Extraction, formulation and characterization of an *in vitro* and e*x-vivo* evaluation of *Thymus serpyllum* L. (Thymus oil) from topical preparations using dialysis cellulose membrane and natural rabbit skin

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Abstract: Herbal remedies like the *Thymus serpyllum* L. is useful in traditional medicine for the treatment of many diseases especially congestion, and bronchitis. The purpose of this study was to formulate a micro-emulsion, a gel and an ointment containing the plant hydro distilled thymus oil extracted from *Thymus serpyllum* L. collected from Ziarat, Balochistan. The prepared formulations were subjected to *in-vitro* and *ex vivo* study release, High performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), to justify their suitability for topical use. The *in-vitro and ex-Vivo* release was studied using Franz Cells and using two different kinds of membrane synthetic dialysis cellulose membrane and natural rabbit skin, and the amount of drug released was determined by HPLC at λ 274nm. The three formulations result obtained through dialysis cellulose membrane showed the faster release than the natural rabbit skin. However, the micro-emulsion, gel formulation showed the same release except ointment. The release from the above mentioned formulation can be arranged in the following descending order. micro-emulsion > Gel > Ointment. The best fit of release kinetics was achieved by Krosmeyer- Peppas, the TLC and HPLC identifies the Thymol, isolation and quantification of the marker. This study demonstrates that it is necessary to assess the impact of release and permeability pattern of different formulations. *In vitro* and *ex-vivo* diffusion cell experiments can be utilized to develop formulations of traditional medicines identifies.

Keywords: *Thymus serpyllum* L., Thymus oil, topical preparations, dialysis cellulose membrane.

INTRODUCTION

Many respiratory diseases are spreading in society, among them bronchitis, asthma is the most common diseases in cold climate countries. Different type of herbal remedies is used as household treatment for Bronchitis and asthma in many countries *Thymus serpyllum* L. (Thymus oil) are topically applied to treat many diseases (Gul *et al.,* 2017). In current years there has been an increasing interest in the therapeutic usage of herbal medicine and Phytopharmaceutical products (Sombra *et al.,* 2005; Mesbah *et al.,* 2005; Gul *et al.,* 2017 & accepted). The reasons of such increasing interest can be referred due to the fears of abuses and misuses of synthetic drugs and its adverse effects and incompetence of conventional drugs to gain the conventional medicine treatment is not in access to the whole population and the proposal related the safety and efficacy of natural formulations (Rates, 2001). Genus *Thymus* (Lamiaceae) family comprises about 400 species of hardy, perennial, and aromatic evergreen or semi-evergreen herbaceous plants (Safaei-Ghomi *et al.,* 2009). Many species of *thymus L.* genus are scattered in Balochistan, Pakistan (fig. 1). *Thymus serpyllum* L. is a perennial shrub, its specific name "*serpyllum*" is resulting from the Greek word meaning "to creep," because of wild thyme's irregular habit. *Serpylli herba* belongs to the European Pharmacopoeia (EP). The EP standard to the essential oil content is not less than 3mL/kg. The essential oil of several species of genus *Thymus* was reported to have antibacterial (Hazzit *et al.,* 2009; Rasooli and Mirmostafa 2002; Ebrahimi *et al.,* 2008) and antifungal activities (Al-Fatimi *et al.,* 2010). The majority of which are mediated by thymol and carvacrol, as the phenolic components of the oil, Thymol is generally more potent (Hudaib *et al.,* 2002; Loziene 2009). *Thymus* oils and extracts are extensively used in pharmaceutical, cosmetic and perfume industry as well for flavoring and preservation of numerous food products. In traditional medicine, leaves and flowering parts of *Thymus* species are generally used as tonic and herbal tea, antiseptic, anti tussive and carminative as well as for treating colds. Thyme mouth washes are also used against gum infections. Superficially it is applied to clean the skin against acne (Ebrahimi *et al.,* 2008; Olowosulu *et al.,* 2005). The inclusion of a phytomedicine in a suitable form will promise the formulation of the herbal medicine in a well-designed dosage form. This is also at important step in the scientific evaluation and standardization of

