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YEDİTEPE UNIVERSITY

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DEPARTMENT OF COSMETOLOGY

CHARACTERIZATION STUDIES OF LIPID BASED DELIVERY SYSTEM CONTAINING NATURAL ANTIOXIDANT

MASTER THESIS

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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 the Administrative Board of Institute with decision dated ..... and numbered .....

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DECLARATION

I proclaim that this work constitutes my own work and that it contains no material previously published or written by another, and that it has not previously been relied upon for the award of any other degree, diploma or other qualification except as appropriately acknowledged in the text.

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SHAKARCHI . H

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LIST OF SYMBOLS AND ABBREVIATIONS

MeOH	Methanol
nm	Nanometers
L	Liters
ml	Milliliters
ul	Microliters
g	Grams
mg	Milligram
M	Molar
mM	Millimolar
ug	Microgram
°C	Celsius
DPPH	2,2-diphenylpicrylhydrazyl
CUPRAC	Cupric Reducing Antioxidant Capacity
FRAP	Ferric ion reducing antioxidant power
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
TPTZ	Triphenyltetrazolium chloride
UV	Ultraviolet
FTIR	Fourier-transform infrared spectroscopy
MBAR	Millibar Pressure Unit
cP	Centipoise
Rpm	Rotations per minute

SEM	Scanning Electron Microscope
Pa s	Pascal second
SPC	Soybean Phosphatidylcholine
UL	Unloaded Liposome
DLS	Dynamic light scattering

ABSTRACT

SHAKARCHI, H. (2022). Characterization studies of lipid based delivery systems containing natural antioxidants. Yeditepe University, Institute of Health Science, Department of Cosmetology, MSc thesis, Istanbul.

Antioxidants are well familiar in conversations or discussions that include topics such as aging and healthy skin, especially in the fields that concern cosmetics industries. Antioxidants are proven to diminish free radical concentration in the body by different types of mechanisms. Free radicals are considered the cause of many diseases and conditions and skin aging included. Antioxidants, Phenols, and flavonoids all showed great effectiveness through depleting ROS (Reactive oxidative stress) levels and scavenging free radical molecules, natural antioxidants have a tendency of being unstable, these instabilities might affect their penetration through the stratum corneum or generally total antioxidant activities. This research aimed to encapsulate natural antioxidants extracted from the fruit commonly named Indian gooseberry or latinly named *Emblica officinalis* from the family of Euphorbiaceae into small phospholipid nanocarriers. It's believed that liposomes will not only provide assistance for antioxidant transportation through the skin but also it will have enhanced bioavailability and stability. In the research, the final cosmetic formulation was chosen as hydrogel (Carbopol 934) due to controlled release and enhancing the stability of the liposomal formulation. Four different methods (DPPH, ABTS, FRAP, and CUPRAC) for measuring the total antioxidant amount of the extract and formulations were evaluated. The results showed that approximately 43% of total antioxidants are in 1 mg of extract. Moreover, phenols and flavonoids showed 12% of total phenols per 1 mg of extract. Design-Expert® was used to obtain the liposomal optimal formulation. Liposomes were characterized by zeta potential, particle size, and polydispersity index. All the samples' particle sizes were shown to be <108 nm and zeta potentials were shown all to be negative and the polydispersity index was presented to be less than 0.3 indicating monomodal size. The optimal formulation 13 contained 1.8% of soybean and 0.07% of cholesterol. FTIR, SEM, and rheological studies were done for further evaluation and analysis. *In vitro* and *ex vivo* comparison studies between loaded liposomes with gel formulation and *Emblica officinalis* extract only with gel formulation were done using Franz cell diffusion method. *In*

vitro results showed 42.28% cumulative drug release for samples that were incorporated with liposomes which is 23.44% more than samples that were not. On the other hand, *ex vivo* results were considered lower but nonetheless satisfactory where samples that were incorporated with liposomes showed 21% cumulative drug release which was a 6% difference for samples that were not.

Keywords: Loaded liposomes, Gel, Free radicals, Antioxidant, *Emblica officinals*

ÖZET

SHAKARCHI, H. (2022). Doğal antioksidan içeren lipid bazlı taşıyıcı sistemlerin karakterizasyon çalışmaları. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Kozmetoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Antioksidanlar, yaşlanma ve sağlıklı cilt gibi konuları içeren konuşmalarda veya tartışmalarda, özellikle kozmetik endüstrilerini ilgilendiren alanlarda oldukça tanıdıktır. Antioksidanların vücuttaki serbest radikal konsantrasyonunu farklı mekanizmalarla azalttığı kanıtlanmıştır, bu serbest radikaller birçok hastalığın ve durumun nedeni olarak kabul edilir ve cilt yaşlanması da tartışmanın geri kalanına yabancı değildir. vitaminler, fenoller ve flavonoidlerin tümü ROS seviyelerini düşürerek ve serbest radikal moleküllerini temizleyerek büyük etkinlik göstermiştir, doğal antioksidanların kararsız olma eğilimi vardır, bu kararsızlıklar stratum corneum'a nüfuz etmelerini veya genel olarak toplam antioksidan aktivitelerini etkileyebilir. Bu araştırma, Euphorbiaceae familyasından Hint bektaşi üzümü veya Latince adıyla Emblica officinalis olarak adlandırılan meyveden elde edilen doğal antioksidanları lipozom olarak da bilinen küçük fosfolipid nanotaşıyıcılara dahil etmeyi ve kapsüllemeyi amaçlamıştır. lipozomların sadece antioksidanın cilt yoluyla taşınmasına yardımcı olmakla kalmayıp aynı zamanda biyoyararlanım ve stabiliteyi de arttıracağına inanılmaktadır. Seçilen kozmetik formül, kontrollü salım ve toplam formülün stabilitesinin arttırılması gibi nedenlerle hidrostatik veya hidroelastik jel (Carbopol 934) olarak dikkatli ve uzun bir değerlendirmeden sonra belirlenmiştir.Meyvenin toplam antioksidan miktarını ölçmek için 4 farklı yöntem uygulanmıştır. DPPH, ABTS, FRAP ve CUPRAC. 1 mg ekstraktta toplam antioksidanların yaklaşık %43'ünü gösteren DPPH, 4 yöntem arasında en doğru olanıdır. fenoller ve flavonoidler hem ölçülmüş hem de değerlendirilmiş ve 1 mg ekstrakt başına toplam fenollerin %12'sini göstermiştir. lipozom yöntemlerinin karakterizasyonu, 3 farklı yanıtın değerlendirildiği bir DLS cihazı kullanılarak gerçekleştirilmiştir. zeta potansiyeli, partikül boyutu ve polidispersite indeksi. Tüm örneklerin partikül boyutunun <100 nm olduğu ve zeta potansiyelinin hepsinin negatif olduğu ve polidispersite indeksinin monomodal boyutu gösteren 0,3'ten az olduğu gösterilmiştir. tüm veriler optimum formülasyonu hesaplamak için bir Design-Expert® ile çalıştırılmıştır. in vitro ve ex vivo karşılaştırma, yöntemler franz hücre difüzyon yöntemi

kullanılarak yapılmıştır. in vitro sonuçlar, lipozomlarla birleştirilen örnekler için %42,28 ilaç kümülatif salınımı göstermiştir, bu da olmayan örneklerden %23,44 daha fazladır. Öte yandan, ex vivo okumalar daha düşük ancak yine de tatmin edici olarak değerlendirilmiştir; lipozomlarla birleştirilen örnekler, birleştirilmeyen örneklerden %6 farklı olarak %21 ilaç kümülatif salınımı göstermiştir. Daha ileri değerlendirme ve analiz için FTIR, SEM ve reolojik çalışmalar yapılmışt

Anahtar Kelimeler: Yüklü lipozomlar, Jel, Serbest radikaller, Antioksidan, Emblica officinals

1. INTRODUCTION

There has been a lot of interest in the topic of free radical chemistry in recent years. In order to tackle our thesis's main goal we first need to grasp a better understanding on the bases of free radical chemistry and antioxidants' role in diminishing or minimizing them. Free radicals are byproducts of normal human metabolism, they are defined as molecules or atoms that are one electron short in their orbital vacancy (odd electron number) and are able to exist with their current unstable state [1]. Its unstable state causes it to become highly reactive and withdraw electrons from normal molecules making them free radicals themselves thus creating a chain reaction till it damages the overall total cell activities [2]. Reactive oxygen species and reactive nitrogen species (ROS, RNS) are subsets of free radicals [3]. In low or moderate concentrations they can be somewhat harmless or even so beneficial such as the involvement in a variety of physiological functions like the immune function and in a number of many cellular signaling pathways, in mitogenic response and in redox regulation [4] but in high concentrations they can cause what is called oxidative stress or nitrosative stress. A condition where these unstable molecules damage DNA protein and lipids resulting in multiple diseases such as diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, cataracts, cardiovascular diseases, respiratory diseases as well as in the total aging process. You can acquire these free radicals from internal sources which are basically as mentioned above from normal human metabolism including mitochondria, xanthine oxidase, inflammation, phagocytosis, and ischemia [5], or externally from radiation, ozone, smoking, and environmental pollutants.

To understand the dire situation, we need to have a better apprehension of the words "oxidative stress". The damage resulting from ROS and RNS can be so versatile from cardiovascular diseases, cancer, inflammatory diseases such as arthritis ischemic diseases (heart diseases, stroke, intestinal ischemia), hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurological disorders (Alzheimer's disease, Parkinson's disease, muscular dystrophy), and most importantly an accelerated aging process [6] which we will weigh in heavily on it in this research. Our cells are in a constant phase of regeneration or repair.

These processes require healthy lipids and proteins created from stable molecules and electrons. Research showed that reactive oxidative damage affects cells and leads them to a path where they are prone to aging [7] aging process alongside other diseases was shown to have either a direct or indirect connection with free radicals causing oxidative damage [8] aging mainly is caused by damaged DNA or cellular function damage [9] research proved that one of the best ways to battle aging is reducing and diminishing free radicals to safe levels with the help of antioxidants. Methods such as lifestyle modification including a plant-based diet shown to have a significant sum of antioxidants [10], ingesting supplements orally to a more local specific effect on the desired area such as skin creams or gels with the antioxidant being placed or spread topically.

1.1 Antioxidants Background

Antioxidants are the opposite of free radicals in being stable molecules on top of that, their ability to donate an electron to a very unstable free radical molecule thus helping in the reduction of the potential damage the free radical molecule could cause [11]. These low molecular particles are able to interfere with and stop the oxidative damage chain reaction, we must keep in mind that these antioxidants are not exclusive to the human body. The body has the ability to produce in normal condition sufficient sums of different types of antioxidants to combat any excessive production of free radicals. Antioxidants such as glutathione, ubiquinol, and uric acid, the same as free radicals are also all produced during normal metabolism in the body [12]. Theoretically, our body seems to be able to combat these free radicals but when it comes to supplying itself with antioxidants, our unfortunate environmental circumstances such as UV damage from the sun, air pollutants from vehicles and industrial facilities, combined with the processed food diet that is not only having damaging ingredients to the body overall but detouring the human need or craving for healthier options that are antioxidant sufficient. The healthy human diet demands the body to be more familiar with and exposed to various types of antioxidants such being vitamins such as vitamin E (α-tocopherol) and vitamin C (ascorbic acid), B-carotene, and anthocyanins. These antioxidants are obtained mainly from the diet [13]. Antioxidants act

as radical scavengers, hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, synergists, and metal-chelating agents. Both enzymatic and nonenzymatic antioxidants exist in the intracellular and extracellular environments to detoxify ROS [14].

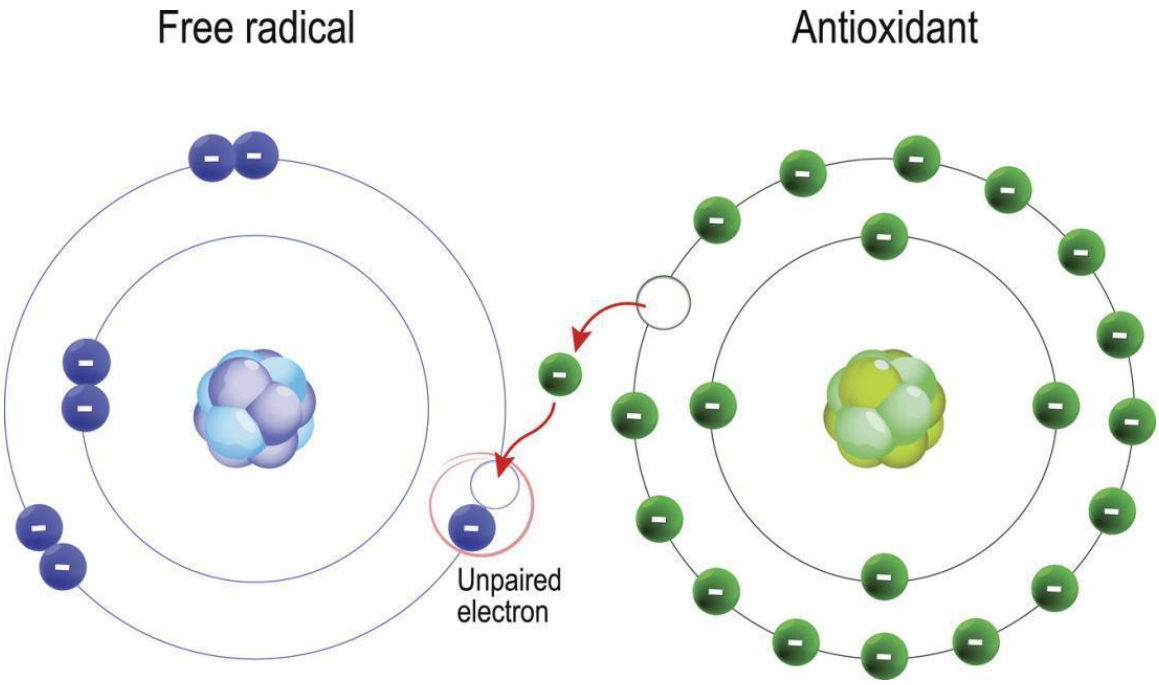

Figure 1.1. Basic antioxidant mechanism in combating free radicals[15].

1.2 Natural Antioxidants and Cosmetics

Plants have long been utilized for therapeutic purposes, we are currently witnessing more products that contain natural oils, herbs, and fruits hitting the cosmetic industry market more commonly. This goes back to the fact that natural products are more appealing to consumers [16]. Plants were the major source of all cosmetics prior to the development of synthetic chemicals with similar qualities [17]. Natural plant compounds continue to excite researchers' curiosity. Using extracts, on the other hand, needs strict attention to the extraction processes such as plant-to-solvent ratios, and active-ingredient levels. Consumers, who are more concerned about purchasing eco-friendly and minimal chemically involved products, want the usage of plant extracts in skincare products[18]. Botanical goods are high in vitamins, antioxidants, essential oils and oils, hydrocolloids,

proteins, terpenoids, and other bioactive components that if extracted correctly from the plant will maintain their antioxidant activities and levels and be useful in creating competent cosmetic formulas. Natural antioxidants used in the cosmetic business include versatile substances and extracts derived from a wide range of plants, grains, and fruits, and are capable of lowering oxidative stress on the skin or protecting cells in general from oxidative degradation [19]. Plant extracts may be found in a wide range of patents and commercial cosmetic products. Plant extracts are typically included in cosmetic formulations such as green tea, rosemary, grape seed, basil grape, blueberry, tomato, acerola seed, pine bark, and milk thistle. These extracts contain natural antioxidants such as polyphenols, flavonoids, flavanols, stilbenes, and terpenes (including carotenoids and essential oils)[20]. According to their function, antioxidants are categorized as primary or natural antioxidants and secondary or synthetic antioxidants. Primary antioxidants include minerals (such as selenium, copper, iron, zinc, and manganese), vitamins (C and E), and Phyto-antioxidants. A mineral antioxidant, in general, is a cofactor of enzymatic antioxidants[21-25]. Secondary or synthetic antioxidants bind to free radicals and prevent the chain reaction from continuing. Secondary antioxidants include BHA, BHT, propyl gallate, metal chelating agents, tertiary butylhydroquinone, and nordihydroguaiaretic acid[26,27].

