 ± 2.5	6.5 ± 0.3	0.96	20	7	8
F16	++	5.2 ± 0.3	Nil	16696 ± 2.5	5.4 ± 0.1	0.92	18	8	8
F17	+++	5.1 ± 0.3	Nil	17354 ± 2.5	5.8 ± 0.3	0.94	18	8	7
F18	++	5.5 ± 0.3	Nil	17167 ± 2.0	5.4 ± 0.3	0.92	19	8	7
F19	++	5.2 ± 0.3	Nil	17279 ± 2.6	5.5 ± 0.3	0.95	19	8	7
F20	+++	5.0 ± 0.1	Nil	17489 ± 3.0	5.4 ± 0.3	0.91	19	8	7
-ve control*	-	-	-	-	-	0.90	18	7	7
+ve control**	-	-	-	-	-	0.99	28	12	14

* Formulation without enhancers.

** Marketed Formulation.

SOLUBILITY STUDIES

Clotrimazole was slightly soluble in aqueous medium as compared to organic solvents. Clotrimazole showed highest solubility 89 mg/ml in ethanol as compared to other organic solvents a shown in Table IV.

ANTIFUNGAL ACTIVITY

Antifungal activity of clotrimazole gel indicated that Clotrimazole gel was not only effective against *Candida albicans* but also produced fungicidal effect against *Aspergillus Niger* and *Mucor*. Optimized formulation 8 produced prominent zone of inhibition against all the three strains as shown in Figure 1.

TABLE IV
Solubility Studies of Clotrimazole in various Solvent Systems at 37 ± 2˚Cfor 48 hours.

Solvent System	Solubility (mg/ml) at 37 ± 2˚C
Water	0.02
Ethanol	89
Methanol	85
Chloroform	87
Di- ethyl ether	14

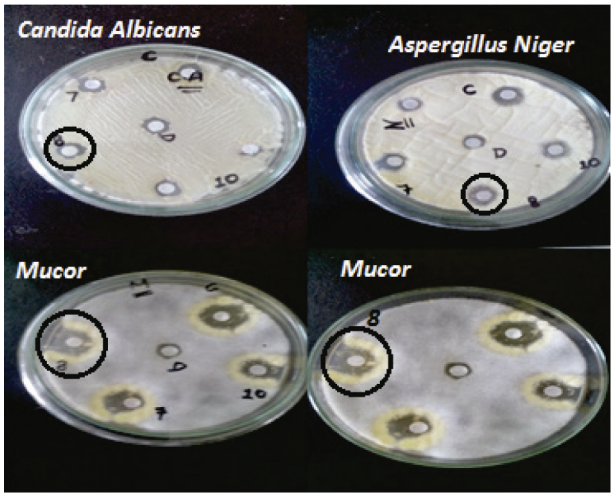

Figure 1 - Anti-fungal activity of clotrimazole gel (optimum formulation F8 is encircled).

STABILITY STUDIES

Stability studies of developed formulation were performed according to International Conference on Harmonization (ICH) guidelines and stability data of optimized formulation F8 is shown in Table V. It indicates that optimized formulation exhibit good stability behavior regarding pH 5.7, appearance (no clog present), homogenity (+++) and percentage drug content (0.97 mg/g).

EX VIVO SKIN PERMEATION STUDIES AND OPTIMIZATION OF GELS

Ex vivo skin permeation studies of developed formulations were performed through rat skin by using modified Franz diffusion cell system. It is cleared from Table VI that formulation F8 exhibited highest

flux 2.17 µg/cm^2/min, permeability coefficient 0.0019 cm/min and input rate 1.543 µg/cm^2/min, along with moderate lag time 77.27 min and diffusion coefficient 0.063 cm^2/min as compared to other developed formulations.

DATA ANALYSIS THROUGH POLYNOMIAL EQUATION.