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Phytomedicine (Cheesbrough, 2002). The *British Herbal Pharmacopoeia* classifies this species as a medicinal plant and among the indications for its use it mentions bronchitis, bronchial catarrh, whooping cough and sore throats. Whooping cough is singled out as a specific indication. In the monograph, recommendations are given for combining it with other plants. As a gargle for acute pharyngitis, it is recommended in combination with the leaves of blackberry (*Rubus fruticosus* L.) or *Echinacea (Echinacea* sp.) (B.P, 2015). According to the PDR for Herbal Medicines, wild thyme is a component in various standardized preparations with anti tussive effects, while alcohol extracts are integral components of drops used for coughs and colds (Thomson, 2004). It is use topically in Vicks vaporub for cold and Bronchitis. The recommended daily dose of this drug is 4-6g. The essential oil of *L. sidoeides* (EOLS) showed potential topical anti-inflammatory activities when use at different concentrations. Due to antioxidant activities, it is believed that the major compound was thymol responsible for the biological activities of the (EOLS). Thymol (84.9%) (Monteiro *et al.*, 2007).

Fig. 1: *Thymus serpyllum* L.

The objective of this study was to formulate three topical dosage forms, a micro emulsion, a gel, and an ointment, using *Thymus serpyllum* L. (Thymus oil) subjected to evaluate their release from topical preparation using Franz cells, dialysis cellulose membrane and Natural rabbit skin. To develop traditional medicine and increase its bioavailability.

MATERIALS AND METHODS

Chemicals

Thymol was chosen as the analytical standard for *Thymus serpyllum* L. was gifted from (Merck Serono Quetta, Factory, Pakistan). Wool fat, cetostearyl alcohol, hard paraffin was purchased from (Sigma Aldrich). Oleic acid from (Merck Germany), CarbopolP934 gifted from (Merck Sereno Quetta Factory, Pakistan). Acetonitrile, Methanol, disodium hydrogen phosphate, potassium dihydrogen phosphate, triethanolamine and ethanol (95 %) were purchased from (Sigma Aldrich). glacial acetic acid and Polysorbate 80 were purchased from Fisher scientific, dialysis cellulose membrane (Width/Sheet 128 mm and length/Sheet 345-mm, thickness 11.5um (CUPROPHAN)^{R} made in Germany. All chemicals were used of high purity grade.

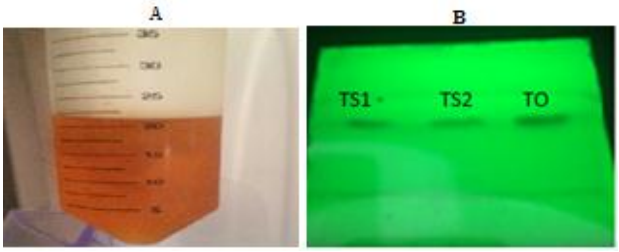

Fig. 2: (A): Thymus oil (B) TLC of Thymol at 252 nm (TS1) and (TS2) are standards (TO) Thymus oil

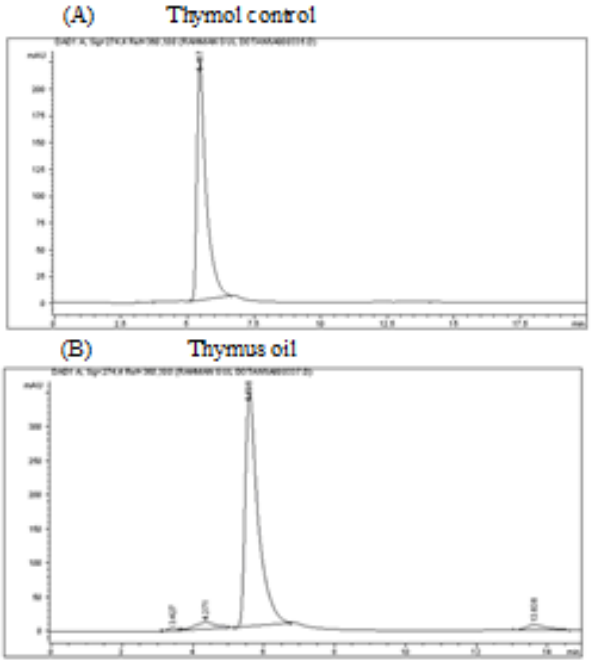

Fig. 3: Chromatogram (A) Thymol control Chromatogram (B) Thymus oil.

