An-Najah National University

Faculty of Graduate Studies

Synthesis of enhanced lipid solubility rutin derivatives, formulation and development of validated analytical method

By

Abeer Yousef Mahmoud Mahmoud

Supervisors

Dr. Murad Abulhasan

Dr. Mohyeddin Assali

This Thesis is Submitted in Partial Fulfillment of the Requirements for the Degree of Master in Pharmaceutical Sciences, Faculty of Graduate Studies, An- Najah National University, Nablus, Palestine

2020

Synthesis of enhanced lipid solubility rutin derivatives, formulation and development of validated analytical method

By

Abeer Yousef Mahmoud Mahmoud

This Thesis was defended successfully on 10/12/2020 and approved by:

Defense Committee Members		Signature
1. Dr. Murad Abualhasan	/ Supervisor	.....
2. Dr. Mohyeddin Assali	/Co-Supervisor	.....
3. Dr. Fuad Alrimawi.	/ External Examiner	.....
4. Dr. Asma Radwan	/ Internal Examiner	.....

Dedication

To the greatest tenderness and love in this world mother, and to the most generous in this universe, my beloved father and to my husband who supported me in completing my career in this field ...

To my sisters and brothers who carried me in all my circumstances and provided me with support and aid.

To my doctors, friends and every person have given me the drive and discipline to tackle any task with interest and determination.

I dedicate this work….

Acknowledgement

Firstly, I thank the holy god for giving me the patience, qualifications strength and the health to complete this research. Hence, I would like to thank my thesis supervisor: Dr. Murad Abualhasan for his advice, guidance and support for me greatly in my research. In addition, special thanks to Dr. Mohyeddin Assali for his scientific and technical support and constructive advice.

Special thanks to the pharmacy department at Al- Najah National University and to everyone who helped me and contributed with me to obtaining this success, especially the supervisor of the laboratories of the College of Pharmacy, Mr. Mohammad Arar and the lab workers Tahreer Shtayeh and Linda Arar. I also thank the members of the examination committee for reading my thesis and their valuable comments and suggestions. Special thanks to Dr Numan Malkieh to help me in my research.

Thanks to the College of Science at the University of Jordan for their cooperation in measuring NMR. Thanks to my family, who will not fulfill their right to my words, and I am grateful for their psychological and moral support throughout my school years and my work in my research.

Abeer Yousef Mahmoud Mahmoud

الإقرار

أنا الموقعة أدناه، مقدمة الرسالة التي تحمل العنوان:

Synthesis of enhanced lipid solubility rutin derivatives, formulation and development of validated analytical method

أقر بأن ما اشتملت عليه الرسالة هو نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه حيثما ورد، وأن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أي درجة أو لقب علمي أو بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provide in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالب:** عبير يوسف محمد محمد

Signature: **التوقيع:**

Date: **التاريخ:** ١٠/ ١٩/ ٢٠١٨

Table of Contents

List of Table

List of Figures

List of Schemes

List of Abbreviations and Unites

MN	Micronuclei
HPLC	High performance liquid chromatography
PCL	Photo chemi luminescence
K562	Human erythroleukemic cell line
R2	New derivative of rutin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
HRs	Hydroxyethylrutosides
Q3G	Quercertin-3-glucoside
UV	Ultraviolet
FDA	Food and Drug Administration
ICH	International Conference on Harmonization
USP	United States Pharmacopeia
LOD	Limit of Detection
ORAC	Oxygen Radical Absorbance Capacity
HCL	Hydrochloric acid
DMAP	4-Dimethylaminopyridine
DIPEA	N,N-Diisopropylethylamine
TEA	Triethylamine

Synthesis of enhanced lipid solubility rutin derivatives, formulation and development of validated analytical method

By
Abeer Yousef Mahmoud Mahmoud
Supervisors
Dr. Murad Abulhasan
Dr. Mohyeddin Assali