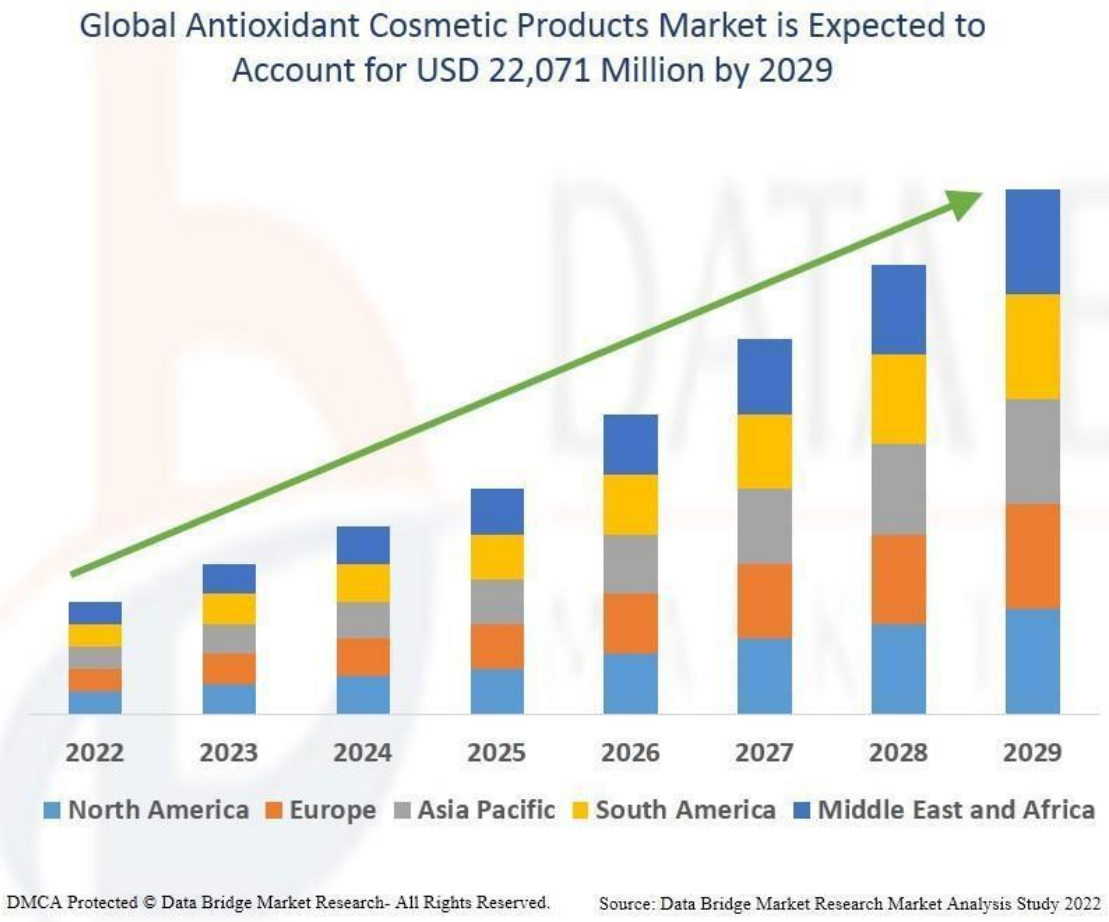

Figure 1.2. Antioxidant cosmetics market size, shares and massive growth[28].

1.3 *Emblica officinalis*

Commonly named as Indian gooseberry this fruit has a long history in Ayurvedic medicine, which is one of the oldest medical systems that originated in India 3000 years ago, *E. officinalis* is widely distributed in tropical and subtropical countries like China, India, Indonesia, and southeast Asia [29] its is believed to have a number of therapeutic benefits and is used as a nutritional supplement. It's a small to medium-sized fruit belonging to *Euphorbiaceae* family. *Emblica officinalis* fruits are considered to have a very rich source of ascorbic acid, further more, the Indian fruit has powerful antioxidant, immune-modulating, and anticancer properties because of its content of vitamin C, tannins, and flavonoids. The fruit extract has been shown to have hypolipidemic, anti-diabetic, anti-

inflammatory, and inhibitory effects on the growth of tumors, gastric ulcers, and retroviruses like HIV-[30,31]. The aqueous extract of the *Emblica* fruit is a powerful inhibitor of lipid peroxidation and a radical scavenger, exhibiting outstanding antioxidant effects.

Figure 1.3. Visual representation of both Dried and Fresh *Emblica officinalis.*

In recent studies, it was shown that *Emblica officinalis* stimulates the formation of pro-collagen and prevents MMP-1 in mammals[32]. Flavonols, such as quercetin, myricetin, and kaempferol, make up the majority of the phenolic chemicals found in gooseberry fruits. Caffeic, coumaric, hydroxybenzoic, and ellagic phenolic acids also have been found in gooseberry fruits[33].

Table 1.1. *Emblica officinalis* antioxidant content (mg/g) [34]

	Antioxidants and general contents	**Percentage(%)**
1	Moisture	81.2%
2	Protein	0.5%
3	Fat	0.1%
4	Carbohydrates	14.1%
5	Fibre	3.4%
6	Calcium	0.05%
7	Mineral matter	0.7%
8	Phosphorus	0.02%
9	Iron	0.0012%
10	Nicotinic acid	0.0002%
11	Vitamin C	0.6%

Table 1.2. *Emblica officinalis* antioxidant content after drying[35].

	Antioxidant content	**Percentage%**
1	Gallic acid	1.32%
2	Tannin Gum	13.57%
3	Albumin	13.08%
4	Crude cellulose	17.08%
5	Mineral matter	4.12%
6	Moisture	3.8%

1.4 Ascorbic Acid and Cosmetology

One of the naturally occurring antioxidants in nature is vitamin C [36]. The majority of plants and animals can produce vitamin C in their living systems from glucose. Because L-glucono-gamma lactone oxidase, a necessary enzyme for the in vivo production of vitamin C [37], since this enzyme is absent in humans and certain other vertebrates, they must obtain vitamin C from natural sources thus classifying ascorbic acid as an essential vitamin, The most prevalent antioxidant in human skin is vitamin C [38], it is able to form multiple mixtures of enzymatic and non-enzymatic antioxidants that work together to shield the skin against reactive oxygen species (ROS) not only that but also acts as a cofactor for collagen biosynthesis[39]. Since ascorbic acid is highly water soluble[39]. It finds itself inside the cell compartment where its antioxidant mechanism is favorable. Once the skin has its share of UV light exposure, ROS in many forms starts affecting the cells, that's where ascorbic acid plays a vital role in combating these free radicals with the donation of electrons creating a more stable molecule rather than a radical thus subsiding the damage resulting from the UV light[40].

1.5 Ascorbic Acid and *Emblica officinalis*

*Embilica officinal*s is a very healthy fruit that is high in vitamin C, amino acids, and minerals[41]. Since It is a very high source of vitamin C, with 30 times the amount found in oranges. The edible *Emblica officinalis* fruit tissue has three times the protein concentration and 160 times the ascorbic acid awareness of an apple[42]. The fruit also has a greater awareness of most minerals and amino acids than apples. Chromium, zinc, and copper are found in *Emblica officinalis* (amla) fruit ash. It is an apoptogenic substance that boosts immunity. It includes a variety of chemical compounds like tannins, alkaloids, phenols, and many more. Among all hydrolyzable tannins, emblicanin A and B, gallic acid, and ellagic acid have been linked to biological activity.

1.6 Stability of Ascorbic Acid

Controlling ascorbic acid's instability is a critical difficulty in the creation of cosmetic formulations, it requires special conditions to be prepared or stored in, hence a range of preparations containing ascorbic acid or its derivatives have been researched to not only evaluate their stability and distribution through the skin but reassuring utmost antioxidant activity and concentration[43-45]. Ascorbic acid is highly sensitive to air and light; and to achieve its stabilization in cosmetic preparations, it has been suggested to use ascorbic acid in microencapsulation form[46], careful consideration should be taken while combining ascorbic acid with other chemical moieties because its high instability it is easily oxidized in aqueous solutions and cosmetic formulations. This will be shown in the purpose section further in this research.

1.7 Phenols and Stability

studies found that *Emblica officinalis* contains an abundant amount of polyphenolic compounds majorly responsible for its strong antioxidant activities[47]. Since it forms a huge part of the fruit we must understand what are these compounds in nature, what they do, and their stability conditions if we were ever to create a cosmetic formulation. phenolic compounds are secondary constituents found in many plants and fruits that we are familiar with in our day-to-day lives, they have a significant contribution to how plants taste and smell and not only that but for apparent color as well[48]. The beneficial properties of phenols mainly focus on antioxidant activities, they can either work as radical scavengers or chain breakers depending on their chemical structure[49,50]. All and all phenolic compounds are considered stable when it's present naturally inside the plant or fruit but when it comes to the extraction process or producing formulas containing these compounds some factors might drastically change or alter the concentration or activity of the phenolic compounds, these factors include extraction solvent temperature storage condition and pH[51]. An appropriate delivery system such as lipid nanoparticles (liposomes) have a great effect on stabilizing phenolic compounds, studies showed that encapsulation of phenolic compound contributes greatly to their bioavailability, stability, and solubility through the conduction of several *in vitro* and *ex vivo* tests [52]. By applying the liposomal

encapsulation technique not only we will benefit from more stabilized phenols but also we will observe a better absorption of these phenolic compounds leading to the treatment of many skin issues that concerns the cosmetic industries such as pigmentation, skin aging, and solar exposure.

1.8 Skin Anatomy

The stratum corneum, the outermost layer of the skin, is a selectively permeable, heterogeneous epidermal layer that provides protection against dryness and environmental damage while retaining sufficient moisture to function. The skin serves several purposes. It acts as a defense against water, microbial invasion, mechanical and chemical harm, and UV radiation damage. The skin is made up of two layers: the inner dermal layer, which assures strength and suppleness and supplies the epidermis with nutrients, and the outer epidermal layer, which is highly cellular and serves as a barrier. The barrier nature of skin, on the other hand, makes it difficult for substances to be transferred or penetrate through it. Even with small dispersed molecules that theoretically easily penetrate the stratum corneum we still need the incorporation of a competent nano-delivery system that ensures utmost stability and penetration.

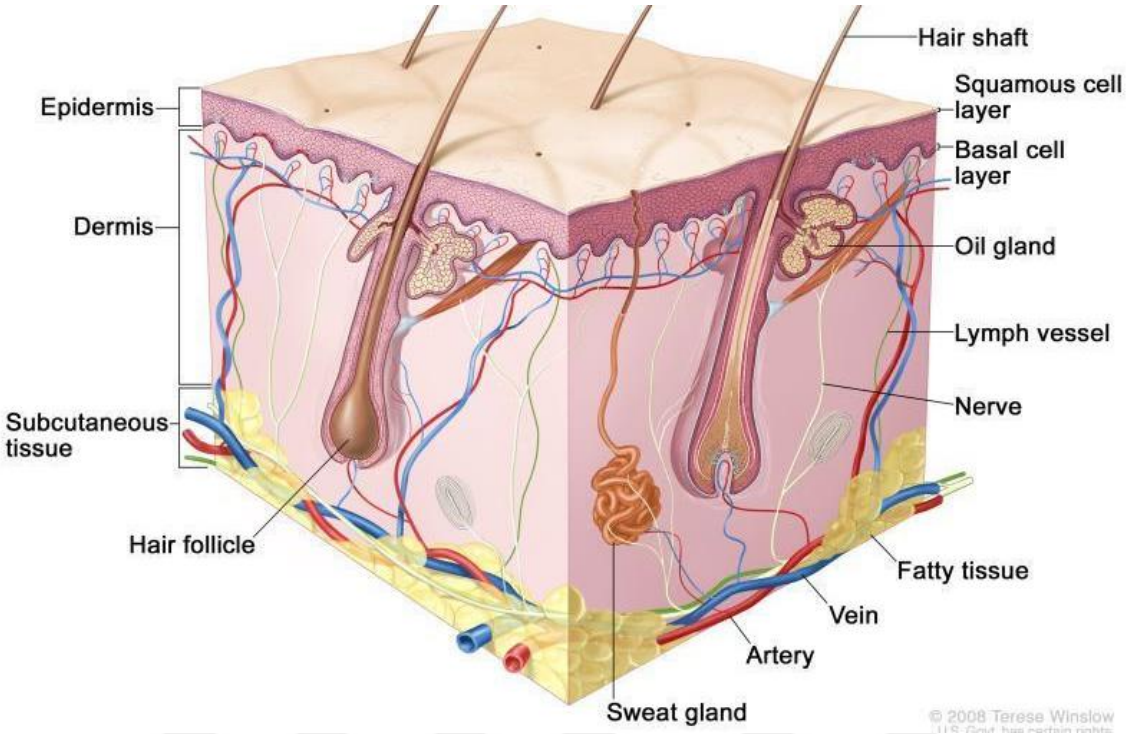

Figure 1.4. Schematic structure of the skin and detailed epidermis[53].

1.9 Liposomes as a Delivery System

Drug delivery system by definition is the method or process of providing a pharmaceutical substance a way to achieve a therapeutic effect in people or animals. These methods, especially for peptide and protein therapies, offer intriguing alternatives to parenteral drug administration. Several medication delivery devices have been developed and are being tested for this purpose. The stratum corneum, the skin's outermost layer, contains flattened dead epidermal cells (corneocytes) enclosed in hydrophobic lipid domains, which serve as the primary barrier[54]. Stratum corneum corneocytes are completely enclosed by crystalline lamellar lipid areas. The cornified envelope, or cell border, is a tightly cross-linked protein structure that inhibits drug absorption into cells[55]. A liposome is a spherical vesicle with a phospholipid and cholesterol bilayer membrane. Liposomes are small vesicles in which an aqueous volume is completely encapsulated by a lipid molecule membrane This structure allows lipophilic drugs to be incorporated into lipid bilayers and hydrophilic drugs to be incorporated into the aqueous compartment[56].

Liposomes can improve cutaneous drug distribution while limiting systemic absorption because their lipids are identical to those of the epidermis[57]. UV radiation has a wide range of acute and chronic impacts on the skin. Erythema is the skin's acute reaction to UV exposure. Long-term UV exposure causes photoaging and photocarcinogenesis in the skin, which is thought to be caused by immunological suppression and mutations[58]. The increased interest in photoprotectants such as sunscreens has resulted from a recent focus on the detrimental effects of UV radiation on the skin. UV-blocking effects in lipidic delivery systems like liposomes are depending on the lipid content and particle size. The higher the sunscreen action, the smaller the particle size[59].