Plant collection and essential oil extraction

The plants of *Thymus Serpyllum* L. were collected from Ziarat, Balochistan in June to August 2015. The plant was identified and authenticated by Prof. Dr. Rasool Baksh Tareen, Botany Department, University of Balochistan, Pakistan. Dried plant material from *T. Serpyllum* L. was hydro distilled for 3h in a Clevenger type apparatus according to the European Pharmacopoeia. The obtained oil was subsequently dried over anhydrous Na_2SO_4 and kept at 4°C until analysis.

Isolation and identification

Thin-layer chromatography

In the testing of thyme oil components, the following solvent have been used as mobile phase (B.P, 1993 and Wagner *et al*., 1984).

- $C_2H_4Cl_2$: EtOAC (9.0:1.0)
- Toluene: EtOAC (9.5:0.5)
- Toluene: EtOAC (9.3:0.7)
- $C_6 H_6$: EtoAC (9.0:1.0)

Utilizing a glass and aluminum plate of silica gel GF254 as the coating substance at dimension (2cm X 8cm) and width 0.25mm.The most excellent separation of active compounds of Thymus oil is the mixture of toluene and ethyl acetate (9.5:0.5), the chromatogram shows Thymol, has been fully identified. Each zone has been scratched, isolated and dissolved in ether, which on filtration and removal of the solvent gave the desired compounds. The identification was done by comparison of R_f value of standard with the R_f valve of Thymus oil under the same situation.

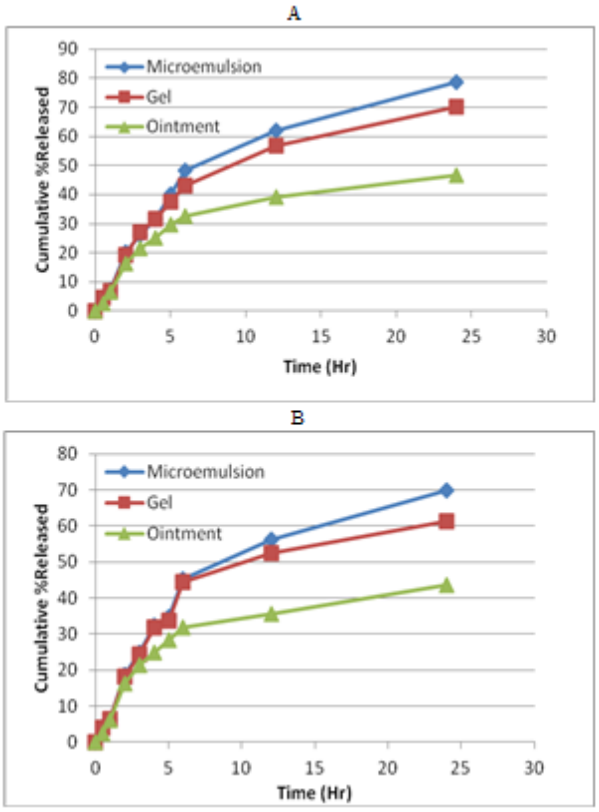

Fig. 4: Comparison of the release profiles of Thymol from three topical dosage forms containing *Thymus serpyllum* L. using (A) dialysis cellulose membrane (B) natural rabbit skin.

High performance liquid chromatographic quantization of thymol from thymus serpyllum L. extracted oil

Instrumentation

HPLC experiment was performed using System (Agilent Technologies, 1100 Series, USA with LC-10 AT VP pump). Equipped with, DGU-AM 14 degasser, manual injector system and SPD-10 AVP UV-VIS detector, Hypersil BDS C 18 (250 X 4.6mm) column. Agilent software use for the data collection and data processes. The chromatographic conditions used for analysis were as follows: the mobile phase consisted of acetonitrile, Water, orthophosphoric acid at ratios of (60:38:2) respectively, the flow rate was 1mL/min, injected volume 20 µL and samples were detected by an ultraviolet-visible detector at a wavelength of 274 nm. using 0.5g plant essential oil. The retention time of Thymol was 5.596 minutes. The limit of detection was 0.015µg/mL and the limit of quantification was 0.050µg/mL.

