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Development and Investigation of Hyperpigmentation Activity of a quercetin loaded emulsion

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ABSTRACT :

The development and investigation of a quercetin-loaded emulsion for hyperpigmentation activity involved comprehensive preformulation studies, emulsion characterization, stability assessments, and evaluation of tyrosinase inhibitory effects. Quercetin, a bioactive flavonoid with known antioxidant and depigmenting properties, was successfully incorporated into an oil-in-water (o/w) emulsion system. The pH of the formulations (F1-F5) ranged between 5.5 and 6.5, which is physiologically compatible with skin application, minimizing irritation risks. Stability tests, including dilution, dye solubility, and cobalt chloride tests, confirmed the o/w nature of stable formulations (F1, F2, F3, F5), while F4 exhibited phase separation, indicating instability. Thermodynamic stability assessments via centrifugation and freeze-thaw cycles further validated the robustness of F2 and F3, which resisted phase separation and precipitation under stress conditions. Zeta potential measurements revealed moderate colloidal stability, with F2 and F3 exhibiting negative surface charges (−11.35 mV and −12.5 mV, respectively). Although these values are below the ideal ±30 mV threshold, they suggest sufficient electrostatic repulsion to prevent aggregation. Encapsulation efficiency (EE) of 68.72 ± 2.37% indicated effective drug loading, while in vitro release studies demonstrated a biphasic pattern: an initial burst release (~51.84% in 3–4 hours) attributed to surface-associated quercetin, followed by sustained release (~86.49% over 10 hours) from the lipid matrix. This biphasic profile is advantageous for both immediate and prolonged therapeutic effects. Stability studies under varied conditions (4°C, 25°C, 40°C) confirmed minimal changes in pH, appearance, and drug content over four weeks, underscoring the formulation's shelf-life suitability. The tyrosinase inhibition assay highlighted quercetin's dose-dependent efficacy, with F2 achieving 67.12% inhibition at 300 µg/mL, albeit less potent than kojic acid (86.35%). IC50 values for F2 (96 ± 0.5 µg/mL) and F3 (92 ± 1.2 µg/mL) suggest moderate inhibitory activity, likely due to quercetin's ability to chelate copper ions at the enzyme's active site. While kojic acid remains the gold standard, quercetin's natural origin and safety profile make it a promising alternative or adjunct for hyperpigmentation therapy.

Introduction:

1. **Skin: Anatomy and physiology**

1.1 **Skin:** The skin, the body's outermost layer, serves as a protective barrier for internal tissues and shields the body's organs from environmental factors. When challenged by microorganisms such as bacteria and fungi, the skin's immune system can become activated, leading to conditions like psoriasis, which is characterized by severe inflammation and excessive skin cell production (Baker 2019)

1.2 **Biology of the Skin:** The skin is the body's largest organ, accounting for 16% of body weight (between 2 and 6 kg) and covering an area of approximately 1.8 m². It provides a crucial defense against harmful external factors, including bacteria, microorganisms, UV radiation, and mechanical pressure on tissues. Additionally, the skin plays a vital role in various bodily functions, such as sensation, touch, temperature regulation, vitamin D synthesis, and waste elimination through sweating (DeSaix, Betts et al. 2013).

1.3 **Skin Anatomy:** The skin is a dynamic organ, constantly renewing itself by shedding the outer layer and replacing it with cells from deeper layers. It also includes various appendages like nails, hair, sebaceous glands, and apocrine glands (Gawkrodger 2016). Skin thickness varies depending on its location; for instance, the skin on the soles of the feet and palms of the hands is notably thicker. The skin is composed of three essential layers, as illustrated in Figure 1, each serving to protect various body organs:

- Epidermis
- Dermis
- Hypodermis

Epidermis and Its Layers

The epidermis is the outermost layer of the skin, ranging in thickness from approximately 0.1 to 1.4 mm. It primarily consists of keratinocytes, which produce keratin proteins, along with other types of cells such as Langerhans cells, melanocytes, and Merkel cells. This layer undergoes continuous cell

proliferation and shedding, with millions of cells being lost every 40 minutes. Over a lifetime, this amounts to around 18 kg of dead skin cells. This shedding process, known as desquamation, typically takes about 28-30 days, from the stratum basale to the stratum corneum (Nguyen and Soulika 2019).