Three dimensional plots were constructed for all responses as shown in Fig. 2 and were analyzed through polynomial equation. A positive sign indicates additive effect which favors the response optimization, while negative sign indicates decreasing relationship between factors and responses.

TABLE V
Stability study for Optimized Formulation (F8).

Parameters	0 day	30 days	**60 days**	**90 days**
Appearance	no clog present	no clog present	no clog present	no clog present
Homogeneity	+++	+++	+++	+++
pH	5.8	5.8	5.7	5.7
Drug content (mg/g)	0.99	0.99	0.98	0.97

TABLE VI
Predicted and Actual values of all Responses Calculated by *Ex Vivo* Skin Permeation Study.

Std	**Flux** Actual **µg/cm^2/min**	**Flux** Predicted **µg/cm^2/min**	**t_{lag}** Actual **min**	**t_{lag}** Predicted **min**	**D** Actual **cm^2/min**	**D** Predicted **cm^2/min**	**K_p** Actual **cm/min**	**K_p** Predicted **cm/min**	**Input rate** Actual **µg/cm^2/min**	**Input rate** Predicted **µg/cm^2/min**
1	1.264	1.227	46.202	59.296	0.037	0.048	0.0012	0.0012	0.996	0.956
2	1.839	1.740	47.34	78.185	0.038	0.063	0.0012	0.0013	0.959	1.042
3	1.568	1.384	115.85	129.77	0.094	0.105	0.0015	0.0014	1.236	1.151
4	1.481	1.334	143.29	143.63	0.117	0.117	0.0014	0.0013	1.167	1.040
5	1.065	1.034	148.10	155.14	0.120	0.126	0.0010	0.0011	0.839	0.875
6	1.577	1.584	118.01	111.47	0.096	0.091	0.0015	0.0015	1.243	1.237
7	1.907	1.829	143.70	120.24	0.117	0.098	0.0019	0.0016	1.503	1.329
8	2.17	1.818	77.272	71.562	0.063	0.058	0.0019	0.0018	1.543	1.493
9	1.281	1.392	107.22	104.48	0.087	0.085	0.0012	0.0014	1.010	1.122
10	1.672	1.813	87.136	79.429	0.071	0.064	0.0016	0.0016	1.318	1.333
11	1.137	1.146	147.01	124.15	0.120	0.101	0.0011	0.0010	0.896	0.808
12	1.233	1.475	137.43	149.85	0.112	0.122	0.0012	0.0015	0.972	1.188
13	1.22	1.412	112.03	80.983	0.091	0.066	0.0012	0.0012	0.961	1.018
14	1.598	1.656	80.362	100.96	0.065	0.082	0.0015	0.0016	1.259	1.331
15	1.299	1.291	73.034	73.348	0.059	0.059	0.0012	0.0012	1.023	1.019
16	1.299	1.291	73.134	73.348	0.059	0.059	0.0012	0.0012	1.023	1.019
17	1.299	1.291	73.031	73.348	0.059	0.059	0.0012	0.0012	1.023	1.019
18	1.299	1.291	73.032	73.348	0.059	0.059	0.0012	0.0012	1.023	1.019
19	1.299	1.291	73.034	73.348	0.059	0.059	0.0012	0.0012	1.023	1.019
20	1.299	1.291	73.034	73.348	0.059	0.059	0.0012	0.0012	1.023	1.019
-ve control*	1.0625	-	73.002	-	0.0009	-	0.0005	-	0.045	-
+ve control**	2.006	-	73.04	-	0.059	-	0.0020	-	1.6329	-

* Formulation without enhancers.
** Marketed Formulation.