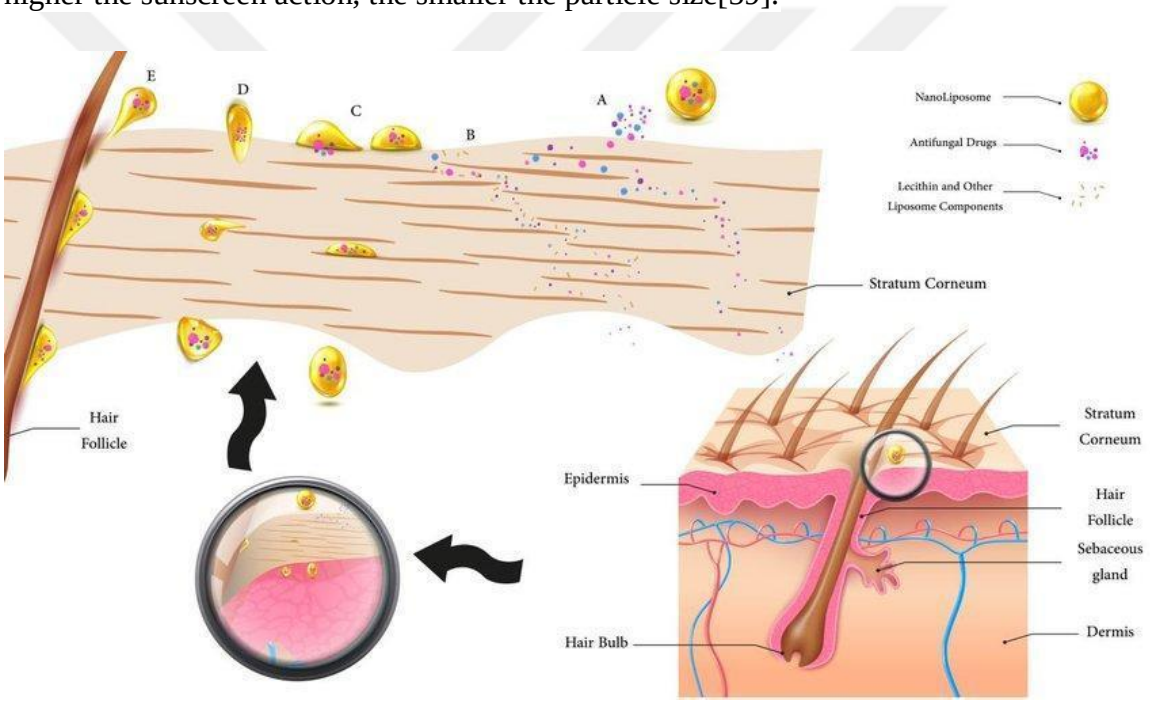

Figure 1.5. A summary on the mechanism of action of liposomes[60]

1.10 Significance of Liposomes

Some studies showed that liposomes acted as a much better delivery system than other conventional formulations, the study tested the octyl methoxycinnamate a UV absorber substance showing clearly better SPF quality having the substance being distributed in the stratum corneum with less penetration to the deeper areas and less systemic absorption[61]. Aging of the skin is a multifaceted process combining genetic, environmental, and hormonal factors. Both intrinsic and extrinsic aging is influenced by free radicals. Cell

metabolism produces free radicals during the chronologic aging process, whereas exogenous causes such as UV exposure, cigarette smoking, and alcohol consumption produce them during the extrinsic aging process[62]. The main difficulty for topical delivery of antioxidants is its stability in the formulations and efficient penetration into the skin. Liposomes have been suggested as a suitable carrier for these purposes many studies conducted for the sake of proving the effectiveness of liposomal and antioxidant combination one of those were Sodium ascorbyl phosphate which is known as a potent free radical scraper antioxidant, it was loaded with liposomes and showed better stability and penetration through the stratum corneum[63]. Other antioxidant substances showed great affiliation with the liposomal delivery system such as Dispersed ascorbyl palmitate, it was incorporated into liposomes with the help of cathodal electric assistance it was shown to have a very effective penetration throughout the skin [64].

Table 1.3. Benefits of loading drugs in liposomes[65].

1. Solubility improvement of amphiphilic and lipophilic drugs
2. non-toxic, flexible, biocompatible, and non-immunogenic
3. Supplying a sustained release method for liposomes that are delivered locally or systemically.
4. increase efficacy and therapeutic index of drugs
5. Site-specific targeting
6. Improving the transport of hydrophilic and charged molecules
7. increase of drug\molecule stability

1.11 Gel Dosage Forms Application in Cosmetics

Gels are semisolid systems that have a liquid phase inside a three-dimensional polymeric matrix (made of natural or synthetic gums) with a high degree of physical (or occasionally chemical) cross-linking. Some of these systems look as clear as water, visually appealing as gelatin desserts, while others are turbid looking. Gels are made up of a two-phase system[66], in which inorganic particles are not dissolved but rather scattered throughout the continuous phase and big organic particles are dissolved in the continuous phase and randomly coiled in the flexible chains. Ideally, gel formulation should have the following properties. First and foremost Ideally, the gelling agent must be inert and safe and it cannot react with the active ingredients or other constituents found in the formulation for safety and stability reasons. Second, the gelling agent should be able to produce a visible and sensible solid-like form at the time of storage which can be easily broken or disrupted when exposed to shear forces at the time of topical application. It should also have a suitable anti-microbial agent in case of a bulky dosage. The topical gel must not be overly sticky or adhesive for ease of use. The apparent viscosity or gel strength increases with an increase in the effective crosslink density of the gel. However, a rise in temperature may increase or decrease the apparent viscosity, depending on the molecular interactions between the polymer and solvent. gels also should exhibit the mechanical characteristics of the solid state. Each component should show continuity throughout the system. High degree of attraction amongst the dispersed phase and water medium should be present so the gel remains equally uniform upon standing and doesn't freely settle[67-70]. Gels in their nature have many advantages It has the ability to avoid first-pass metabolism. They are normally easy to prepare and Convenient to apply compared to other topical dosage forms such as patches, etc. They can also evade the risks and inconveniences of intravenous therapy and of the varied conditions of absorption, like pH changes, presence of enzymes, gastric emptying time, etc. since gels are easier to apply there is a form of Achievement of efficacy with lower total daily dosage of the drug by continuous drug input. It has the ability to avoid fluctuation in drug levels, inter- and intra patient variations. Ability to easily terminate the medications, when needed. Gels have a relatively larger area of application in comparison with the buccal or nasal cavities, gels have the ability to deliver and hold drugs more selectively to a specific site. Avoidance of gastrointestinal

incompatibility. Providing utilization of drugs with a short biological half-life, narrow therapeutic window, improving physiological and pharmacological response and patient compliance and Providing suitability for self-medication.

1.12 Purpose of This Research

Our goal would be to improve the penetration and stability of the antioxidants and phenols found inside the fruit of *Emblica officinalis*, getting them through the stratum corneum into the depth of the epidermis where antioxidants are much needed in the role of diminishing free radicals. Very few articles have studied the effect of liposomes delivery system combined with the fruit of *Emblica Officinalis* in a cream formulation, although results were satisfactory some articles suggest that the main constituent ascorbic acid requires very sensitive conditions in a cream formulation such as the choice of emulsion, choice of oil phase, emulsion consistency, pH level and choice of emulsifying agent to achieve optimal stability[71]. Hence our research will be conducted with the goal of testing the optimal effectiveness in gel form since some articles believe ascorbic acid is most stable in formulations containing thioxigel and so on[72].

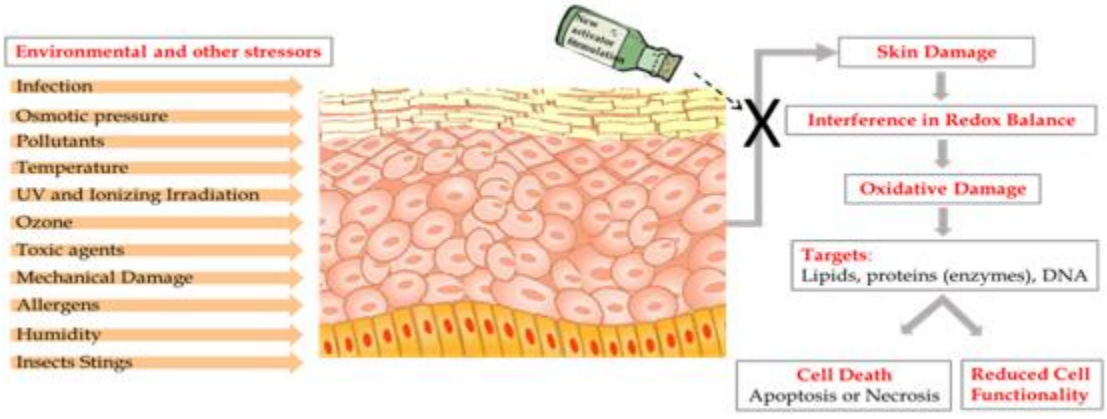

Figure 1.6. Types of free radicals and their residual areas in the skin[73].

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment and Devices Used

The following laboratory equipments and devices were all used respectively and accordingly to help achieve the required methods in this research.

Table 2.1. All equipments and devices used in this research and their manufacture details.

Equipments and devices	Model\Company	Country of manufacture
Rotary evaporator	Heidolph	Germany
Freeze drier	Christ alpha	Northern Ireland
Orbital shaker	Ika ks50\Sigmaaldrich	Germany
Magnetic stirrer	Scilogex	USA
Microcentrifuge	spectrafuge 7m	Germany
Vortex mixer	WN-2800 \Weightlab	Turkey
Analytical balance	Ohause explorer	Switzerland
FTIR spectrometer	NICOLET IS50\Thrmo scientific	USA
Zetasizer	Nano ZS 3600 malvern panalytical	USA
Franz diffusion cell	Permegear	USA
Microplate reader	Thermo scientific varioskan\thermo fisher	USA

Ion sputter	Mc100\Hitachi High Tech	USA
Rehometer	Kinexus\Malvern instrument	USA
Scanning electron microscope	Zeiss EVO 40	USA
Viscometer	Brookfield DV2	USA
Rheometer	Anton paar mcr 302	USA
Probe sonicator	Bandelin Sonoplus HD 2200\Istec	Germany
Labrotary mill	Arcelik K3104	Turkey

2.1.2 Chemical and Compounds used

The following chemicals were all used respectfully and accordingly in the aim of achieving this research

Table 2.2. All chemicals used in this research.

Chemical\Compound\Reagent	**Company of manufacture**
Ethanol	Merck-millipore (Germany)
Methanol	Merck-millipore (Germany)
Chloroform	Merck-millipore (Germany)
Cholesterol	Merck-millipore (Germany)
SPC	Merck-millipore (Germany)
Copper (II) Chloride	Merck-millipore (Germany)

Neocuproin	Merck-millipore (Germany)
Ammonium acetate pH(7)	Merck-millipore (Germany)
Trolox	Merck-millipore (Germany)
Potassium Bromide	Merck-millipore (Germany)
Carbopol 934	Serva (Germany)
Quercetin	Merck-millipore (Germany)
Gallic acid	Merck-millipore (Germany)
10% Aluminum Chloride	Merck-millipore (Germany)
Sodium Hydroxide	Merck-millipore (Germany)
DPPH	Sigma Aldrich (Germany)
Iron Trichloride	Merck-millipore (Germany)
TPTZ	Merck-millipore (Germany)
Sodium Acetate pH(3.6)	Merck-millipore (Germany)

2.2 Methods

2.2.1 Plant Material

The dried fruits of *Emblica officinals* were bought in Pakistan Punjab region from a Bazar located in pinanwal district (32°39'29.3" N 73°17'01.4" E) in the 20th of April. The dried fruit was packed in tightly sealed nylon bags and was stored in a dry environment with a relative humidity of 30 ± 10% away from any light exposure for future preservation prior to the analysis and use.

2.2.2 Extraction Method

35.7 grams of the dried fruit of *emblica officinalis* were weighed and measured with an analytical balance, then collected and grinded using a laboratory mill, after that the grinded extract was weighed at 35.4 grams, 500 mL of 50% ethanol was measured in a graduated cylinder then shortly after it was mixed with the dried grinded extract, covered the bottle with aluminum foil alongside the cap with parafilm and put into orbital shaker set to 150 rpm and left overnight for relatively 14 hours. Filter papers with pore size 20~25 μm were used to filter the extract. At that point, we evaporated the ethanol phase using a rotary evaporator under the temperature 45°C and pressure was used initially at 200 mbar and gradually reduced to 80 during the evaporation process. The extract was frozen in a laboratory fridge after that for one night. Christ freeze dryer was used to lyophilize the extract under -55°C degree and 0.200 mbar overnight for approximately 9 hours, extract powder was then measured at 13.8g which yielded 39% of total initial plant weight extract powder was then poured inside an amber bottle for further light protection and stored inside a laboratory refrigerator of -4°C for further use.

2.2.3 Design Optimal Experimental Design

Using D-optimal mixture experimental design, the ideal liposomal formulation was found. D-optimal design was used to assess the effects of four independent variables on response variables, including zeta size, mean particle size, and polydispersity index. The independent variables were the amount of soybean (A), cholesterol (B). Previous studies were used to identify the parameter range for the current inquiry. In order to pick the best fitting model and improve the process, Design-Expert® (version 13, Stat-Ease Inc., Minneapolis, MN, USA) was utilized. **Table 3.2** displays the variables and their levels that were employed in the design.

The analysis of the variance tables was conducted, and the effect and regression coefficients of the various linear models, as well as the correlations between the variables, were all calculated, determined, and planned accordingly. The experimental design resulted in combinations. Based on comparisons of many statistical characteristics offered by Design Expert® software, including the coefficient of variation, the multiple correlation

coefficient (R^2), and the adjusted multiple correlation coefficient (adjusted R^2), three optimal formulations were the results of the calculations, and one desired formulation was chosen. By conducting experiments under the circumstances listed in **Table 3.4**, the ideal variances were confirmed.

2.2.4 Liposomes Characterization and Preparation

Unloaded liposomes were prepared for further characterization tests with the following measurement **Table 3.2** according to the design expert. The Bangham method of preparation was used to prepare unloaded liposomes[74]. Cholesterol and soybean phosphatidylcholine were used in all of the preparation of the unloaded liposomal samples.

The two components were all measured under an analytical balance, after that 10 mL of chloroform alongside 5 mL of methanol were used to mix and dissolve both cholesterol and soybean phosphatidylcholine. Rotary evaporator was then used to evaporate the organic phase under the constant rotary movement of 80 rpm, 60 ˚C and 600 mbar the organic phase was fully evaporated, topped up with 10 mL of distilled water the samples were all put into an ultrasonic probe sonicator device for 15 minutes and 7 cycles each. 1 mL of each sample was then taken and added into eppendorf tubes prior to further characteristic analysis. A zeta sizer device also known as DLS was used to determine three characteristics and responses which were zeta potential, particle size, and polydispersity index all under the temperature of 25 °C [75]. One drop of each sample was taken and diluted with 2 drops of purified water inside a cuvette. Each sample underwent triplicate scans, giving 33 results.