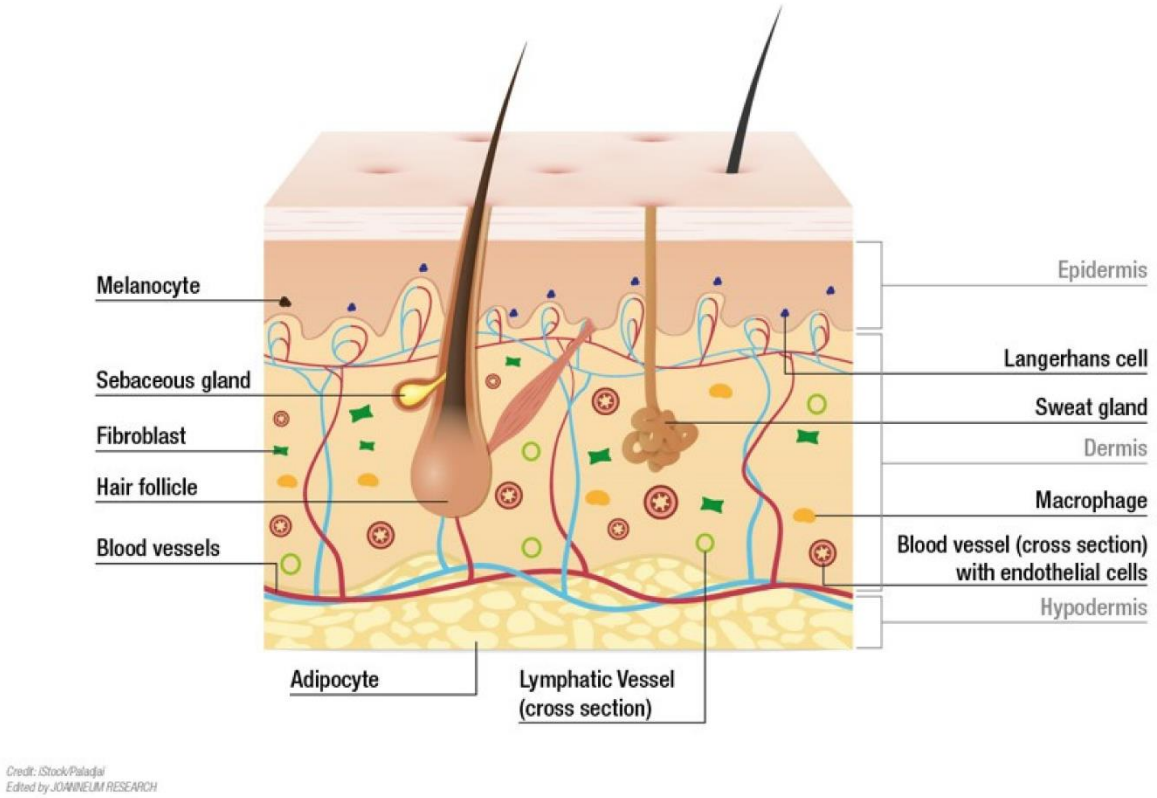


Figure 1: Anatomy of skin ((Hofmann, Schwarz et al. 2023)

- **Stratum Basale**: The basal layer of the epidermis contains dividing and non-dividing keratinocytes, which produce keratin, and melanocytes, which produce melanin. It also houses Merkel cells, which are involved in touch sensation
- **Stratum Spinosum**: In this layer, daughter cells from the basal layer migrate upward to form a polyhedral cell layer connected by desmosomes. This layer primarily contains Langerhans cells, which are involved in immune responses.
- **Stratum Granulosum**: Cells in this layer become flattened and lose their nuclei. There is significant lysosomal activity for cell digestion and disintegration, with keratohyalin granules and lipid-filled vesicles present. The lipids include 40% ceramides, fatty acids, phosphates, and proteins.
- **Stratum Lucidum**: Located between the stratum granulosum and stratum corneum, this layer is found only in thick skin areas like the palms and soles, where it provides additional density.
- **Stratum Corneum**: The final stage of keratinocyte maturation occurs in this layer, where the cells, now called corneocytes, are flat and devoid of nuclei, forming a tough, protective outer layer (Montagna 2012).

Dermis

The dermis is a robust connective tissue layer providing physical support and nourishment to the epidermis. It consists of a matrix of water, protein fibers, and a mix of large organic molecules, including polysaccharides and proteins. Glycosaminoglycans (GAGs), such as hyaluronic acid, are common in this matrix, which is referred to as the extracellular matrix (Eckhart, Lippens et al. 2013).

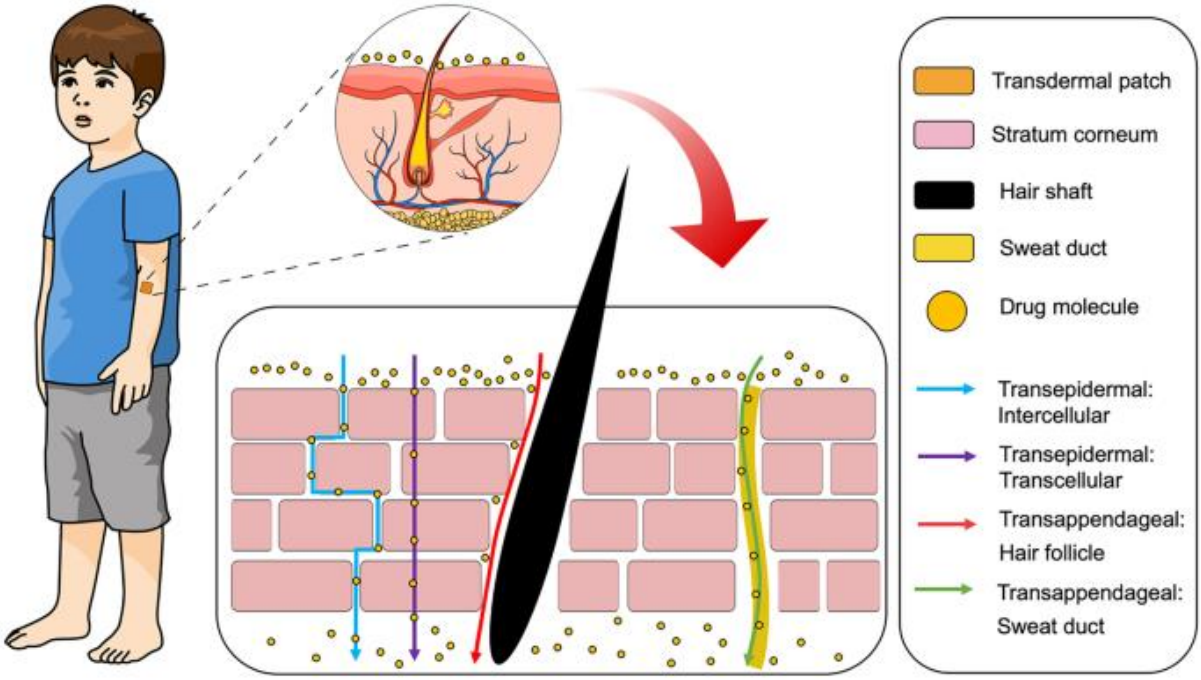
Hypodermis (Subcutaneous Tissue)

Located beneath the dermis, the hypodermis primarily consists of fat cells and connective tissue, serving as protection for internal structures. It functions as an insulator to regulate body temperature and as a cushion to protect against trauma.

