T.C
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF DRUG AND COSMETIC PRODUCTION TECHNOLOGIES

PERCUTANEOUS ABSORPTION ENHANCEMENT OF THIOCOLCHICOSIDE WITH DIFFERENT APPROACHES: IN VITRO AND EX VIVO EVALUATION

MASTER THESIS

MUNA ALZAWATI

ISTANBUL- TURKEY, 2022

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

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Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my original own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

02.08.2022

Muna Al-Zawati

DEDICATION

I dedicate this thesis to my father's soul (Samir Abdullatif Alzawati R.I.P), and to my adorable mother & supporter (Feryal Masoud), my brothers & sisters, finally to my sisters and brothers in law and relatives, I will be always grateful for their support to me in all aspects of my life, encouraging me to trans my goals into reality and to be the best version of myself. And for all of the teachers and doctors whom have taught me during my life and for their efforts to build my knowledge.

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Finally, I am grateful to all the academicians and technicians who have helped me in the laboratories of Yeditepe University -Faculty of Pharmacy.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abs	UV Absorbance
cm^{-1}	Inverse centimeter
DC	Drug content
DS	Droplet size
EE%	Entrapment efficiency for liposomes
F1	Formulation 1
F2	Formulation 2
F3	Formulation 3
F4	Formulation 4
F5	Formulation 5
F6	Formulation 6
F7	Formulation 7
F8	Formulation 8
F9	Formulation 9
g	Gram
h	Hours
HPMC	Hydroxypropylmethylcellulose
ICH	International Conference on Harmonization
In vitro	in the laboratory
Jss	Steady state flux
Kp	Permeability Coefficient

mean	The Average Value
mg	Milligram
min	minutes
ml	Milliliter
mp	melting point
Muscoflex	A brand name of Thiocochicoside cream
ng\mL	Nanograms per milliliter
nm	Nanometer
pH	Potential of Hydrogen
ppm	Parts Per Million
Rpm	Rounds per minute- Revaluation per minute
R %	Recovery (%)
RSD %	Relative Standard Deviation
R^2	Correlation Coefficient
S	Slope
SC	Subcutaneous.
SD±	Standard deviation
TEA	Triethanolamine
THC	Thiocolchicoside
Tyoflex	A brand name of Thiocochicoside ointment
USP	United states pharmacopeia
w/v	Weight by volume
v/v	Volume by volume

w/w	Weight by weight
ZP	Zeta potential
µm	Micrometer
µg /cm²	Microgram per square centimeter
µg/mL	Micrograms per milliliter

ABSTRACT

Al-zawati, M (2022). Percutaneous Absorption Enhancement of Thiocolchicoside with Different Approaches: *In Vitro* and *Ex Vivo* Evaluation. Yeditepe University Institute of Health Sciences, Drug and Cosmetics Production Technology Master Program, İstanbul.

Thiocolchicoside (THC) as analgesic, anti-inflammatory, muscle relaxant drug, hydrophilic (relatively high MW, 563.3) have been chosen to present the behavior of hydrophilic and relatively high MW drugs which is not favorable for the permeation through the skin. To achieve our thesis aim which is study and evaluate the skin permeation enhancement of hydrophilic drugs and their topical penetration capability by using different drug delivery approaches, THC gel formulations were prepared by using 3 different gel polymers (HPMC, Chitosan, Carpobol) with 3 different percentages of each polymer, the permeation study was performed by Franz cells diffusion apparatus using the artificial membrane to choose the best percentage of each polymer, then with pig skin as permeation membrane to choose the best gel polymer type as suitable THC gel vehicle. In the third step, we hypothesized that preparing THC-loaded liposomes (one of the new drugs nanovesicles carriers' delivery systems) would increase THC permeation. Final step was (pig skin microporation) by Dermaroller® (mechanical microneedles) to create microchannels in pig skin as permeation membrane. Commercial marketed cream and ointment were involved in every step to compare the results with them and evaluate the skin permeation enhancement of prepared gel formulations. Samples analyzed by UV spectrophotometer; data statistically analyzed by GraphPad Prism v.5.04 program. Final result showed that THC in chitosan gel 2.5% formulation is most promising THC topical formulation, and has the highest drug release profile across pig skin after Dermaroller® application. We suggest the use of different types, sizes, and lengths of Dermaroller® must be evaluated with the same formulation in future studies.

Keywords: Thiocolchicoside; Franz diffusion cell ; Liposomes; Dermaroller; Topical formulation; HPMC; Chitosan; Carpobol.

ÖZET

Al-Zawati, M (2022). Tiokolşikozit Etken Maddesinin Perkütan Absorpsiyonunun Değişik Yaklaşımlar ile Artırılması: *İn Vitro* ve *Ex Vivo* Değerlendirilmesi.Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, İlaç ve Kozmetik Üretim Teknolojileri Yüksek Lisans Programı, İstanbul.

Yüksek molekül ağırlıklı ve hidrofilik moleküllerin deriden penetrasyonu sınırlı olduğu için, bu tür moleküllerin deriden geçiş özelliklerini araştırmak amacı ile analjezik, anti-inflamatuar, kas gevşetici etkiye sahip ve nispeten yüksek molekül ağırlıklı ve hidrofilik özellikte bir molekül olan Tiyokolşikozid (THC) çalışmamızda model etken madde olarak seçilmiştir. Bu tür özellikteki moleküllerin deriden penetrasyonlarını artırmak için farklı yaklaşımlar denenmiştir. İlk yaklaşım farklı özellikteki jel yapıcı polimerler (Hidroksipropilmetil selüloz, kitozan ve karbopol 934) farklı konsantrasyonlarda kullanılarak jel formülasyonları hazırlanmış ve franz difüzyon hücresi kullanılarak ilk önce yapay membran daha sonra da domuz derisi kullanılarak formülasyonlardan etken madde salımı değerlendirilmiştir. Salım özellkleri en iyi olan kitozan formülasyonu ile etken maddenin liposomal dispersiyonu birleştirilerek yeni bir formülasyon yapılmıştır ve dermaroller ile gözenek açılmış domuz derisi kullanılarak etken maddenin deriden geçiş özellikleri incelenmiştir. Örneklerdeki THC miktarı spektrofotometrik yöntem kullanılarak analiz edilmiş, istatistiki değerlendirme GraphPad Prism v.5.04 programı ile yapılmıştır. Deneysel çalışmalar sonucunda % 2.5 kitozan formülasyonunun en iyi salım özelliği sergilediği ve mikroporasyon için kullanılacak dermaroller cihazının farklı tipteki iğne boyutlarına sahip modelleri ile deriden geçiş parametrelerinin ilerideki çalışmalarda araştırılması sonucuna varılmıştır.

Anahtar Kelimeler: Tiyokolşikozid; Franz hücre difüzyon hücresi; lipozom; Derma roller; topikal formülasyon; HPMC; kitozan; Karpobol;

1. INTRODUCTION AND PURPOSE

1.1. Aim of the Study:

This thesis aims to enhance the skin permeability of hydrophilic drugs and to achieve this purpose, we used Thiocolchicoside (THC) as a hydrophilic, relatively high molecular weight (MWT) topical analgesic, anti-inflammatory, muscle relaxant drug [1,2].

THC semisolid pharmaceutical formulations are available in the market as creams and ointments. We prepared several gel formulations to investigate the enhancement effect of using various gel polymer types as vehicles and to identify the most promising polymer type for THC gel, then we prepared THC loaded liposomes to evaluate their permeation enhancement capability and compared with unloaded THC-gel formulations. As well as the potential of combined microneedle application and THC loaded liposomes. All the formulations compared with the marketed THC cream and THC ointment as control formulations to evaluate the enhancement of each formulation, the permeation experiments were done by using Franz Cell Diffusion Apparatus with artificial membrane first, then across pig skin as permeation membrane with and without microneedle application.

1.2. Dermal as Route of Administration:

For many years, the dermal drug delivery system has been used as an alternative drug delivery route to oral, intravascular, intramuscular, and subcutaneous routes due to potential advantages such as large absorption surface area and access to various placement options [3, 4], the avoidance of hepatic first-pass metabolism and enzymatic degradation of drug, accessibility to the site of action, and ease of administration are all advantages of topical drug delivery. Safeguarding naive cells from toxic drug administration, Desire discontinuity, controlled drug administration, fixed plasma drug concentrations (transdermal delivery), painless non-invasive rout especially for the patients with needles phobia.

Economic advantages and no need for medical intervention for drug administration means better patient compliance and adherence, prolongs drug administration, etc [5].

1.3 Skin:

The skin is the largest organ in our bodies and serves numerous functions. It covers the entire body and protects us from external elements. It shields the body from UV rays, microorganisms, chemicals, and physical traumas. The fat in the deep skin layers and the sebum secreted by the sebaceous gland (the skin's microscopic exocrine gland that opens into hair follicles) prevent water loss and lubricate the skin and hair follicles.

The skin is an impermeable barrier, and it consists of 3 layers: epidermis, dermis, and hypodermis. In some dermatology books, they consider the stratum corneum as a separate layer, the most superficial layer above the epidermis, and other books consider it as a sub-layer of the epidermis layer [6, 7].

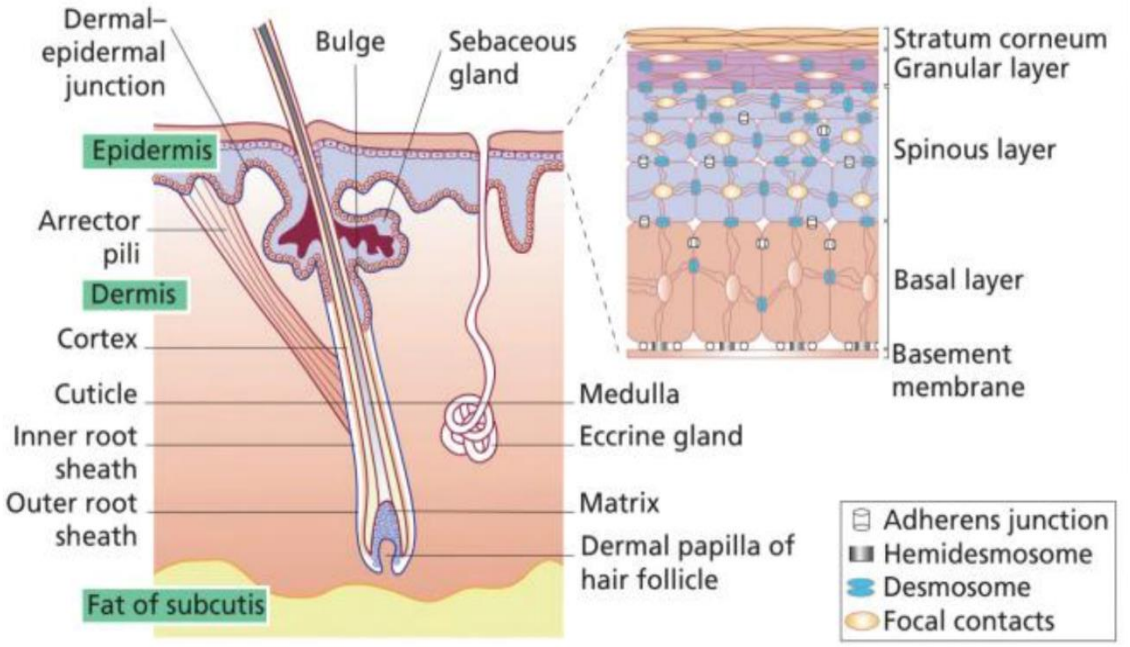


Figure 1. The skin anatomy (McGrath 2004), [8].

1.3.1. Epidermis:

The epidermis is the skin's outermost protective layer and is made up of several layers, including the stratum basale, stratum granulosum, stratum lucidum, stratum spinosum, and stratum corneum.

1.3.2. Stratum Corneum (SC):

The final sub-layer mentioned is SC, which is the epidermis's uppermost layer and most superficial portion [9]. Because of the stratum corneum's selectivity barrier function, drugs cannot easily penetrate by passive diffusion (concentration gradient), and consider as a rate-controlling barrier, dead keratinocytes in this layer secrete defensins, which are part of our first immune defense. [6] Several studies have found that the stratum corneum is to blame for the restricted skin permeability of topically applied drugs. Other studies show that the underlying dermal tissue contributes to drug transport resistance and is not only limited by the stratum corneum. [10, 11].

1.3.3. Dermis:

The epidermis is separated from the dermis layer beneath it by a basement membrane. The dermis is a tough connective tissue that contains hair follicles and sweat glands.

1.3.4. Hypodermis:

The subcutaneous tissue hypodermis is located beneath the dermis layer and contains blood vessels, sensory neurons, fat, adipose cells, and connective tissue.

1.4. Drug penetration routes through the skin [12, 13]:

In general, drugs penetrate the skin via two major pathways:

1- Transcellular (intracellular) route.

2- Intercellular route.

Schematic representation of penetration routes of drugs throughout the skin showed in (figure 2).

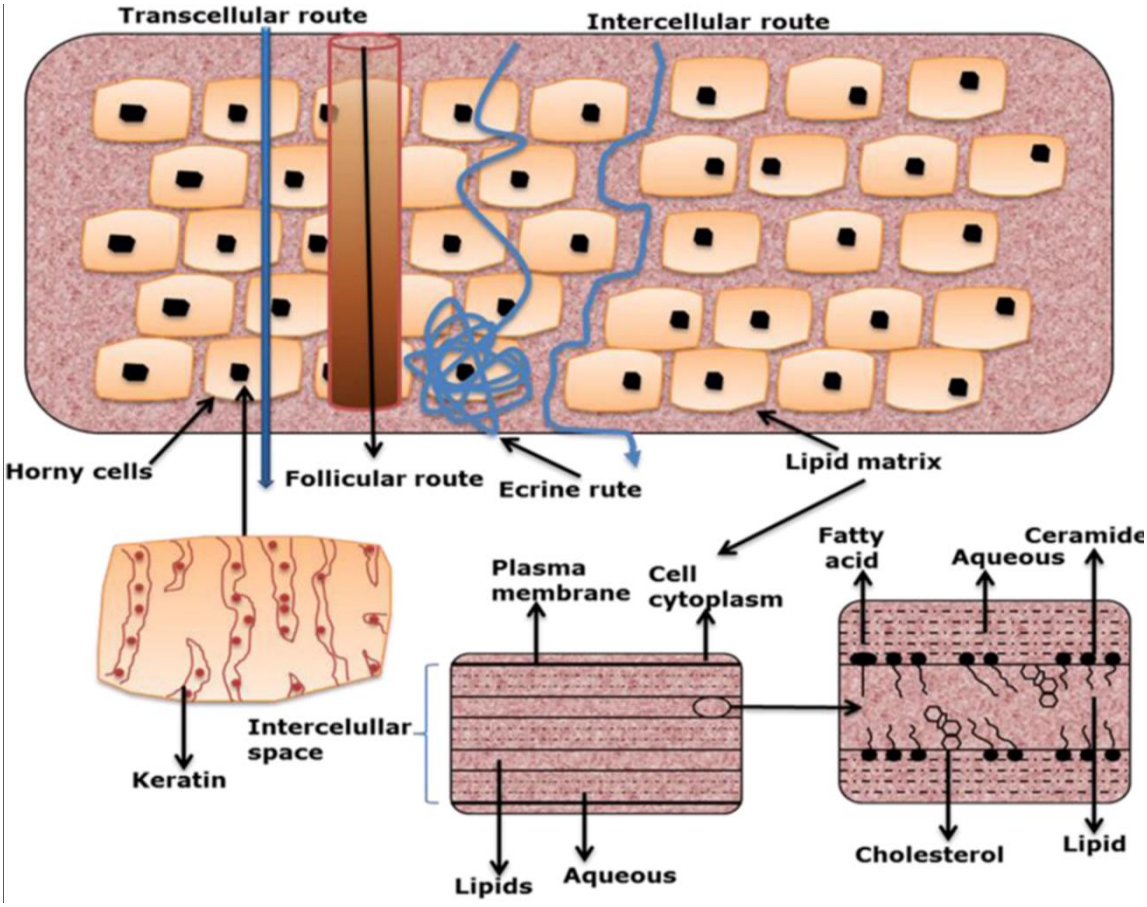


Figure 2. Diagram of drug penetration routes through the skin [12].

1.5. Challenges of topical drug delivery [5]:

1- Permeability challenges: because of barrier feature of stratum corneum layer in the top of skin.
2- Physiochemical properties of the drug: low aqueous solubility, PH dependent instability, high MWT and log P of drug.
3- Drug side effects: photosensitivity, allergic reactions erythema and irritation.

1.5.1 Physicochemical Skin Permeation Limitations:

Pharmaceutical developers and researchers are interested in dermal drug permeation. Aside from its advantages as an alternative drug delivery pathway, the skin has physicochemical constraints.

To be able to pass through the SC, the drug must be lipophilic and have a low molecular weight (500 Da) [14], as well as a low therapeutic dose to be administered dermally.

These limitations affect the permeation of many drugs through the skin and are regarded as a challenge that must be overcome by using available approaches to improve formulations and develop new transdermal drug delivery systems.

1.6. Pervious Succeeded Methods to Overcome Skin Permeation Limitations:

Hundreds of pharmaceutical studies have been conducted to improve and enhance the dermal drug delivery and facilitate the drug penetration into the skin and succeeded to overcome some of the obstacles to which the drug is usually exposed, either by improving the drug formula features to increase its permeability or by decreasing the resistance of the skin layers. Different approaches are used, such as adding some chemical penetration enhancers or changing the vehicle-type [15, 16],

as we did in this study by using different gel polymers (Chitosan, hydroxypropyl methylcellulose (HPMC), and Carpobol) instead of the existing available commercial semisolids vehicles for THC (cream ointment, and foam), as we noticed When the researchers Compared between the use of once-daily calcipotriol and betamethasone dipropionate gel vs. ointment formulations in patient with psoriasis vulgaris after 52-week treatment period, Greater treatment satisfaction was reported in patients using gel compared to those using ointment. Gels are considered easier to use, faster to apply and more convenient [17].

Also, in-vitro release studies for flurbiprofen were conducted on various gel formulations (Carbopol 934P, poloxamer 407, and eudragit S100) and ointment formulations (emulsion and polyethylene glycol).

The study concluded that hydrogels (Carbopol 934P and poloxamer 407) released more drug than hydrophobic eudragit S100 gel and ointments, and could be suitable vehicles for the rapid release and onset of flurbiprofen after topical application, Carbopol 934P gels released three times more flurbiprofen than other formulations [18].

At 37°C, dialysis through a cellulose membrane into phosphate buffer pH 6.8 was used to study piroxicam in vitro release from oil-in-water creams and hydroalcoholic gel topical formulations.

The results show that a hydroalcoholic gel formulation with hydroxypropylcellulose as the gelling agent performs better than an O/W cream formulation for piroxicam [19].

In other study, it has been discovered that adding chemical enhancers (lauric acid) to the formula effecting the permeation of THC by altering skin protein confirmation, as well as increasing the methanol percentage in the aqueous donor vehicle will increase THC affinity to the skin [10].

Researchers, on the other hand, are attempting to develop and discover new transdermal drug delivery systems and improve their drug permeation ability by using carriers that mimic the structure of the skin anatomy, such as the phospholipid bilayer of nano-vascular-carrier systems, lipid carriers (liposomes), transferosomes [20] non-ionic surfactant vesicles (Niosomes), transferosomes, ethosomes, etc.

Many studies have been conducted in order to increase drug penetration by widening or multiplying drug delivery pathway routes through the skin (transcellular, intracellular routes) using high voltage electrical sources (electroporation) [10]. Alternatively, the skin can be exposed to mechanical (microneedles) [21], ultrasounds (sonophoresis) [22], or both or more of the preceding methods at the same time [22], to develop new drug-passing routes.

1.7. Novel drug delivery systems (NDDS):

Drug delivery systems rely on drug formulation, drug release mechanisms, and the use of a medical device or dosage form/technology to transport the drug inside the body.

The traditional method of drug delivery is to formulate the drug into a suitable form, such as a compressed tablet for oral administration or a solution for intravenous administration.

These dosage forms have serious limitations in terms of higher dosage requirements, lower effectiveness, toxicity, and adverse side effects. To meet the needs of the healthcare profession, new drug delivery systems have been developed or are being developed to overcome the limitations of conventional drug delivery systems.

A significant improvement in drug delivery performance can be observed when an existing drug molecule is converted from its conventional form to a novel delivery system.

The term "novel drug delivery systems" refers to methods, formulations, technologies, and systems for transporting pharmaceutical compounds in the body as needed to achieve the desired therapeutic effects and safely. It could be scientific site-targeting within the body or facilitating systemic pharmacokinetics; in any case, it is usually concerned with both the quantity and duration of drug presence.