Abstract

Background: Interest in flavonoids has increased significantly overtime because of its significant pharmacological and biological activities. Rutin has features that make it used in several benefits such as antioxidant, anti-inflammatory, chemotherapeutics activity, various disease condition such as hemorrhoids, varicose vein, and allergic contact dermatitis. Rutin is available in the market as a topical formulation for the treatment of several diseases such as internal bleeding, hemorrhoids and varicose veins. However, these gels have a low solubility and limited bioavailability due to hydroxyl groups in rutin that decreased a lipid solubility, and therefore limits its application. In this study our aim is to synthesize a potentially novel lipophlic rutin prodrug. The suggested library of these prodrugs of rutin is to change the solubility profile in order to facilitate rutin transport across biological barrier and therefore improve drug delivery through topical application.

Method: Six derivatives of the Rutin based on ester pro-drug strategy were synthesized, then Octanol-water partition coefficients (LogP) of these compounds were determined. The synthesized compounds were formulated into a topical ointment and their permeability through Franz

diffusion was measured. UV analytical method was developed in our labs to quantify rutin derivatives both as a raw material and in the final dosage form. The analytical method was then validated. The validation was performed according to ICH which tested all validation parameters like: Range, Specificity, Linearity, Accuracy, Precision (Repeatability, Intermediate Precision, Reproducibility, Quantification limit, Detection limit and Robustness.

Results: The synthesis of the six derivative esters of the Rutin was successfully achieved. The logP values of these derivatives indicate a better lipid solubility compared to rutin and the results indicate that the structural modification done on rutin will improve its transdermal bioavailability. The results Franz diffusion showed that the transdermal permeability was best for decaacetylated rutin compared to the other esterified rutins. A simple analytical method for the analysis of the formulated rutin ester was developed and validated. The developed method was found to be linear, precise, accurate, stable and selective. The formulated ointment of decaacetylated rutin in our research laboratory were found to be stable under stability accelerated conditions.

Conclusion: An improvement of the transdermal permeability of rutin was obtained by performing esterification of the hydroxyl groups of rutin. Acetyl esterification showed the best dermal diffusion. A validated analytical method for the synthesized decaacetylated ester of rutin was developed. The formulation ointment of the synthesized compound was stable under both stress and shelf life condition.

Chapter one
Introduction

1.1 General background

Compounds derived from natural products from plants is of interest to many scientific researchers and pharmaceutical companies [1]. Polyphenols are the most commonly founded natural products in plants and form the basic and most important human diet elements. Flavonoids which characterized are main class of these polyphenolic compounds usually have significant antioxidant effects, antiviral as well as anti-inflammatory and anticancer activities [2, 3]. Rutin which is an interested type of flavonoids nowadays represent a promising group of nutraceutical formulations that are being tested as protective agents against several environmentally illustrated insults [4].

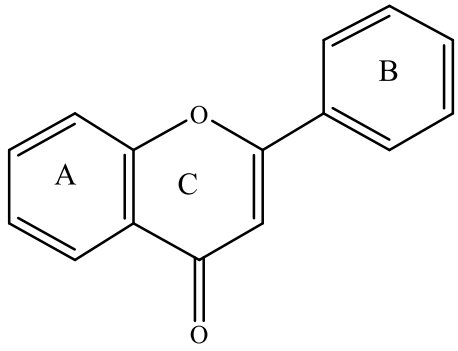
Various semisynthetic derivatives have been prepared from these naturally occurring compounds in order to develop their therapeutic effects; for example Acylation was the first adopted method for modification which was ready to be tested, it has been shown than in 1980's, with the goal of facilitating "sticky" molecule like quercetin which is a member in flavonoids group to be entered into cells by performing of some decoration on it with lipophilic short fatty acid chains [5]. Various acylated semisynthetic quercetin derivatives were then prepared and investigated for activity in several biochemical tests [6] .In addition different rutin derivatives and its well-known metabolite quercetin which are considered

as main examples of flavonoids are used for cardiovascular chronic pathology in some clinics, as they additionally showed some *in vivo* antiulcer effect and *in vitro* antiproliferative and anti-mutagenic effects. Quercetin unfortunately as well as rutin are poorly absorbed from the gastrointestinal tract and also in irregular absorption manner, almost because of their very low solubility in water and slow dissolution rate so this point increased scientific attention to improve their solubility and dissolution profiles using prodrug strategy [7].