Drug absorption via skin: Drug absorption through the skin's stratum corneum (SC) can generally be categorized into two pathways: transepidermal and transappendageal, as illustrated in Figure 2. The primary absorption route is the transepidermal pathway. This pathway takes advantage of the SC's large surface area, allowing drugs from transdermal patches to spread across the skin and penetrate either through the cells (transcellular) or the spaces between cells (intercellular) (Haque and Talukder 2018).

The transepidermal route can be further divided into two sub-pathways: transcellular and intercellular. In the transcellular pathway, drugs diffuse through the SC cells, requiring passage through lipid bilayer membranes. This route is predominantly used by hydrophobic drugs due to the lipid-rich

nature of the cell membranes in the SC. In the intercellular pathway, drugs diffuse through the lipid matrix within the spaces between keratinocytes in the SC. This route is favoured by hydrophilic compounds or small molecules, enabling them to reach the vascular capillaries in the dermis. The intercellular route is the primary pathway for drug absorption and relies on a balance between the drug's lipid and aqueous solubility (Zhang, Jung et al. 2017).



Schematic representation of transdermal drug delivery mechanisms (Ramadon, McCrudden et al. 2022)

In the transappendageal route, drug is delivered via hair follicles or sweat glands. This pathway is particularly important for transporting polar or ionizable compounds and large macromolecules that struggle to pass through epidermal cells due to their size and partitioning properties. However, the use of this pathway is somewhat limited because it covers a much smaller area (~0.1% of the total skin surface) compared to the transepidermal route. Consequently, researchers have developed various methods to improve drug absorption through the skin by modifying the structure of the SC, using chemical, physical, or combined approaches. The subsequent sections will explore the advancements in transdermal products and technologies designed to enhance drug absorption through the skin(Verma, Jain et al. 2016)

Topical Drug Delivery Systems: Drug delivery systems are designed to ensure that medications reach their intended targets within the body effectively. These systems must address various factors, including ease of application and drug efficacy. Numerous companies specialize in creating these delivery methods, either developing proprietary systems for pharmaceutical companies or marketing their own solutions. Many of these systems are patented and proprietary (Lu and Gao 2010).

When administering a drug, precise dosing is crucial to ensure the body can properly utilize it, necessitating a delivery system that allows for accurate dosage. Additionally, drug delivery systems must account for how a drug is metabolized. For instance, some drugs are degraded in the intestinal tract and thus cannot be administered orally. Others might pose risks if given in large doses, necessitating controlled release methods to ensure patient safety (Bhowmik, Gopinath et al. 2012).

Topical drug delivery systems introduce medications directly to the body's surface in a form that can be absorbed. Examples include skin patches, nasal sprays, inhalation aerosols, eye drops, and creams applied to the skin. These methods are often favored by patients for their ease of use and minimal discomfort.

Overall, the aim of any drug delivery system is to deliver the correct dosage to the appropriate site in the body. Patients generally prefer methods that are painless and user-friendly, which is why topical and enteral methods (for oral administration) are popular. In clinical settings, parenteral routes are more commonly used, especially for controlled substances, as they offer more precise control over drug administration and timing (Zolghadri, Bahrami et al. 2019).

Material and Method:

Several important factors that should be considered while doing visual observations to identify drug-excipient and excipient-excipient interactions include whether there are any physical changes, such as colour changes, phase separation, precipitation, or crystallisation. Next, texture and consistency should be noted, including any changes in the mixture's consistency and whether it becomes sticky, hard, or brittle. There are factors related to physical states, such as efflorescence, deliquescence, and hygroscopicity. There should not be the creation of clumps or aggregates. Odour changes or the creation of new smells may be signs of chemical breakdown or reactions. There should not be any signs of sedimentation or phase separation. Potential interactions that could impact the final pharmaceutical product's stability, efficacy, and safety can be found by keeping account of these factors.

e) Melting point: The melting point of quercetin was determined using the capillary method. It was filled in a small quantity in a capillary which was inserted in melting point apparatus (MR-VIS, Labindia, Mumbai, India) and as the temperature raised the melting point of the drug was noted. The temperature at which the drug started melting was noted and a temperature at which the drug was completely melted was noted. The melting point was reported as a temperature range.

f) Partition coefficient: To evaluate the drug's hydrophilicity and lipophilicity, the partition coefficient must be determined. For this, the Shake Flask method was applied. A particular quantity of the medication that is Quercetin was dissolved in a mixture of equal parts of octanol and distilled water in a flask. After agitating the flask to reach equilibration, phase separation was accomplished by allowing it to rest. UV spectrophotometry was used to determine the quercetin amounts in both phases upon separation. The samples were scanned at 256 nm. With the obtained absorbance, the concentration was calculated. Using the following formula, the partition coefficient (K) was determined: (Cumming H and Rucker C, 2017)

$$Ko/w = Co/Cw \qquad \text{(Eq 4.1)}$$

Where, K- partition coefficient
Co - concentration of quercetinin organic solvent
Cw - concentration of quercetinin distilled water.