Physicochemical properties of a drug, acute versus chronic therapy, route of administration, target sites, the patient, and the disease state or disease level are all factors that can affect NDDS.

1.7.1. The benefits of NDDS:

These new systems have the following therapeutic benefits:

1- Increased drug permeability and efficacy.

2- The capability of drug delivery carriers to transport both hydrophobic and hydrophilic medicines.

3- Controlled drug release systems.

4- Site specific delivery, targeted drug delivery systems.

5- Decreased toxicity/ side effects.

6- Increased convenience, and improved patient compliance.

1.7.2. Pharmaceutical drug delivery carriers [5]:

There are various types of pharmaceutical drug delivery carriers available (Figure 3), including:

1. Vesicular carriers:

Ex: Liposomes, Niosomes, Ethosomes, Transfersomes.

2. Micellar carriers:

Ex: Polymeric micelles, Mixed micelles, Phospholipid based micelles.

3. Particulate carriers:

Ex: Solid lipid nanoparticles, Nanostructured lipid carriers, Polymeric nanoparticles.

4. Emulsified carriers:

Ex: Nano-emulsions, Lipid emulsions, Microemulsions.

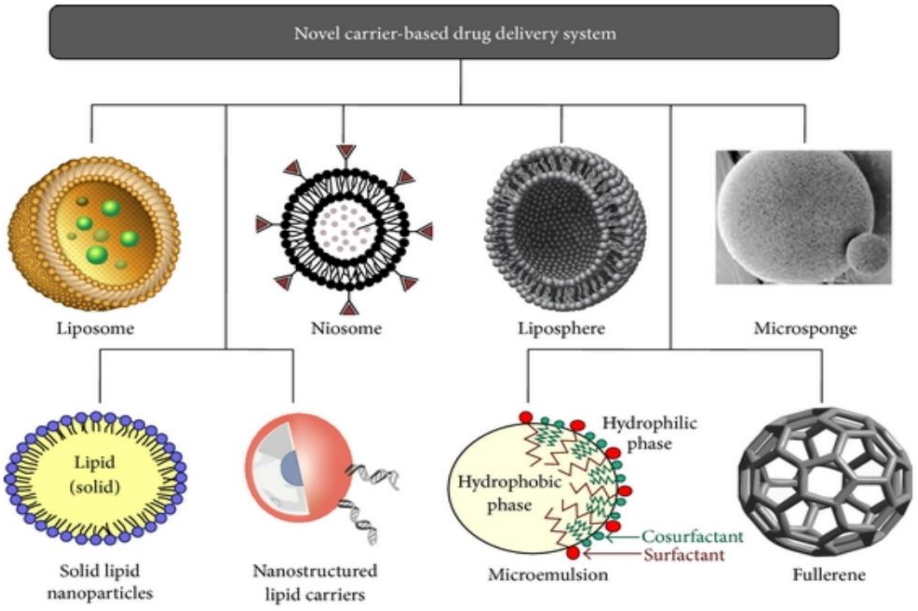


Figure 3. Novel carrier-based drug delivery systems (Vyas A.2014), [23].

1.7.2.1 Nanoparticles:

Nanoparticles, which are colloidal drug carriers with diameters ranging from 10–400 nm, are one of the novel drug delivery systems.

The goal of developing these formulations is to create systems with optimized drug penetration, loading and release properties, a long shelf life, and low toxicity.

2.7.3. Liposomes, Properties and Advantages:

In the 1960s, the challenge between the physicochemical properties of drugs and the anatomy of skin was one of the subjects that gained a lot of attention to be overcome in parallel with the discovery of liposomes as a carrier for many drugs [20].

Alec Bangham discovered liposomes at the Babraham Institute, University of Cambridge, when he realized that phospholipid bilayer membranes can form sphere-shaped vesicles with an internal hydrophilic compartment by introducing phospholipids into water

solution.Bangham and colleagues defined liposomes years ago as small vesicles with spherical shapes that can be made from phospholipids, cholesterol, and non-toxic surfactants [24].

Liposomes are carriers commonly used in transdermal drug delivery. These closed phospholipid bilayer vesicular systems have the capability of encapsulating hydrophilic drugs in their core and can carry the lipophilic compound between the two phospholipid bilayer membranes of their shell (Figure 4), liposomes can be formulated in the cosmetics and pharmaceutical industries [25, 26, 27].

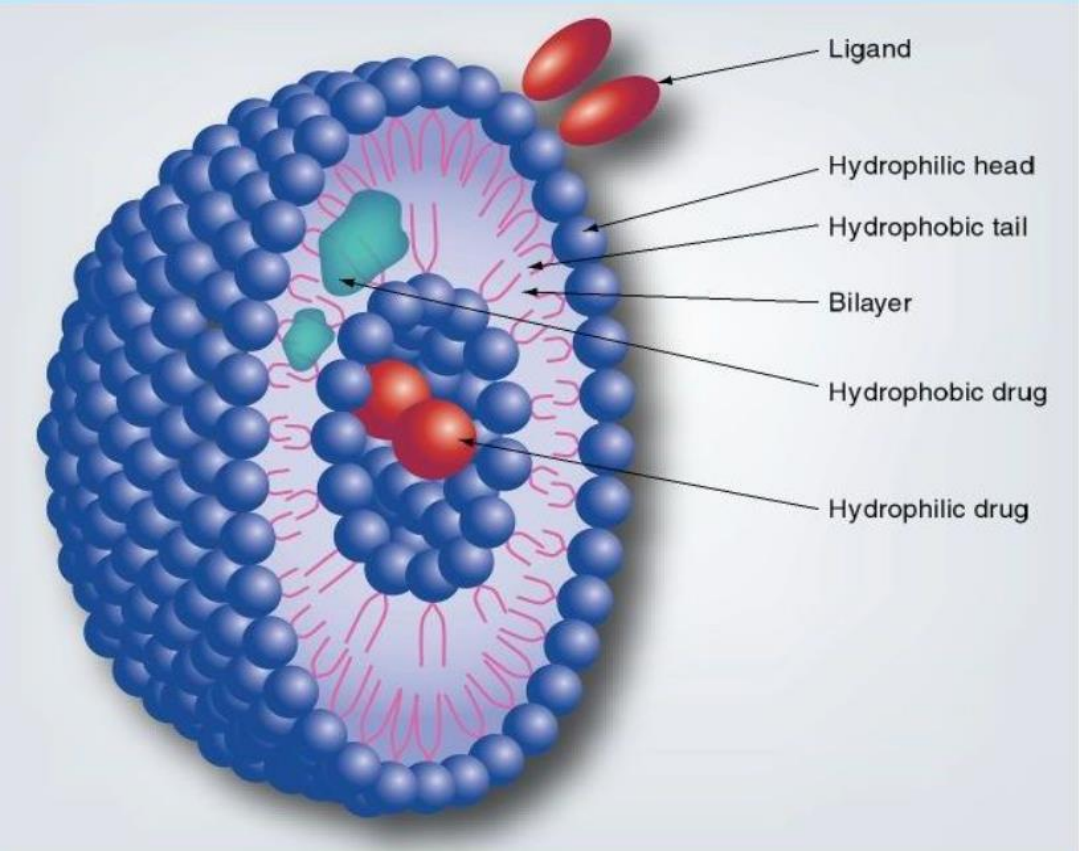


Figure 4. Liposome's structure (Bei D, Meng J. 2010) [28].

It is used to carry molecules that are not allowed to pass through the stratum corneum SC with the possibility of having local or systemic pharmacological effects [25].

Since the liposomes one type of NNDS which have many ways to pass through the targeted area, and in the case of skin it can pass through the resistance SC and can stay in-between the layers and act as reservoir [5], shown in (Figure 5).

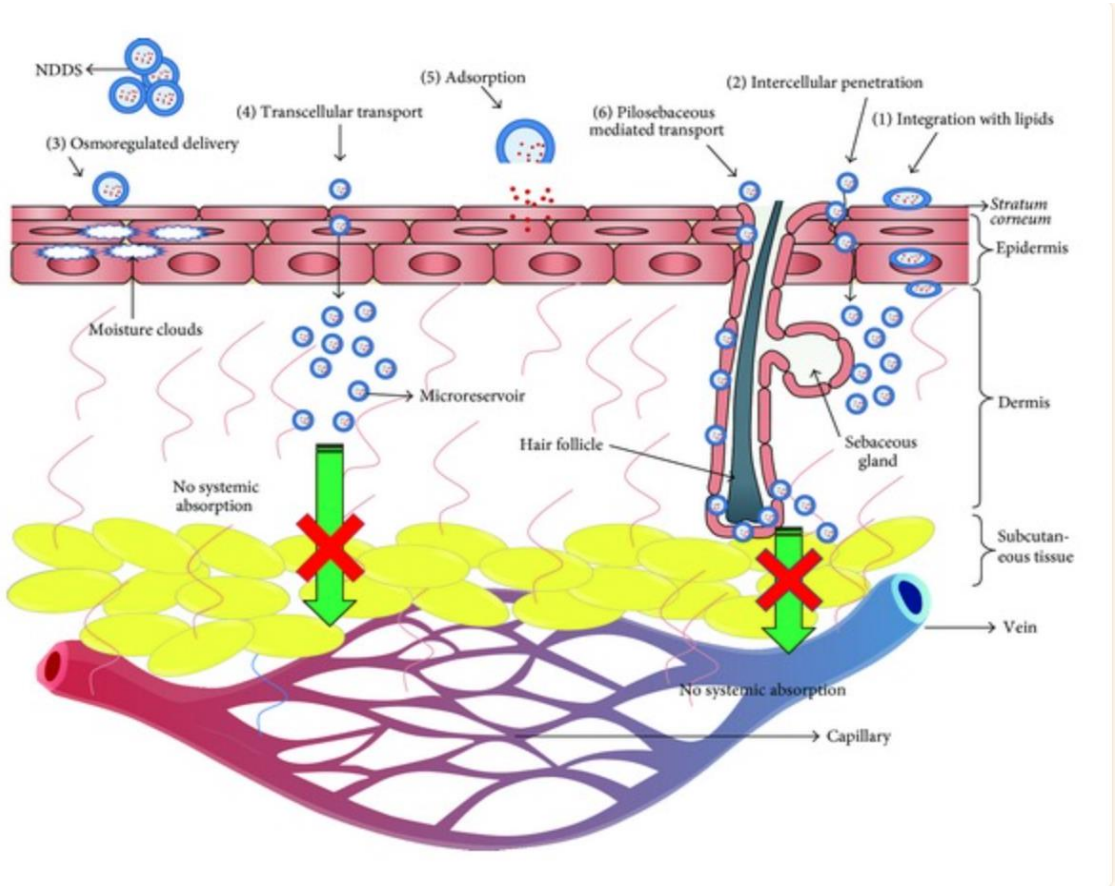


Figure 5. The penetration of NNDS ways through the skin (Raza K. 2014), [5].

Simultaneously, we are attempting to gain additional advantages over conventional routes, such as improved drug solubility, bioavailability, stability, and pharmacological benefits and responses with fewer adverse effects, and we believe that there are many more solutions to be discovered in the near future.

1.8. Microneedles (MN) device (derma roller ®) (D.R):

For the first time in 1995, Orenterich developed the concept of using MN to induce a wound healing like response within the skin layers.

Many in-vitro skin penetration enhancement studies for hydrophilic drugs have been done using chemical or physical methods. MN are one of the physical methods applied to the skin to improve the penetration and permeation of drugs that can't be passively passed through the skin because of the impermeable barrier properties of the SC against the drug's physiochemical characteristics.

Only after overcoming the barrier function of SC by reducing or disrupting it, can the hydrophilic and/or relatively high molecular weight drugs (> 500 Dalton) [14] pass through, penetrate, and be absorbed by the skin. In general, drugs administered via dermal rout can enter the bloodstream after reaching the hypodermis layer.

Due to their ease of use and low cost, micro-needling devices (derma rollers®) have piqued the interest of many scientists for over 20 years since first application [29].

The procedure has both therapeutic and cosmetic benefits. The concept behind its use is to make small repetitive punctures in the skin, creating small open channels from the surface to the dermis layer depending on the length of the microneedle used with minimum damage to the epidermis layer (perforation of the SC with MN), and this lead to improve penetration and absorption of topical agents applied to the skin, which is the benefit we seek in pharmaceutical researches. MN used for the administration of many substances such as vitamins, insulin, and vaccines [30].

In cosmetic, after treating the skin with the MN device the derma roller®, the immune system and the growth factors will be stimulated and deal with the created pores in the same way that the skin acts when is exposed to trauma or wounds and starts to build up the skin and stimulate the production of collagen and normal wound healing response. A collagen synthesis cascade is induced under the epidermis (leading to the production of collagen and elastin in the papillary dermis) [31], which is the purpose of the application of MN in some dermatological cases such as scars [32], acne [33, 34], stretch marks, alopecia, and wrinkles [31, 35].

Even if the fact of SC is about 15 layers [36], the average thickness of SC in human skin is between 10–30 m [37], the MN manufactured with arrays long enough to cross the barrier SC, permeation of drugs into the lower dermis aiding in the systemic uptake, and shorter than the sensory nerves which are located in the deep tissue, so they aren't nerve stimulators with a pain-free active ingredient delivery method [29], and even though there is an inter-variation for pain threshold, applying a little amount of local anesthetic such as Emla® for a few minutes before the application of the derma roller may be more than enough, which is considered an extra advantage of MN.

1.8.1. Types of MN:

Different types of MN are available and under studies, in which the drug can be loaded in arrays shaped like selfies as dissolving microneedles or the MN can be coated with the drug. The choice of which type to use depends on where to apply and the purpose of application [29, 35].

1- Solid microneedles.

2- Coated microneedles.

3- Hollow microneedle.

4- Dissolving microneedles.

5- Microneedle liquid injection system.

MN arrays are made from metal, glass, ceramic, stainless steel, and silicon [35]. And many types also available and depends how and where we want to apply the drug, in this study we apply the formulations for permeation evaluation by using pig skin after MN application with commercially available solid unloaded-drug titanium with microneedles length of 0.30 mm (total 680 needles) derma roller ® taken from UREW cosmetics® showed in figure 7.

One of the benefits of Titanium needles is its long shelf-life which mean cost-effective added value for the patients [35].

Figure 6. Derma-roller® taken from UREW cosmetics.

In the sections that follow, some literature review of our API (THC) and the enhancements made to our API (THC) following its loading in the study's gel polymers, in liposomes as a novel drug delivery system, and following the MN application method were discussed.

2. LITERATURE REVIEW

2.1. Thiocolchicoside:

The Thiocolchicoside (TH) is N-[(7S, 12aRa)-3-(β-D-Glucopyranosyloxy)-1,2-dimethoxy-10- (methyl-sulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[a] heptalene- 7-yl] acetamide. Contain 96.0 per cent to 102.0 per cent (anhydrous substance). It contains a variable quantity of water. Appearance is like yellow crystalline powder, slightly hygroscopic. Solubility is sparingly soluble in water, slightly soluble in ethanol (96 percent), practically insoluble in acetone. mp: about 272 °C [38], the chemical structure of THC is shown in (Figure 7).

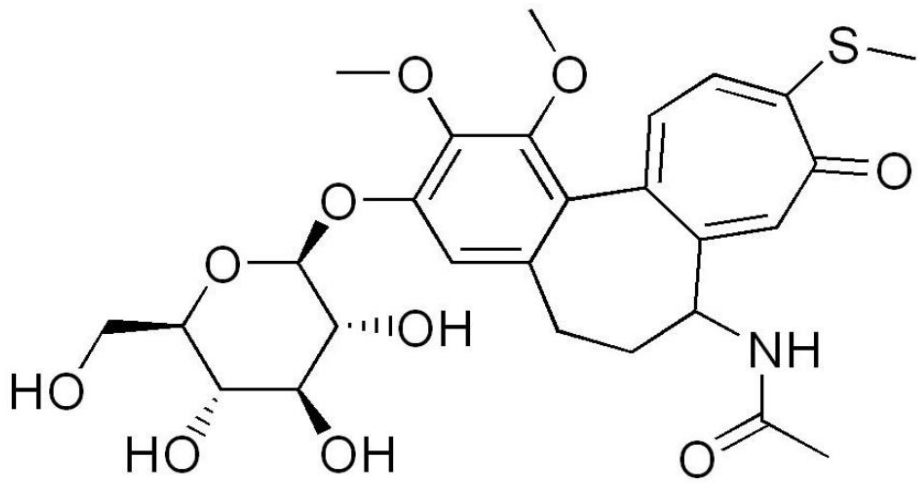


Figure 7. Structural formula of Thiocolchicoside (THC).

THC has a similar mechanism of action to the glycine receptor and competitive antagonist properties to the gamma-aminobutyric acid (GABA) receptor, with a lower effect on nicotinic acetylcholine receptors [39].

THC is also useful in the treatment of acute and chronic lumbar and sciatic pain, post-traumatic and post-operative pain, cervicobrachial neuralgia, and persistent torticollis [39, 40].

2.2. Gels:

Gels are semi-solid, three-dimensional polymeric matrices that consist of small amounts of solid dispersed in a relatively large amount of liquid but have a more solid-like character. These systems combine to form a three-dimensional polymeric matrix with a high degree of physical reticulation [41].

Gel formulations outperform cream and ointments in terms of application and stability [42], the main differences between ointment, cream and gel are shown in the following (Table 2.2).

Table 2.2. Comparison between Gel, Ointment and Cream [43]:

Ointment	**Cream**	**Gel**
Hydrocarbon-based greasy semi-solid Made of less than 20% water and volatile substances, and more than 50% of hydrocarbons (waxes, or polyols) as the vehicle	mostly water-based where the drugs are loaded W/O or O/W emulsion	Semisolid systems in which the liquid phase is trapped within a three-dimensional polymeric Matrix
oil-based formulation	Water or oil-based formulation	water-based formulation
Greasy	Less greasy	Non-greasy
Very stiff richer and thicker than cream	Rich and thick compared to the gel	light and watery
white, off-white or colored	white, off-white or colored	Translucent and transparent
Slowly absorbed	Slowly absorbed	Rapidly absorbed
Spread ability is less	Easy to spread	Spread ability is high

2.2.1. Gel Polymers Types:

• Gel polymers used in pharmaceuticals and cosmetics are classified into three types [44]:

1. Natural polymers.

2. Semisynthetic polymers.

3. Synthetic polymers.

2.2.2 Gel Polymers and Preservative Used in Formulations in This Study:

• We chose one of each type for this study:

1. Natural polymer: Chitosan.

2. Semisynthetic polymer: Hydroxy Propyl Methyl Cellulose (HPMC).

3. Synthetic polymer: Carbopol 934.

2.2.2.1. Hydroxy Propyl Methyl Cellulose (HPMC):

Cellulose methyl ether, Hydroxy Propyl Methyl Cellulose (HPMC) is a semisynthetic polymer that is odorless and tasteless, white, fibrous powder or granules, nontoxic, nonallergenic, and nonirritant, and is used to thicken topical creams, ointments, and gels as a viscosity-increasing agent in pharmaceutical preparations. HPMC concentrations ranging from 1.0 to 5.0 percent are used in pharmaceutical topical preparations. During formulation, HPMC is dissolved in water and the viscosity is increased by increasing the concentration of HPMC. Furthermore, increasing the temperature reduces the viscosity of solutions until gel formation occurs at 50–60 °C. HPMC solutions exhibit pseudoplastic flow (discussed in rheology section) [45].

2.2.2.2 Chitosan:

Chitosan is a deacetylated derivative of Chitin which is considered as second most founded natural polysaccharide on earth after cellulose, chitosan is white creamy odorless powder [46], biodegradable, non-toxic, non-irritant [46, 47] Chitosan is used as a viscosity increasing agent in cosmetics and pharmaceutical preparations, and many articles mentioned some of chitosan pharmacological activity such as: anticancer [48] anti-inflammatory [48,49] antimicrobial [50] wound healing [49] anti-fungal and antioxidant [51], the main sources of chitin and chitosan are crabs, shrimps, fungi [46,47] and insects [47, 52] chitosan acts as a pseudo-plastic material the viscosity decrease when the rate shear increasing [16] (discussed in rheology section).

2.2.2.3. Carbopol 934:

Known as Carbomers are synthetic high molecular weight polymers white colored slightly odor acidic hygroscopic powders, used as rheology modifier in liquids and semi-liquids pharmaceutical cream, ointments, gels topical, rectal, Opthalmic, vaginal preparations and cosmetics, carbomers concentration used as gelling agent is between 0.5-2.0 %, carbomers disperse in water and form an acidic solution needs to be naturalized by using one of base agents include: potassium hydroxide or sodium bicarbonate or organic amines or amino acids or triethanolamine because neutralized aqueous gels are more viscous at PH 6-11 [53] In this study, triethanolamine (TEA) was used to obtain PH 8, which made the solution more basic and facilitate gel formation [53].