2.2.5 Sample Preparation for Total Antioxidants, Phenols, and Flavonoids

6 samples were prepared, 3 of them containing liposomes loaded with our extract and the other 3 were liposomes loaded with extract mixed with gel formulation. All samples consisted of Soybean phosphatidylcholine, cholesterol, and extract powder. They were then dissolved in 5mL of methanol then topped with 10 mL of chloroform for dissolving

liposomes and was put into a bath sonicator for complete dissolution. Samples were then poured into flasks and put under an evaporation method (known as bangham method)[74] with a rotary evaporator. After full evaporation samples were mixed with the aqueous phase adding distilled water then sonicated with a probe sonicator for 7 cycles for 15 minutes. Gel formulations were prepared using 2.6 g of carbopol mixed with 100 mL of water and stirred with a magnetic stirrer. 10 grams out of each sample were measured and collected and topped with 20 grams of gel 8 mL of each sample were taken and mixed with 8 mL of meOH 1:1 poured respectively inside eppendorf tubes centrifuged then filtered and stored inside vials for further tests. The samples were labeled as (G1, G2, G3, L1, L2, L3).

Table 2.3. Samples measurement that were used in total phenols, antioxidants and flavonoids tests

Sample	**Cholesterol**	**Soybean**	**Extract**	**Gel (Carbopol)**	**Total size**
G1	70mg	180mg	250mg	20mL	30mL
G2	140mg	320mg	500mg	20mL	30mL
G3	140mg	320mg	750mg	20mL	30mL
L1	70mg	180mg	250mg		10mL
L2	140mg	320mg	500mg		10mL
L3	140mg	320mg	750mg		25mL

Figure 2.1. Sample preparation regarding further total antioxidant and phenol tests.

2.2.6 Total Phenolic Content

50 ul of water, 50 ul of 10% folin-ciocalteu reagent and 100ul of 0.35 M sodium hydroxide were added to microplates containing 50ul of either sample solution, 50ul of gallic acid (4-125 ug/mL) or blank (ethanol 50%). After 3 minutes absorbance was measured at 760 nm wavelength by a microplate reader [76].

2.2.7 Total Flavonoids Content

50 ul of either sample solution, quercetin (16-250 ug/mL) or blank (ethanol50%) were added inside a microplate each cell was then topped with 30 ul of aluminum chloride, 30 ul of 1M sodium acetate and 130ul of distilled. Solution was kept at room temperature for 30 minutes furthermore absorbance was measured at a 415 nm wavelength [77].

2.2.8 Total Antioxidants Activity

Ferric reducing antioxidant power assay (FRAP) was performed according to the procedure of benzi and stern. Frap reagent containing ($2 \square 10^{-2}$M $Fecl_3$), $1 \square 10^{-2}$ M TPTZ and sodium acetate buffer (pH3.6) were mixed with the ratio of 1:1:10) total reagent volume was 280 ul. It was then added to cells containing 20 ul of either sample solution,

Trolox (4-128 ug/mL), or blank (ethanol 50%), the plate was kept incubated for 6 minutes after that the reaction was measured, and detected at a wavelength of 595 nm.[78]

280ul of 0.1mM DPPH methanolic solution was prepared for the determination of DPPH radical scavenging activity 20 ul of sample solution, trolox (6-250 ug/mL), or blank (ethanol 50%) were topped with the DPPH reagent, 30 minutes was the time it was given to achieve a reaction before measuring the absorbance at a 520 nm.[79]

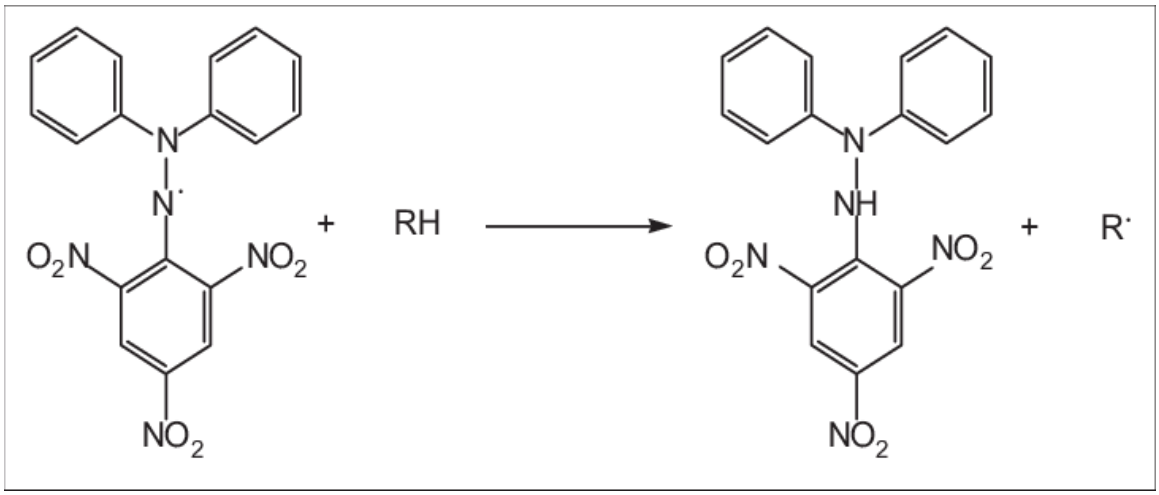

Figure 2.2. Structure of DPPH and its reduction by an antioxidant

ABTS radical scavenging reaction method was conducted where 20 ul of test solution which either was blank (methanol), Trolox (4-125 ug/mL), or sample was added into microplates topped with 280 ul ABTS reagent which was prepared with the following measurement ($7 \square 10^{-3}$ M ABTS and $2.45 \square 10^{-3}$M potassium persulfate were mixed and kept for 12-16 H in room temperature then the solution was diluted with the ratio of 1:10 with MeOH absorbance was then measured at 734 nm.[80]

Cupric reducing antioxidant capacity (CUPRAC) procedure was prepared and done with the following 1 mL of $1 \square 10^{-2}$ M copper (II) chloride 1 mL of $7.5 \square 10^{-3}$ M neocuproine 1 mL of 1 M ammonium acetate pH(7) mixed in vortex then 1 mL of sample solution, blank

(methanol) or trolox (8-128 ug/mL) and 0.1 mL of water were added on top microplates were left for 30 minutes before measuring absorbance at wavelength 450 nm.[81]

2.2.9 Fourier Transform Infrared (FTIR) Analysis

All 6 samples were prepared and freeze-dried before conducting the analysis samples were as follows (loaded liposomes, unloaded liposomes, loaded liposomes with gel, extract with gel, extract alone and gel alone). Using Fourier transform infrared at a wave number between 400 and 4000 cm^{-1} the spectrum was measured. Samples were cut using a scalpel blade and were added to the pellet mixed and grinded with potassium bromide. It was then added to the crystal where the absorbance was measured.

2.2.10 *In vitro* Formulation Release Test

Franz cell diffusion method was conducted for this test, 6 cells were used 3 for each sample, the samples of choice were 2 (loaded liposomes with gel formulation and extract with gel formulation). The release membrane used in this experiment was artificial cellulose acetate (Spectra / Por Regenerated Cellulose, Molecular weight cut off 8-10 kDa). The diffusion area was 5.29 cm^2 and the volume of the receiving phase was 20 mL of 0.9% isotonic solution of NaCl pH (7.8) with a temperature of (37°C ± 0.5°C) to simulate human conditions. 2 g of each sample was placed on the membrane respectfully covered with parafilm, injectors were used to draw 1 mL from the receiving phase in these following time intervals (5 min, 15 min, 30 min, 60 min, 120 min, 180 min, 240 min and 300 min). Samples were labeled in eppendorf tubes and stored for further DPPH radical scavenging activity method.[82][83]

2.2.11 *In vitro* Radical Scavenging Activity Test

0.1 mM of ethanolic DPPH was prepared by weighing 3.92 mg of DPPH and dissolved with 100 mL of absolute ethanol. The mixture was then poured into a volumetric flask,

covered with foil, and sealed tightly to prevent oxidation from the air and degradation from light. 20 ul of blank (ethanol) or samples were taken and topped with 280 ul of DPPH reagent, microplates were then covered with foil and a time of 30 minutes was given for a reaction. Absorbance was then measured by a microplate reader under 520 nm wavelength. A change of color was apparent indicating a strong reaction and successful release.

2.2.12 Skin Preparation for Franz Cell Diffusion Method

In order to further simulate the penetration of the formula to the human skin. Pig ear skin samples were needed for reasons its resemblance to human skin. Pieces were obtained from Acibadem experimental research center and kept in a -80 °C freezer for 24 hours. Skin samples were then taken out the next day and underwent preparation. It was carefully done by using a sharp knife to cut the layers of fat underneath the skin to assist the samples with more convenient penetration. Razors were then used carefully not to damage the stratum corneum and to rid the skin of the hairs that might affect the absorption of the formula. All skin samples were disinfected with ethanol and washed carefully with distilled water then wrapped in aluminum foil and stored inside the freezer for further use.

Figure 2.3. Prepared membrane samples prior to franz cell diffusion method

2.2.13 *Ex vivo* Formulation Release Test

In order to further simulate the penetration of the formula closest to the human skin. pig ear skin was used for reasons being it shares resemblance to the human skin. Pieces were obtained from Acibadem experimental research center. Preparation was carefully done by using a sharp knife to cut the layers of fat underneath the skin to assist the samples with more convenient penetration. Razors were then used carefully not to damage the stratum corneum and to rid the skin of the hairs that might affect the absorption of the formula. Formulation of choice were loaded liposomes with gel and plain extract with gel. Similar to the *in vitro* method. Franz diffusion cells were used. The skin was spread carefully on top of the cell topped with 2 grams of each sample. Receiver phase was isotonic NaCl pH(7.8) with the temperature of the bath set to (37°C ± 0.5°C) for further human condition simulation. Injectors were used to draw 1 mL of each cell with the following time intervals (5 min, 15 min, 30 min, 60 min, 120 min, 180 min, 240 min, and 300 min) keeping in mind refilling each cell with fresh solution every time a sample was collected. Samples were then collected and labeled inside eppendorf tube for

further DPPH radical scavenging tests.

Figure 2.4. Franz diffusion cells set up (3X3 loaded liposomal gel formulations and extract with gel formulation)

2.2.14 *Ex vivo* Radical Scavenging Test

0.1 mM of ethanolic DPPH was prepared by weighing 3.92 mg of DPPH and dissolved with 100 mL of absolute ethanol. The mixture was then poured into a volumetric flask, covered with foil, and sealed tightly to prevent oxidation from the air and degradation from light. 20 ul of blank (ethanol) or samples were taken and topped with 280 ul of DPPH reagent, microplates were then covered with foil and a time of 30 minutes was given for a reaction. Absorbance was then measured by a microplate reader under 520 nm wavelength. A weaker change of color was noticed compared to the *in vitro* method but nonetheless, the results were satisfactory.

2.2.15 Rheological Behavior Estimation of the Formulation

Rheometer was used to further study and determine the rheological properties and behaviors of the formulations of choice being (gel with loaded liposomes, gel with extract and blank gel alone). The rheometer is of the Couette type. The measuring system that was used was C18 concentric cylinders. The sample total volume was about 2 mL. Formulations were added to the surface of the sample with the goal of preventing any further evaporation of the solvent. Extended shear analysis of each formulation was conducted. In shear rate ramp mode and using parallel steel plate (CP4/40) (0.8 mm of the gap). Flow curves were measured ranging from 0,1 s^{-1} to 100 s^{-1}. The measurements are done at room temperature, 25.0°C and body temperature 37°C To ascertain the maximum strain amplitude for the gel, also strain-controlled measurements were conducted on each sample. The three-dimensional network of the gel is destroyed over a specific strain amplitude. As a result, measurements above this point do not capture the physical characteristics of a gel. The greatest strain was used for all subsequent rheological property measurements.

2.2.16 Liposomal Morphology

The properties of the liposomes were analyzed using a scanning electron microscope (SEM). To prevent electron charging, All liposomal samples that underwent proper lyophilization were sputter-coated with Au-18k at a thickness of 20 nm (Ion sputter mc1000, Hitachi High Tech, USA). Carbon tape was used to further maintain the lyophilized liposome powders in the SEM stub. Before Obtaining the final images, any extra powder was removed by lightly hitting the stub.

3. RESULTS AND DISCUSSION

3.1 Total antioxidants, Phenols, and Flavonoids

Total phenols regarding the experiment, absorbance was measured and noted for each sample as triplicate, and a calibration curve was calculated using Gallic acid (4-125 ug/mL) where R^2 value was closest to 1 and the plot of the standards was linear providing the necessary formulation to further conduct calculations.

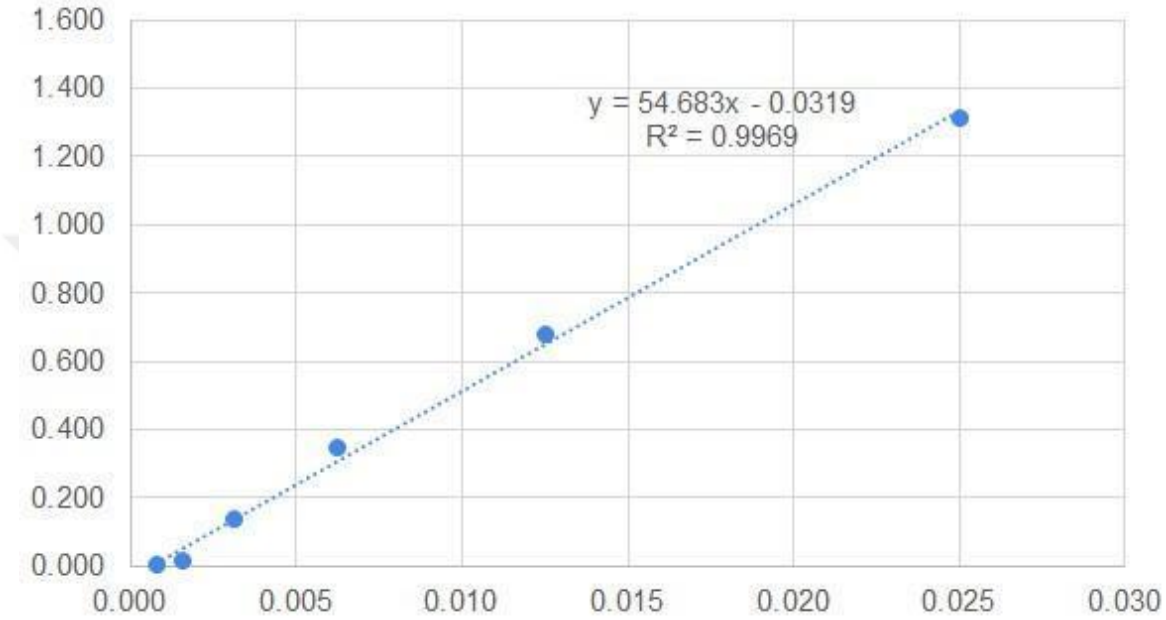

Figure 3.1. Calibration curve of gallic acid at an absorbance of 760 nm.

Results were considered as follows (mg/sample gallic acid equivalent total phenol content)

Total flavonoids regarding the experiment, absorbance was measured and noted for each sample as triplicate, a calibration curve was calculated using Quercetin (16-250 ug/mL) where R^2 value was closest to 1 and the plot of the standards was utmost linear providing us with the necessary formulation to further conduct calculations.