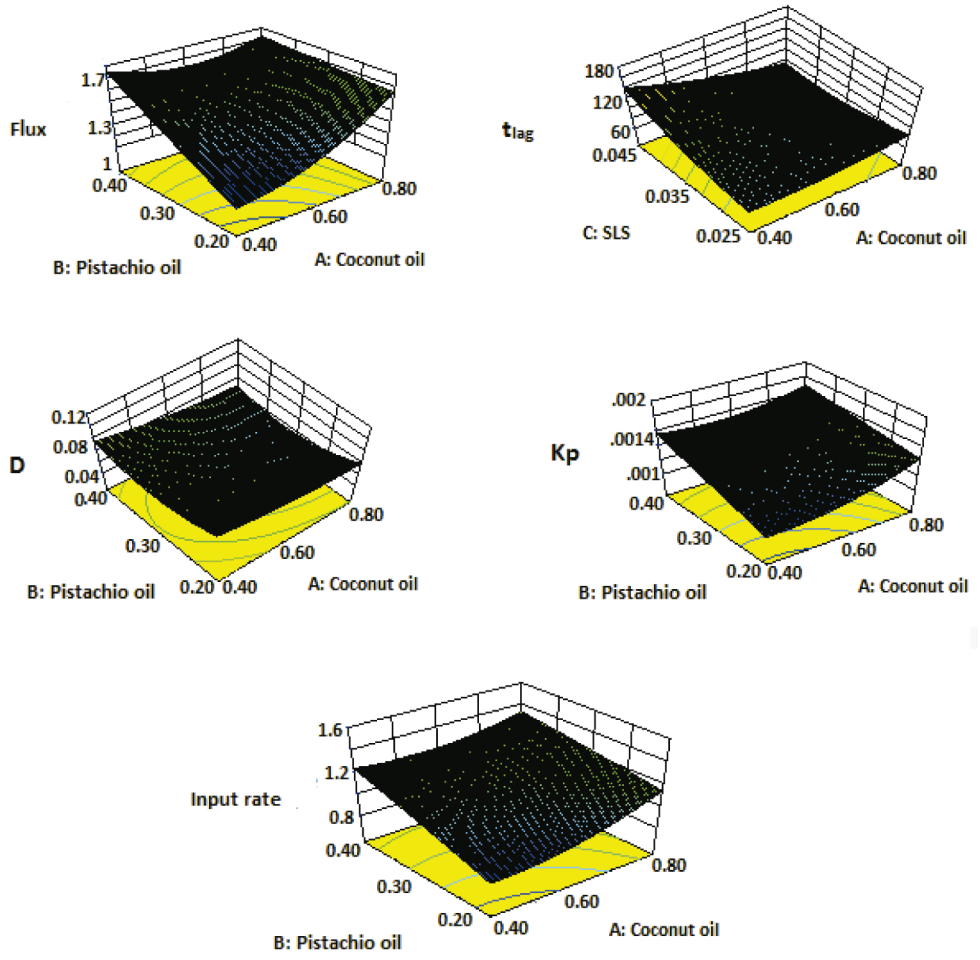

Figure 2 - Three Dimensional (3D) Plots of all Five Responses.

Response Y_1

$$Y_1 = 4.413288 - 0.792X_1 - 2.805X_2 - 187.562X_3 - 7.020X_1X_2 + 7.129X_1X_3 + 239.644\,X_2X_3 + 2.748X_1^2 + 0.674\,X_2^2 + 1930.681X_3^2 \quad (7)$$

In Eq. 7, Y_1 denotes flux while X_1 is concentration of propylene glycol, X_2 is concentration of tween 80 and X_3 is concentration of sodium lauryl sulphate. As per Eq. 7, coconut oil along with pistachio oil have non-significant decreasing effect on flux of clotrimazole while sodium lauryl sulphate along with coconut oil and pistachio oil have non-significant additive effect on flux of clotrimazole.

Response Y_2

$$Y_2 = -162.593 + 155.622X_1 + 15.031X_2 + 10901.16X_3 - 62.756X_1X_2 - 11728.6X_1X_3 - 39513.2X_2X_3 + 164.473X_1^2 + 2250.455X_2^2 + 140230.1X_3^2 \quad (8)$$

According to polynomial Eq. 8, all given permeability enhancers i.e. coconut oil, pistachio oil and sodium lauryl sulphate alone has non-significant additive effects while coconut oil along with pistachio oil and sodium lauryl sulphate have non-significant decreasing effect on lag time of clotrimazole.