Developing transdermal prodrug has an increased interests during the last decade because of several advantages. Improvement of an effective means of transdermal delivery will usually improve drug concentrations, a decrease systemic distribution and thereby preventing certain limitations oforally administered products [8].

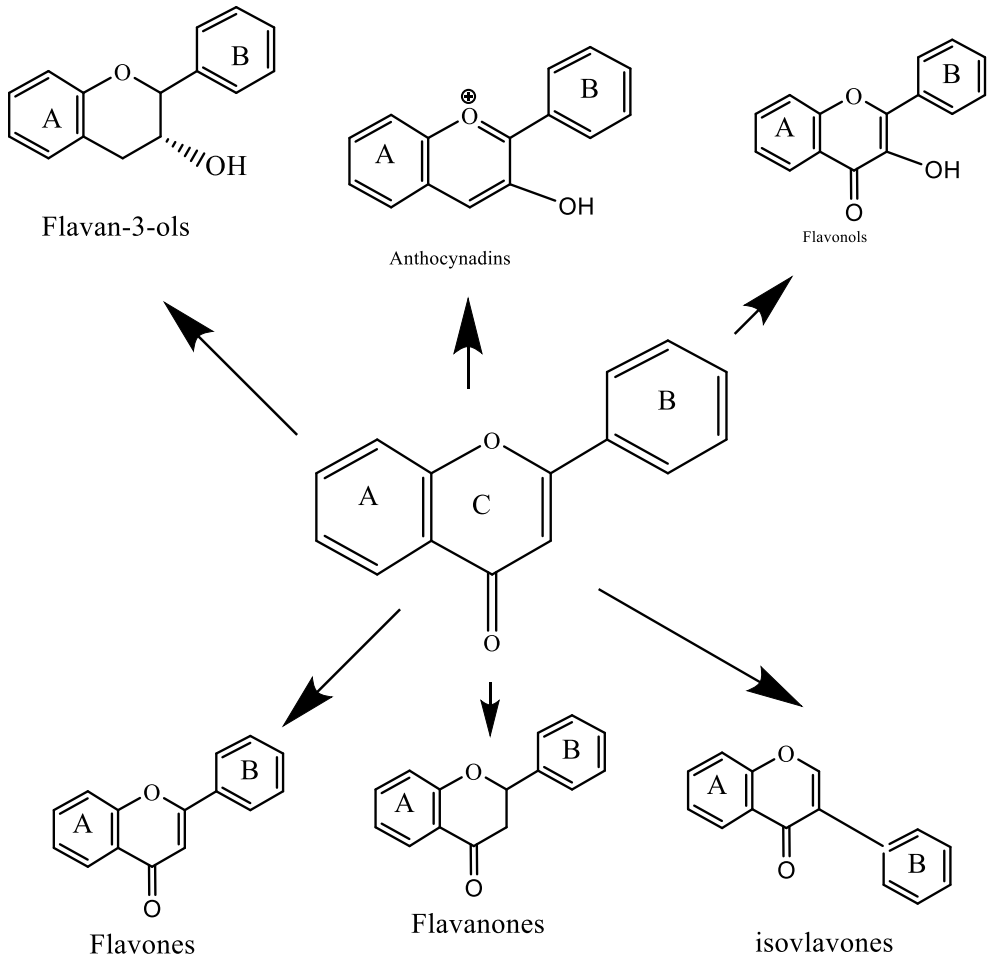
1.2 Flavonoids

The important polyphenol groups in natural products are flavonoids which are classified as plant secondary metabolites [9]. They are presented in plant extracts as derivatives of 2-phenyl-benzo-γ-pyrone. It consists of two benzene rings, known as A and B, which are connected by pyrene ring (C). Flavonoids skeleton is based on flavan main structure as shown in Figure 1.1 in which the main flavonoid nucleus appeared [10].