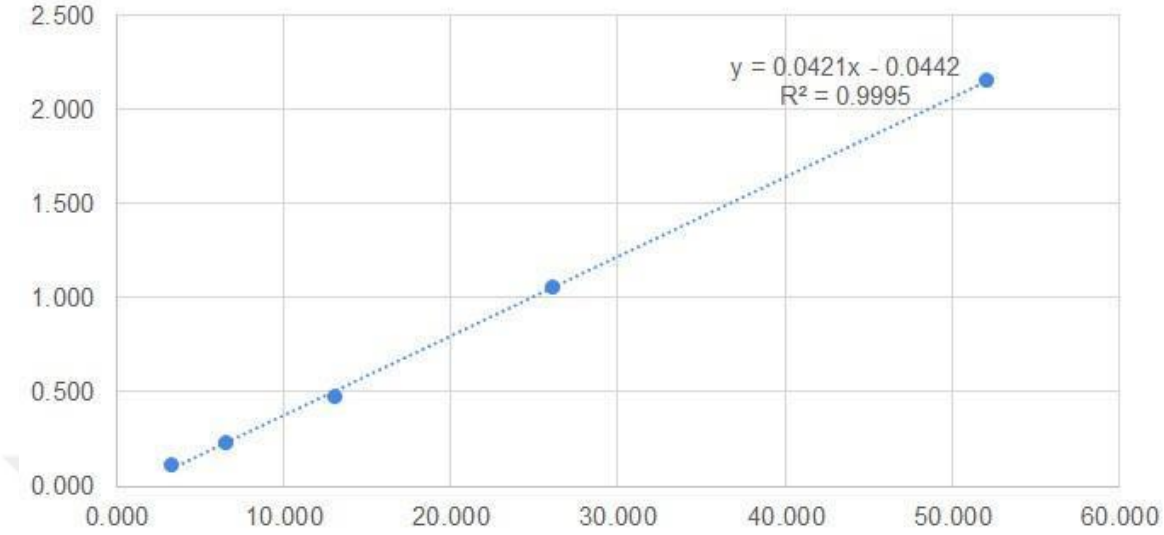

Figure 3.2. Calibration curve of quercetin at an absorbance of 415 nm.

Results were considered as follows (mg/sample Quercetin equivalent total flavonoid content)

Total antioxidant (DPPH, FRAP, CUPRAC, ABTS). Regarding these 4 variable tests absorbance was measured respectively for each sample as triplicate (different amount of absorbance for each test). Calibration curve was deduced using Trolox where R^2 value was closest to 1 and the plot of the standard was most linear.

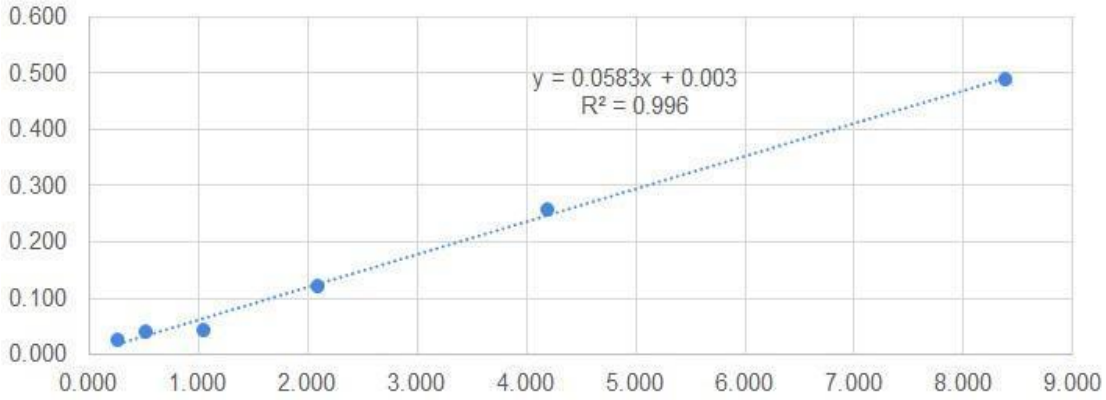

Figure 3.3. Calibration curve of trolox at an absorbance of 520 nm.

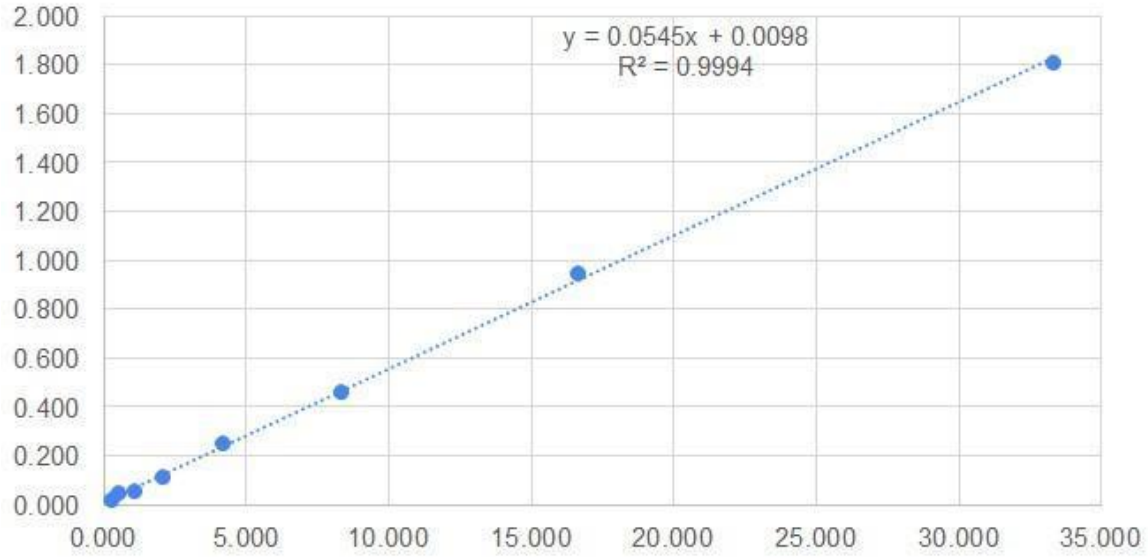

Figure 3.4. Calibration curve of trolox at an absorbance of 450 nm.

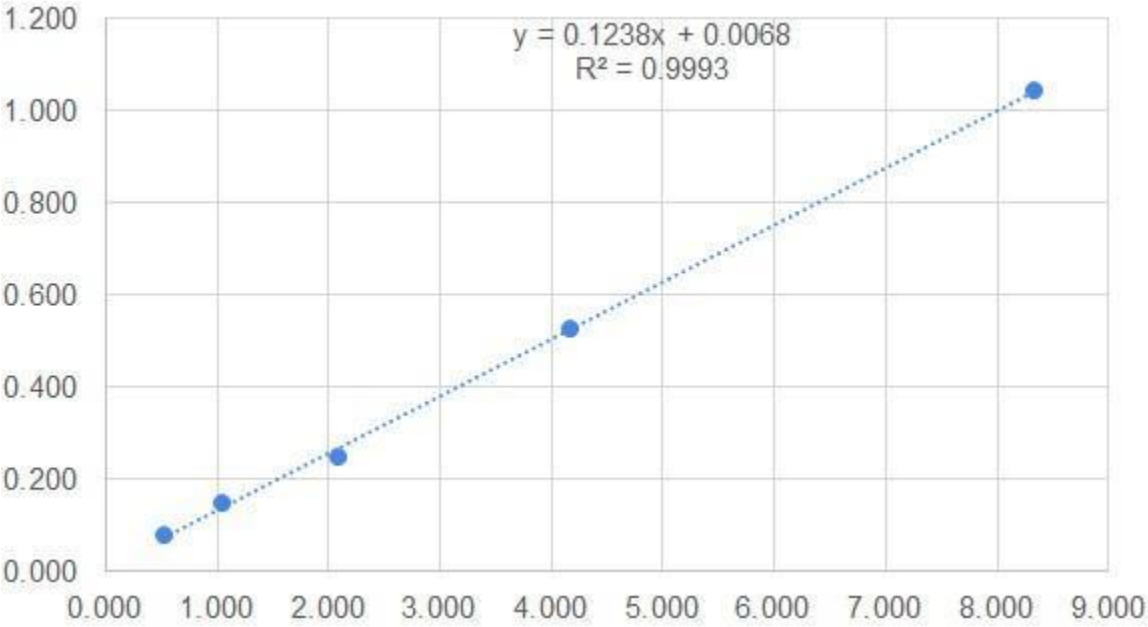

Figure 3.5. Calibration curve of trolox at an absorbance of 595 nm.

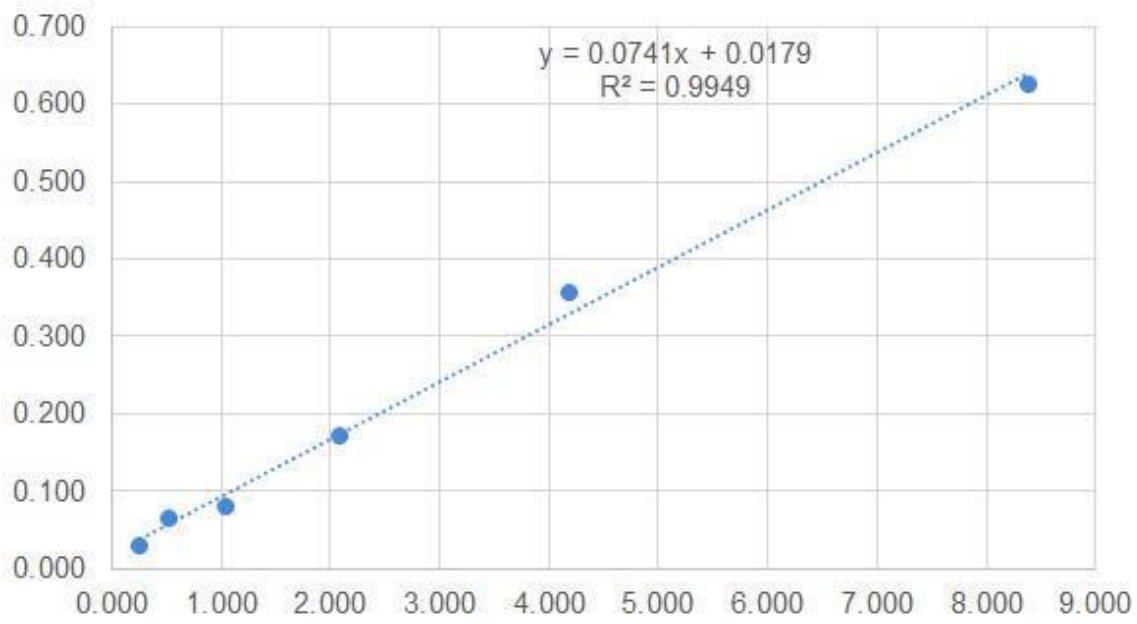

Figure 3.6. Calibration curve of trolox at an absorbance of 734 nm.

Results were considered as follows (mg/sample Trolox equivalent total Antioxidant content).

Table 3.1. Total phenols, falvonoids and antioxidants results are presented as mean±SD

Total phenols, flavonoids and total antioxidants (mg/sample)						
Samples	total Phenols[a]	total Flavonoids[b]	DPPH[c]	FRAP[c]	CUPRAC[c]	ABTS[c]
G1	29.7±0.8	0.52±0.01	103.7±4.4	59.3±4.2	90±5.9	96.1±6.4
G2	67.1±0.46	0.94±0.02	211.3±26.4	142.2±6.4	208.4±4.06	244±14.6
G3	119.7±2.52	1.7±0.06	370±25.05	217±13.05	358.4±16.8	384±22.7
L1	33.8±2.3	0.4±0.002	102±6.7	80.5±6.5	120±9.1	92.5±2.4

L2	70.1±5.1	0.74±0.001	209±11.8	181.6±9.4	263.2±20	140±13
L3	91.2±5.2	1.15±0.005	360±30.6	262.4±19.7	486.7±15	222±18.1

[a] mg/g gallic acid equivalent total phenolic content

[b] mg/g quercetin equivalent total flavonoids content

[c] mg/g Trolox equivalent total antioxidant content

DPPH method was the most accurate among all the other antioxidant methods. The results were very close to samples that had similar extract amounts. This goes back to the possibility of the free radicals being the least unstable compared to the other radicals used in further methods. DPPH method is fast, invasive, and cost-effective the only drawback the method has is the inability to fully react with phenols and flavonoids. DPPH does not react with flavonoids without substituted OH in the B-ring furthermore, it could be said that DPPH scavenging mechanism counts highly on the degree of electron delocalization in a structure-activity study[84,85].

FRAP on the other hand showed a different amount per sample from the other methods because it is believed that Plant extracts antioxidant potential is greatly enhanced by the presence of phenolic compounds the ferric reducing potential is related to the phenolic content[86,87]. In another article, it was observed that the phenolic content in some of the extracts showed a much higher correlation with reducing power than the radical scavenging activity. It could be estimated that the phenolic compounds present in the extracts act as antioxidants directly through the mechanism of reduction rather than the scavenging mechanism[88].

ABTS is considered the most sensitive out of all the methods thus explaining the difference in the amounts, the reason being that filtration, centrifuge, and storage all had a role in the outcome of this method. And this goes in agreement with many articles coming to the conclusion that ABTS is more sensitive and preferable than DPPH method [89,90]. This

goes back to the fact that extract does well with reduction reaction rather than scavenging and also the fact that the free radicals used in ABTS are generated and unstable. The only drawback when it comes to the ABTS method is that it's time-consuming having to wait for 12-16 H for the radicals to generate under very precise circumstances.

There are numerous methods for determining the antioxidant potential of various samples. However, no single method is appropriate for all situations, and there is no quick way to determine antioxidant activity. The DPPH method has been widely used for estimating antioxidant activity among all available methods; however, its applications should be carried out while keeping in mind the method's basis and the need to establish the stoichiometry for reaction wherever possible, so that the antioxidant activity can be related to the structure of the substrate molecule.

3.2 Liposomal Characteristics

D-optimal experimental design using Design-Expert® was used to identify the two different materials involved in the composition of liposomes. The two independent variable components were basically the amounts of soybean and cholesterol. Zeta size, polydispersity index and mean particle size were all studied based on the variable change in the amount of SPC and cholesterol and were evaluated as presented in **table3.2** where Table lists the fitting models, equations, and statistical parameters. $P < 0.05$ was utilized to determine the significance level. Design Expert®. The optimal parameters for the formulations were then generated using the desirability approach. The polydispersity index values were all less than 0.3 for all formulations, meaning the presence of monomodal size distributions. Also, the particle size of the unloaded liposomes was relatively small, the mean particle size of all formulations was smaller than 110 nm meaning the particles are all well made to conveniently penetrate the stratum corneum. particles smaller than 120 nm all show significant penetration through the skin[91]. Furthermore, in this research, zeta potential values were all shown to be negative for all of our unloaded formulations, negative values are preferable when it comes to penetration and release on the nanoparticles. A study once showed that negatively charged particles yield better release

than positively charged particles[92]. In general, the characteristics were all satisfactory because they were small enough, negatively charged, and densely coated, these nanoparticles seemed ideal to further conduct our research based upon it.