Response Y_3

$$Y_3 = -0.133 + 0.127X_1 + 0.012X_2 + 8.892X_3 - 0.051X_1X_2 - 9.578X_1X_3 - 32.265\,X_2X_3 + 0.135X_1^2 + 1.839X_2^2 + 114.658X_3^2 \quad (9)$$

As per polynomial Eq. 9 all three permeability enhancers coconut oil, pistachio oil and sodium lauryl sulphate alone has non-significant additive effects while coconut oil along with pistachio oil and sodium lauryl sulphate have non-significant decreasing effect on diffusion coefficient.

Response Y_4

$$Y_4 = 0.005 - 0.004X_1 - 0.0006X_2 - 0.171X_3 - 0.003X_1X_2 + 0.0654X_1X_3 + 0.123X_2\,X_3 + 0.003X_1^2 - 1.2 \times 10^{-5}X_2^2 + 1.775X_3^2 \quad (10)$$

According to polynomial Eq. 10, coconut oil along with pistachio oil have non-significant decreasing effect on permeability coefficient while sodium lauryl sulphate along with coconut oil and pistachio oil have non-significant additive effect on permeability coefficient of clotrimazole.

Response Y_5

$$Y_5 = 0.005 - 0.004X_1 - 0.0006X_2 - 0.171X_3 - 0.003X_1X_2 + 0.065X_1X_3 + 0.123X_2\,X_3 + 0.003\,X_1^2 - 1.2 \times 10^{-5}X_2^2 + 1.7751X_3^2 \quad (11)$$

It is cleared form polynomial Eq. 11, that coconut oil along with pistachio oil have non-significant decreasing effect while sodium lauryl sulphate along with coconut oil and pistachio oil have non-significant additive effect on input rate of clotrimazole.

Quadratic model was best fit to all responses and the comparative values of regression coefficients are described in Table VII.

TABLE VII
Regression Analysis and Their Value of all Five Responses.

Regression Coefficient	Flux	t_{lag}	D	Kp	Input rate
Model (p-value)	0.0088	0.0123	0.0122	0.0149	0.0159
Coefficient of variation	10.48176	20.83931	20.84093	10.99389	10.80187
R^2	0.82179	0.807649	0.807717	0.79891	0.795883
Adjusted R^2	0.6614	0.634533	0.634662	0.617929	0.612178
PRESS	1.707236	32097.18	0.021405	1.77×10-6	1.096865
F-value	5.123715	4.66536179	4.667407	4.414334	4.332398
Lack of fit (p-value)	< 0.0001	< 0.0001	< 0.0001	0.0008	< 0.0001
Std. Dev	0.149906	20.31082	0.016587	0.000153	0.119085

DISCUSSION

The penetration enhancers are used to get improved permeation of active pharmaceutical ingredient across the skin. The enhancers have distinguishing function, which relies upon the nature of the drug, the vehicle used, the concentration of enhancers, and other factors (Aungst 1989). Coconut and pistachio oil are natural permeability enhancers, which potentiate the permeation of the drug by producing alteration in the lipid

layer of stratum corneum resulting in the improved Topical permeation of the drug (Hussain et al. 2012). While sodium lauryl sulphate is an anionic surfactant which acts by perturbation of intercellular lipids and protein domain integrity (Velmurugan and Dodla 2013). Anionic surfactants interfere greatly with keratin as well as with lipid layer of stratum corneum changing the permeability of the skin. These agents are involved in the uncoiling and extension of the helical filaments of the stratum corneum lipids. These changes causes the expansion in the membrane leading to increased permeability (Pandey et al. 2014), thus these agents were aimed to possibly enhance the permeation of clotrimazole.