Preparation of micro-emulsion

The micro-emulsion was formulated as mentioned by Chen *et al*. A (surfactant) mixture of polysorbate80 and (co surfactant) ethanol was mixed in a ratio 2:1. A 4.6-g aliquot of the surfactant mixture was added to 0.5g of (oil) oleic acid and stirred with magnetic stirrer properly. Thymus oil Plant extracts 0.5g were added to the surfactant mixture and then into oleic acid stirred until fully dissolved. Then 4.4g of filtered, de ionized water was added slowly under continuous stirring 1200 rpm at ambient temperature (Gul *et al*., 2017; Chen *et al.,* 2006).

Preparation of a gel

The formulation of Carbopol gel was done by Proniuk *et al.,* (2001) a carbopol powder 934P was gradually dissolved in distilled water under continuous stirring (1200 rpm) at ambient temperature to form a 1% homogeneous solution. (TEA) Tri ethanolamine was added drop wise to initiate the gel and increase the pH. The 0.5g plant extracts were added and then pH was determined. (Gul *et al*., 2017)

Preparation of ointment

The Simple B.P ointment was formulated as described by Marriot *et al*. Melting 4.75g of hard paraffin at 60^0c. Then added 4.75g of wool fat. Followed by 4.75g cetostearyl alcohol. Cooled and stirred the prepared ointment at room temperature. Then white soft paraffin 80.75g and 5g thymus oil extract were added (Gul *et al*., 2017; Marriott *et al.,* 2006).

Determination of pH, Viscosity, Centrifugal tests and Drug Uniformity

The pH of all formulations were determined by digital pH meter.

Viscosity of all formulations were determined at room temperature (25+/-1^{o}C) using a cone and flat type viscometer (DV-III Ultra, Brookfield engineering laboratory, USA).

Centrifugal tests were determined for all formulations immediately after preparations the centrifugation tests 5 g sample were carried out at 25^{o}C for 10 minutes at 5000rpm (Ueda *et al.*, 2009).

The drug content of all formulations were determined, approximately 100 mg sample was dispensed in 100mL Acetonitrile in a conical flask and stirred with the help of magnetic stirrer for 2 hours until well dissolved completely. The solution was filtered (0.2µm) and filter and was analyzed by a Validated HPLC method at 274 nm and the drug concentration was calculated.

Table 1: Physical parameters values for Thymus oil formulations

Formulations parameters		pH	Viscosity(cps)	Drug content (%)
Thymus Serpyllum L.	Micro emulsion	5.9	21.32 ±0.003	98.20
	Gel	5.8	29.22±0.002	97.57
	Ointment	5.6	141.57±0.002	97.33

Table 2: factor *f2* analysis for three dosage forms *Thymus serpyllum* L. (Thymol) from (0-24h)

Formulations	Cellulose membrane		Natural rabbit skin	
	0-6h	0-24h	0-6h	0-24h
Micro emulsion	75	61	94	66
Gel	49	33	53	36
Ointment	55	39	55	42

Table 3: *Thymus serpyllum* L. (Thymol) released from different formulations via dialysis cellulose membrane.

% of drug Released after 24 hrs	Base	Amount of drug release in Amount in mg/1.5 cm^2 after the following time interval (minutes).								
		0.5 hr	1 hr	2hr	3hr	4hr	5hr	6 hrs	12 hrs	24 hrs
78.5607	Micro emulsion	0.18883	0.27780	0.79202	1.0321	1.24699	1.58158	1.90874	2.45251	3.10315
70.0004	Gel	0.18328	0.26682	0.76307	1.06453	1.25160	1.48146	1.69779	2.24492	2.76518
46.5564	Ointment	0.106676	0.251011	0.64120	0.85412	0.98598	1.16606	1.29394	1.54027	1.83898

Table 4: Kinetics Data of *Thymus serpyllum* L. released from different formulations by using dialysis cellulose membrane.