1.4 Formulation of Quercetin loaded emulsion:

The emulsion was prepared using corn oil as oil phase, Tween 20 as surfactant, Propylene Glycol as co-surfactant and water as continuous phase. The emulsion prepared was an O/W emulsion. Tween 20 and Propylene Glycol were mixed in equal ratio that is 1:1 to get a mixture of surfactant: co-surfactant which is called as Smix. The corn oil was taken first and Smix was added in it in a certain quantity and mixed properly. 2 to 3 drops of rose water (fragrance) were added during this stirring time to give good fragrance to the emulsion. Then the water was added slowly in the required quantity with continuous magnetic stirring at 500 rpm to make an emulsion. Care must be taken during formulation development so that no turbidity is seen and foam formation has to be avoided. In the above-mentioned method, the quantities of ingredients that needs to be added was decided on the basis of ratios selected from the developed Pseudo – Ternary Plot. According to it five different ratios were selected as given in **table** and blank formulations were developed that is without adding drug. After detecting the stability, of these formulations three formulations were selected which were F1, F4 and F5 and they were developed again by adding drug. Corn oil was taken and drug was added into it and was mixed properly after which the Smix was added following which the water in the required quantity was added slowly with continuous magnetic stirring at 500 rpm. (Patel SK et al., 2013; Afzal O et al., 2023; Herneisey M et al., 2016; Balata et al., 2016).

Table 3: Different batches of the formulation made

Formulation	Oil (corn oil) (%)	Smix (1:1) (%)	Water (%)
F1	9	25	69
F2	8.5	35.5	56
F3	7.5	37	55.5
F4	10	39	51
F5	13	30	67

1.5 Characterization of Quercetin loaded emulsion (F1 to F5)

1.5.1 pH determination:

The pH of the quercetin emulsion was determined with a digital pH meter. The electrode was fully immersed in the emulsion to ensure complete coverage. Measurements were conducted in triplicate, and the average value of these readings was recorded.

Dilution test. It was performed by diluting the formulated emulsion sample (Emulsions A and B) with oil or water. This test depends on the fact that no phase separation is possible when a dispersion medium is added to anEmulsion. For example, when water is added to o/w (oil in water) Emulsion, it is freely miscible with the Emulsion, and no phase separation occurs. Similarly, the addition of oil to water in oil (w/o) Emulsion shows miscibility in the case of the o/w type of Emulsion; if it is diluted with water, it will remain stable as water is the dispersion medium. Still, if it was diluted with oil, the Emulsion will break as oil and water will not be miscible with each other.

Dye solubility test. Two dyes were used: amaranth (water-soluble dye) and scarlet red (oil-soluble dye). This test is based on the principle that the dye can disperse uniformly throughout the phase in which it is more soluble, i.e., amaranth in the aqueous phase, whereas scarlet red in the oil phase. Two

samples of emulsion A (2 ml) were taken in two different test tubes. In one of the samples was added or sprinkled amaranth dye while in another piece was added the scarlet red paint and after little mixing both the elements was observed under the microscope (LEICA, Model: DM 300, German). The same was performed for emulsion B [6]. Therefore, in the case of the o/w type, the water-soluble dye miscible indicates the o/w, whereas oil-soluble pigment shows immiscibility and vice versa for the w/o type of Emulsion. Hence, if the continuous phase takes up the water-soluble dye, it represents o/w Emulsion. Still, if not taken up by the ongoing phase, that means the oil is the continuous phase (w/o Emulsion), the dye will remain as a cluster on the system's surface

Cobalt (II) chloride (CoC12)/filter paper test. This test involved the use of filter paper. The filter paper was first impregnated with CoC12 and dried (it appeared to be blue). This dried filter paper was then dipped in the formulated emulsion sample (A and B) and observed for any color change, if any. The color change from blue to pink indicates an o/w type of Emulsion. This test may fail if the Emulsion is unstable or break in the presence of an electrolyte.

1.5.2 Thermodynamic Stability and Precipitation assessment

1.5.2.1 Centrifugation test:

Accelerated ageing was done using centrifugation and freeze-thaw cycles to evaluate thermodynamic stability. The formulations that had "good" dispersion properties were centrifuged for 30 minutes at 5,000 rpm, and any indications of phase separation, including creaming or cracking, were noted. For the ensuing freeze-thaw testing, only the formulations that passed the centrifugation test were chosen (Afzal O et al., 2023, Harshal M et al., 2011).

1.5.2.2 Freeze – Thaw test:

The chosen formulations were made to go through four freeze-thaw cycles, each lasting 24 hours, with the freezing phase lasting at -4°C and the thawing phase lasting at 40°C and the changes were noted (Afzal O et al., 2023, Harshal M et al., 2011).

1.5.2.3 Precipitation assessment:

After 24 hours, precipitation was detected by visually examining the resultant emulsion. The formulations were categorised as non-clear (turbid), clear (transparent or transparent with a bluish tinge), stable (no precipitation after 24 hours) and unstable (precipitation detected within 24 hours) (Afzal O et al., 2023, Kumar Gupta S, nd.)

4.2.6.2 Viscosity measurement:

The viscosity of the developed emulsion was measured at varying shearing rates at room temperature using a MCR 102e, Modular Compact Rheometer, Anton Paar India Pvt. Ltd (Nawaz et al., 2022).

4.2.6.3 Spreadability:

The parallel plate method was used to assess the quercetin-loaded emulsion's spreadability. A precisely weighed 0.5 g sample was spread into a circle with a designated diameter of 1 cm on a glass plate. Subsequently, the gel sample was carefully covered with a second glass plate. The sample was then allowed to spread out on the plates by applying a 500 g weight for five minutes. The increase in diameter brought on by the gel spreading was measured after the allotted amount of time. To guarantee that the results could be repeated and are accurate, this experiment was carried out in triplicate. A linear scale was used to measure the extension in order to quantify the spreadability. The following formula was used to calculate the spreadibility of organogel (Nawaz et al., 2022).

Spreadibility (S) = m . l t (Eq 4.3)

Where, S represents spreadibility m is mass applied

l is length of the spread of emusion in cm (average diameter) t is time in seconds

4.2.4.4 Entrapment efficiency (EE):

Entrapment efficiency of the quercetin emulsion was determined using centrifugation method. Here the formulation was developed by adding 400 mg of drug to make a 10mg/ml concentration. Then this formulation was centrifuged at 10,000 rpm for 5 minutes. Supernatant was separated and added in sufficient quantity of methanol to dissolve the drug in methanol. This sample was then tested in UV spectrophotometer to get the absorbance and concentration was calculated from it. The detected concentration was of un associated drug. Using the below formula the entrapment efficiency (EE %) was calculated. Experiment was performed in triplicate (Hamzawy et al., 2017).