Table 3.2. Optimal design displaying Component and Response Variables for the Preparation *Emblica officinalis* loaded liposomes

	Component 1	Component 2	Response 1	Response 2	Response 3
Sample	A: Soybean %	B: Cholesterol %	Zeta size	Mean particle size (nm)	PDI
1	1.85	0.65	-45	77.1	0.27
2	1.9	0.6	-48.8	80	0.282
3	1.93333	0.566667	-49	103.2	0.233
4	1.9	0.6	-48	80	0.28
5	1.9	0.6	-48.2	81.23	0.286
6	2	0.5	-50.2	75	0.26
7	1.8	0.7	-50.7	74.62	0.3
8	1.95	0.55	-50.8	108.3	0.234
9	1.8	0.7	-50	74.65	0.3
10	2	0.5	-50.4	75.23	0.26
11	1.86667	0.6333	-45	78.7	0.275

Table 3.3. Fitting Models, Equations, and Statistical Parameters of the Experimental Design

Response	Model	R^2	R^2 Adjusted	R^2 Predicted	Final equation
Zeta size	Quartic	0.9473	0.9122	0.7940	= +1.34343x10^5 A +7.64661x10^6 B +51.66782 C -81205.78985 A * B -1394.87178 A * C -76622.24486 B * C
Mean particle size (nm)	Quartic	0.9836	0.9726	0.9332	1/(mean particle size) = -43028.00352 A -5.14307x10^7 B +0.435772 C +7.58012x10^5 A * B +658.76614 A * C +7.73231 x10^5 B * C -5018.19548 A * B * C -2432.57808 A * B * (A-B) +2.28838 A * C * (A-C) +2586.71053 B * C * (B-C)
PDI	Quartic	0.9388	0.898	0.7240	1/(PDI) = +3.96465 x10^7A +3.60533Ex10^{10} B -360.25365 C -5.37382 x10^8 A * B -6.06968 x10^5 A * C -5.41924 x10^8 B * C +3.57648 x10^6 A * B * C +1.76484 x10^6 A * B * (A-B) -2108.08769 A * C * (A-C) -1.81212E+06 B * C * (B-C)

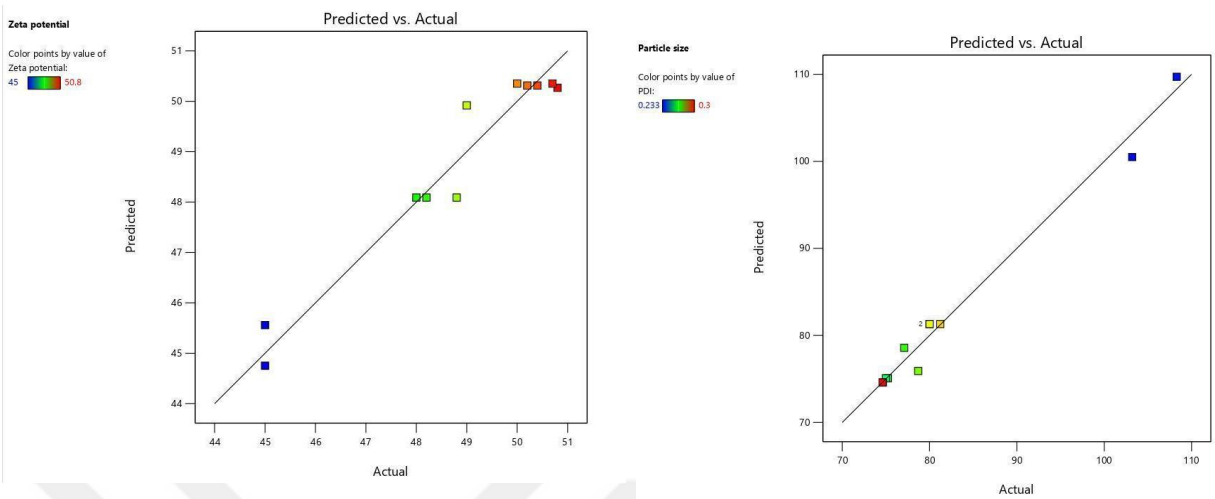

Figure 3.7. Influence of liposomal component (soybean and cholesterol) on particular responses such as zeta potential and particle size

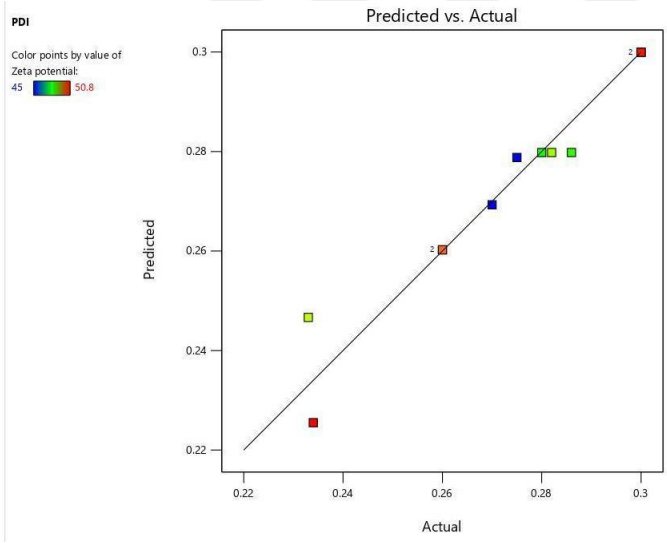

Figure 3.8. influence of liposomal component (soybean and cholesterol) on polydispersity index

3.3 Choosing the Desired Formulation

Equations and calculations were all conducted in the means to optimize a desired formulation depending on the three responses we got from the design expert after. Based on the criteria of achieving the smallest value of particle size with significant zeta and PDI value, the best formulation was chosen. Utilizing the extensive grid search and feasibility

search features offered by the Design Expert program, the final desired experimental parameters were determined.

Table 3.4. Optimization of components amount using desirability function.

Independent variables	Amount value	Predicted amount value
Soybean	1.85-2	1.8
Cholesterol	0.6-0.7	0.7
Response variables		
Mean particle size	74.62-108.3	74.606
Zeta potential	45-50.8	50.352
PDI	0.233-0.3	0.3

Desirability 1.

The results showed that experimental values did not significantly differ from the predicted values ($p > 0.05$).

Table 3.5. Values of the optimal formulation.

Optimal formulation	
Soybean(%)	**Cholesterol**(%)
1.8	0.07

3.4 Liposomal morphology

The surface morphology of the liposomes was evaluated using SEM analysis. The lyophilized samples were placed on adhesive carbon pads that were rested on aluminum

and were sputter coated with gold using a sputtering system and then later on imaged by a scanning electron microscope at 100 nm with a 250X magnification and 20 nm with 500X magnification. The results showed spherical or near-round nano-sized liposomes fully intact[93] as shown in **Figure 3.9**. the shape seems to be capable of transferring and encapsulating the required antioxidants

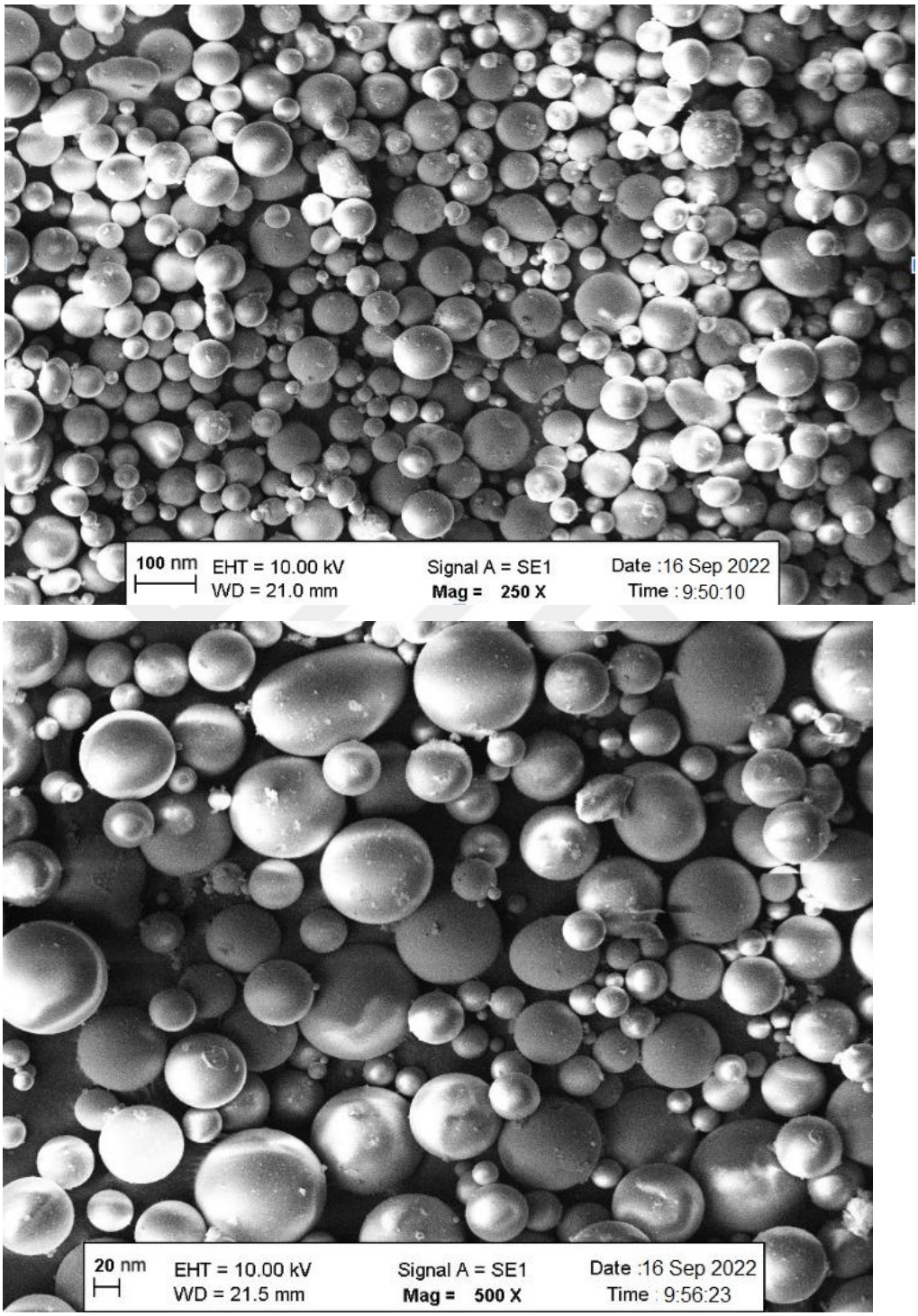

Figure 3.9. Liposomal morphology imaged by scanning electron microscope at magnifications 250 X and 500 X.

3.5 Rheological Studies

In order to fully understand our gel formulation structure we took a closer look at its rheology behavior. Measurement conditions were chosen to be conducted under the following ambient conditions of (25°C ± 0.5°C) and (37°C ± 0.5°C). from room temperature to body temperature in order to fully track the rheological changes. Shear rate, which is the difference of velocity of two layers within the bulk of material divided by a distance, was applied to the formulation from 10 rpm to 100 rpm while viscosity was monitored and noted. The formulations showed proper textbook non-newtonian pseudoplastic properties. Viscosity was reported to be around +800 cps which is the reference range for carbopol hydrogel formulations in cosmetics[94]. A change in viscosity was observed after a certain shear rate allowing better flowability an obvious increase in viscosity relative to the increase in temperature was noted similar to another paper that was studying rheological behaviors or carbopol [95]. The increase of viscosity with the increase of temperature can be interpreted in many ways but one article explained that with temperature the solvent-polymer interaction grows stronger thus explaining the increase of viscosity[96] results were expressed in cPs and Pa s

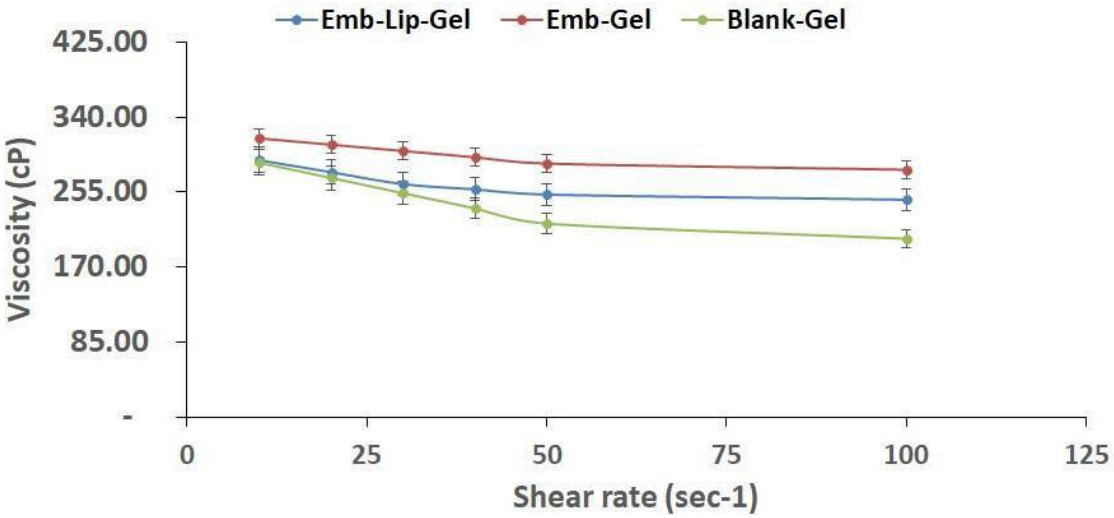

Figure 3.10. Viscosity and flowability under the influence of gradual shear rate at 25°C ± 0.5°C temperature (in terms of cPs)

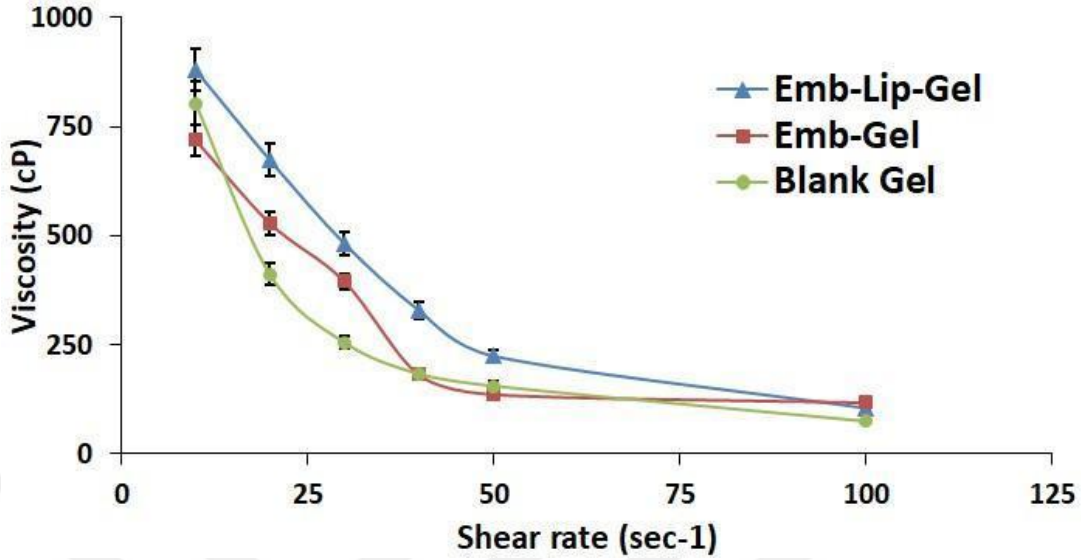

Figure 3.11. Viscosity and flowability under the influence of gradual shear rate at 37°C ± 0.5°C temperature (in terms of cPs)