In this study, the impact of permeation enhancers i.e. the independent variables, was clearly understood by generation of three dimensional contour plots for the responses. These three dimensional graphs constituted the base of the model polynomial function which was further used to analyze and investigate the interactive impact of the three variables i.e. the three enhancers, which were coconut oil, pistachio oil, and SLS. The inferences so attained are discussed here, as indicated in Fig. 2 that the pistachio oil, coconut oil and SLS have considerable influence on the permeability of clotrimazole.

It can be deduced from the Figure 2 that with higher levels of pistachio oil and coconut oil and lower level of SLS, non-significant ($P > 0.05$) increase in flux was observed. It could be due to the fact that the coconut and pistachio oil, like other unsaturated fatty acids, increase the skin‘s permeation through disruption of the anatomical structure of the ordered lipids of the stratum corneum (Benson 2005). These changes promote the fluidization of stratum corneum lipids simultaneously by lowering the lipid transition temperature (Ongpipattanakul et al. 1991) resulting in increase in flux. Similar results for the improved released profile of the drug by coconut oil have been reported earlier (Patel et al. 2014).

With increase in SLS concentration with coconut and pistachio oil, significant ($P < 0.05$) increase in flux of clotrimazole was observed, because SLS causes swelling of the stratum corneum and produces the uncoiling and extension of α-keratin helices, which results in the opening up of protein-controlled polar pathways (Barry 1987). Another mode of action of SLS for penetration enhancement of drug through skin is associated with the hydrophobic interaction of SLS with skin lipid chains leaving the end sulphate group exposed. This generation of extra sites in the stratum corneum leads to an increase in skin hydration (Rhein et al. 1986). Conclusively, the increased fluidization of stratum corneum by the unsaturated fatty acids present in coconut and pistachio oil produces aadditive effect on the permeability of skin in the presence of SLS, which is involved in the increased skin hydration as well as opening of protein controlled pathways. It alters the membrane permeability leading to significant increase in flux in aadditive manner. Similar results of increased permeability by SLS have already been documented (Jocelyne et al. 2000).

Figure 2 shows that with increase in concentration of SLS as compared to coconut oil and pistachio oil, there is significant ($P < 0.05$) decrease in t_{lag} since coconut oil, pistachio oil, and SLS work simultaneously increasing flux and decreasing time taken by drug to be absorbed, as explained above. Moreover, there is non-significant ($P > 0.05$) decrease in t_{lag} by increasing coconut or pistachio oil amount as compared to that of SLS.

Mathematical models (Equation 10) depict that increasing amount of SLS with coconut and pistachio oil show increased level of Kp. Permeability coefficient of the stratum corneum is directly related to the drug’s partition coefficient (Roberts 1997). The permeability enhancers causes the fluidization of stratum corneum and opens the protein dependent pathway leading to increase in the skin’s permeability coefficient. It is reported that an increase in drug’s partition coefficient is also accompanied with increase in Kp (Roberts 1997).

Moreover, contour plots for input rate shows increasing trend in input rate with increase in level of enhancers used. Equation 11 shows that a combination of pistachio and SLS and coconut and SLS results in the increased input rate. Due to fluidization of stratum corneum (Ongpipattanakul et al. 1991) and hydration in the skin by SLS, the permeability of the skin is increased, which cases increase in input rate of the drug (Rhein et al. 1986).

CONCLUSION

Topical clotrimazole gel formulations with increased penetration rate were aimed, formulated, and optimized by a three factor central composite design. Formulation F8 consisting of coconut oil (0.8 ml), pistachio oil (0.4) and SLS (0.04) came out to be the optimized formulation, since it exhibited maximum flux and a high degree of stability. It exhibited the largest zone of inhibition against the tested fungal strains. In conclusion, the present data and permeability studies confirm the feasibility of developing the clotrimazole gel with excellent Topical permeation properties.