Entrapment Efficiency (EE%) = Total amount of drug – amount of unbound drug X 100 Total amount of drug

4.2.6.4 Stability studies:

In order to make sure that the formulation created is stable and safe, three samples of Quercetin loaded emulsion were prepared and all the samples were subjected to a high temperature of 40°C for a period of 1 month. The samples were withdrawn from each of the three developed emulsion at regular intervals of time that is at 0,2 and 4 weeks, to check for appearance, pH and concentration of drug to ensure that there are no major changes in these characteristics. UV Spectrophotometry was used to perform the drug assay of formulations using methanol as solvent. Appearance was checked visually and 81 the pH was checked using a digital pH meter (IG-10PH, PH Meter, IGene Labserve Pvt. Ltd.) (Afzal O et al., 2023, Harshal M et al.,

2011).

In vitro Tyrosinase inhibition assay

Tyrosinase inhibitory activities of MBV, MBR and MBT were determined according to the method described previously with slight modifications (Manosroiab et al., 2010). L-tyosine and mushroom tyrosinase were purchased from Sigma–Aldrich (St. Louis, MO, USA). Briefly, 400 µl of 3 mM of L-tyosine solution was added to 700 µl of 50 mM of phosphate buffer (pH 6.8) with methanol extract of each sample separately and incubated at room temperature (25 0C) for 5 min. Then, 600 µl of mushroom tyrosinase (50 U/ml) was mixed and subsequently the absorbance of was measured at 256 nm after 0 min and 15 min of incubation. Kojic acid was used as reference compound. The experiments were carried out in triplicate. The IC50 value is the concentration of the sample that inhibits 50 % of the enzyme activity and was determined. The percentage of tyrosinase inhibition was calculated using following formula:

Inhibitory activity (%) = × 100 Where, A1= Absorbance of the blank at 0 minute A2= Absorbance of the blank after 15 minutes B1= Absorbance of the sample at 0 minute

5.1 Preformulation studies

5.1.1 Organoleptic properties The drug powder was physically examined and the following observations were recorded. The recorded observations of physical state, colour and powder odour of the drug were found to be similar to the reference reported in official literature.

Table 5.1: Observed organoleptic properties of Quercetin

Properties	Quercetin
Physical form	Amorphous powder
Colour	Yellow
Odor	Odourless

5.1.2 Solubility studies: In solvents: It was experimentally found that quercetin had highly soluble in methanol and the resultant order of solubility is ethanol>methanol>DMSO. In excipients: Using UV/VIS spectroscopy, the solubility of quercetin in a variety of excipients. Drug was found to be soluble in corn oil, and tween 80, hence these excipients are selected to be used in formulation development.

Table 5.2: Experimentally obtained solubility values of quercetin in different excipients

Excipients	**Concentration**
Methanol	6.316 ± 0.41 mg/ml
Ethanol	8.147 ± 0.35 mg/ml
DMSO	6.289 ± 0.29 mg/ml
Corn oil	5.374 ± 0.61 mg/ml
Tween 80	4.418 ± 0.23 mg/ml

Determination of Absorption maxima (λmax) and construction of Calibration curve of Quercetin :

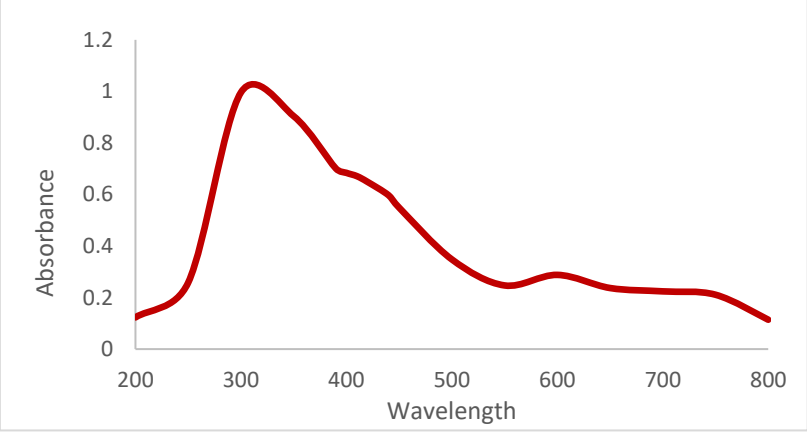


Figure : Absorption maxima of quercetin in Methanol Solvent Absorption maxima (λ max)

5.1.3.1.B Construction of calibration curve of Quercetin:

Using the different dilutions that were made, absorbance values of Quercetin at different concentrations were determined and these values along with concentration values were plotted on a graph to get the calibration curve. The regression value was calculated and was found to be 0.9904.