2.2.2.4. Methyl- Paraben:

Methyl-4-hydroxybenzoate, known as Methyl-paraben, its empirical formula ($C_8H_8O_2$), Parabens are non-mutagenic, non-teratogenic, and non-carcinogenic and are used in pharmaceutical preparations, cosmetics, and food products as antimicrobial preservatives. The concentration of methyl-paraben used in pharmaceutical topical preparations is between 0.02- 0.3% [54]. WHO estimates the upper daily intake amount of methyl-paraben is 10 mg/kg of body weight [55], in this study, 0.2 % of methyl-paraben was added to the

formulation as a preservative, the same percent used in the marketed commercial cream and ointment.

2.3. Liposomes, Vesicle-Forming Ingredients:

2.3.1. Phosphatidylcholine:

Phospholipids are the main components of the cell membrane [56, 57], Composed of glycerol (backbone) and a polar moiety, amphiphilic (can be naturally cationic or anionic in charge), phospholipids can be obtained naturally from a variety of sources, including eggs and soybeans, non-toxic, biologically inert phospholipids are used in pharmaceutical formulations for forming different types of liposomes [56]. In our study, reddish brown granules of L-phosphatidylcholine (from soybean) were used as a membrane forming agent in the preparation of liposomes.

2.3.2. Cholesterol:

Lanolin contains cholesterol. Cholesterol a white odorless substance powder is used as one of the vesicle-forming ingredients in liposomes. Cholesterol plays a significant role in membrane rigidity, stability, fluidity, and permeability [57].

2.3.3. Liposomes' characterization:

Liposome characterization relies on morphology, vesicle size, and Zeta Potential. Which were examined by scanning electron microscopy (SEM) (Zeiss Evo40, Germany) [58].

3. MATERIALS and METHODS

3.1 Materials Used in study:

3.1.1. The active ingredient:

Thiocolchicoside (THC), From Abdi Ibrahim Drug Company.

3.1.2. The excipients:

3.1.2.1 Gelling agent and viscosity increasing agents, polymers:

1- Natural polymer: Chitosan.

2- Semisynthetic polymer: Hydroxy Propyl Methyl Cellulose (HPMC).

3- Synthetic polymer: Carbopol 934.

3.1.2.1. Additives:

1- Preservative:

Methyl- Paraben.

2- PH Adjuster:

Liquid Tri-ethanolamine.

Acetic acid.

3.1.3. Composition of Liposomes:

3.1.3.1 Vesicle-Forming Ingredients Used in Preparing Liposomes:

1- Phosphatidylcholine.

2- Cholesterol.

3.1.3.2. Solvents:

1- Distilled water for the hydrophilic phase.

2- Chloroform and methanol for the lipophilic phase.

3.1.4. Materials of the In-vitro and Ex-vivo Release Studies:

3.1.4.1 Artificial Membrane (A.M):

Cellulose acetate membrane filter (ISO OLA13 1.104.01.01 2.100 REF).

3.1.4.2. Pig Skin (P.S):

Local slaughterhouse. Excised from acıbadem university research center – Istanbul-Turkey.

3.1.4.3. Dermaroller (D.R):

We used solid unloaded-drug titanium with a microneedle length of 0.30 mm (total 680 needles) for the derma roller® taken from UREW cosmetics®, (Figure 6).

3.2. Devices and Instruments:

Table 3.2. Devices and Instruments Used in This Study:

Device\Instrument name	**Company name**	**Origin country**
Franz cell diffusion apparatus	Perme Gear	USA
Rotary Evaporator. -Rotary Vacuum Evaporator	Heidolph	Germany
Spectrophotometer	(Agilent 8453 UV-VIS)	USA
Viscometer	Brookfield	Canada
Ultrasonic-Homogenizer	Bandelin Sono Plus Ultrasonic Homogenizer	Germany
Homogenizer	Heldol PH Silent Crusher M Homogenizer	Germany
Ultrasonic bath	Bandelin Sonorex Super RK 156 BN	Germany
Hot plate	Heidol ph (MR 3004 Safty)	Germany
Water shaking bath	1) Wise Bath	1) Japan
Water shaking bath	2) GFL	2) Germany
Weighing Device	Analytical plus OHAUS	USA
PH Meter	Mettler Toledo Quattro PH Meter	Switzerland
Ultrapure Type I Water Machine	Simplicity UV Merck Life Science	Germany
Vortex Mixer	Velp Scientifica ZX3 Advanced Vortex Mixer	Italy
DSC	Diffrential Setaram DSC 131	USA

3.3. Methods:

Several in-vitro and ex-vivo permeability studies of THC through artificial membrane and pig skin have been done with Franz cells diffusion apparatus.

The study went through many steps, in first step we prepared 9 gel formulations by using 3 different suitable types of polymers with 3 different percentages of each polymer which all contains THC as API and the rheology of each were measured by using Brookfield, the permeation experiments were performed by Franz cells diffusion apparatus by using artificial membrane and 2 marketed products (cream and ointment) also were tested in this step to compare the result of gels formulations with them as control formulas.

After that, statistical analysis was done by using GraphPad Prism v.5.04 program (GraphPad software Inc., La Jolla, CA) to choose the best percentage of each polymer.

The second step was performed by using 3 formulations that had been chosen in the first step, and the same two marketing products were also tested by performing the permeation measurements with Franz cell diffusion apparatus using pig skin as permeation membrane, which is thankfully offered by Acıbadem University Research Center – Istanbul-Turkey. After statistical analysis, one formula was chosen. The permeation enhancement of THC-loaded liposomes in gel formula were tested with pig skin by the Franz cell diffusion apparatus. The last step was derma roller ®, MN application to the pig skin and testing the enhancement effect of using it with cream, ointment, and THC-loaded liposomes in chitosan-gel and THC chitosan-gel without liposomes.

To compare the enhancement effect of the gel formulations and the liposome formulation and micro-needling effect, we prepared our formulations with the same THC concentration of the marketed cream and marketed ointment which were used in this study as control groups. All the preparations contain THC 0.25% W/W.

3.3.1. Preparation of Gel Formulations:

3.3.1.1. Preparation of HPMC Gel Formulations:

It depends on the concentration percentage of the polymer wanted, the amount of HPMC powder added, methyl paraben as preservative and THC as API, and in e.q., 25 g of type 1 distilled water mixed by 10x40 mm magnetic stir to avoid the formation of air bubbles on the hotplate, 60 °C, 350 rpm for 1 hour (h) until the gel formation occurs. The amount used of each component is represented in (Table 3.3.1).

3.3.1.2. Preparation of Chitosan Gel Formulations:

Because the Chitosan dissolves readily in dilute or concentrated aqueous acids [46, 59] in 25 ml of 0.5% v/v acetic acid solution and depends on the concentration percentage of the polymer, methyl paraben as a preservative, and THC were added. Because chitosan is a natural polymer, the solution was placed on a hot plate that did not exceed 40 °C. For 1 h until the gel texture was formed, 350 rpm continuous mixing by 10x40 mm magnetic stir was used to avoid the formation of air bubbles. The amount used of each component is represented in (Table 3.3.1).

3.3.1.3. Preparation of Carbopol Gel Formulations:

The amount of THC plus methyl paraben as preservative, and amount of Carpobol powder calculated depends on the concentration wanted to be prepared in e.q. 25 g of PH adjusted distilled water after adding 2 drops of 1-2 drops of 2% triethanolamine (TEA) to reach PH 7-8, the PH required to form Carbopol gel, and mixed by a lab homogenizer (17 rpm) for a few minutes until the gel is formulated. The amount used of each component is represented in (Table 3.3.1).

All the formulations used in this study contain: 0.25% THC w/w.

- F1= marketed cream.
- F2 = marketed ointment.
- F3 = HPMC 2.5%.
- F4 = HPMC 3%.
- F5 = HPMC 3.5%.
- F6 = Chitosan 2.5%.
- F7 = Chitosan 3%.
- F8 = Chitosan 3.5%.
- F9 = Carbopol 0.5%.
- F10 = Carbopol 1%.
- F11 = Carbopol 1.5%.
- F6-L = THC loaded liposomes in 2.5% chitosan gel.

❖ **1st Step:**

In step one, we measured the permeation of marketed cream and marketed ointment next to the papered 9 formulations by using an artificial membrane (A.M) in Franz's cells diffusion apparatus.

We gave them codes according to the following:

- F1 (A.M) = marketed Cream across Artificial Membrane.
- F2 (A.M) = Marketed Ointment across Artificial Membrane.
- F3 (A.M) = HPMC 2.5% across Artificial Membrane.
- F4 (A.M) = HPMC 3 % across Artificial Membrane.
- F5 (A.M) = HPMC 3.5% across Artificial Membrane.
- F6 (A.M) = Chitosan 2.5% across Artificial Membrane.
- F7 (A.M) = Chitosan 3% across Artificial Membrane.
- F8 (A.M) = Chitosan 3.5% across Artificial Membrane.
- F9 (A.M) = Carbopol 0.5% across Artificial Membrane.

- F 10 (A.M) = Carbopol 1% across Artificial Membrane.
- F11 (A.M) = Carbopol 1.5% across Artificial Membrane.

❖ **2nd Step:**

As the rheology results and after the UV analysis the statistical analysis results showed the best polymer percentage of each polymer gel. In this step, we measured the permeation of cream and ointment alongside these chosen gels. Their permeation enhancement was measured in this step across pig skin.

- F1 (P.S) = marketed Cream across pig skin.
- F2 (P.S) = Marketed Ointment across pig skin.
- F3 (P.S) = HPMC 2.5% across pig skin.
- F6 (P.S) = Chitosan 2.5% across pig skin.
- F9 (P.S) = Carbopol 0.5% across pig skin.

❖ **3ed Step:**

After the UV analysis of the Franz cells experiments samples and the statistical data analysis, the best formula was chitosan gel 2.5% across the pig skin membrane, and because of Chitosan nanoparticles considered as a valuable tool for novel drug delivery systems in the current scenario due to their biodegradability, biocompatibility, improved stability, low toxicity, and simple preparation methods [60], in this step we decided to evaluate the liposome permeation enhancement effect for THC permeation, so we prepared THC-loaded liposomes in chitosan 2.5% (F6-L), and the permeation measurement was made again with pig skin (P.S), and we gave the formulation a code as following:

- F6-L (P.S) = THC Loaded Liposomes in chitosan 2.5% across pig skin.

❖ **4th Step:**

As a final step, we study the permeation of marketed cream and ointment and the THC in chitosan gel 2.5% and the THC-loaded liposomes in chitosan gel 2.5% across the pig skin after the MN application by using Derma roller ® (P.S-D. R).

- F1 (P.S- D.R) = Marketed Cream across pig skin after derma roller application.
- F2 (P.S- D.R) = Marketed Ointment across pig skin after derma roller application.
- F6 (P.S- D.R) = THC in chitosan gel 2.5% across pig skin after derma roller application.
- F6-L (P.S- D.R) = THC-loaded liposomes in chitosan gel 2.5 % across pig ski n after derma roller application.

Table 3.3.1 Composition of the Tasted Formulations:

Component	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F6-L**
HPMC (4000 Cps) (mg)	625	750	875							
Carbopol 941 (mg)							125	250	375	
Chitosan (High viscosity) (Mg)				625	725	875				625
THC (Mg)	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
Distil water e.q (g)	25	25	25	25	25	25	25	25	25	25
Paraben (mg)	50	50	50	50	50	50	50	50	50	50
Acetic acid (ml)				0.125	0.125	0.12				

Tea (drop)							2	2	2	
Phosphatidyl-choline (mg)										750
Cholesterol (mg)										150
Methanol (ml)										10
Chloroform (ml)										10

F3= HPMC 2.5 %, F4= HPMC 3%, F5= HPMC 3.5%, F6= CHITOSAN 2.5%, F7= CHITOSAN 3%, F8= CHITOSAN 3.5 %, F9= Carbopol 0.5%, F10= Carbopol 1%, F10 =Carbopol 1.5% F6-L= THC Loaded Liposomes in 2.5% chitosan gel.

3.3.2 Rheology Measurement:

Rheology describes the flow of liquid and the measurement of viscosity, which indicates resistance of a fluid to flow.

Because of Its high measurement accuracy, good repeatability, stable speed, the gel formulations were evaluated by (BROOKFILED RHEOMETER DV3T) [61] for providing an immediate calculation of the viscosity value, and by using (Spindle # RV- 07) at (10,20,30,40,50,100, and 200 rpm) at 25°C ± 0.5°C. This viscosity determination confirms whether the formulation has non-Newtonian pseudoplastic flow.

3.3.3. Analytical Method of Thiocolchicosoid:

Several studies investigated analytical procedures for the determination of THC. These procedures include using UV spectrophotometry, HPLC, liquid chromatography, gas chromatography, and capillary electrophoresis techniques.

3.3.3.1 Spectrophotometric Methods:

For its low-cost, and as it is an easy widely used techniques A double beam UV-Visible spectrophotometer (Agilent 8453 UV-VIS) was selected as the analysis instrument. Simple, precise, and cost-effective methods to determine THC in bulk and commercial pharmaceutical preparation by spectrophotometric methods [62]. The drug follows Beer-Lambert's law, Acharjya et al in her article tried 4 methods for THC analysis UV-Visible spectrophotometer, THC shows with (first method) λ max at 259.0 nm in zero spectrum, (second method) 252.0 nm in the first spectrum, and (third method) 260.0 nm in second spectrum. (Fourth method) is based on the calculation of area under the curve (AUC) for analysis of THC in the wavelength range of 254.0 – 264.0 nm. The fourth method showed no significant difference when applied to a pharmaceutical preparation. The study also shows that all four methods were simple, sensitive, accurate, and precise. Also, they can be used for routine determination of THC in drug dosage forms [62].

3.3.3.2. Selection of wave length:

THC solutions were scanned in the 200-400nm range to determine the wavelength for measurement.

(Figure 8) Shows that THC was found to exhibit a maximum absorption peak (λmax) at 260 nm

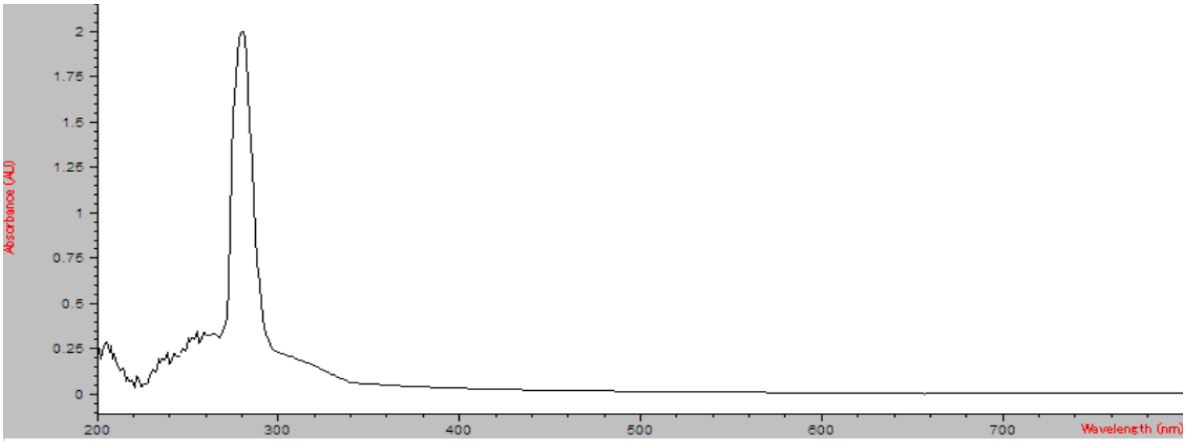


Figure 8. Scanning UV spectra of THC.

3.3.3.3 Calibration Curve and the Linearity Equation:

The calibration curve, an important step in analytical methods, is the preparation of a set of standards containing a known amount of the analyte of interest, measuring the instrument response for each standard and establishing the relationship between the instrument response and analyte concentration. It is essential to know how to evaluate the results obtained [63, 64].

To make the calibration curve, serious of the analyte solutions, calibration standard with at least seven different concentrations (including a blank) should be prepared. So in our study, Dilution Serious of THC solutions were prepared starting with the high concentration 1000 ppm (1mg\ml) by dilute 10 mg of THC in 10 ml of normal saline in 10 ml volumetric flask as stock solution (calibration standard) then 100, 80, 60, 50 ppm were prepared by dilute specific volume of stock solution with appropriate volume of normal saline following M1xV1=M2xV2 which M1 is the concentration of stock solution and V1 is the volume that must be taken from the stock solution to prepare the wanted concentration solution , M2 is the concentration want to be prepared and V2 is the volume used to obtain the next solution.

From the solution of 50 ppm, we prepared 10, 20, 40 ppm solutions to avoid the errors chances obtained by prepare very low concentration solutions from relatively higher concentration solutions.

After that, the analysis of the calibration standard and series of diluted samples was done by using UV spectrophotometry. The blank was normal saline, which was used to dilute the samples.

The results were applied to the XL program and the instrument response was plotted as the Y axis and concentration (mg L^{-1}) (ug/ml) of the prepared samples in (ppm) were plotted as the X axis.

With R Square (R^2) = 0.9921, which is the correlation coefficient (the related parameters R^2 or adjusted R^2) is a measure of the correlation strength degree between the y and x values. The highest absorption of THC was 260 nm.

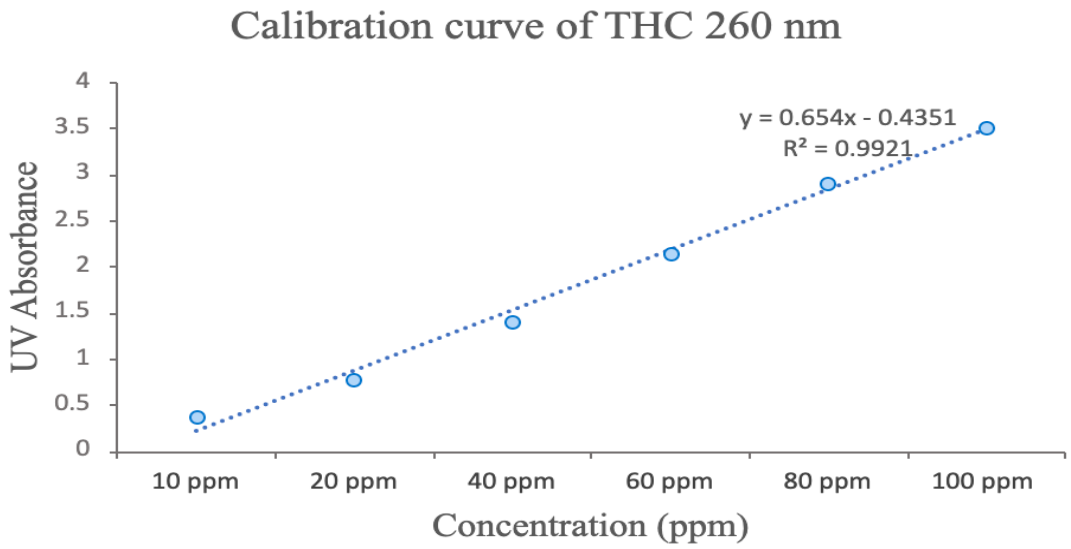


Figure 9. THC calibration curve and the linearity equation and the correlation coefficient (R^2).

The equation gained from the calibration curve is used to determine the concentration of an unknown THC concentration in an aqueous solution of permeation experiment samples.

3.3.4. The Penetration Measurement Method:

Experiments were carried out using the Franz diffusion cell diffusion apparatus with a diffusion area of 2.883 cm2, thermo-stated at 37°C.

In the first step, an artificial membrane was used, and in the subsequent steps, pig skin was placed with the stratum corneum facing the donor phase, between the donor and receptor compartments of the Franz cell diffusion apparatus.

2 g of (0.25% w/w) of each formulation and the control formulations (marketed cream and marketed ointment) were placed in the donor chamber. And the top of doner covered by parafilm M to avoid the effect of out pressure and magnetic bars was used to continually stir 20 mL of isotonic saline solution (pH 7.4) into the receptor chamber.

Sampling was at various times (5, 10, 15, 30 minutes, 1, 2, 3, 4, 5, 6 hours) and replaced with new normal saline to maintain the sink condition. The collected samples were analyzed with a UV spectrophotometer (200-400 nm), [65, 66, 67].