R^2(Coefficient of determination)						
Formulations	Zero order	First order	Higuchi equation	Hixon crowell equation	Krosmeyer-Peppas	Best fitting model
Microemulsion	0.6517	0.6619	0.9569	0.6585	0.9599	Peppas
Gel	0.5810	0.5913	0.9583	0.5878	0.9584	Peppas
Ointment	0.3571	0.3659	0.9205	0.3629	0.9319	Peppas

Table 5: *Ex-Vivo* release of *Thymus serpyllum* L. (Thymol) from different formulations by using natural rabbit skin

% of drug Released after 24 hrs	Base	Amount of drug release in Amount in mg/1.5 cm2 after the following time interval (minutes).								
		0.5 hr	1 hr	2hr	3hr	4hr	5hr	6 hrs	12 hrs	24 hrs
69.73620	Micro emulsion	0.16277	0.24885	0.73927	0.98305	1.27639	1.37854	1.78748	2.21779	2.75458
61.30101	Gel	0.15661	0.24733	0.75542	0.96271	1.27886	1.33331	1.75012	2.07438	2.42139
43.64936	Ointment	.09436	0.24419	0.64038	0.84022	0.98352	1.17004	1.25397	1.4051	1.72415

In vitro release studies of thymol from the prepared formulations via cellulose membrane

The *in vitro* drug transport through the artificial dialysis cellulose membrane was carried out using Franz diffusion cell. Franz diffusion cells (Perme Gear, Bethlehem USA) are characterized by an effective diffusion surface area of 1.5cm^2 and a receptor cell volume of 12mL. The static receptor cell was filled with 12mL absolute alcohol and Phosphate Buffer (30:70) and was stirred with a small magnetic bar at a speed of 600 rpm for uniform mixing. The receptor compartment was maintained at 32±0.5°C using a circulating water bath.

Formulation of a micro emulsion, a gel and an ointment 3.95mg of Thymol in amounts of 1 g were placed on the dialysis cellulose membrane surface facing the donor compartment and 100µL samples were withdrawn from the receptor compartment at predetermined time points of hours 0.5, 1, 2, 3, 4, 5, 6, 12 & 24. The 100µL sample withdrawn was replaced by fresh alcohol, Buffer and maintained at 32±0.5°C (Ueda *et al.,* 2009). The drug content in the collected samples was determined by a validated high-pressure liquid chromatography method. All experiments for each sample were carried out in triplicate. The chromatographic conditions used for

analysis were as follows; the mobile phase consisted of acetonitrile water and orthophosphoric acid at ratios of 60:38:2, respectively. The flow rate was 1mL/min, injected volume 20µL and samples were detected by an ultraviolet-visible detector at a wavelength of 274nm.The calculation was adjusted for withdrawn sample quantity (Shah *et al.*, 2006).

In Ex-vivo release studies via the natural rabbit skin

Animals

It is best to use the human skin for *in vitro* permeation of drug to get the *in vivo* performance of drug. However, human skin sample is not easy to obtain and use of human body organs have limitations from ethical committees. Therefore, in current study rabbit skin was used to evaluate the in vitro permeation profile of Thymol from formulated topical formulations. Previously, rabbit model was used successfully by various researchers to monitor the permeation of numerous lipophilic and hydrophilic drugs through transdermal route of administration (Ogiso *et al.*, 2001; Gamal and Maghraby, 2007). White rabbits weighing 2.20-2.70kg were obtained from the animal store of the Department of Pharmacy, the Islamia University of Bahawalpur, Pakistan.

The study on rabbit was approved by the Institutional Ethics Committee, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan (Ref. No.29-2015/PREC).