Scheme 1.1: Basic carbon skeleton of flavonoid.

The term flavonoid involves the following commonly subclasses that shown in figure (2): flavanones, flavones, flavan-3-ols, flavonols, isoflavones and anthocyanins. Flavonoids can directly act as antioxidants and can be direct free radical scavengers, and additionally have ability to modulate enzymatic biological activities and to stop also cell proliferation cases. As known flavonoids are in general available in glycosylated forms in medicinal herbs, having sugar moieties which are important part to determine their bioavailability [11]. Flavonoids activities depend mainly on their structures. The chemical characteristics of flavonoids are usually based on their structural class, degree of hydroxylation, also some substitutions and presenting of few conjugations and polymerization degree [12].



Scheme 1.2: Basic structures of flavonoid subclasses.

Recently significant interest in these substances has been encouraged by the potential benefits of them for human health is due to the antioxidant effects of the polyphenolic compounds present in the plants. Hydroxyl groups in flavonoids chelate metal ions and hence have an antioxidant activity [13, 14].

A long history of medicinal use of Flavonoids have been suggested, mainly because these compounds improve the health of blood vessels. They are in addition reported as anti-spasmodic as well as anti-inflammatory natural remedies [15]. Furthermore, flavonoids have existed for over one

billion years and exerted anti-thrombic effect [16], anti- hypertensive activity was also reported in previous studies [17] and protection against prostate cancer after consumption of phytoestrogens which classified as Isoflavone group of flavonoids was also observed in some cases [18].

The antioxidant activity of flavonoids especially rutin and quercetin is due the presence of an o- dihydroxy of the catechol ring in their structures, and also because of the presence of double bond in conjugation with a 4-oxo function, as well as due to hydroxyl groups that are available in positions 3, 5 and 7 [19, 20].

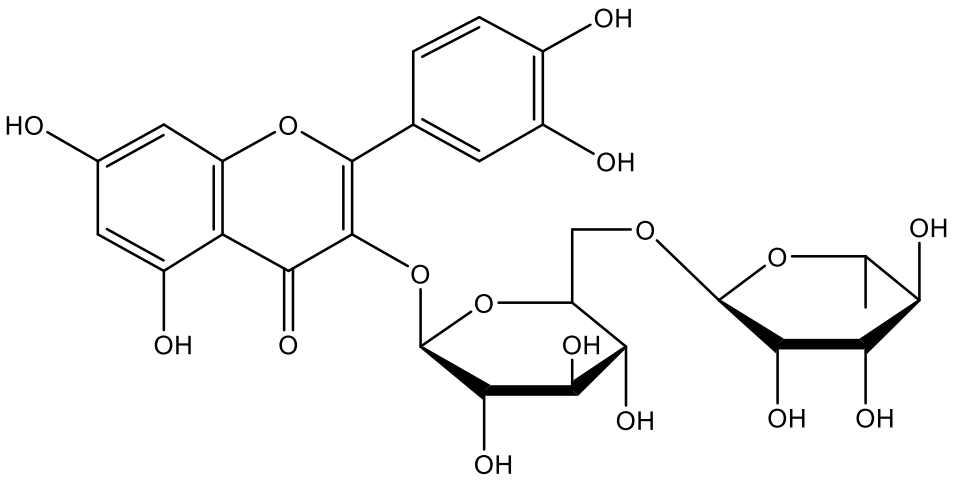
Trans esterification reactions can be achieved in some industries especially pharmaceutical type to prepare flavonoid semisynthetic derivatives, developing their physical and chemical characters and so these antioxidant compounds will be absorbed better. Acylated rutin derivatives have modifications in their physical and chemical properties which make their penetration to cell membranes more easy and efficient. Acylation of the flavonoid rutin through hydrophobic addition resulted in new characters on this compound.