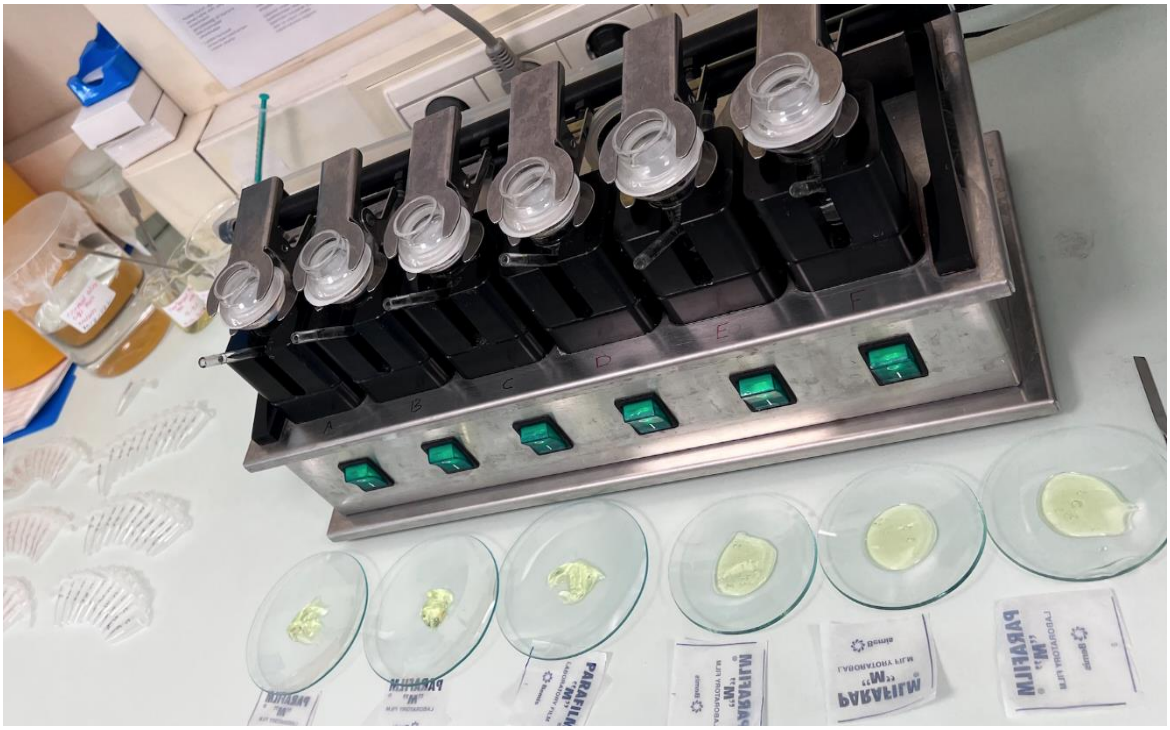
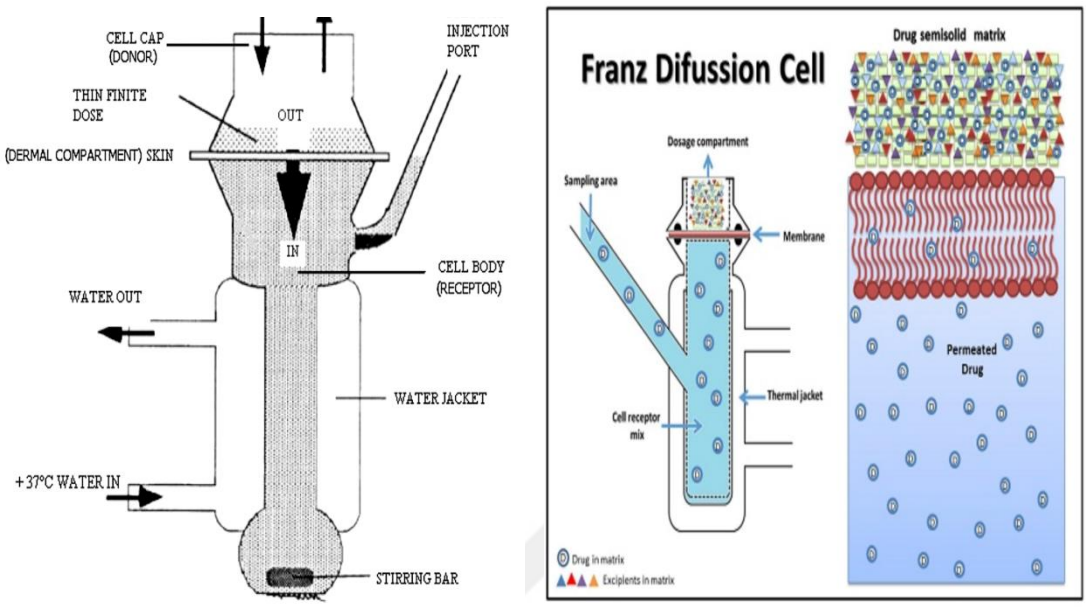


Figure10. Preparation of the Permeation Experiments with Franz Diffusion Cell Apparatus Used in the Lab for This Study.



(a) (b)

Figure 10.1. (a) Franz diffusion cell apparatus (Pineau 2012), [65]. (b) Franz diffusion cells apparatus (salamanca CH. 2018), [66].

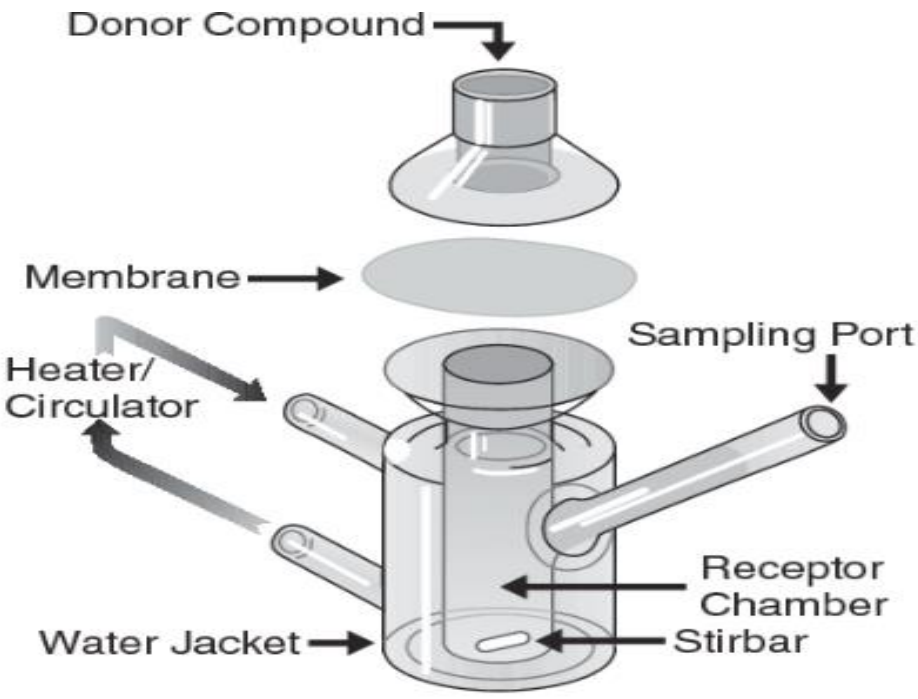


Figure 10.2. Franz diffusion cell apparatus compartments (Finnin 2011), [67].

3.3.4.1. Tissue Preparation:

Before starting the permeation experiment on one night, the pig skin was taken out of the freezer. Pig skin hair was shaved off with a razor, and the fat layer was removed with a knife and cut in size to fit the diffusion area between the doner and receptor chamber of the Franz cell diffusion apparatus.

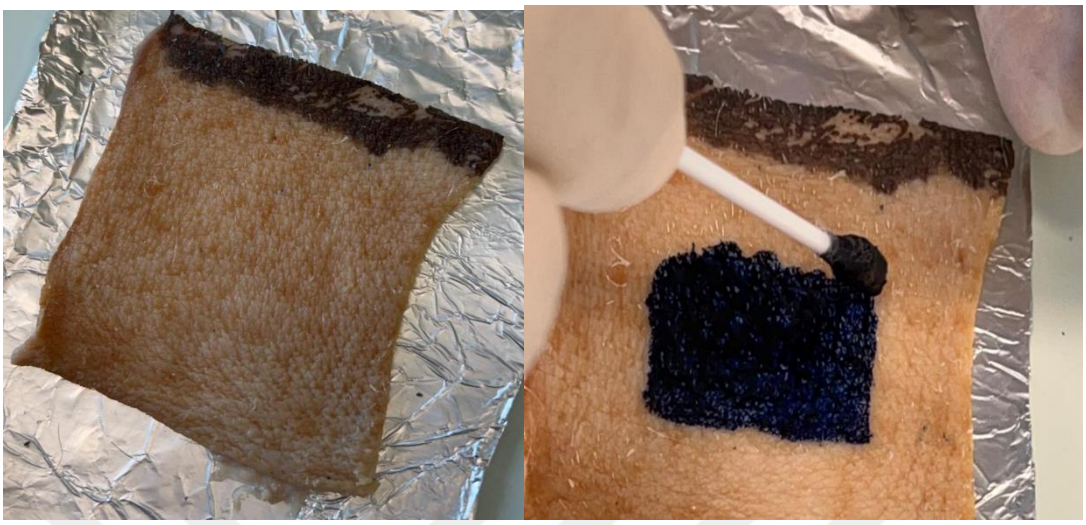
Afterward, the skin was immediately mounted on the Franz-type diffusion cell apparatus for the run of the experiment.

3.3.4.2. Skin Poration with Derma roller:

In the final step, in which we evaluated the effect of derma roller application on permeation enhancement of THC, we prorated the pig skin and made the small pores with derma roller application by rolling it on the pig skin's upper surface.

3.3.5. Methylene Blue Dye Application to Pig Skin Poration with Derma Roller:

Pig skin after derma roller application and waiting for 5 minutes to be sure of methylene blue penetration through the holes created after derma roller application, which is a sign and indication that the drug will penetrate through the holes (Figure 11).



(a) (b)

Figure 11. Tissue preparation and Methylene Blue Dye Application to Pig Skin Poration with Derma Roller.
(a) Clean and shaved prepared tissue (pig skin) for Franz cell diffusion apparatus experiments.
(b) Applying methylene blue dye to the pig skin after derma roller application.

3.3.6. Liposome Preparation Method:

We prepared our liposome formula based on the Thin Film Hydration Technique/Rotary Evaporation-Sonication Method (BANGHAM METHOD).

Firstly, for the hydrophobic phase, we weighed the (edge activator components) (vesicle-forming ingredients 1:5 ratio) phospholipids, L-Phosphatidylcholine (soybean phosphatidylcholine (sigma) and cholesterol (fluka) dissolved in a round-bottom flask using a volatile organic solvent mixture in a suitable ratio (v/v) 1:1) chloroform and methanol with the aid of a bath sonication, and then, in order to form a thin film, the organic solvent is evaporated above the lipid transition temperature (It is important that the temperature is below the melting temperature), by using a rotary vacuum evaporator, keep it under reduced pressure and under observation to ensure that no bubbles occur. The heat side (hot jacket) was at 30–40 °C and the cool side (water bath) at 10 °C, 150 rpm was used. On the other hand, an aqueous solution (hydrophilic phase) was prepared in another beaker. We

added our API, which is THC, after dissolving with an appropriate solvent (distilled water e.q. 25 ml) and kept it for 5 minutes in bath sonication for better dissolution.

After chloroform and methanol are completely evaporated in a rotary evaporator, the thin layer lipid film will be formed. At this stage, the organic phase is mixed with the aqueous phase, resulting in a two-phase system. The hydrophilic phase, which contains our hydrophilic API THC was added into the round-bottom flask which contain the condensed thin film layer (hydrophobic phase). The mixture was sonicated at a specified sonication frequency (70 kHz and 7 cycle) for 5–7 minutes at room temperature in a probe sonicator by using (KE 76) prop size to obtain small vesicles (liposomes). At this stage, a milky suspension is obtained and the liposome particles have been formed and the nano level has been reached. We add the amount of chitosan and the paraben preservative to the formula to make it in chitosan gel 2.5% gel formulation (F6-L).

The liposome preparation was transported to a dynamic light scattering particle size analyzer (DLS) device for measurement of size and zeta potential [58].

3.3.6.1. Calculation of entrapment efficiency for liposomes (EE %):

The entrapment efficiency % shows the percentage of drug amount entrapped inside the liposomes in the formulation [58].

After preparing the liposomes, ultracentrifugation for formulation must be done to separate free drug (unentrapped drug) from the liposome vesicles. Liposomes will precipitate, and the free drug will be in the supernatant. In the direct method, methanol must be added and shaken with a vortex mixer to release the drug from precipitated liposomes, disrupt the shell of liposomes, and find the amount of the entrapped drug after analysis.

The entrapment efficiency in (direct method) will be calculated by this equation:

% Entrapment efficiency= (Amount of the entrapped drug /Total amount of the drug added) ×100 %.

In this study, we used indirect method to determine the free drug in supernatant first then the amount of drug loaded in liposomes and the entrapment efficiency can be calculated by this equation:

% Entrapment efficiency= ((Total amount of the drug added − Amount of the free drug) / Total amount of the drug added) ×100 %.

3.3.7. Statistical Evaluation:

The results are expressed using the overall average and standard deviation. The data was statistically examined using the GraphPad Prism v.5.04 program (GraphPad Software Inc., La Jolla, CA). Furthermore, for each experiment, samples were used in triplicate. During statistical analysis, triplicated means were employed. The significance level [$p < 0.05$] was adopted for all formulations utilized in the permeation test.

4. RESULTS

4.1 Rheology results:

All the formulations are non-Newtonian systems exerted by the polymer in the formulation shown in (Table 4.1).

They are shear thinning, and their viscosity decreases with an increase in shear rate [61].

Table 4.1 Rheology Measurement Results:

rpm	F3 (cps)	F4 (cps)	F5 (cps)	F6 (cps)	F7 (cps)	F8 (cps)	F9 (cps)	F10 (cps)	F11 (cps)
10	4800 ±0.059	9200 ±0.219	12400 ±1.029	800 ±2.219	5600 ±0.019	4800 ±2.177	12000 ±1.319	19200 ±0.881	24400 ±0.568
20	4400 ±1.251	8200 ±0.807	11000 ±1.173	800 ±1.017	3600 ±2.403	3200 ±1.219	8000 ±1.023	11000 ±0.913	14200 ±1.029
30	4133 ±0.703	7600 ±1.006	10130 ±1.004	800 ±2.301	2800 ±1.225	2533 ±1.328	4933 ±1.116	7867 ±1.029	10130 ±1.488
40	4000 ±1.008	7100 ±1.213	9300 ±0.445	700 ±0.003	2300 ±0.917	2100 ±0.717	4000 ±1.004	6200 ±1.458	8000 ±1.003
50	3760 ±1.226	6560 ±0.717	8720 ±0.519	640 ±2.618	2000 ±0.329	1840 ±1.025	3280 ±1.913	5200 ±1.919	6800 ±0.611
100	3040 ±0.019	5160 ±0.007	6800 ±0.078	560 ±0.907	1440 ±0.674	1320 ±1.003	1920 ±1.066	3080 ±0.081	4000 ±0.027
200	2360 ±0.107	3820 ±0.059	4880 ±1.002	440 ±0.122	1000 ±1.283	900 ±1.424	1140 ±1.773	1800 ±0.797	2260 ±1.604

F3= HPMC 2.5 %, F4= HPMC 3%, F5= HPMC 3.5%, F6= CHITOSAN 2.5%, F7= CHITOSAN 3%,

F8= CHITOSAN 3.5 %, F9= Carbopol 0.5%, F10= Carbopol 1%, F10 =Carbopol 1.5%, rpm = rounds per minutes, cps = centipoise rheology unit.

4.2. Liposomes Results:

4.2.1. DLS Results of Liposomes:

Table 4.2.1 DLS results of liposomes (F6-L), n = 3, standard deviation SD±:

Parameter	Values
DS (nm)	98.76 ± 1.49
PDI	0.24 ± 0.04
ZP (mV)	- 29.3 ± 3.8

(DS, Droplet size; PDI, The polydispersity index; ZP, Zeta potential,

L: liposomes in F6: Chitosan 2.5% formula).

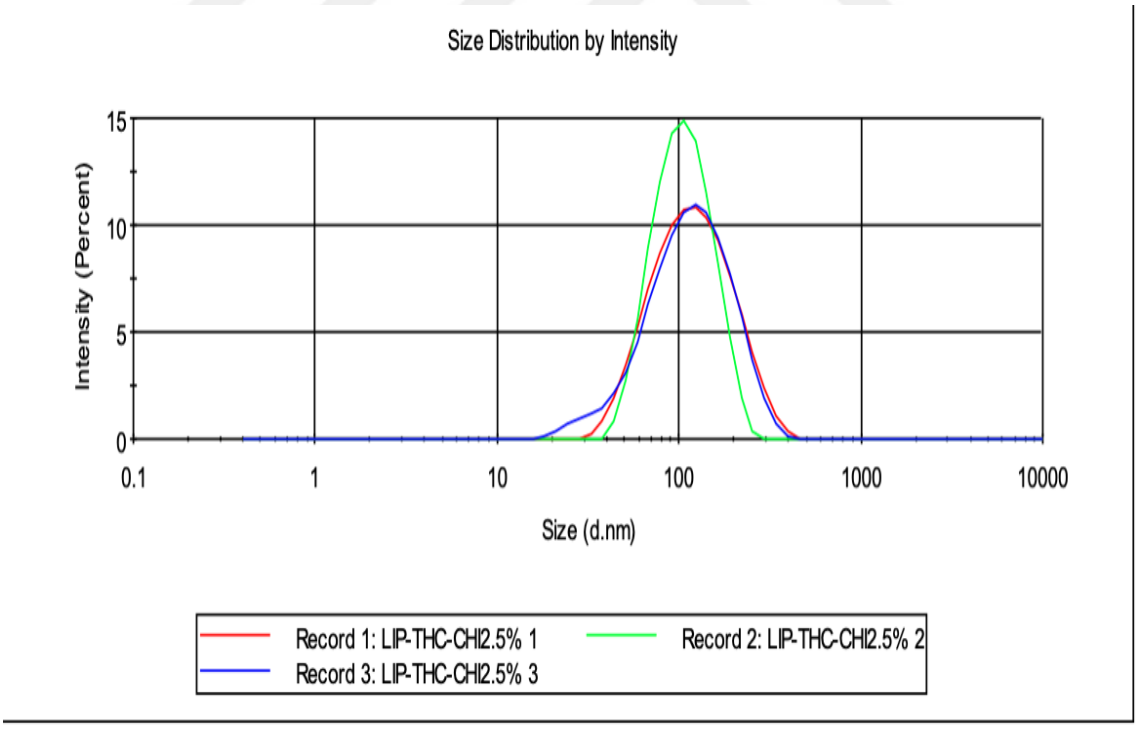


Figure 12. Size distribution of liposomes in F6-L formulation, F6-L: THC loaded liposomes in Chitosan 2.5% gel.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -26.0	Peak 1:	-28.4	92.9	5.24
Zeta Deviation (mV): 10.3	Peak 2:	6.11	7.1	2.59
Conductivity (mS/cm): 0.0112	Peak 3:	0.00	0.0	0.00