Skin preparation for franz diffusion cell

Carefully examine and shaved the dorsal region of a white male rabbit with an electric hair clipper. In order to avoid any cut or damage to skin. The shaved area of the skin was depilated by depilatory (Anne French cream, Pakistan). After 5minutes, skin was cleaned with water and cotton swab. The treated animal was left for 24 hours in order to heal of any cut or abrasion during the shaving, return back the skin to its original physiological position. After it marked the treated skin and sacrificed the animal. And processed skin was excised from the rabbit with the help of surgical Scissors. After it skin subcutaneous fat was removed with the help of knife and epidermis removed by heat separation method, by putting the skin in hot water at 60^{o}C for a minute and carefully removed the epidermis from dermis. Washed the removed epidermis with distilled water, packed in aluminum foil and stored at -50^{o}C in ultra-low temperature, freezer, Sanyo Japan, was used for study (Pellett *et al.*, 1997; Shah *et al.*, 2006). The frozen excised skin was brought to room temperature, and thereafter fitted between the donor and receptor compartments of the diffusion cell, with the dermal side facing the receptor compartment and the stratum side facing the receptor medium side. All other experimental steps were consecutively followed as mentioned above. Conducted on dialysis cellulose membrane samples tested.

Pharmacokinetic studies of Thymol via the rabbit skin

The quantity of drug (mg) in the receptor medium was analyzed (0-24hours) by HPLC and the released amount of drug was identified and computed, then linearity regression analyses and parameters of the drug permeation for each formula, were analyzed in which, the (R^2) correlation coefficient was calculated for each formula, by each kinetic equation, to assess whether the permeation of the drug through the natural skin follows zero order, first order, Higuchi, Krosmeyer- Peppas or Hixon-Crowell diffusion release model. All of these calculations were carried according to the following kinetics equations. using validated DD solver software, program (Zhang *et al.*, 2010).

Model	**Equation**
Zero order	$Q_t = Q_o + K_o t$
First order	$\ln Q_t = \ln Q_o + K_1 t$
Higuchi	$Q_t = K_H \sqrt{t}$
Krosmeyer-Peppas	$Mt/M\infty = k_t^n$
Hixon-Crowell	$Q_t/Qo = K_k t^n$

STATISTICAL ANALYSIS

The analysis of variance (ANOVA) was used evaluate the effect of dialysis cellulose membrane and Rabbit skin using SPSS 18 software (Yyksel *et al.*, 2000). For comparison of the formulations, the *f*2 factor analysis was used (Gohel *et al.*, 2005). The release of all data of formulations was fit to the release kinetic model Krosmeyers-Peppas and then compare with one another. Flux (j) was calculated as µg/h.cm^2 (Kreilgaard *et al.*, 2000).

RESULTS

The pH, Viscosity and drug contents of the three formulations Micro emulsion, Gel and Ointment were found stable its results as shown in table 1.

Many chromatographic analyses presently available, thin-layer chromatography has become commonly adopted for the fast and positive analysis of essential oil. The results of the tests showed the thymus oil contain Thymol at value (R_f =0.65), and known compound at (R_f =0.65). The best separation of active compounds of volatile oil is mixture of 5 volumes of ethyl acetate and 95 volumes of toluene, the chromatogram shows three zones, and this system is suitable for the separation.

HPLC (high performance liquid chromatography)

The High performance liquid chromatography technique was used to deduct the Thymol presence in the essential oil of *Thymus serpyllum* L. The results showed the retention time of reference was 5.46 and sample 5.596. The quantification of the sample was 3.95mg of Thymol/g thymus. oil.

Table 6: Kinetics data of *Thymus serpyllum* L. released from different formulation by using natural rabbit skin.

R^2(Coefficient of determination)						
Formulations	Zero order	First order	Higuchi equation	Hixon crowell equation	Krosmeyer-Peppas	Best fitting model
Microemulsion	0.5969	0.6067	0.9509	0.6035	0.9514	Peppas
Gel	0.4759	0.4862	0.9275	0.2793	0.9291	Peppas
Ointment	0.2735	0.2822	0.8952	0.2793	0.9161	Peppas

Table 7: Flux values of formulations

Formulations	Cellulose membrane µg /cm^2/h	Natural rabbit skin µg /cm^2/h
Micro emulsion	7.10	6.10
Gel	5.02	4.12
Ointment	3.80	2.40