REFERENCES

AGGARWAL G AND DHAWAN S. 1990. Development, fabrication and evaluation of transdermal drug delivery system-A review. Pharmainfo Net 7: 1-28.

AKHTAR N, PATHAK K AND CAVAMAX W. 2012. Composite ethosomal gel of clotrimazole for improved topical delivery: development and comparison with ethosomal gel. AAPS PharmSciTech 13: 344-355.

ALLEN L, POPOVICH NG AND ANSEL H. 2004. Pharmaceutical dosage forms and drug delivery systems. Lippincott Williams & Wilkins, USA.

AUNGST BJ. 1989. Structure/effect studies of fatty acid isomers as skin penetration enhancers and skin irritants. Pharm Res 6: 244-247.

BARRY BW. Dermatological formulations: percutaneous absorption. 1st ed., Info health care 1983: 18-23.

BARRY BW. 1987. Mode of action of penetration enhancers in human skin. J Control Release 6: 85-97.

BENSON HA. 2005. Transdermal drug delivery: penetration enhancement techniques. Cur Drug Delivery 2: 23-33.

BOX GE AND DRAPER NR. 1987. Empirical model-building and response surfaces. Wiley New York, USA, p. 424.

BUCHANAN CM. 2007. Solubilty and dissolution studies of antifungal drug: hydroxybutenyl-β-cyclodextrin complexes. Cellulose 14: 35-47.

CHAUDHARY H, ROHILLA A, RATHEE P AND KUMAR V. 2013. Optimization and formulation design of carbopol loaded Piroxicam gel using novel penetration enhancers. Int J Biol Macromol 55: 246-253.

CORCORAN J, LANGE A, CUMMING RI, OWEN SF, BALL JS AND TYLER CR. 2014. Bioavailability of the imidazole antifungal agent clotrimazole and its effects on key biotransformation genes in the common carp (Cyprinus carpio). Aquat Toxicol 152: 57-65.

ELISABETTA E, LAURA RI, CATIA C, ANDREA C, MARKUS D AND DAMIANO R. 2013. Clotrimazole nanoparticle gel for mucosal administration. Mater Sci Eng 33: 411-418.

FLOREY K. 1992. Analytical profiles of drug substances and excipients. Academic press, UK, p. 20.

GOPALA KAG, GAURAV RASB, PRASANTH KPK AND PREETI C. 2010. Coconut oil: chemistry, production and its application - a review. Ind Cocunut J 8: 15-27.

HELAL A, EL-REHMAN DA, ABDEL-HALIM SA AND EL-NABARAWI MA. 2012. Formulation and evaluation of fluconazole topical gel. Int J Pharm Pharm Sci 4: 302-310.

HUSSAIN A, KHAN GM, SHAH SU, SHAH KU, RAHIM N AND WAHAB A. 2012. Development of a novel ketoprofen transdermal patch: Effect of almond oil as penetration enhancers on in-vitro and ex-vivo penetration of ketoprofen through rabbit skin. Pak J Pharm Sci 25: 227-232.

IANNELLI A, DE SOUSA G, ZUCCHINI N, SAINT-PAUL MC, GUGENHEIM J AND RAHMANI R. Anti-apoptotic pro-survival effect of clotrimazole in a normothermic ischemia reperfusion injury animal model. J Surg Res 171: 101-107.

JOCELYNE P, ANDRÉ D, HÉLÈNE C, JULIE L, PIERRETTE G AND JULIANNA J. 2000. Sodium lauryl sulfate increases the efficacy of a topical formulation of foscarnet against herpes simplex virus type 1 cutaneous lesions in mice. Antimicrob Agents Chemother 44: 2263-2270.

KÖFELER HC, FAULER G, WINDISCHHOFER W AND LEIS HJ. 2000. Effect of cytochrome P-450 inhibitors econazole, bifonazole and clotrimazole on prostanoid formation. Br J Pharmacol 130: 1241-1246.