Result quality : See result quality report

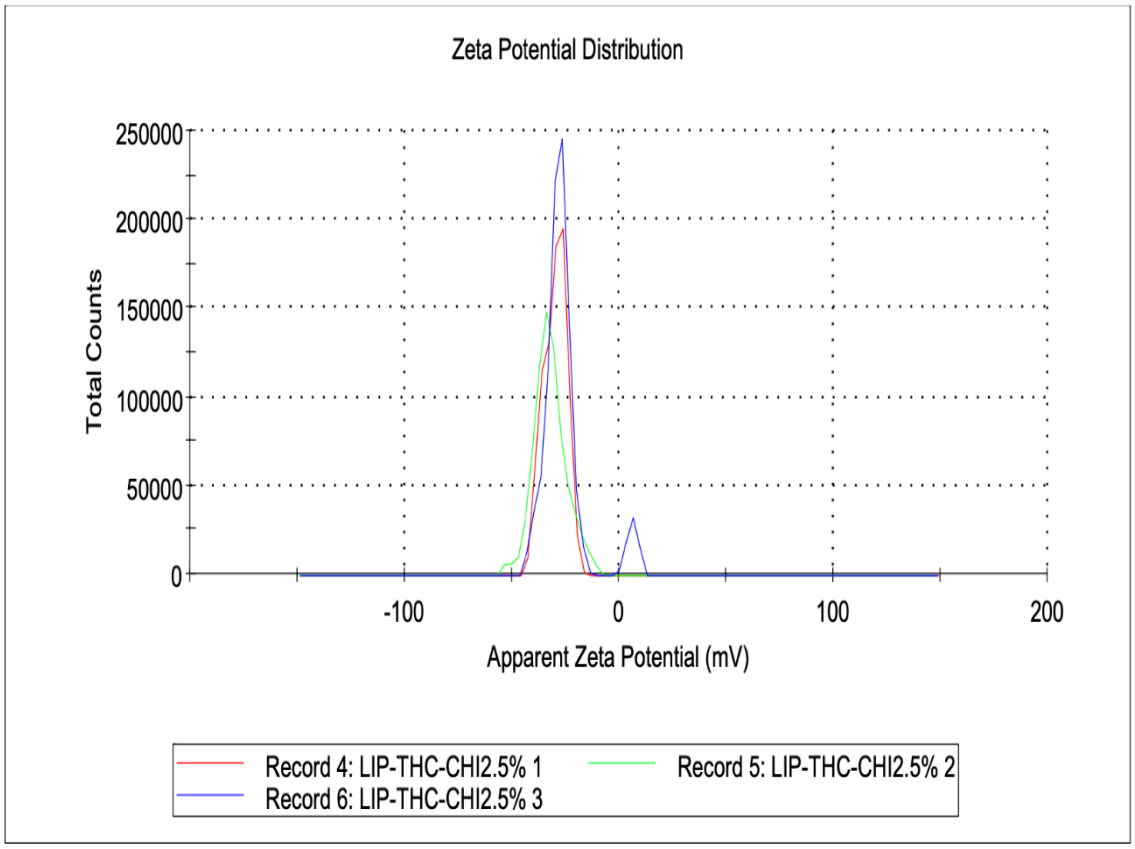
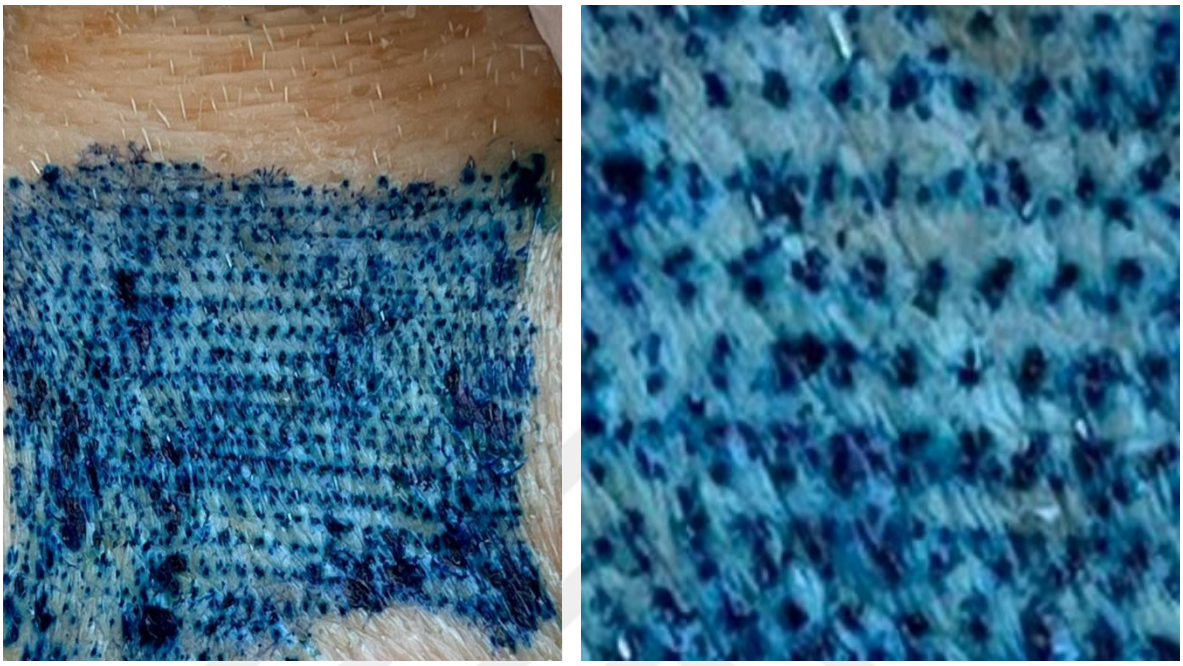


Figure 13. Zeta potential distribution of liposomes in F6-L formulation, F6-L: THC loaded liposomes In Chitosan 2.5% gel.

4.2.2 Entrapment efficiency for liposomes (EE %) Results:

- Unloaded THC = 14.9 %.
- Loaded THC = 85.1%.

4.3. Methylene Blue Dye Application to Pig Skin Poration with Derma Roller Result:



(a) (b)

Figure 14. Methylene blue dye to pig skin after derma roller application.

(a) Methylene blue penetration through the holes created after derma roller application.

(b) Closer view for the holes created after derma roller application.

4.4. Statistical Analysis Results:

Table 4.4.1. Statistical Analysis for HPMC Gel Formulation through Artificial Membrane:

Formulation codes	Jss	Cumulative amount of THC Released %	Permeability coefficinet
F3 (A.M) vs F4 (A.M)	ns	ns	ns
F3 (A.M) vs F5 (A.M)	ns	ns	ns
F4 (A.M) vs F5 (A.M)	ns	ns	ns

Table 4.4.2. Statistical Analysis for Chitosan Gel Formulation through Artificial Membrane:

Formulation codes	Jss	Cumulative amount of THC Released %	Permeability coefficinet
F6 (A.M) vs F7 (A.M)	ns	ns	ns
F6 (A.M) vs F8 (A.M)	ns	*	ns
F8 (A.M) vs F9 (A.M)	ns	ns	ns

Table 4.4.3. Statistical Analysis for Carbopol Gel Formulation through Artificial Membrane:

Formulation codes	Jss	Cumulative amount of THC Released %	Permeability coefficinet
F9 (A.M) vs F10 (A.M)	ns	***	ns
F9 (A.M) vs F10 (A.M)	ns	***	ns
F9 (A.M) vs F10 (A.M)	ns	ns	ns

Table 4.4.4. Statistical Analysis and Comparing between Formulations through Pig Skin:

Formulation codes	jss	Cumulative amount of THC Released %	Permeability coefficient (Kp)
F3 (P.S) vs F6 (P.S)	ns	ns	ns
F3 (P.S) vs F9 (P.S)	ns	ns	ns
F6 (P.S) vs F9 (P.S)	ns	ns	ns

Table 4.4.5. Statistical Analysis for Chitosan Formulations through Different Membranes:

Formulation codes	Jss	Cumulative amount of THC Released %	Permeability coefficient (Kp)
F6 (A.M) vs F6 (P.S)	***	***	***
F6 (A.M) vs F6 (P.S-DR)	***	***	***
F6 (P.S) vs F6 (P.S-DR)	**	ns	**
F6 (P.S) vs F6-L (P.S)	ns	ns	ns
F6 (P.S-DR) vs F6-L (P.S-D.R)	**	*	**
F6-L (P.S) vs F6-L (P.S-DR)	ns	ns	ns

Table 4.4.6. Statistical Analysis for Cream Formulation through Different Membranes:

Formulation codes	Jss	Cumulative amount of THC Released %	Permeability coefficient (Kp)
F1 (A.M) vs F1 (P.S)	*	***	*
F1 (P.S) vs F1 (P.S- D.R)	ns	***	ns
F1 (A.M) vs F3-F11 (A.M)	***	***	***
F1(P.S) vs F3, F6, F9 (P.S)	ns	ns	ns
F1(P.S) vs F6-L (P.S)	ns	ns	ns
F1(P.S- D.R) vs F6 (P.S-D.R)	ns	ns	ns
F1(P.S- D.R) vs F6-L (P.S-D.R)	ns	ns	ns

Table 4.4.7. Statistical Analysis for Ointment Formulation through Different Membranes:

Formulation codes	Jss	Cumulative amount of THC Released %	Permeability coefficient (Kp)
F2 (A.M) vs F2 (P.S)	ns	ns	ns
F2 (P.S) vs F2 (P.S-D.R)	ns	ns	ns
F2 (A.M) vs F3-F11 (A.M)	***	***	***
F2(P.S) vs F3, F6, F9 (P.S)	ns	ns	ns
F2(P.S) vs F6-L (P.S)	ns	ns	ns
F2(P.S- D.R) vs F6 (P.S- D.R)	ns	**	ns
F2(P.S- D.R) vs F6-L (P.S- D.R)	ns	ns	ns

Table 4.4.8. Statistical Analysis and Comparing between Cream and Ointment Formulations through Different Membranes:

Formulation codes	Jss	Cumulative amount of THC Released %	Permeability coefficient (Kp)
F1 (A.M) vs F2 (A.M)	ns	ns	ns
F1 (P.S) vs F2 (P.S)	ns	ns	ns
F1 (P.S- D.R) vs F2 (P.S- D.R)	ns	ns	ns

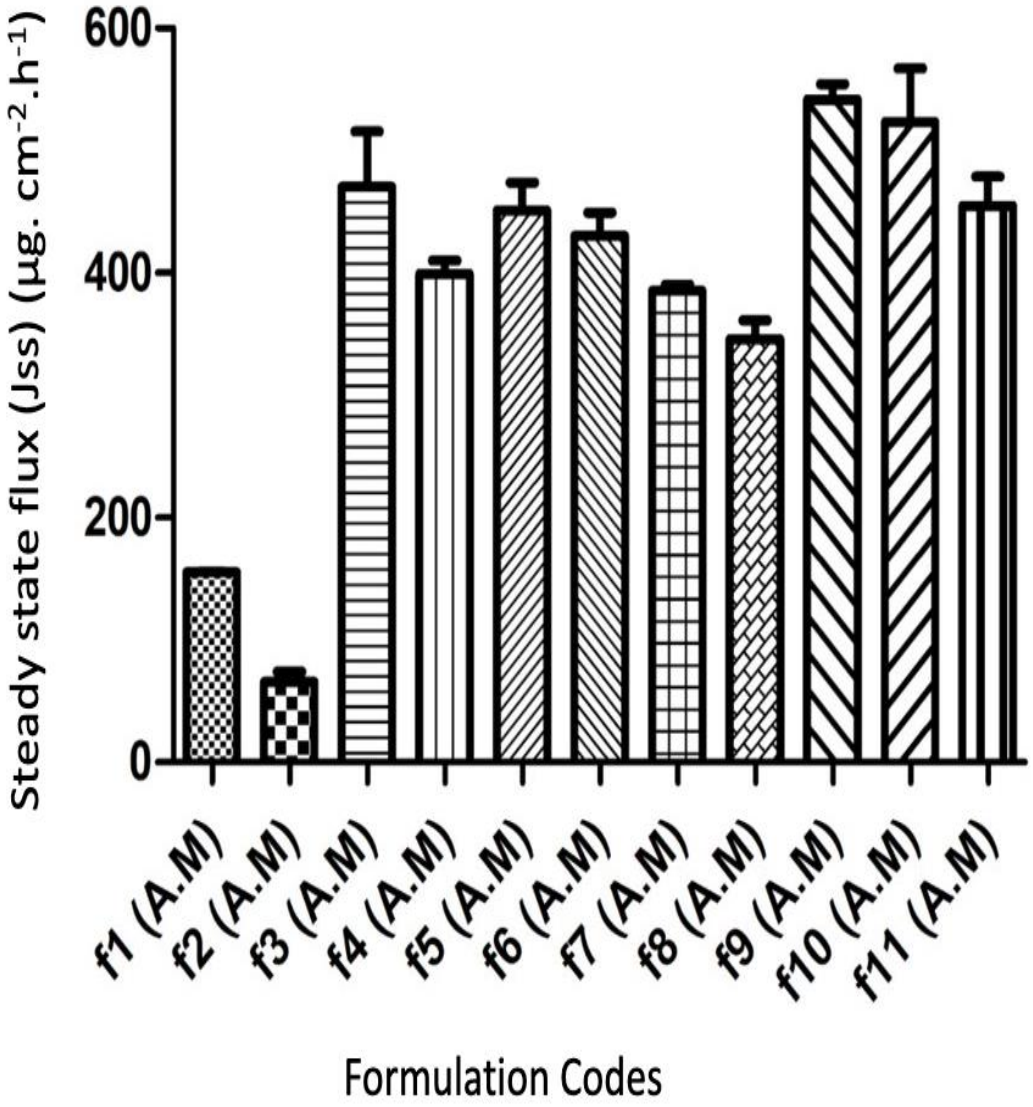


Figure 15. Steady state flux (Jss) (µg .cm^{-2} .h^{-1}) of F1-F11 formulations through artificial membrane (n=3).

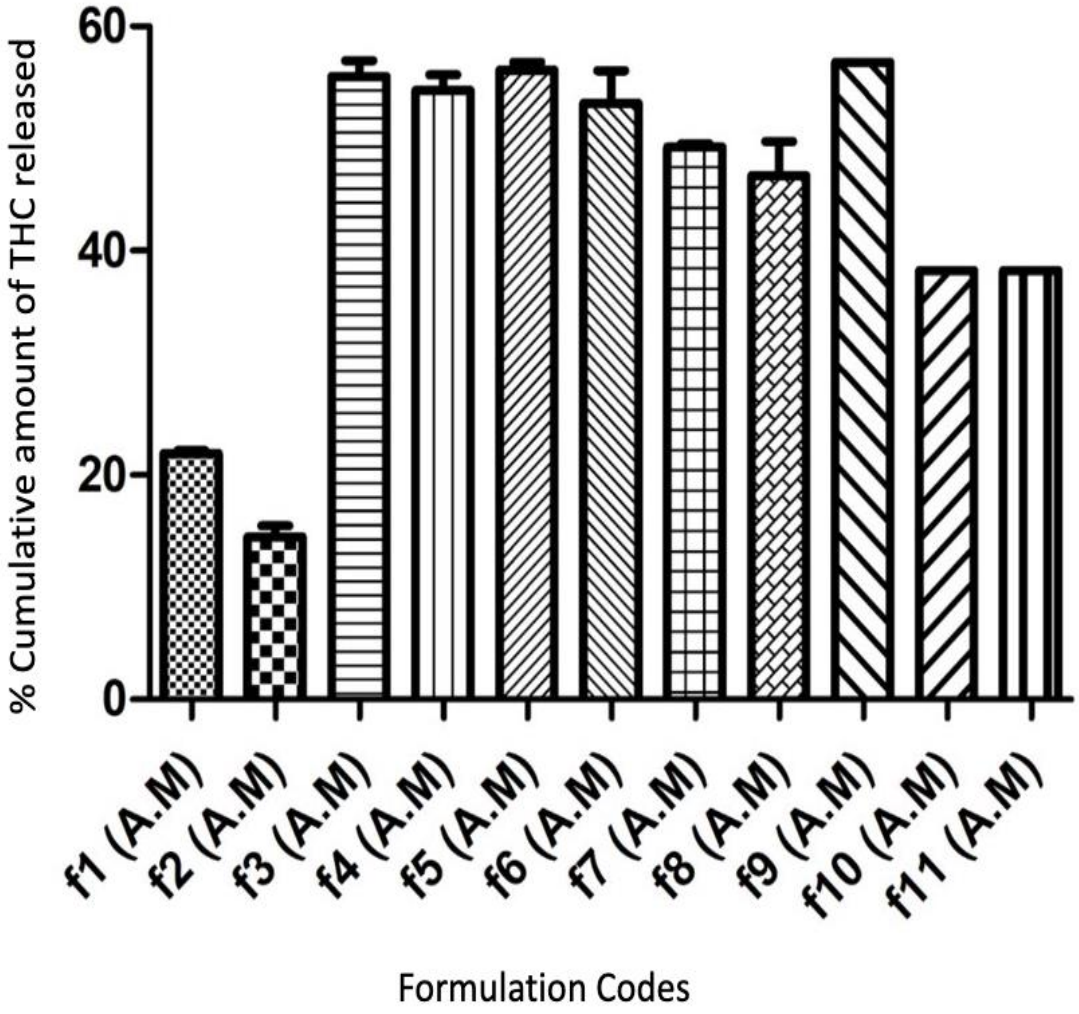


Figure 16. Cumulative amount of THC released % (at the end of 6 hours) of F1-F11 formulations through Artificial membrane (n=3).

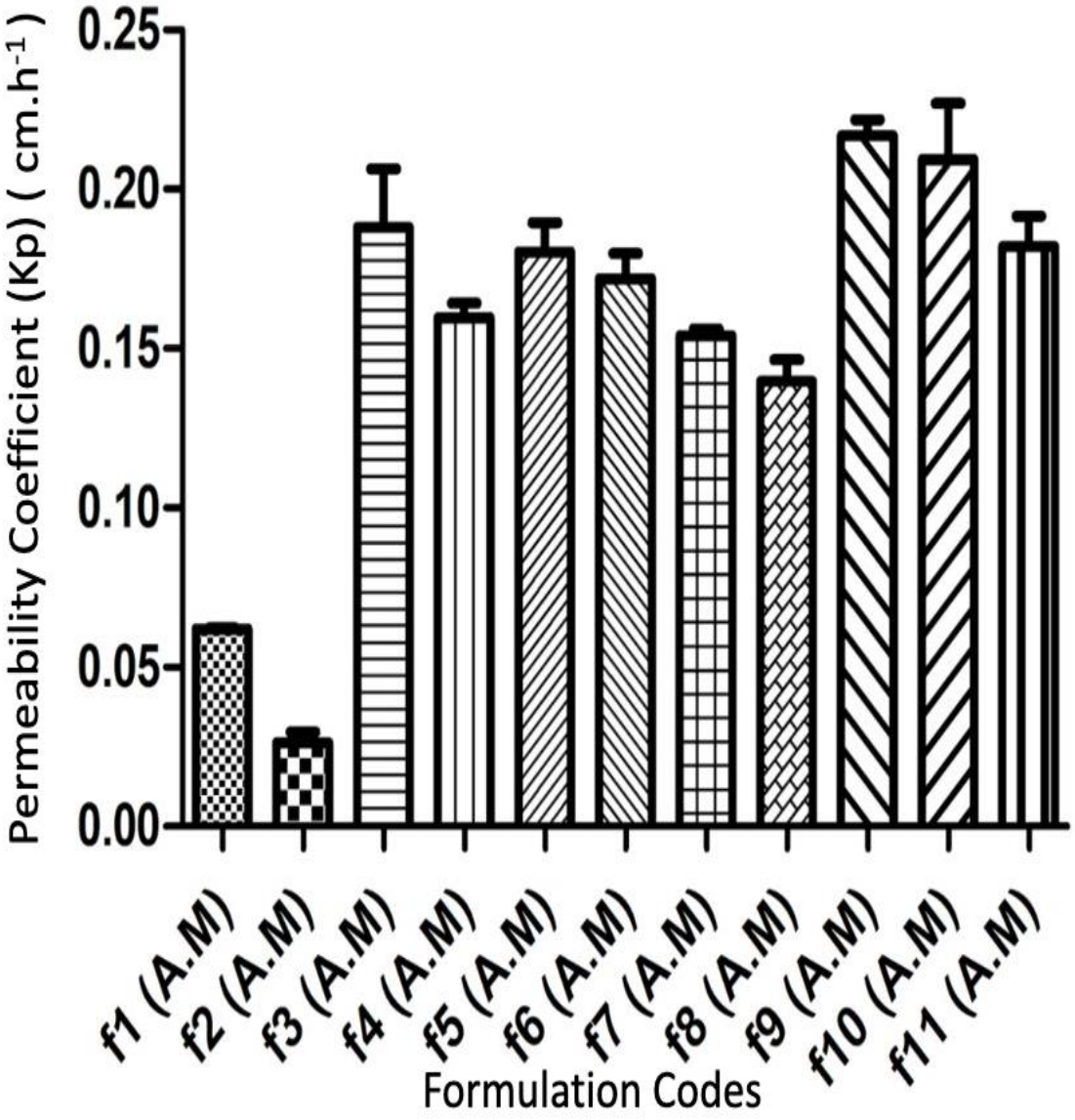


Figure 17. Permeability Coefficient (Kp) (cm .h $^{-1}$) F1-F11 Formulations through Artificial membrane (n=3).