Samples (1g) of topical formulations containing *Thymus serpyllum* L. (thymol) were analyzed using dialysis cellulose membrane and rabbit skin. ig 4, (A),(B) Shows the release profiles of Thymol from three dosage forms the micro-emulsion and gel showed visible release and permeable patterns from cellulose membrane and natural rabbit skin both formulations showed similarity in their release and permeability pattern in table 2,(f_2=75,94) before 6(h) and (f_2=61,66) after 6(h).the release and permeability data fit well to a Krosmeyer-Peppas model .the ointment showed similarity in their release and permeability in first 6(h)(f_2=53) in rabbit skin and (f_2=49)in dialysis cellulose membrane. The ointment showed different release and permeability rates within 24 (h). The f_2 value was below 50 when compare with the micro-emulsion and gel formulations. A Krosmeyer-Peppas release and permeability from the ointment was observed when the dialysis cellulose membrane and rabbit skin was used. The *p* values corresponding to the Wilks Lambda statistics indicates that the membrane nature had a considerable impact on the release and permeability of the tested formation.

DISCUSSION

The pH, Viscosity and drug content of the three formulations Micro emulsion, Gel and Ointment were found stable. In order to assess the release pattern involved in the release from topical formulation is not new but only few studies used to investigate the release of marker from traditionally used medicinal plants. In the current study, the Thymus oil used as a surrogate for the whole extracts. As showed in the graph, at least two separate release profiles for the formulations micro-emulsion and gel were apparent within 24 hours. One from 0 to 6 hours and one from 06 to 24 hours. After 6 hours it is observed change in the behavior of release profiles might be the solubility or the dilution factor of the Thymol extracts in the receptor medium. It might be due to the thickness or pore size of the membranes. The quantity of drug released from the dialysis synthetic membrane in table 3 while using the natural rabbit skin illustrated by the table 5 it is clearly indicating the release behavior in descending order micro-emulsion >gel >ointment. This were also observed in the same way while using the rabbit skin in *ex-vivo* study as shown in fig. 4 (B). However, the release of micro-emulsion and gel were very close and obvious with in the 24 hours. In table 3 the drug release from the micro-emulsion permitted through the dialysis cellulose membrane, observed after 24 hours was 78.560%, gel 70.000% and ointment 46.554%. The drug release from rabbit skin in table 5 micro-emulsion 69.736% gel 61.301% and ointment 43.649%. In order to assess the mechanism involved in the release of Thymol from micro-emulsion, gel and ointment a kinetic method was used by employing different model like Zero order, first order, Higuchi, Krosmeyer-Peppas and Hixon Crowell. The best fit of release model was Krosmeyer-Peppas while using dialysis cellulose membrane and natural rabbit skin as shown in table 4,6. The dialysis cellulose membrane showed the highest flux valve for all the formulations as compare to the natural rabbit skin shown in table 7 in addition, the penetration of Thymol across dialysis cellulose membrane was more than that of penetrate natural rabbit skin which could be the above mentioned reason or the rabbit skin used in this study was thicker or its pore size as compare to the dialysis cellulose membrane which could not allow more drugs to penetrate. Further more studies are needed to perform if the observed effects were only an effect of Enhancer, pore size, or if the nature of the membrane material also contributed to the release behavior.

CONCLUSION

The study demonstrates that *in vitro* and *ex-vivo* release experiments using Franz diffusion cells were successfully applied to traditional medicinal extracts. The three formulations showed similar release rates except ointment, the micro emulsion and gel has the fastest release profiles via dialysis cellulose membrane and rabbit skin compare to the ointment statistical data

showed that both dialysis cellulose membrane and rabbit skin had a significant impact on the marker release. The membrane had more discriminative among formulations .and should be use for further studies. *In vitro* and *ex vivo* diffusion cell experiment can be used to develop improved formulation of traditional medicines application for transdermal drug delivery.

ACKNOWLEDGMENTS

The Author s would like to thank the Higher Education Commission of Pakistan for financial support, Health Department Government of Balochistan (for study Leave). Dean, Faculty of Pharmacy, The Islamia University of Bahawalpur for his support to complete this research.

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