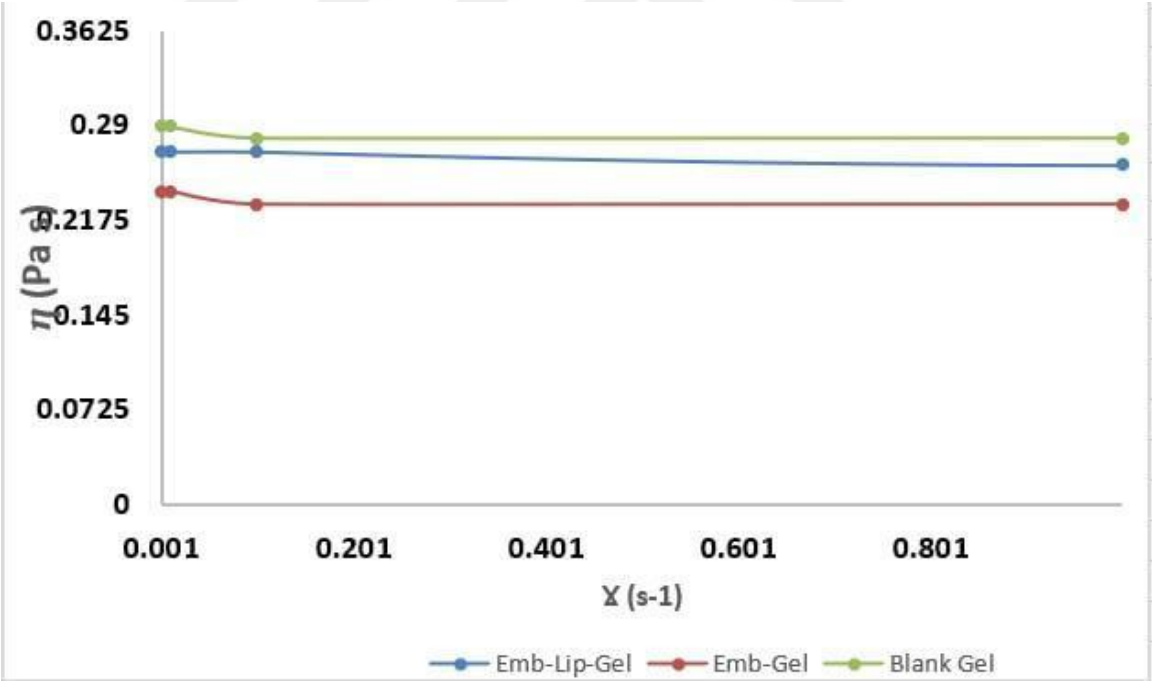

Figure 3.12. Viscosity and flowability changes at 25°C ± 0.5°C temperature (in terms of Pa s)

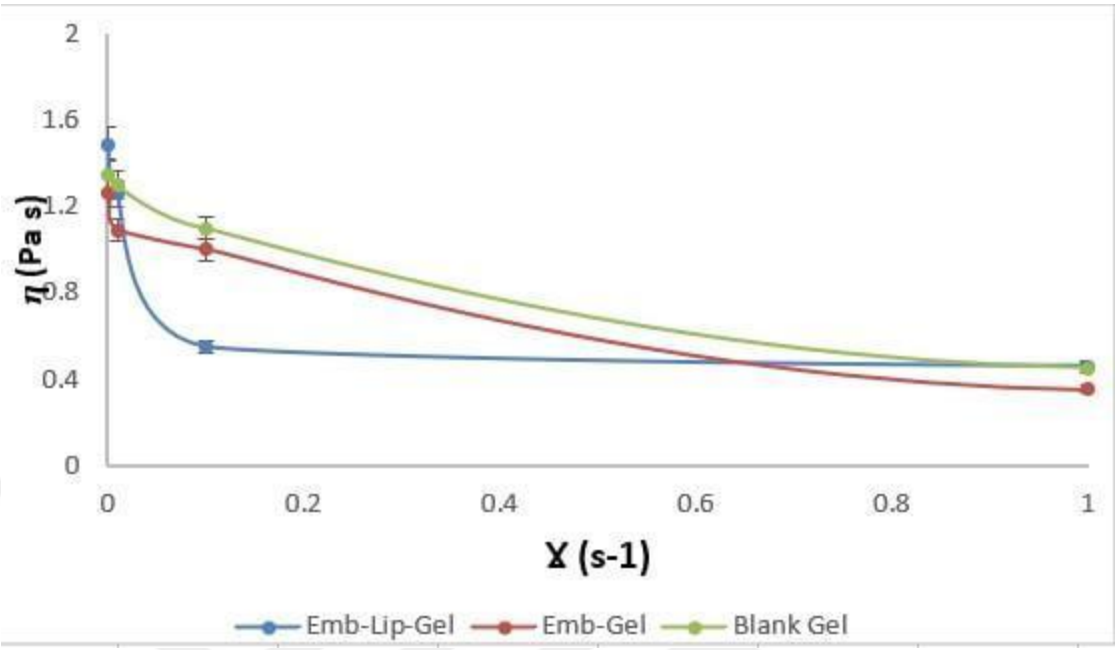

Figure 3.13. Viscosity and flowability changes at 37°C ± 0.5°C temperature (in terms of Pa s)

3.6 Fourier Transform Infrared Spectroscopy (FTIR-ATR) Analysis

FTIR test was conducted for 6 different samples those being (*Emblica officinalis* extract, Loaded liposomes, Unloaded liposomes, Loaded liposomes with gel, *Emblica officinalis* extract with gel and gel only) all previous samples underwent proper lyophilization overnight and had their wavelength scanned respectfully between 400 - 4000 cm^{-1} wavelength. Presence of a hydroxyl group was found in the *Emblica officinalis* extract with the wavenumber of 3348 cm^{-1} which is due to OH stretching vibration[97]. Wave numbers recorded between 1712 cm^{-1},1613 cm^{-1} and 1553 cm^{-1} all indicated the presence of double bonds (C=O, C=C and C=N), Aryl-substituted C=C and Conjugated C=C C=C-C Aromatic ring stretch[97]. wavenumbers were also noted around 1448 cm^{-1}, 1344 cm^{-1} and 1212 cm^{-1} which translates as Aromatic primary amine, CN stretch Primary or secondary, OH in-plane bend Phenol, and C-O stretch[97]. 872 cm^{-1}, 764 cm^{-1} and 628 cm^{-1} wavenumbers were absorbed meaning a possibility of Vinylidene, C-H out-of-plane bend Peroxides, C-O-O- stretch Alkyne. C-H bend 680–610 cm^{-1}. The 1652 cm^{-1} band could indicate the existence of flavonoids stretching vibration of C=O and of C=C,

asymmetric bending vibration of N–H [98]. 1448 cm^{-1} could be related to CH3, CH2, flavonoids and aromatic rings [98].

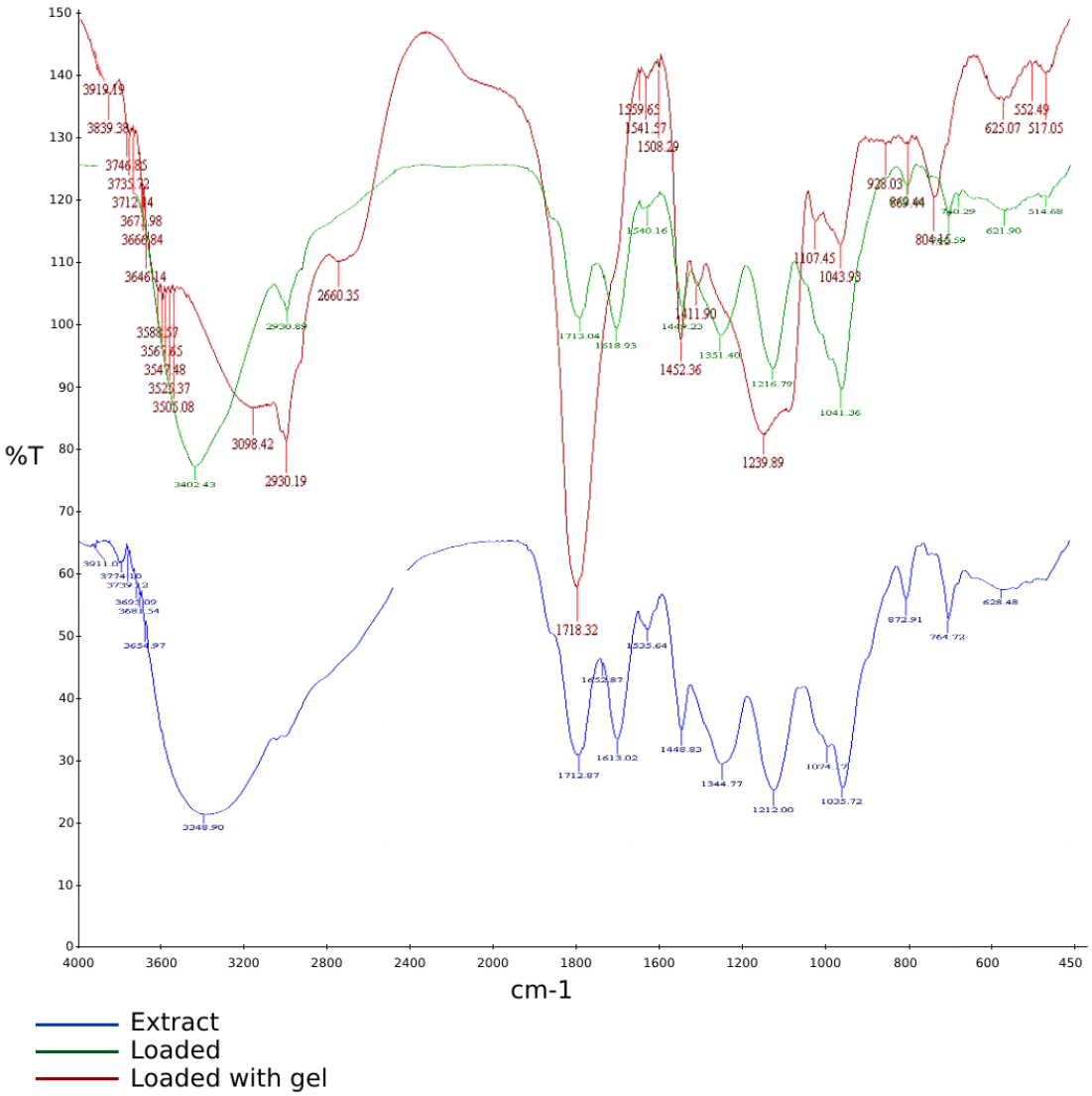

Figure 3.14. FTIR absorbance comparison between following samples (extract only, loaded liposomes, and loaded liposomes with gel)

As noticed in **Figure 3.14.** -OH groups are detected in higher transmittance meaning that most of the extracts phenols were encapsulated inside the liposomal vesicle

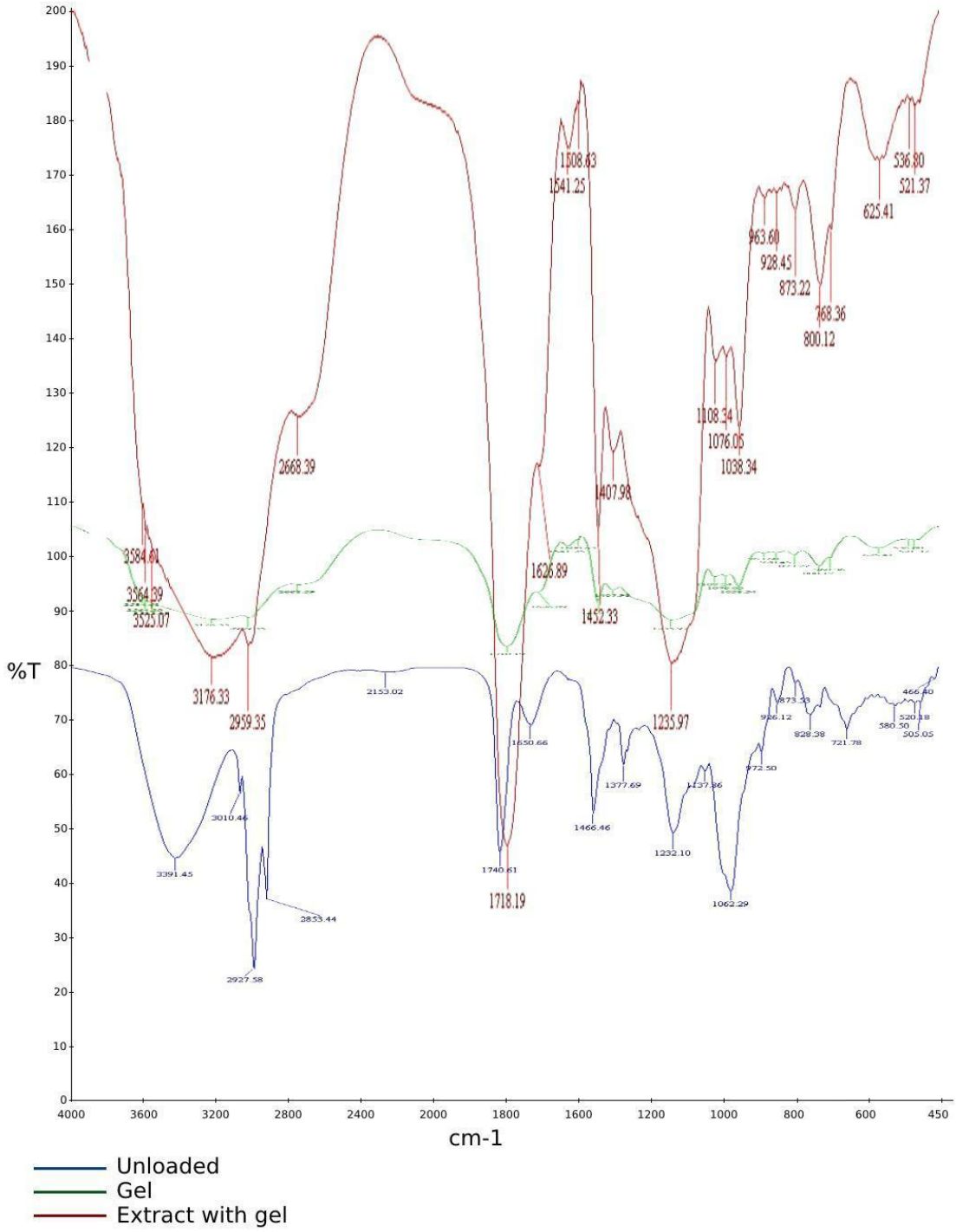

Figure 3.15. FTIR comparison for the following samples (unloaded liposomes, gel only and extract with gel)

3.7 *In Vitro* Release Studies

formulations that were incorporated with liposomes showed higher DPPH radical scavenging activity with higher penetration and significant difference than formulations that weren't. Loaded liposomes formulation showed a peak antioxidant amount of 3018 ug ± 73 per sample after 240 minutes, which is 42.28% ± 2.42 of the total sample that was used[99]. While the formulation that contained only *Emblica officinalis* extract without any liposomal incorporation amount showed no significant difference throughout the whole time reaching a 1345 ug ± 57 after 300 minutes which is 18.84% ± 4.24 of the total initial sample that was used. It is also worth mentioning that a strong change of color was observed while performing DPPH total antioxidant content method regarding the samples that were incorporated with liposomes indicating stronger antioxidant penetration as shown in **Figure 3.16.**