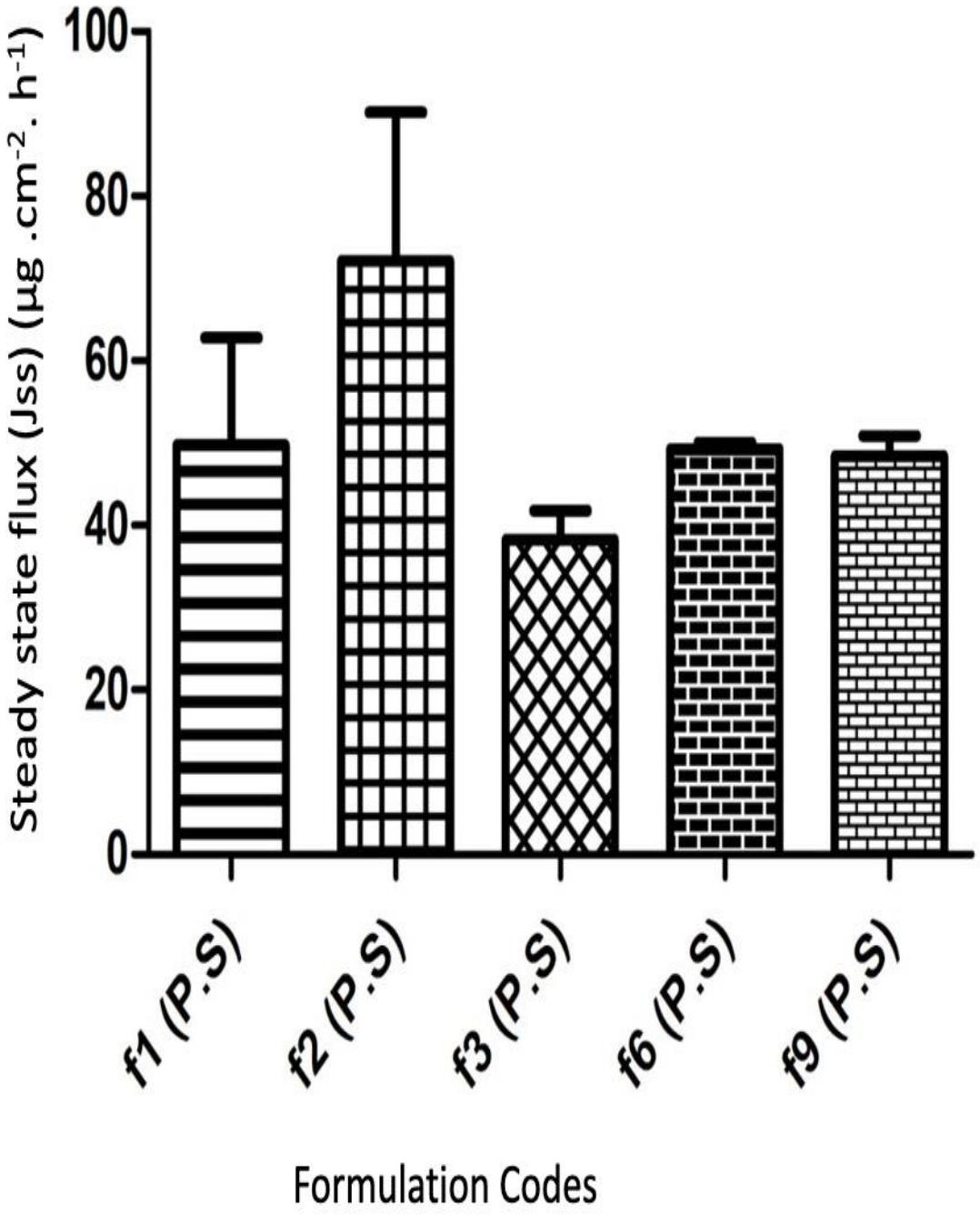


Figure 18. Steady state flux (Jss) (µg. cm^{-2} .h^{-1}) of F1, F2, F3, F6, F9 formulations through Pig Skin (n=3).

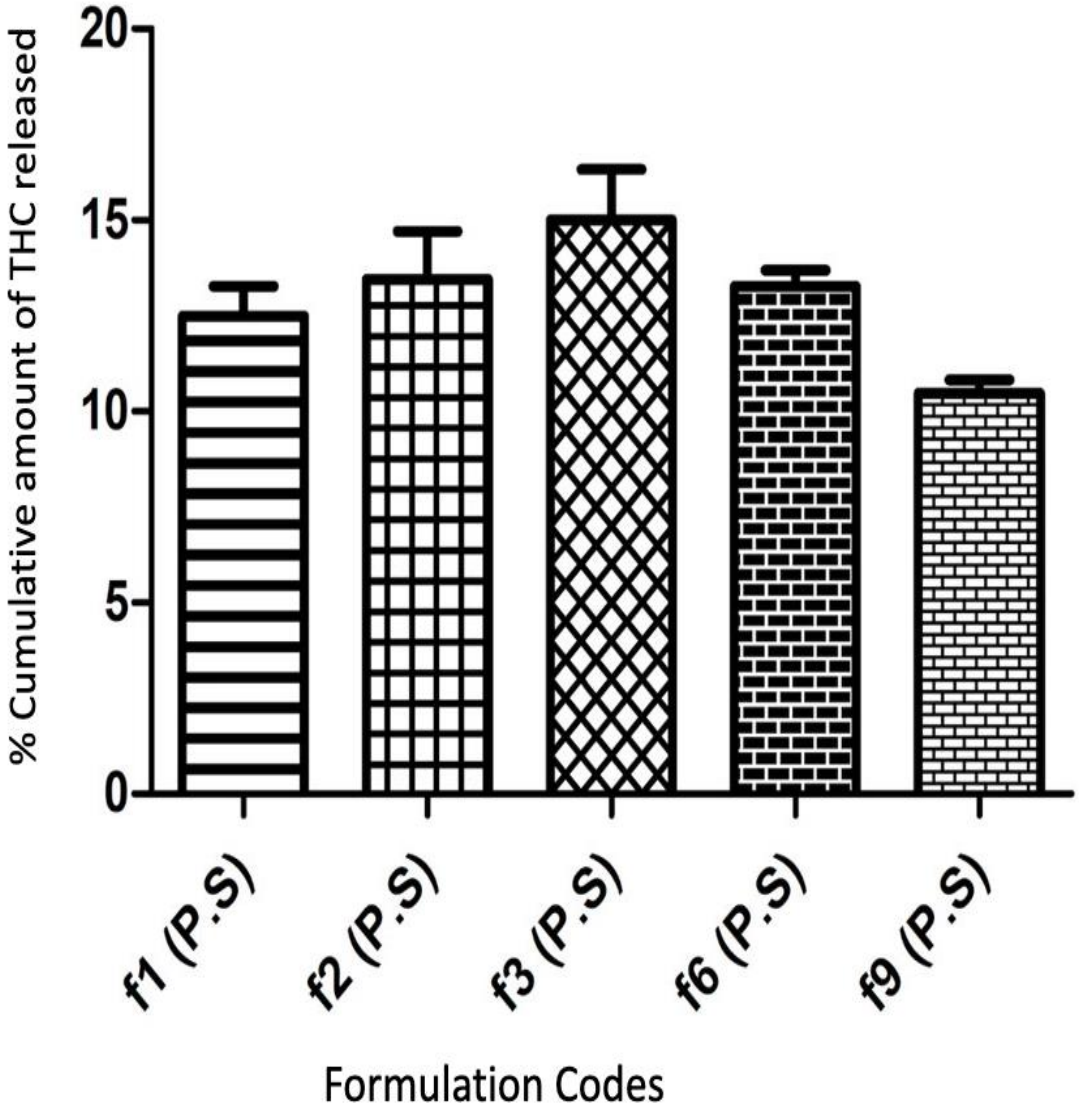


Figure 19. Cumulative amount of THC released % (at the end of 6 hours) of F1, F2, F3, F6, F9 formulations through pig skin (n=3).

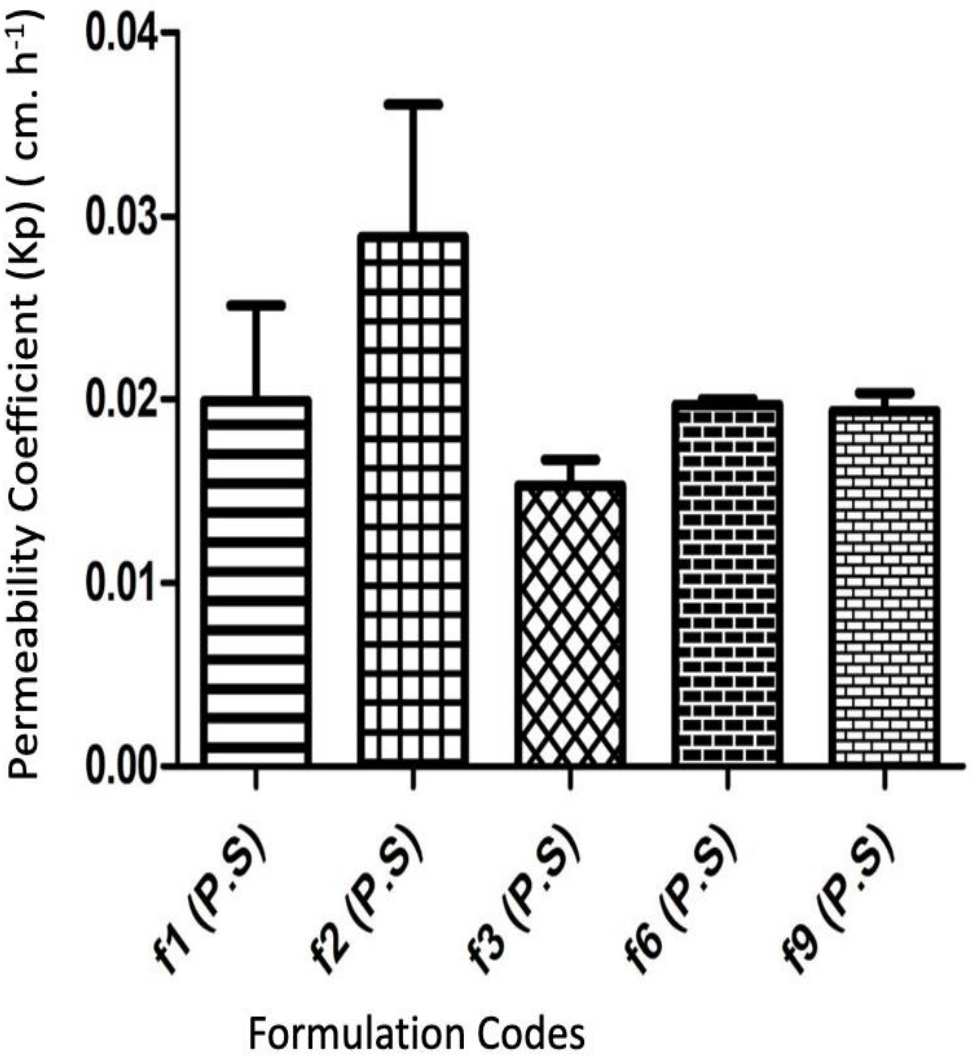


Figure 20. Permeability coefficient (Kp) (cm. h^{-1}) of F1, F2, F3, F6, F9 formulations through pig skin (n=3).

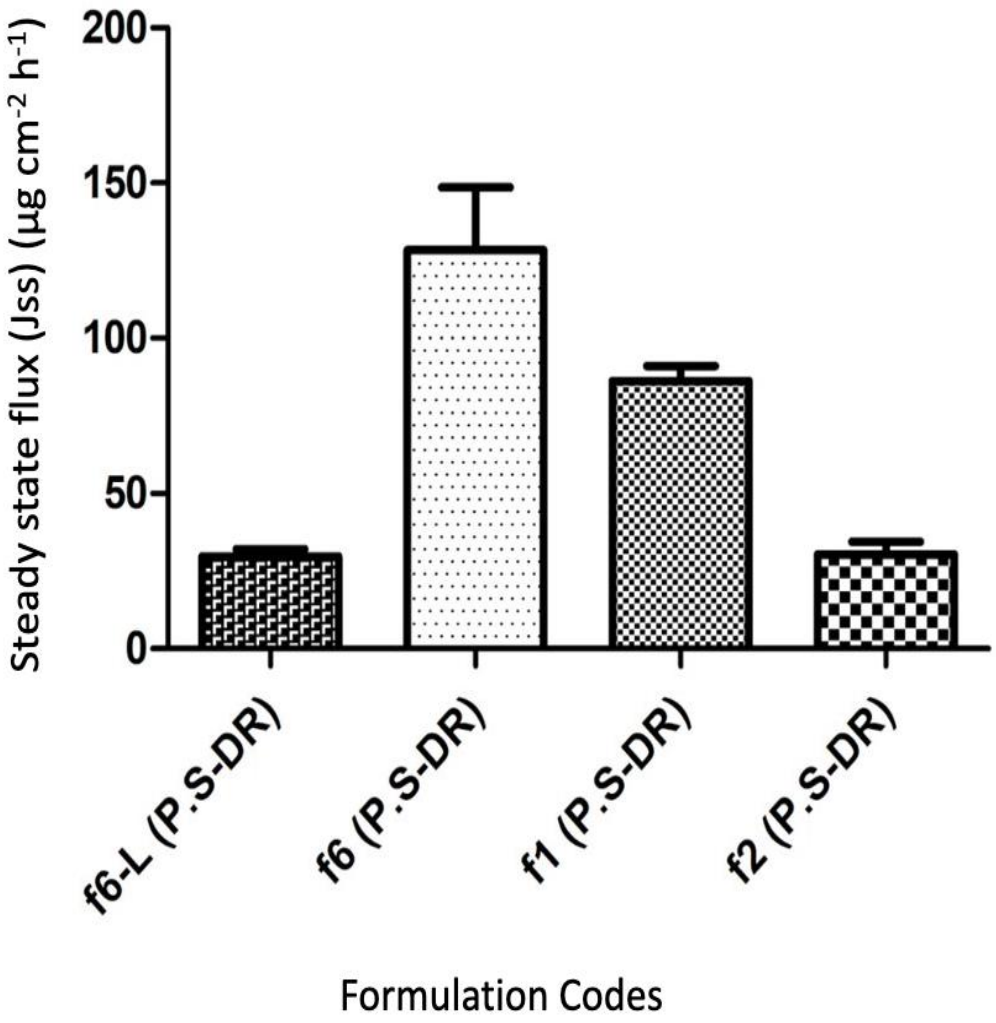


Figure 21. Steady state flux (Jss) (µg. cm^{-2}. h^{-1}) of F1, F2, F6, F6-L formulations through pig skin after derma roller ® application (n=3).

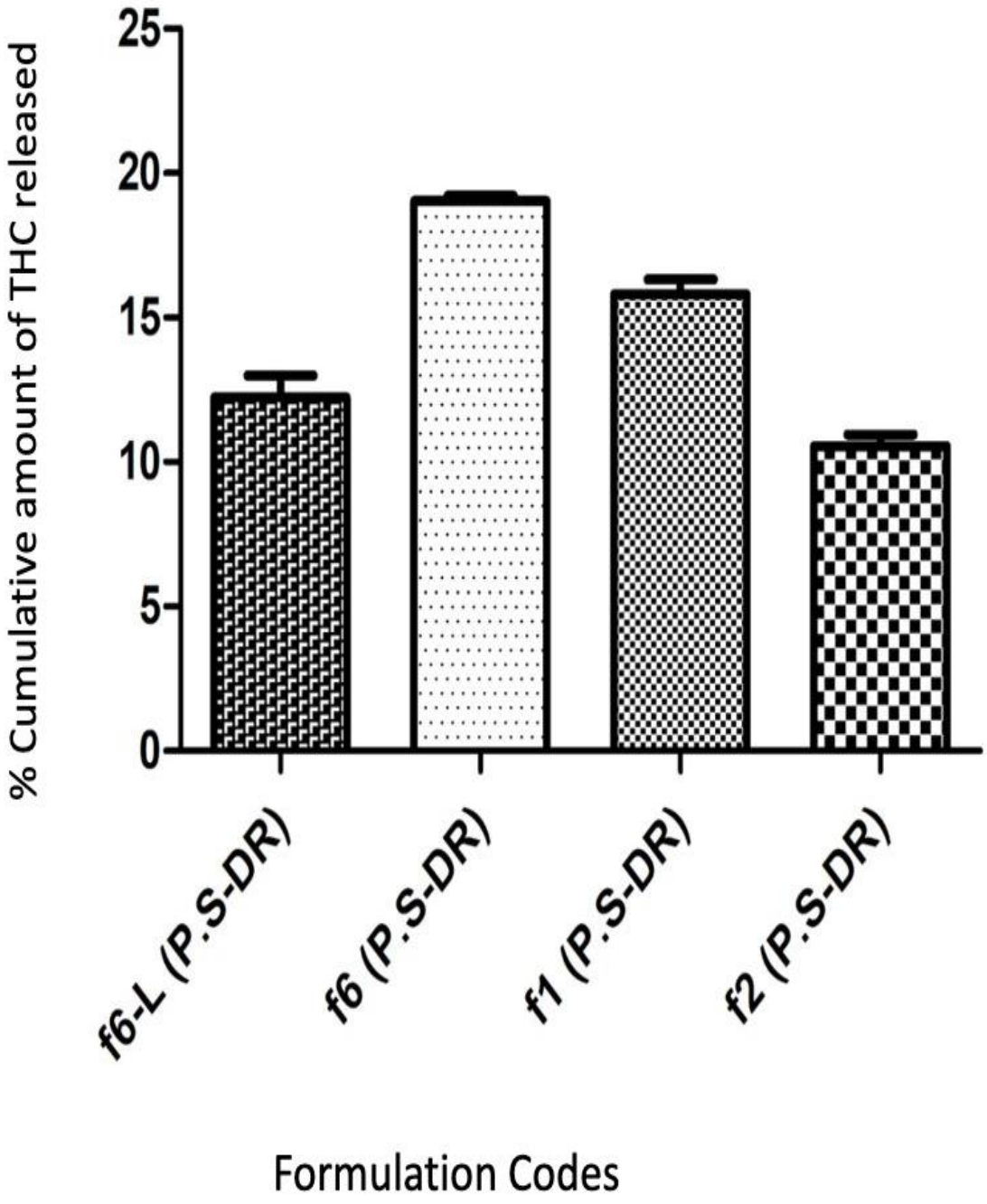


Figure 22. Cumulative amount of THC released % (at the end of 6 hours) of F1, F2, F6, F6-L formulations through pig skin after derma roller ® application (n=3).

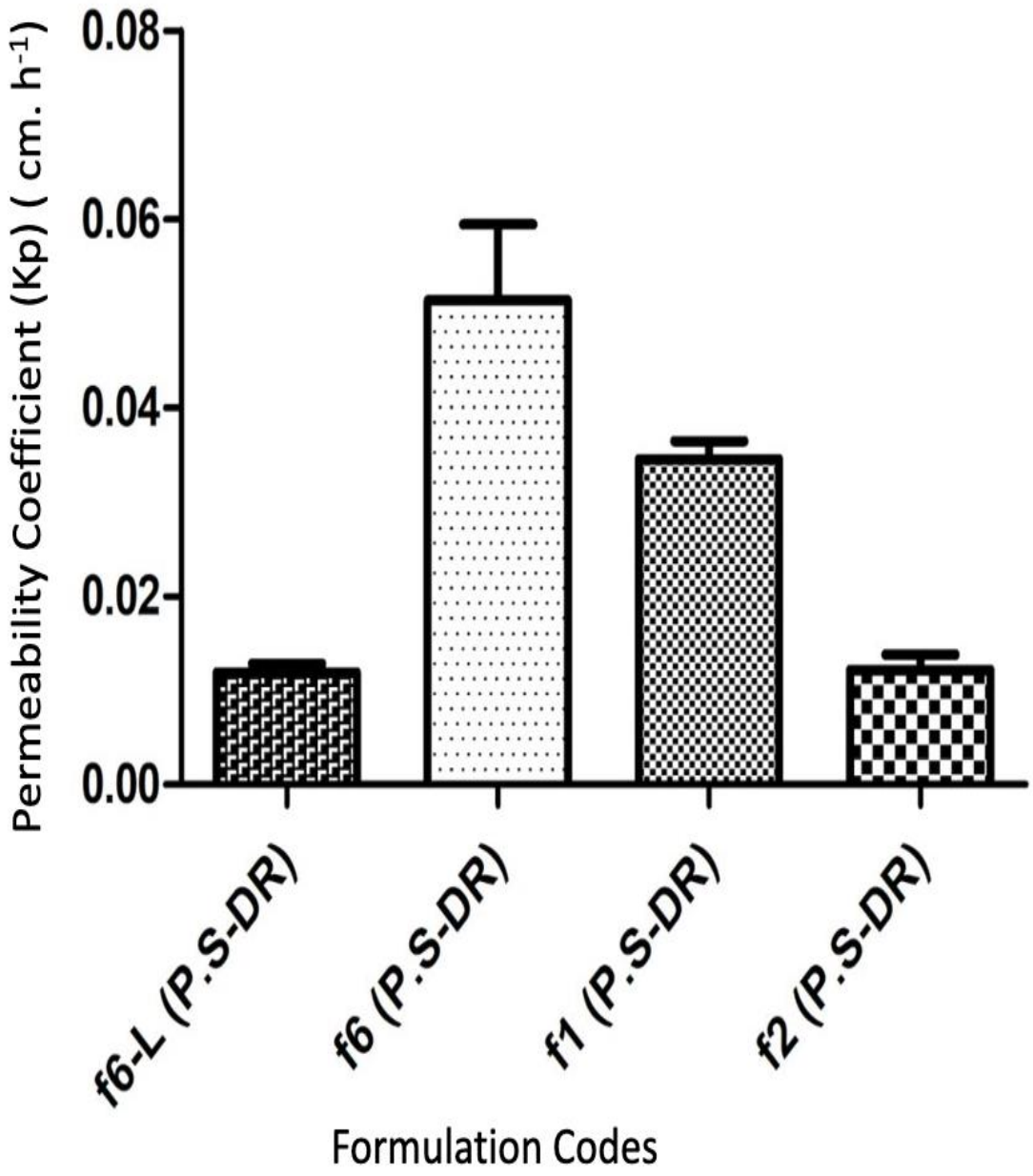


Figure 23. Permeability coefficient (Kp) (cm. h^{-1}) of F1, F2, F6, F6-L formulations through pig skin after derma roller ® application (n=3).