MOHAMMAD IT, NAIR RH, SUSAN C AND CHRISITA A. 2004. Rheological characterization of topical carbomer gels neutralized to different pH. Pharm Res 21: 1192-1199.

NAWAZ A, JAN SU, KHAN NR, HUSSAIN A AND KHAN GM. 2013. Formulation and in vitro evaluation of clotrimazole gel containing almond oil and tween 80 as penetration enhancer for topical application. Pak J Pharm Sci 26: 617-622.

ONGPIPATTANAKUL B, BURNETTE RR, POTTS RO AND FRANCOEUR ML. 1991. Evidence that oleic acid exists in a separate phase within stratum corneum lipids. Pharm Res 8: 350-354.

PANDEY A, MITTAL A, CHAUHAN N AND ALAM S. Role of surfactants as penetration enhancer in transdermal drug delivery system. J Mol Pharm Org Process Res 2(113): 2-7.

PANKAJ S, RINI T AND DANDAGI P. 2013. Formulation and evaluation of proniosome based drug delivery system of the antifungal drug clotrimazole. Int J Pharm Sci Nano Tech 6: 6-12.

PATEL HR, PATEL RB, PATEL GN AND PATEL MM. 2014. The influence and compatibility of vegetable oils and other additives on release of ketoprofen from transdermal films. E C Afr J Pharm Sci 13: 3783-3790.

RAJINIKANTH P AND MISHRA B. 2008. Floating in situ gelling system for stomach site-specific delivery of clarithromycin to eradicate H. pylori. J Control Release 125: 33-41.

RAO S, BAROT T, RAJESH KS AND JHA LL. 2016. Formulation, optimization and evaluation of microemulsion based gel of butenafine hydrochloride for topical delivery by using simplex lattice mixture design. J Pharm Investig 46: 1-12.

RHEIN L, ROBBINS C AND FERNEE K. 1986. Surfactant structure effects on swelling of isolated human. J Soc Cosmet Chem 37: 125-139.

ROBERTS M. 1997. Targeted drug delivery to the skin and deeper tissues: role of physiology, solute structure and disease. Clin Exp Pharmacol Physiol 24: 874-879.

SAWYER PR, BROGDEN RN, PINDER RM AND SPEIGHT TM. 1974. Clotrimazole: a review of its antifungal activity and therapeutic efficacy. Drugs 9: 424-447.

SERA U AND RAMANA M. 2006. *In vitro* skin absorption and drug release–a comparison of four commercial hydrophilic gel preparations for topical use. The Indian Pharmacist 73: 56-360.

SHIVHARE UD, JAIN KB, MATHUR VB, BHUSARI KP AND ROY AA. 2009. Formulation development and evaluation of diclofenac sodium gel using water soluble polyacrylamide polymer. Dig J Nanomater BioS 4: 285-290.

SLOAN K AND WASDO S. 2006. The role of prodrugs in penetration enhancement. Taylor and Francis, NY, USA, p. 51-64.

TEHRANI MS, GIVIANRAD MH, AZAR PA, HUSSAIN SW AND MOHAMMADI SAJ. 2013. Chemical composition of Iran Pistacia atlantica cold press oil. J Chem 20: 1-6.

TIAN M, DONG MQ, CHIU SWL, AU CP AND LI GR. 2006. Effects of the antifungal antibiotic clotrimazole on human cardiac repolarization potassium currents. Br J Pharmacol 147: 289-297.

VELMURUGAN S AND DODLA S. 2013. Buccal penetration enhancers-an overview. Asian J Pharm Clin Res 6: 93-100.

WALKER RB AND SMITH EW. 1996. The role of percutaneous penetration enhancers. Adv Drug Deliv Rev 18: 295-301.

WILLIAMS AC AND BARRY BW. 2012. Penetration enhancers. Adv Drug Deliv Rev 64: 128-137.