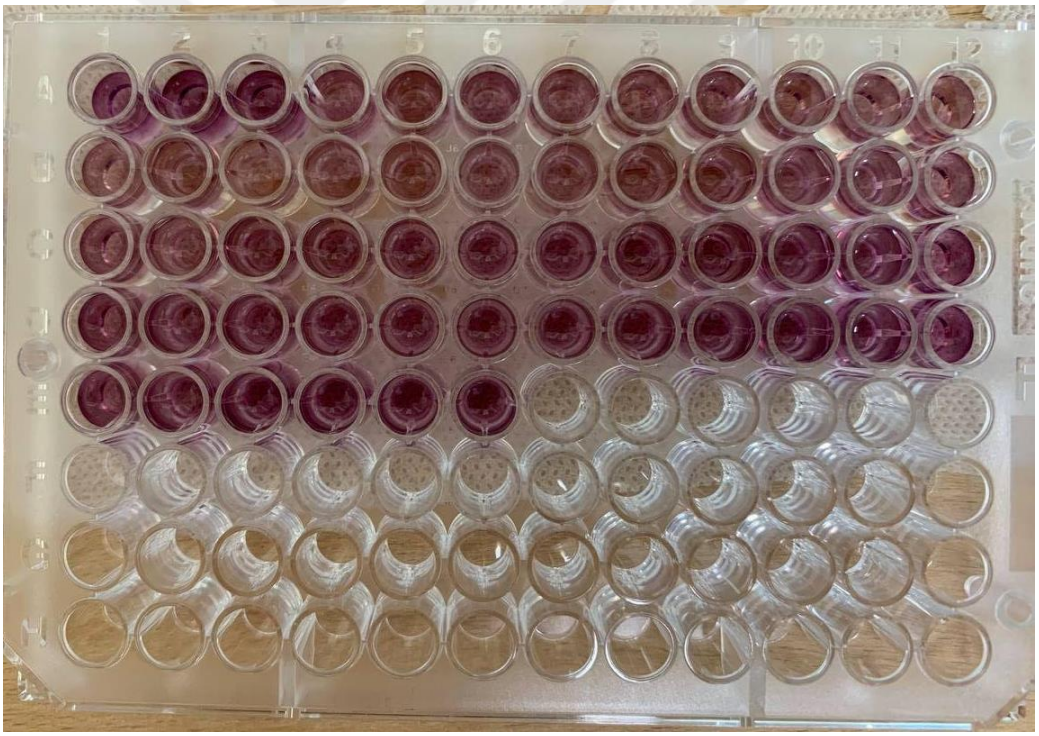

Figure 3.16. DPPH inhibition rate with time relative to color change.

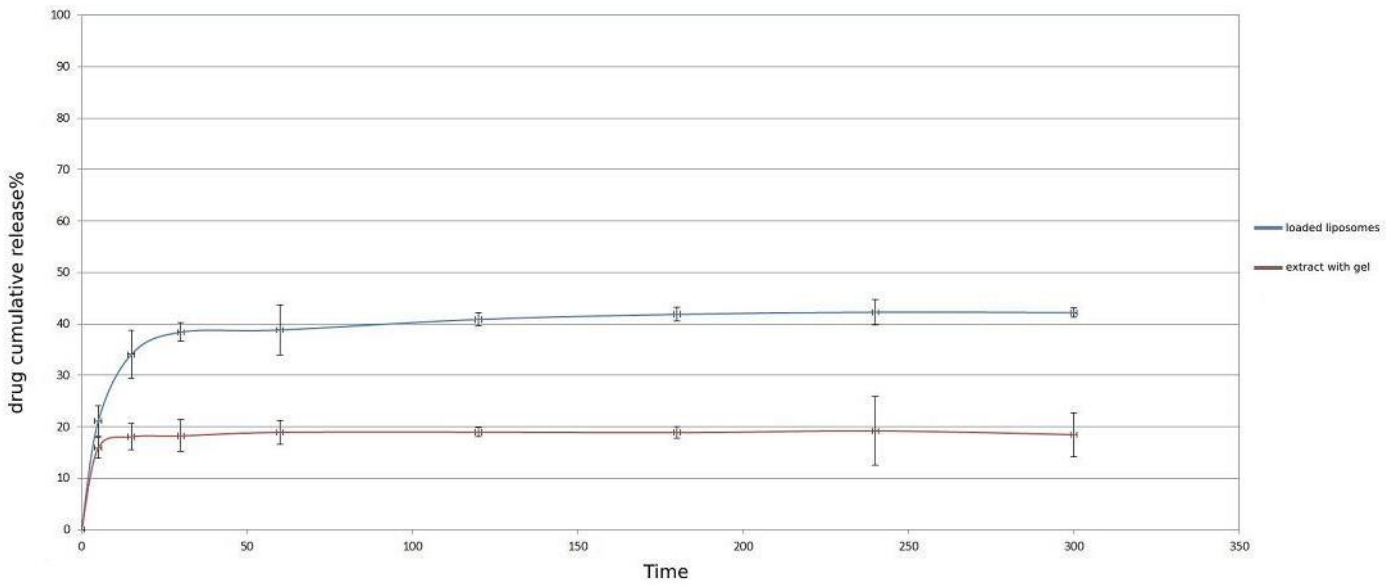

Figure 3.17. *In vitro* drug release comparison between loaded liposomes with gel formulation and *Emblica officinalis* extract only with gel formulation. Graph expressed as total cumulative drug release% ±SD over time

3.8 *Ex vivo* Release Studies

Our *ex vivo* results showed lesser antioxidant penetration with regard to amount than *in vitro* [100]. Results but nonetheless, the formulation that contained liposomes showed better penetration through the pig skin thus yielding a relatively higher antioxidant amount. peak amount was reached after 240 minutes with a total of 1540 ug ± 12.8 per sample which is 21.5% ± 0.78 of total cumulative drug release while on the other hand, the samples that were not incorporated with liposomes had a peak antioxidant amount of 1131 ug ± 35 after 300 minutes, that is 15% ± 3.09 of total cumulative drug release.

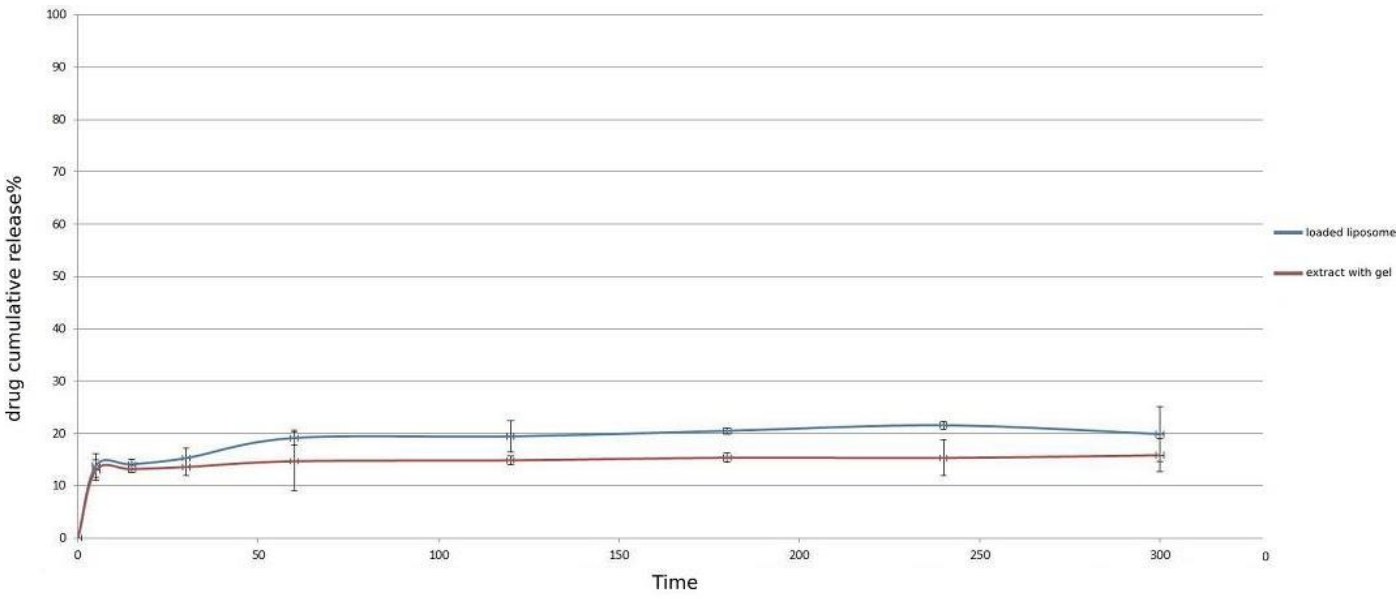

Figure 3.18. *Ex vivo* release study comparison between loaded liposomal with gel formulation and *Emblica officinalis* extract with only gel formulation. Graph expressed as total cumulative drug release% ±SD over time

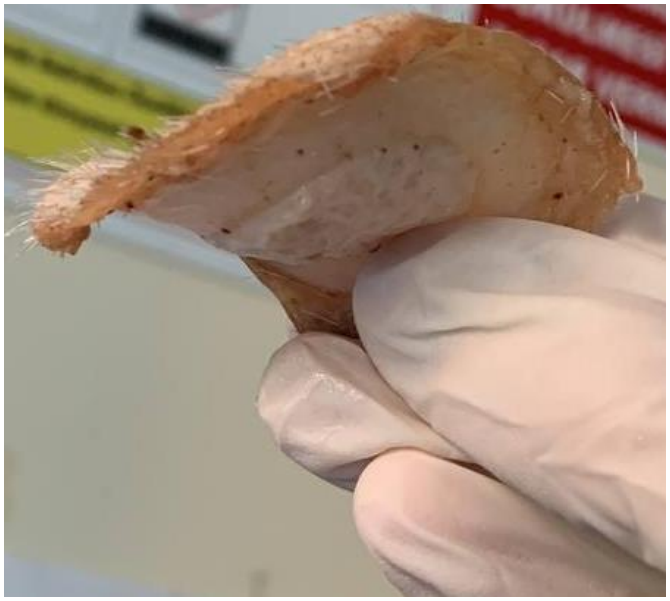

Figure 3.19. Area of liposomal penetration through the skin

3.9 Statistical Analysis

The software program Minitab 17 was used to evaluate the results and statistical significance of multiple outcomes and findings in this research. The results were determined by the use one-way analysis of variance and the order of significance was determined using the Tukey pot-hoc test. Most methods were done in this research in form of triplicates and results were given as mean ±SD. Concerning significant levels it was defined as $p \leq 0.05$ with CI of 95%.

Table 3.6. *In vitro* antioxidant amount relative to time significance level (groups that don't share the same letter are significantly different)

Sample name	N	Mean[a]
loaded gel 300min	3	2995.4^{A}
loaded gel 180min	3	2988.6^{A}
loaded gel 240min	3	2976.5^{A}
loaded gel 120min	3	$2900.0^{A'B}$
loaded gel 30min	3	$2770.4^{B'C}$
loaded gel 60min	3	2698.9^{C}
loaded gel 15min	3	2366.8^{D}
loaded gel 5min	3	1510.3^{E}
extract gel 240min	3	$1371.5^{E'F}$
extract gel 120min	3	$1355.36^{E'F}$
extract gel 60min	3	$1351.0^{E'F}$
extract gel 180min	3	$1347.20^{E'F}$

extract gel 30min	3	$1326.9^{E'F'G}$
extract gel 300min	3	$1320.7^{F'G}$
extract gel 15min	3	$1314.0^{F'G}$
extract gel 5min	3	1147.5^{G}

[a]ug/g Trolox equivalent total antioxidant content.

As shown in the **Table 3.6.** the samples that were revealed to have highly significant differences were the ones that were encapsulated with liposomes (Groups A-D) and highest difference was found at time intervals of 180 min, 240 min, and 300 min (Group A).

Table 3.7. *Ex vivo* antioxidant amount relative to time significance level (groups that don't share the same letter are significantly different)

Sample name	N	Mean[a]
loaded 240min	3	1540.05^{A}
loaded 180min	3	$1464.42^{A'B}$
loaded 300min	3	$1418.9^{B'C}$
loaded 120min	3	$1388.8^{B'C}$
loaded 60min	3	1367.9^{C}
extract 300min	3	1131.2^{D}
extract 180min	3	$1097.26^{D'E}$
extract 240min	3	$1095.9^{D'E}$

loaded 30min	3	$1093.2^{D'E}$
extract 120min	3	$1062.95^{D'E'F}$
extract 60min	3	$1051.3^{D'E'F}$
loaded 15min	3	$1009.37^{E'F'G}$
loaded 5min	3	$990.1^{F'G}$
extract 30min	3	$970.33^{F'G}$
extract 15min	3	942.88^{G}
extract 5min	3	934.8^{G}

[a]ug/g Trolox equivalent total antioxidant content

As shown in **Table 3.7.** highest significance levels were achieved with the samples that were incorporated with liposomes (Groups A-C) With the time interval of 240 minutes, it is shown to have the utmost significance among all the other readings.

Table 3.8. DPPH antioxidant content significance level.

Sample name	N	Mean[a]
G3	3	370.4^{A}
L3	3	360.1^{A}
G2	3	211.3^{B}
L2	3	209.14^{B}
G1	3	103.7^{C}
L1	3	102^{C}

[a]mg/g Trolox equivalent total antioxidant content

As shown in **table 3.8.** samples that had a higher concentration of the *Emblica officinalis* extract presented a more significant difference. It is also worth noting that there was no significant difference between samples that shared the same concentration implicating that liposomes had no negative interaction regarding the total antioxidant amount.

4. CONCLUSION

This research was conducted with the goal of creating a topical antioxidant gel formulation incorporated with a competent delivery system that is liposomes. The method of preparation was easy and neither time nor material-consuming. The gel formulation made from the material carbopol 934 had no negative interaction or implication on the delivery system or antioxidant content. Viscosity was convenient for topical application and reacted upon a certain shear stress with better flowability. The fruit *E. officinalis* lived up to its high antioxidant content reputation alongside its phenolic and flavonoids content according to one out of the four total antioxidant methods that were done to evaluate the fruits, DPPH showed that the fruit contains 0.43 ± 0.02 mg of antioxidant Trolox equivalent per mg of the fruit extract that is 43% antioxidant. The high amount of antioxidants can surely combat and diminish the free radicals that the skin is exposed to in the day to day life. The liposome preparation was fast and invasive, providing us with stable, spherical, negatively charged, and nanosized particles using only cholesterol and SPC performed under the regular Bangham method conditions. When it comes to penetration and target area it was proved in the *ex vivo* and *in vitro* tests that the incorporation of liposomes is important for utmost penetration there was no doubt in the necessity of liposomal incorporation. Samples that did not contain any liposomes were found to have a significant difference from the samples that did. Antioxidants are required to be underneath the stratum corneum inside the cells where they can function and perform their duty in combating the free radicals and creating an anti-aging effect. At the end of this research, we are provided with a functional anti-aging gel that is rich in antioxidants and encapsulated with nano lipid carriers that proved to have higher bioavailability than formulas that didn't.

5. References

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