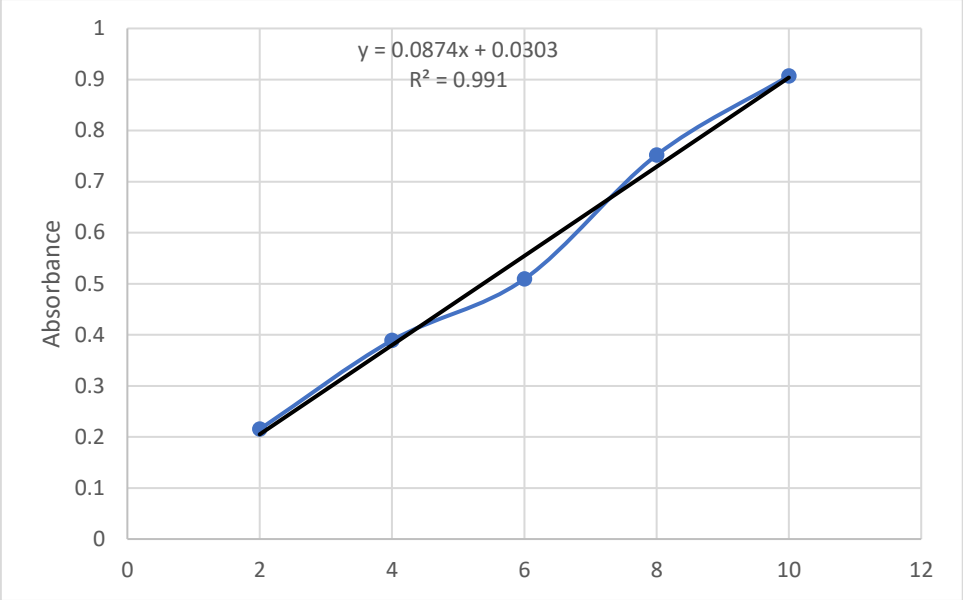


Figure 5.3 depicts the generated calibration curve of Quercetin in methanol.

5.1.3.1.C Construction of calibration curve of Quercetin in PBS (pH 5.5):

Using the different dilutions that were made in PBS of pH 5.5, absorbance values of Quercetin at different concentrations were determined and these values along with concentration values were plotted on a graph to get the calibration curve. The regression value was calculated and was found to be 0.991. Figure depicts the prepared calibration curve of Quercetin in PBS (pH 5.5).

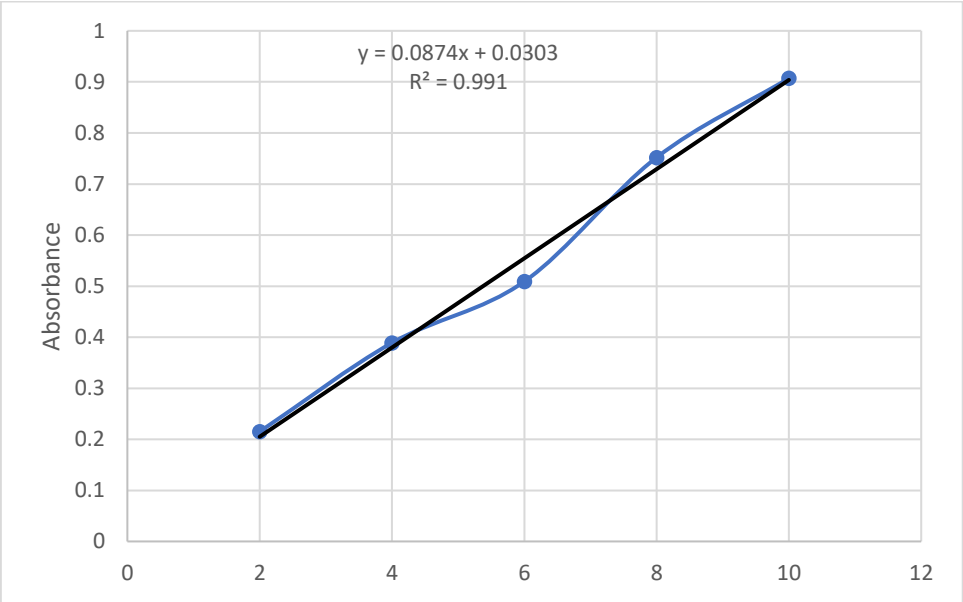


Figure: Calibration curve of quercetin in PBS

5.1.4 Drug – excipient interaction by visual observation:

There were no physical changes, such as colour changes, phase separation, precipitation, or crystallisation. Texture and consistency were also same. No aggregates formation was there. Any new odour was not developed and there was no change in odour with absence of sedimentation or phase separation. These results suggested that the developed formulation is free of any kind of potential interactions and is stable and safe.

5.1.5 Melting point:

The melting point of pure Quercetin was found to be 315 ± 2°C. Since the experimentally obtained melting point is found to be near the actual melting point of Quercetin, it can be suggested that the compound is pure and this is also likely to be confirming the identity of the compound.

5.1.6 Partition coefficient:

To evaluate the drug's hydrophilicity and lipophilicity, the partition coefficient was determined because it is a fundamental parameter which can influence various parameters of formulation development like, solubility, permeability, stability, distribution and more. Absorbance values were determined using UV spectrophotometer of Quercetin in octanol and Quercetin in water, which was used to calculate concentration of Quercetin in these solvents and the formula was used to determine partition coefficient, which was found to be 2.98 ± 0.08.

Table 5.6: Mean absorbance with obtained concentration and calculated partition coefficient

Solvents	**Mean absorbance ± SD(N=3)**	**Concentration ± SD(N=3)**
Octanol	0.271 ± 0.002	3.81 ± 0.13
Water	0.308 ± 0.004	5.58 ± 0.07
Calculated partition coefficient (P) = 0.683 ± 0.003		

pH determination:

The pH of the quercetin emulsion and its different batches were recorded in triplicate and the results are presented in the following table:

Formulation	pH
F1	5.5
F2	6.5
F3	6
F4	6.5
F5	5.5

Dilution test. It was performed by diluting the formulated emulsion sample (Emulsions A and B) with oil or water. This test depends on the fact that no phase separation is possible when a dispersion medium is added to an Emulsion. For example, when water is added to o/w (oil in water) Emulsion, it is freely miscible with the Emulsion, and no phase separation occurs. Similarly, the addition of oil to water in oil (w/o) Emulsion shows miscibility in the case of the o/w type of Emulsion; if it is diluted with water, it will remain stable as water is the dispersion medium. Still, if it was diluted with oil, the Emulsion will break as oil and water will not be miscible with each other.