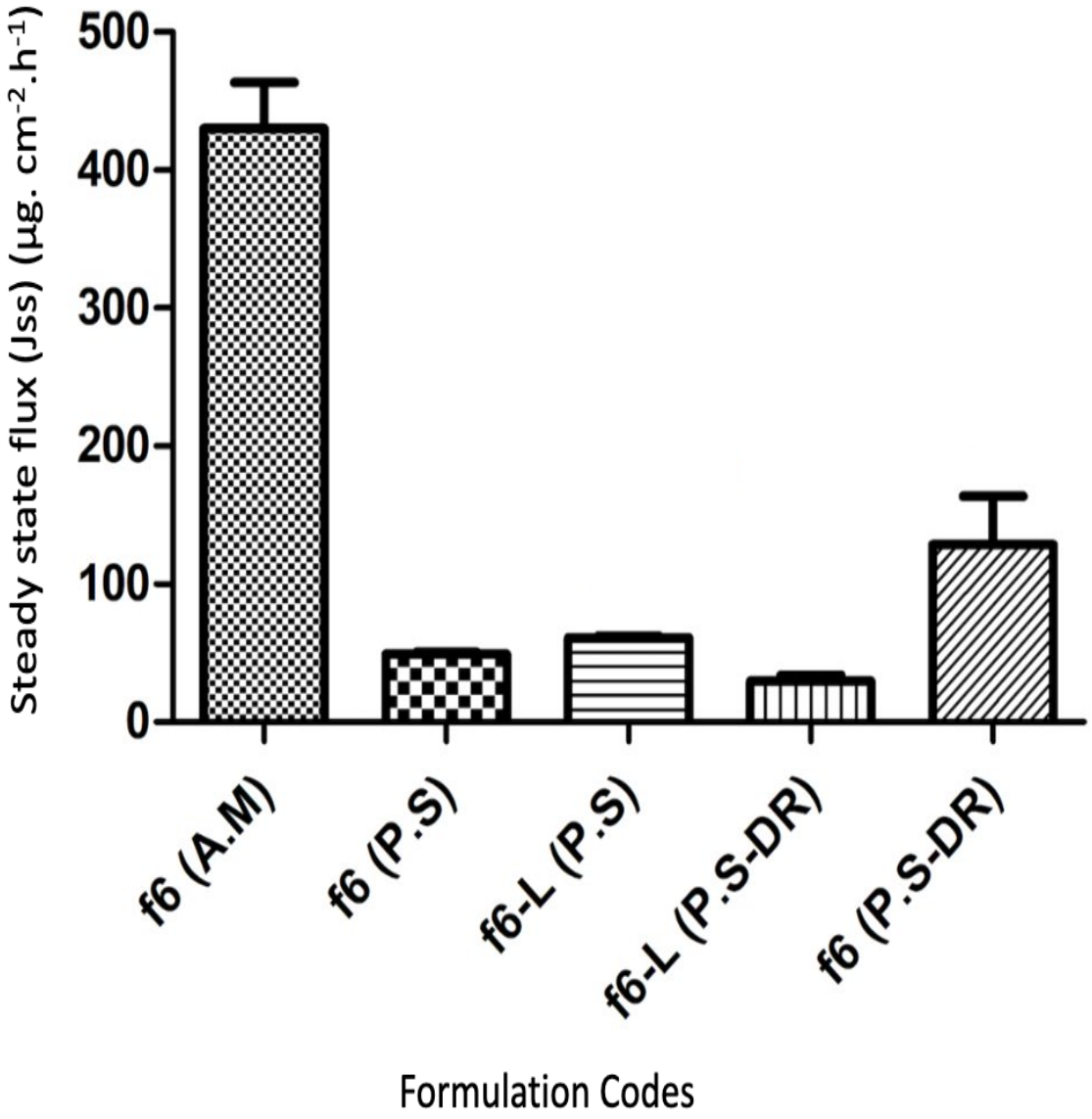


Figure 24. Comparing between the steady state flux (Jss) (µg. cm^{-2}. h^{-1}) of F6, F6-L Formulations through artificial membrane (A.M), Pig Skin (P.S), and pig skin after derma roller ® application (PS-DR) (n=3).

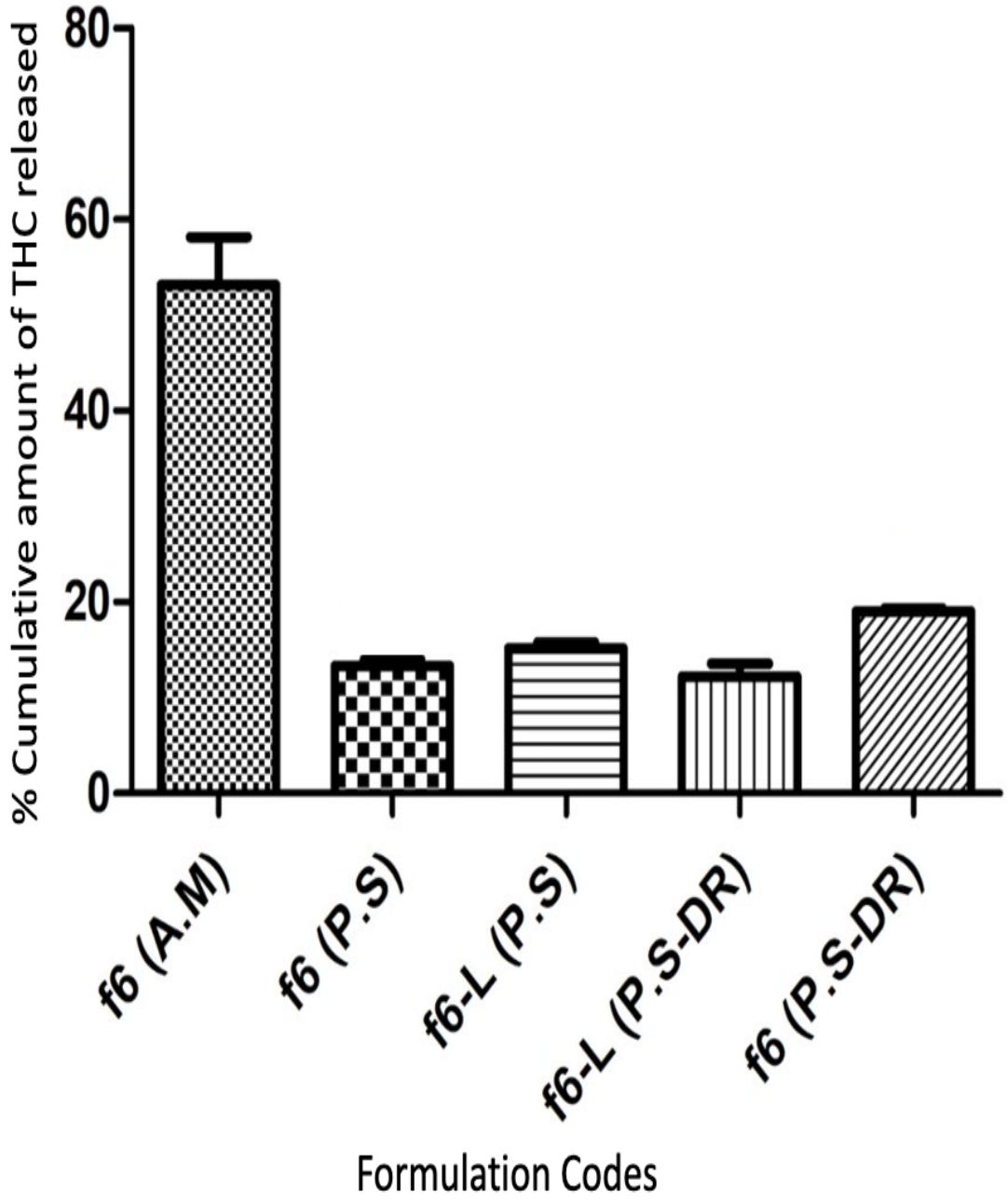


Figure 25. Comparing between cumulative amount of THC released % (at the end of 6 hours) of F6, F6-L formulations through artificial membrane (A.M), pig skin (P.S), and pig skin after derma roller ® application (PS-DR) (n =3).

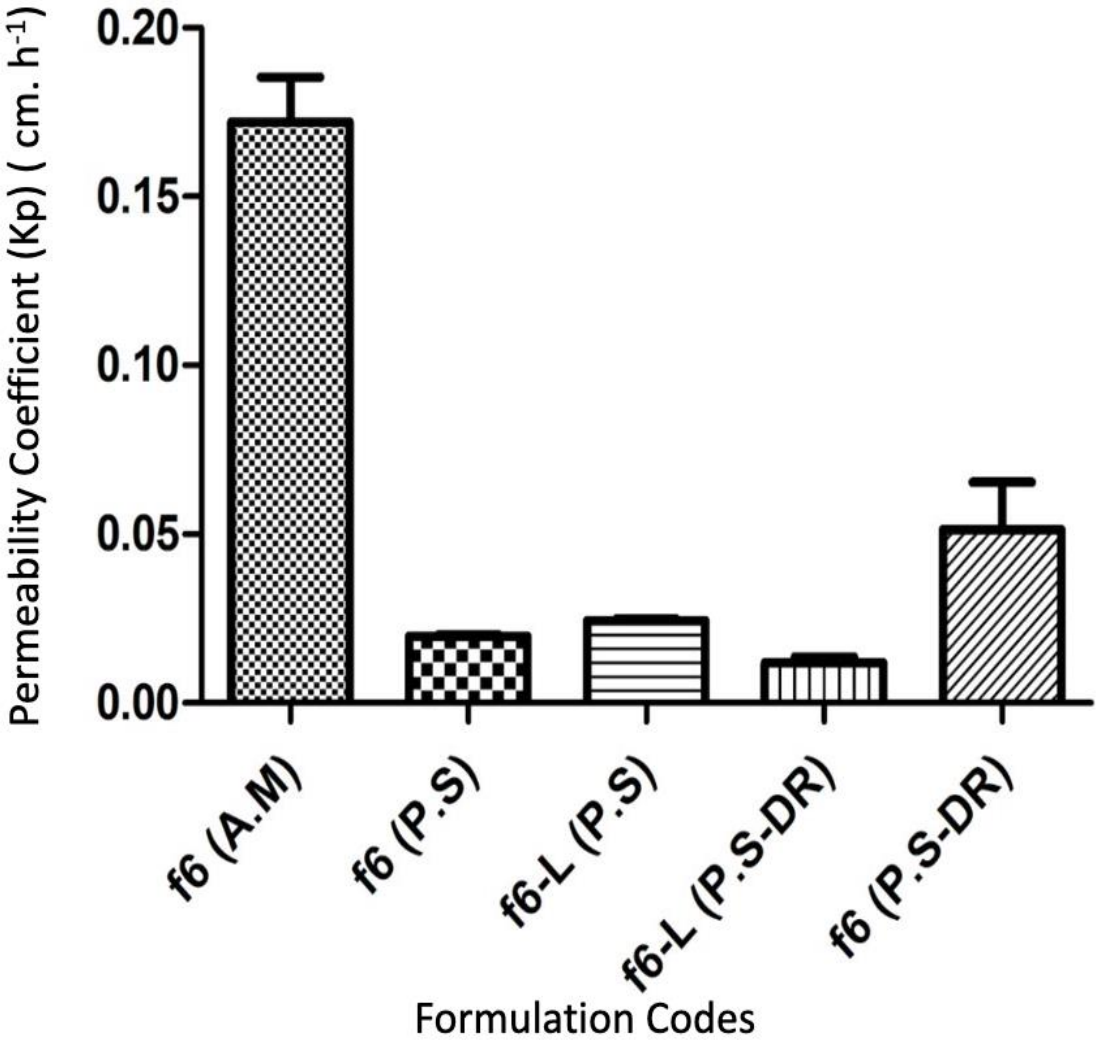


Figure 26. Comparing between the permeability coefficient (Kp) (cm. h^{-1}) of F6, F6-L formulations through artificial membrane (A.M), pig skin (P.S), and pig skin after derma roller ® application (PS-DR) (n=3).

4.5. Amount of THC Released (µg/cm²) from different formulations:

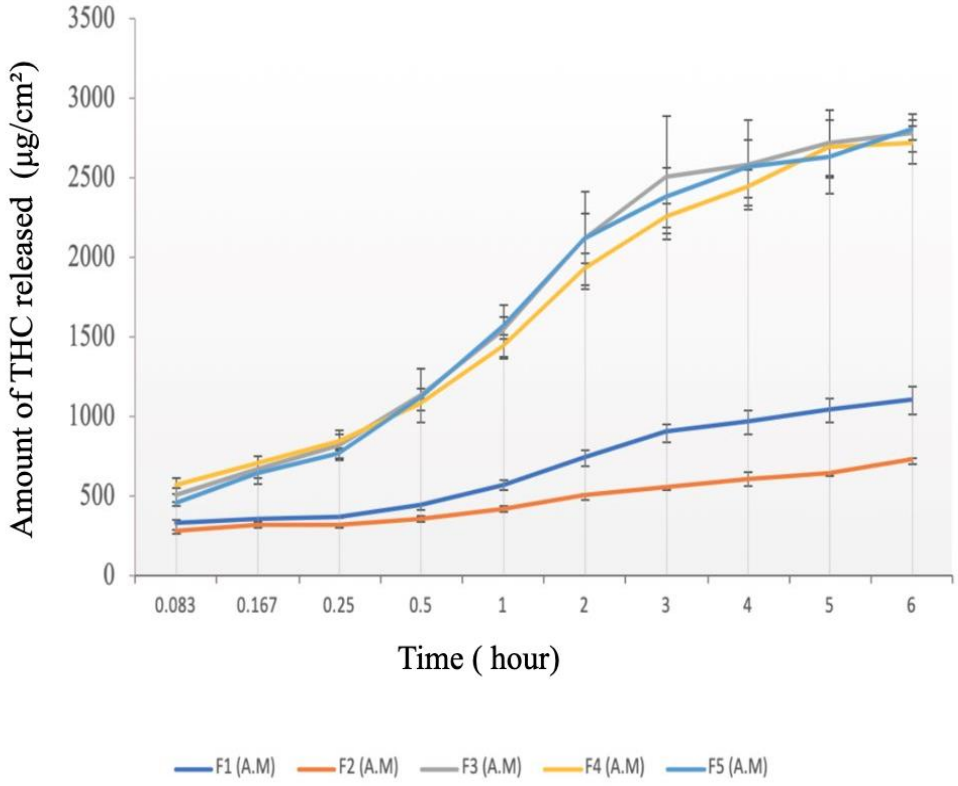


Figure 27. Comparing between the amount of THC (µg/cm²) released from HPMC gel formulations with different concentration (F3, F4, F5) and the amount of THC (µg/cm²) released from F1 and F2 through artificial membrane (A.M) (n=3).

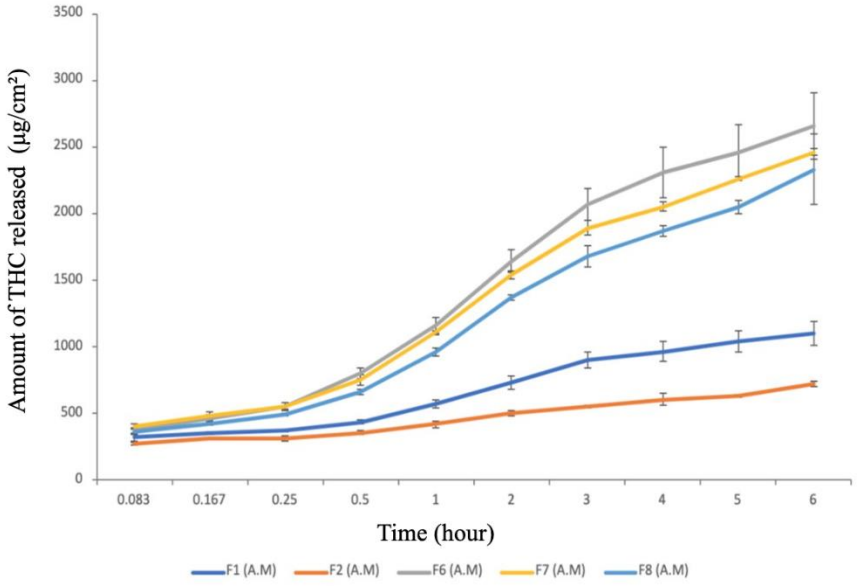


Figure 28. Comparing between the amount of THC (µg/cm²) released from Chitosan gel formulations with different concentration (F6, F7, F8) and the amount of THC (µg/cm²) released from F1 and F2 through artificial membrane (A.M) (n=3).

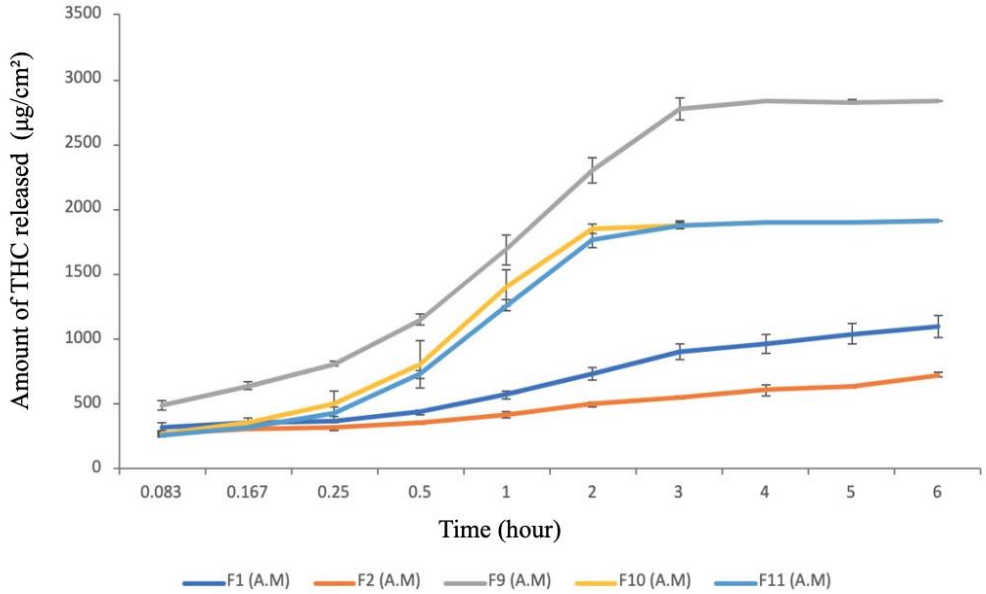


Figure 29. Comparing between the amount of THC (µg/cm²) released from Carbopol gel formulations with different concentration (F9, F10, F11) and the amount of THC (µg/cm²) released from F1 and F2 through artificial membrane (A.M) (n=3).