Formulation	Result of dilution test
F1	No phase separation
F2	No phase separation
F3	No phase separation
F4	**Phase separation**
F5	No phase separation

Dye solubility test. Two dyes were used: amaranth (water-soluble dye) and scarlet red (oil-soluble dye). This test is based on the principle that the dye can disperse uniformly throughout the phase in which it is more soluble, i.e., amaranth in the aqueous phase, whereas scarlet red in the oil phase. Two samples of emulsion A (2 ml) were taken in two different test tubes. In one of the samples was added or sprinkled amaranth dye while in another piece was added the scarlet red paint and after little mixing both the elements was observed under the microscope (LEICA, Model: DM 300, German). Therefore, in the case of the o/w type, the water-soluble dye miscible indicates the o/w, whereas oil-soluble pigment shows immiscibility and vice versa for the w/o type of Emulsion. Hence, if the continuous phase takes up the water-soluble dye, it represents o/w Emulsion. Still, if not taken up by the ongoing phase, that means the oil is the continuous phase (w/o Emulsion), the dye will remain as a cluster on the system's surface

Formulation	Dye used	Observation	Inference
F1	Amaranth/Scarlet red	External phase turns red/Internal globules turn red	o/w emulsion
F2	Amaranth/Scarlet red	External phase turns red/Internal globules turn red	o/w emulsion
F3	Amaranth/Scarlet red	External phase turns red/Internal globules turn red	o/w emulsion
F4	**Amaranth/Scarlet red**	**No distinct color**	**Broken emulsion**
F5	Amaranth/Scarlet red	External phase turns red/Internal globules turn red	o/w emulsion

Cobalt (II) chloride (CoC12)/filter paper test. This test involved the use of filter paper. The filter paper was first impregnated with CoC12 and dried (it appeared to be blue). This dried filter paper was then dipped in the formulated emulsion sample (A and B) and observed for any color change, if any. The color change from blue to pink indicates an o/w type of Emulsion. This test may fail if the Emulsion is unstable or break in the presence of an electrolyte.

Formulation	**Dye used**	**Observation**	**Inference**
F1	Blue cobalt chloride paper	Pink Color	o/w emulsion
F2	Blue cobalt chloride paper	Pink Color	o/w emulsion
F3	Blue cobalt chloride paper	Pink Color	o/w emulsion
F4	Blue cobalt chloride paper	**No color change**	**Broken emulsion**
F5	Blue cobalt chloride paper	Pink Color	o/w emulsion

1.5.2 **Thermodynamic Stability and Precipitation assessment**

1.5.2.1 Centrifugation test:

Accelerated ageing was done using centrifugation and freeze-thaw cycles to evaluate thermodynamic stability. The formulations that had "good" dispersion properties were centrifuged for 30 minutes at 5,000 rpm, and any indications of phase separation, including creaming or cracking, were noted. For the ensuing freeze-thaw testing, only the formulations that passed the centrifugation test were chosen (Afzal O et al., 2023, Harshal M et al., 2011).

1.5.2.2 Freeze – Thaw test:

The chosen formulations were made to go through four freeze-thaw cycles, each lasting 24 hours, with the freezing phase lasting at -4°C and the thawing phase lasting at 40°C and the changes were noted (Afzal O et al., 2023, Harshal M et al., 2011).

1.5.2.3 Precipitation assessment:

After 24 hours, precipitation was detected by visually examining the resultant emulsion. The formulations were categorised as non-clear (turbid), clear (transparent or transparent with a bluish tinge), stable (no precipitation after 24 hours) and unstable (precipitation detected within 24 hours) (Afzal O et al., 2023, Kumar Gupta S, nd.)

Formulation	**Centrifugation test**	**Freeze-thaw test**	**Precipitation test**
F1	Passed	**Did not pass**	Passed
F2	Passed	Passed	Passed
F3	Passed	Passed	Passed
F4	**Did not pass**	**Did not pass**	**Did not pass**
F5	Passed	Passed	**Did not pass**

Zeta potential

The surface charge and colloidal stability of quercetin-loaded liposomes were assessed by measuring their zeta potential. The results revealed a negative zeta potential of **−11.35 mV**, likely due to the anionic nature of phospholipids and other components in the lipid bilayer. This negative charge plays a crucial role in stabilizing the formulation by promoting electrostatic repulsion between particles, thereby minimizing aggregation and enhancing dispersion stability.

While a zeta potential magnitude exceeding **±30 mV** is typically required for optimal colloidal stability, the observed value suggests that the quercetin-loaded liposomes still maintain sufficient stability to resist coalescence and sedimentation. Furthermore, the surface charge may influence interactions with biological membranes, potentially affecting cellular uptake and the overall bioavailability of quercetin

Formulation	Zeta Potential
F2	-11.35
F3	-12.5

Encapsulation efficiency

(EE) refers to the proportion of core material successfully encapsulated within liposomess. Since liposomes can carry both hydrophobic and hydrophilic molecules, EE must be determined for all compounds encapsulated in the vesicles. EE is calculated as the difference between the total amount of the compound added for encapsulation and the amount of non-encapsulated compound, divided by the total compound added. This measure reflects the effectiveness of the encapsulation process and the quality of the liposomes.

Encapsulation efficiency can vary based on the physical state of the compound.

The experiment was performed and using the equation entrapment efficiency of the developed and finalized Quercetin loaded liposomes was determined. The Entrapment Efficiency was found to be 68.72 ± 2.37%. This suggests that a good amount of drug was entrapped in the liposomal formulation.

In-vitro drug release study:

In Vitro Drug Release Study of Quercetin-Loaded Liposomes

An *in vitro* drug release study was performed to assess the release kinetics and sustained-release potential of quercetin-loaded liposomes. The formulation exhibited a **biphasic release pattern**, characterized by an **initial burst release** followed by a **prolonged, sustained release phase**.

- **Initial Burst Release (~51.84 ± 0.14% within 3–4 hours):**
  This phase likely results from the rapid diffusion of surface-associated or loosely bound quercetin molecules, providing an immediate therapeutic effect.
- **Sustained Release (~86.49 ± 0.49% over 10 hours):**
  The subsequent controlled release indicates effective encapsulation within the liposomal bilayer, where quercetin gradually diffuses from the lipid matrix. This sustained release enhances bioavailability and protects quercetin from degradation under physiological conditions.

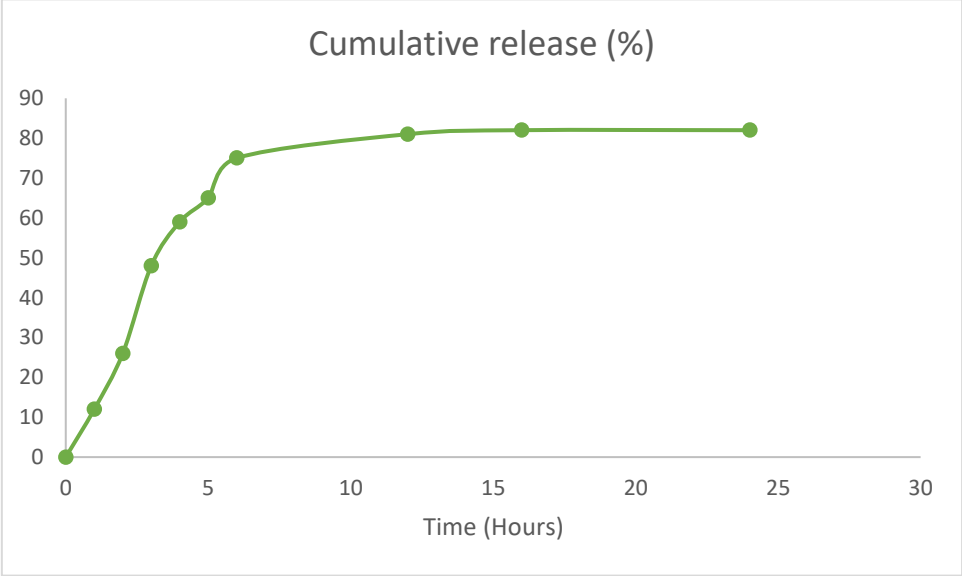


Figure: percentage cumulative release of quercetin from liposomes

Stability study

Stability study of three samples of developed Quercetin loaded organogel was done at a high temperature of 40°C and it was found that the pH of the formulation was consistent with negligible change, appearance does not changed in any manner to suggest any instability in the developed formulation as it was consistent throughout the test period and no change in colour, odour and consistency. Assay of drug was done and it was found that a very minor variation in the concentration of drug was there which did not suggest any degradation of the drug. The results suggests that all the samples of developed formulation did not show any major variations in pH, appearance and drug concentration which provides us evidence to report that the developed formulation is stable and it can be concluded that it is safe to use and its efficacy is also ascertained.

Table: Results of stability studies

Parameter	Time point	Zeta potential	%EE
Set 1 (4°C)	**Week 0**	-11.56	79.54
	Week 2	-14.57	75.75
	Week 4	-18.59	76.32
Set 2 (25° C ± 2° C 60% RH ± 5% RH)	**Week 0**	-10.98	78.79
	Week 2	-15.89	76.52
	Week 4	-17.46	71.59
Set 3	**Week 0**	-11.28	74.97

(40° C ± 2° C 75% RH ± 5% RH)	**Week 2**	-13.68	72.51
	Week 4	-17.54	73.35

Results of Tyrosinase Activity:

Table: Percentage tyrosinase inhibition at various concentrations

Concentration (µg/ml)	**F2 (%)**	**F3 (%)**	**Kojic Acid (%)**
20	16.20	13.78	38.86
40	35.56	27.45	49.87
80	56.01	45.53	61.24
150	63.21	52.28	70.67
300	67.12	64.34	86.35

Table: IC_{50} Values of Extracts and Standard

Compound	**IC_{50} (µg/ml)**
Kojic Acid	38 ± 1.6
F2	96 ± 0.5
F3	92 ± 1.2

The in vitro evaluation of tyrosinase inhibitory activity of F2 and F3 extracts, as compared to the standard kojic acid, demonstrated promising anti-tyrosinase effects in a dose-dependent manner. Tyrosinase is a key enzyme in melanogenesis, and its inhibition is a primary target in managing hyperpigmentation disorders. Among the test extracts, F2 showed the highest percentage of tyrosinase inhibition at all tested concentrations, with 67.12% inhibition at 400 µg/mL, followed by F3 (64.34%). These results were markedly lower than those observed with kojic acid, which exhibited 86.35% inhibition at the same concentration, indicating its superior efficacy.

The IC_{50} values further support these findings, with kojic acid having the lowest IC_{50} (38 ± 1.6 µg/mL), confirming its potent inhibitory effect. F2 showed a moderate IC_{50} of 96 ± 0.5 µg/mL, suggesting it is a relatively effective natural inhibitor. F3 displayed significantly higher IC_{50} values of 92 ± 1.2 µg/mL, respectively, reflecting weaker inhibitory potency. These differences may be attributed to variations in the phytochemical composition of the extracts, particularly the presence and concentration of bioactive flavonoids or phenolic compounds known to interact with the copper ion at the tyrosinase active site.

Overall, the results indicate that while all three extracts possess anti-tyrosinase potential, F2 stands out as the most effective among them. However, kojic acid remains the benchmark for potency. The findings justify further investigation into the active constituents of F2 and their mechanisms of action. Additionally, these extracts could be explored in combination with standard depigmenting agents to enhance efficacy and minimize potential side effects in clinical or cosmeceutical applications.

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