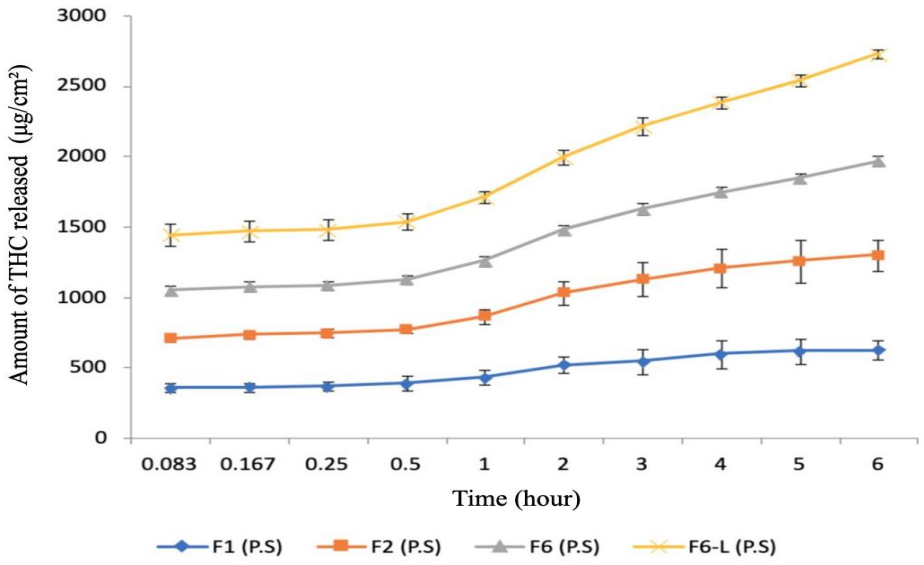


Figure 30. Comparing between the amount of THC released (µg/cm²) from F1, F2 F6, F6-L formulations through pig skin (P.S) (n=3).

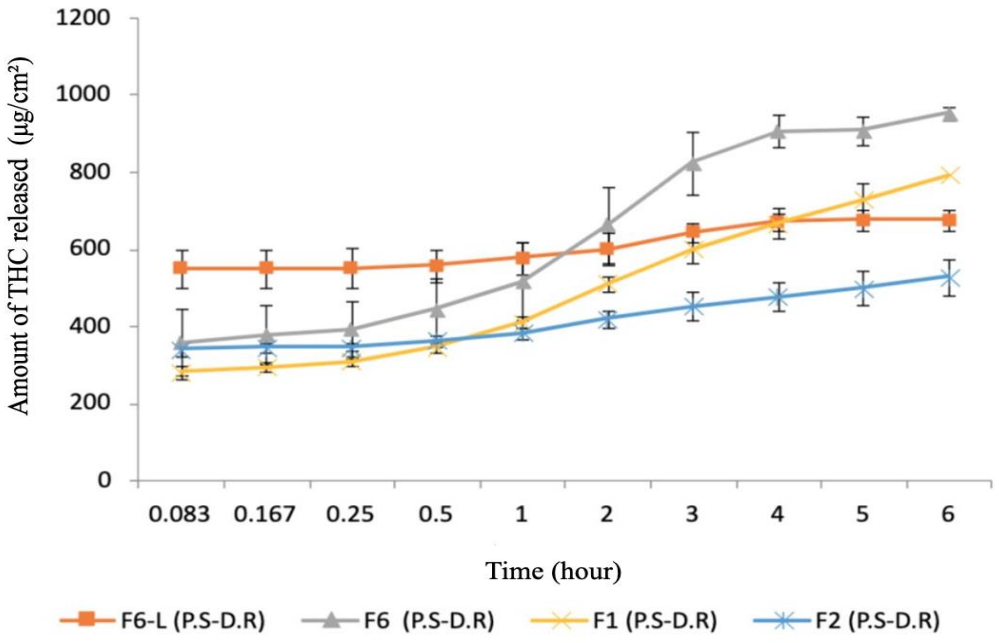


Figure 31. Comparing between the amount of THC (µg/cm²) released from F1, F2, F6, F6-L formulations through pig skin after derma roller ® application (P.S-DR) (n=3).

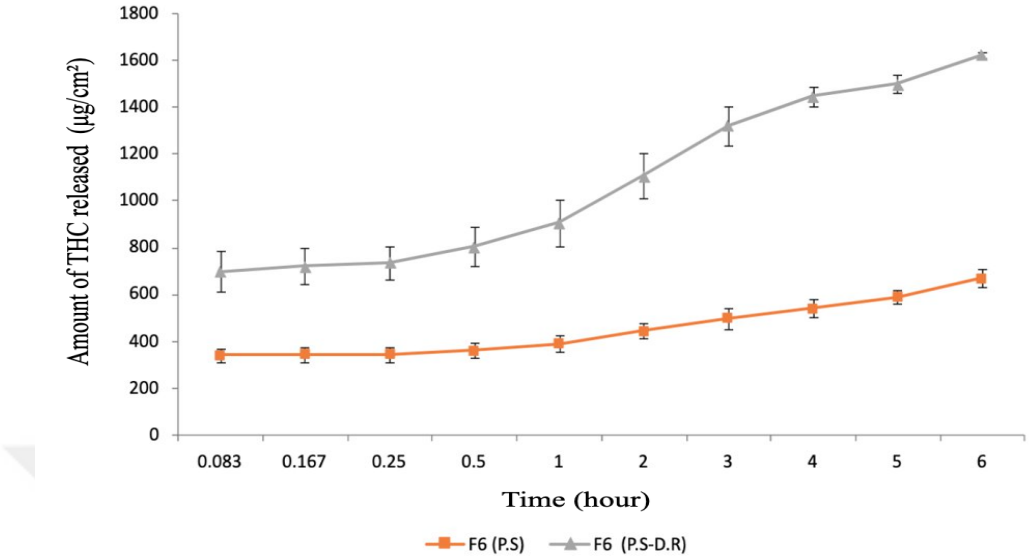


Figure 32. Amount of THC (µg/cm²) released from F6 formulations through pig skin with and without derma roller ® application (n=3).

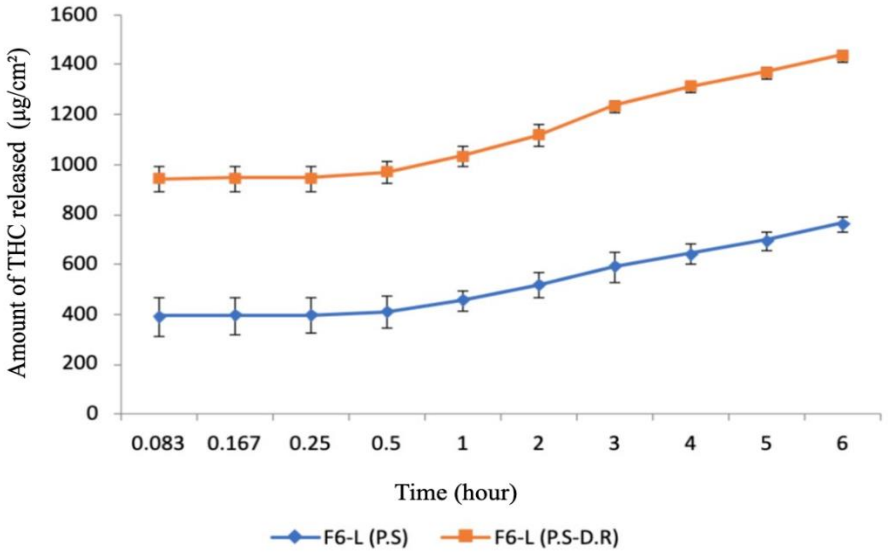


Figure 33. Amount of THC (µg/cm²) released from F6-L formulations through pig skin with and without the derma roller ® application (n=3).

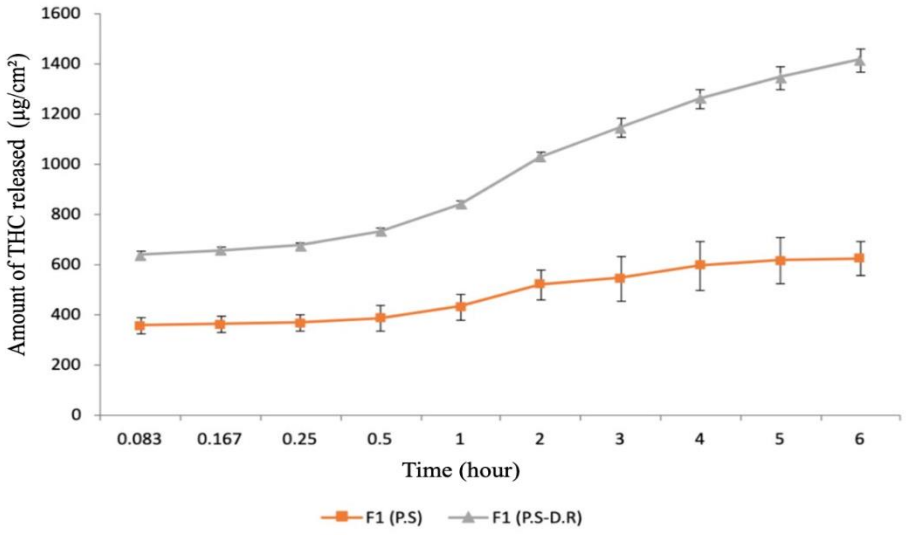


Figure 34. Amount of THC (µg/cm²) released from F1 formulations through pig skin with and without derma roller ® application (n=3).

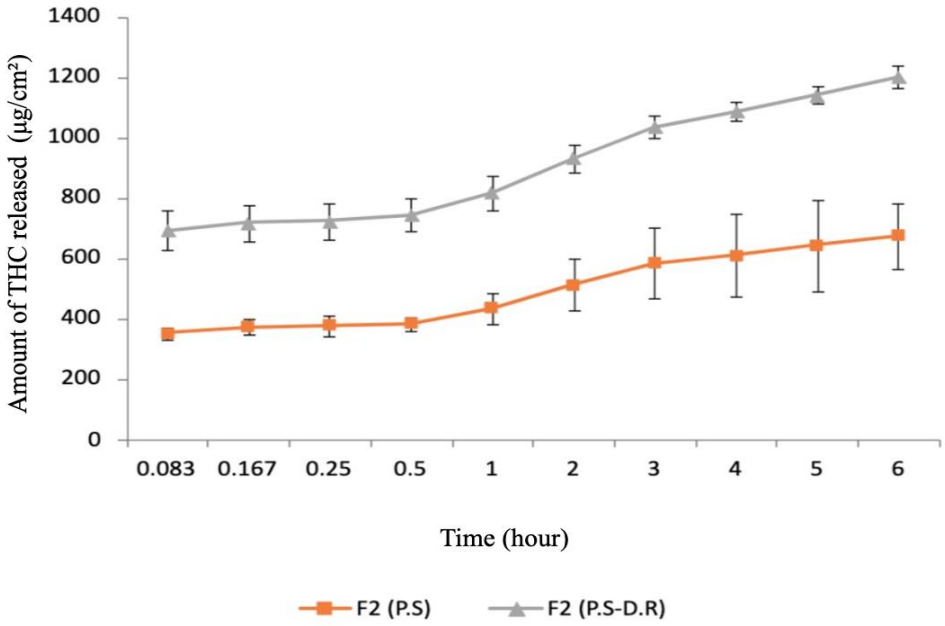


Figure 35. Amount of THC (µg/cm²) released from F2 formulations through pig skin with and without derma roller ® application (n=3).

5. DISCUSSION and CONCLUSION

5.1. Discussion:

The physicochemical properties of THC (TCC) (Relatively high MW, 563.3; low octanol/water partition coefficient: log P= −2.71) [39] is not favorable for skin permeation because of the barrier function of the SC in the superficial dermal layer of the skin, Stratum corneum [68] imposes physicochemical limitations to the type of permeant that can traverse the barrier [8, 9]. For a drug to be delivered passively through the skin it needs to have adequate lipophilicity and also a molecular weight <500 Da [14, 69]. THC was our candidate in this study as active pharmaceutical ingredient (API) to present the skin permeability behavior of hydrophilic drugs, THC has pharmaceutical indications such as analgesic, anti-inflammatory, and muscle relaxant drug. THC can also be used to treat acute and chronic lumbar and sciatic pain, post-traumatic and post-operative pain [39, 40]. and it has been shown to interact with g-aminobutyric acid (GABA) type A receptors (GABAARs) and strychnine-sensitive glycine receptors in the rat central nervous system [70,71]. (Carta et all., Neuropharmacology, 805-815, 2006), THC originates from the flower seeds of Superba gloriosa [72, 73].

Many approaches have been studied and succeeded during the last 20 years to enhance the permeability of poorly absorbed drugs through the skin. A lot of studies have focused on the dermal drug delivery system due to its significant advantages over traditional routes of administration.

All of the approaches chosen had a proven track record of success in cosmetics and medicinal formulations administered dermally [74].

Many articles proved the ability of liposomes and Derma Roller (microneedles) to improve the permeation capability of active pharmaceutical ingredients across the skin, either for animals' skin or human skin [22, 30, 75].

In this thesis, we studied the skin permeation enhancement of THC by using many approaches. We changed the vehicle type [76] from cream and ointment, which are available on the market, and prepared the same formula with gel polymers to evaluate the enhancement of THC in different types of gels as vehicles and try to find a promising formula that can be used in the future.

The type of source influences the selection of polymers used in this study. As for natural-sourced polymers, we have chosen Chitosan, HPMC as a semi-synthetic polymer, and Carbopol as a synthetic one.

Rheology measurements can indicate topical pharmaceutical product spread-ability. It was noticed that increasing the polymer concentration increased the viscosity of the gel preparation, which is not favorable for topically dermally administrated drugs. When we applied the formulations to the rheology measurements, all the investigated formulations revealed pseudo plastic flow non-Newtonian systems exerted by the polymer in the formulation, as shown in (Table 4.1).

They are shear thinning, and their viscosity decreases with the increase of shear rate [61]. The lowest viscosity formula was the Chitosan 2.5% gel. It has the best spreading properties, which indicates an easy spread on the skin and an easy pourability characteristic.

The results demonstrated, as in previous studies, that the Franz Cells Diffusion apparatus was suitable for studying THC penetration into skin [65, 66, 67, 69, 77, 78].

After we chose the best gel polymer as the THC vehicle depending on the highest percent amount of THC release and the rheology results, we incorporated our API into liposomes because of its success and many advantages in the pharmaceutical and cosmetics fields. We added it to the gel and tried to figure out if liposomes approach added value to the formula.

The second alternative for enhancement of THC was to evaluate the effect of derma roller ® (Microneedles) application to pig skin to our gel formulation with and without liposomes as one of the new physical-mechanical techniques used in pharmacy for the same purpose.

In our first step, we measured the in-vitro permeation of our 9 formulations and 2 control formulas Muscoflex (the marketed cream) and Tyoflex (the marketed ointment) by the Franz cell diffusion apparatus with artificial membrane [79].

The results showed that there was a numerical (Figure: 27, 28, 29) and significant increase (*** $p < 0.001$) (Figure: 15,16,17) (Table 4.4.6, Table 4.4.7) by our 9-gel formulations over the control groups, which means enhancement of THC permeation through artificial membrane by gel formulations.

Comparing between HPMC 2.5%, HPMC 3%, and HPMC 3.5% showed there is no significant difference (n.s) (Table 4.4.1) between them, but there is a numerical increase of the cumulative amount of THC release with the least viscous HPMC 2.5% (Figure 27), and the rheology measurements showed also that HPMC 2.5% has the best spreading properties among the higher concentration HPMC gels (Table 4.1).

For the chitosan preparations, Chitosan 2.5% (Table 4.4.2) (Figure 28) and Carpool 0.5% (Table 4.4.3) (Figure 29) were chosen for the same reasons.

The second step was to measure the permeation enhancement capability of the formulations chosen in the previous step (HPMC 2.5%, Chitosan 2.5%, Carpobol 0.5%) and the control groups (F1 = the marketed cream and F2 = the marketed ointment) via pig skin. The results showed that (n.s) (Table 4.4.4) between all formulations, between the gel formulations vs control groups F1 (P.S) and F2 (P.S).

In recent days the conventional drug delivery system has been revolutionized by nanoparticles' ability to manipulate molecules and their structures [80, 81,82], So we decided to study the permeation enhancement of THC through pig skin by using liposomes as a carrier, by formulating THC loaded liposomes and incorporating them into Chitosan 2.5% gel, the highest Jss and permeability coefficient (Kp) value was for the Chitosan 2.5% when compared to other gel formulations (HPMC 2.5%, Carbopol 0.5%) (Figure: 18, 20), and because chitosan is from a natural source, and has good rheology results (Table 4.1), we prepared our formulation THC-Liposomes in Chitosan 2.5% (F6-L) by Thin Film Hydration Technique/Rotary Evaporation-Sonication Method (BANGHAM METHOD) [83].

The ability of liposomes to encapsulate drug compounds is influenced by vesicle size. A high lipid-to-core ratio is preferred for lipophilic and amphiphilic agents, while a larger aqueous core volume is preferred for hydrophilic compound encapsulation. Measurements of vesicular size can be taken in triplicate by the dynamic light scattering (DLS) method [58]. The average of our liposomes vesicular size was (98.76 ± 1.49 d.nm), (Table 4.2.1).

Generally, liposomes produced by the thin film layer method are SUVs. Because size reduction is important in this method and since sonication causes the liposome to shrink, we get an SUV.

A zeta potential value (ZP) determines the surface charge of the liposome particles, which must be measured because of its role in evaluating the physical stability of the particles. [44] Our liposome particles' ZP mean was =-29.3 3.8 mV, (Table 4.2.1).

ZP other than -30 mV to +30 mV is generally considered to have sufficient repulsive force to achieve better physical stability [80].

Results showed through intact pig skin the F6-L (P.S) has higher cumulative amount of THC release among other formulations (Figure 30).

The release profile showed that the THC-liposomes in the Chitosan 2.5% gel F6-L through intact pig skin (P.S) have like sustained and controlled profile and showed higher permeation and release profile compared to the Chitosan 2.5% gel without liposomes F6 (P.S) (Figure 30), but there was no significant difference (n.s) (Table 4.4.5). Between them when the results were applied to the statistical analysis (Figure: 24, 25, 26).

When we applied the Derma roller ® (MN) to the pig skin, we evaluated the permeation enhancement effect on the F1, F2, F6, and F6-L and the statistical results showed that there were no significant differences between the formulations,

Through the pig skin after derma roller application non liposomal formulation F6 (P.S-D.R) showed higher release profile than F6-L (P.S- D.R) (Figure 31) with statistically significant difference (*P < 0.05) (Table 4.4.5), numerical increase for all formulations when comparing between the formulations permeation results through pig skin with and without derma roller applications, the holes created by derma roller facilitated and increased the penetration of the hydrophilic drug THC for all formulations, when

comparing F6 (P.S) vs F6 (P.S-DR) (Table 4.4.5) (Figure 32), And for the liposomal formula F6-L (P.S-DR) which has higher release profile than F6-L (P.S) through intact pig skin without derma roller application (Figure 33) with no significant difference (Table 4.4.5), also for the cream (Figure 34) (Table 4.4.6) and ointment (Figure 35) (Table 4.4.7).

There are two ways to explain this. For example, because of the lipophilicity of liposomes and their bi-lipid structure, liposomes mimic the lipid structure of the skin, which facilitates and increases the permeability and absorption of the drug through intact skin. For the same reason, the liposomes will have the affinity to stay in-between the skin layers act as reservoir. One of the previous studies mentioned that the drugs delivered by liposomes target the upper layers of skin [80].

The statistical results (Figure: 24, 25, 26) revealed a better permeation capability for the F6 (P.S-DR) vs. F6-L (P.S-D. R) (** $p < 0.01$) (Table 4.4.5) after derma roller application. And the cumulative amount of THC released profile was higher with F6 (P.S-DR) against F6-L (P.S-DR) (Figure 31).

No significant difference between cream F1 and ointment F2 through pig skin with or without derma roller application (Table 4.4.8), with higher release profiles after derma roller application for both (Figure 34, 35).

The best results were for the THC-Chitosan 2.5% across pig skin after Derma roller application. (Figure 21, 22, 23, 24, 25, 26, 27, 31), (Table 4.4.5) verify that derma roller application significantly (** $p < 0.01$) enhanced the THC permeation of chitosan gel.

5.2. Conclusion:

THC it has undergone excessive hepatic degradation due to first pass effect; the formulation of pharmaceutical dermal dosage form is an alternative option used for THC administration, the physicochemical properties of THC (relatively high MW, 563.3; low octanol/water partition coefficient: log P= −2.71) are not favorable to the permeation of the drug through the skin, because of the barrier function of the SC, to overcome this obstacle many approaches evaluated in this thesis, the findings confirm our hypothesis that the gel formulation has better permeation profile VS the available marketed THC cream and THC ointment, The best results were for the THC-Chitosan 2.5% across pig skin after Derma roller application. THC loaded liposomes can be a good choice when applying to intact skin and for sustained and control release because the liposomes act as reservoir in the skin layers, and the results also show that the use of derma roller enhance the permeation of the hydrophilic drugs.

The application of the dermaroller® (microneedles) before administration as convenient and easy to apply and perform tool increased the THC permeation from all the formulation (marketed cream and ointment are included) except the liposomal one because we used the very small hight arrays which leads to fast closing of created pores the enhancement was not significantly observed, we suggest the need of future study with different types and arrays lengths of dermarollers with the same formulation.

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