1.3 Rutin overview

Rutin chemically is (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) as shown in figure (3); is a flavonoid glycoside that is widespread in the plant phytochemical compounds, rutin can be also named as; quercetin-3-rutinoside, rutoside, and sophorin [21] .This compound is

founded usually in various food sources especially in oranges, grapes, lemons, limes, peaches and berries [22, 23]. As well as Rutin has been founded in sufficient quantities for industrial extraction in several medicinal plants like *Sophora japonica*, *Eucalyptus macrorhyncha* and buckwheat [24].

Rutin is a fine microcrystalline, yellow, tasteless, odorless powder, soluble in 2 mL methanol and 4 mL water at 100°C, while hardly soluble in alcohol and also practically insoluble in chloroform and ether [25]. The medicinal applications of rutin are relatively limited because of its low water solubility (0.125 g/L) that seemed to restrict rutin usage [26].



Scheme 1.3: Chemical structure of Rutin.

Rutin is a bioactive plant flavonoid that owns significant biological therapeutic uses as its inhibition of free-radical, mediated cytotoxicity and lipid peroxidation. Furthermore, rutin increase the resistance as well as the permeability of capillary blood vessels [27].

It is also has some benefits in hypertension treatment, also builds a protective barrier against infection exerting antibacterial effect, anti-inflammatory as a result of diminishing the formation of pro-inflammatory mediators, as well as diuretic action was observed for rutin, lowers the intensity of cholesterol in blood stream. Rutin can be considered as a remedy for allergies, preventing cataracts and macular degeneration is also reported for rutin compound, disorientation and senility caused by advancing age. Rutin also helps in sustaining collagen synthesis in the tissue just below the skin to improve the epidermis appearance [28, 29].

Rutin treatment was reported to reduce blood glucose levels when elevated as shown previously in a study demonstrated beside that the onset of cardiovascular complications may be reduced and delayed by controlling some metabolic abnormalities [30].

1.3.1 Previous Scientific studies conducted on rutin

Rutin which belongs to flavenol group of flavonoids has low bioavailability due to poor absorption related to their glycoside form and polymers which limited its solubility. Acylation of glycosylated flavonoids increased the antioxidant effect of parent rutin compound [31].

On the other hand a study of the enzyme acetylation reaction was performed between rutin and esculin, to produce rutin and esculin monoacetate ester which has more effective antioxidant compound [32].

A new derivative of rutin was also synthesized and investigated for topical application to evaluate a potential usage of this derivative in the dermatology field and to investigate its antioxidant, antimicrobial, anti-inflammatory effects. Usual studies for stability were taken place by high performance liquid chromatography(HPLC); Photo chemi luminescence (PCL) assay and as shown also Oxygen Radical Absorbance Capacity (ORAC) tests were also performed to assess the antioxidant properties. New derivative of rutin (R2) presented an antioxidant activity highly similar to that of the parent Rutin but owing much higher lipophilic character. Concerned with anti-proliferative actions on the human erythroleukemic cell line (K562), R2 was more effective as reported than Rutin itself. Applied preliminary experiments demonstrated that R2 inhibits Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) action and encouraged cellular apoptosis [33]. In a recent research done by our team rutin prodrug was synthesized in order to improve the rutin solubility by selectively acetylating some of the hydroxyl group. The results were successful and the synthesized rutin showed an improved solubility of at least two fold [34].

1.4 Prodrugs therapeutic for transdermal drug delivery

Prodrug play a significant role in the discovery and designing of new drugs. Several therapeutic areas where in this approach can be applied and utilized. Achieving pro drug preparation will be going to be an emerging approach in years to come. Nowadays huge numbers of drugs are being

developed into prodrugs to reduce unwanted characters obviously [35] When Historical view was taken on prodrug approach it seemed that it was used to develop derivatives that could resist the hepatic metabolism and the introduction of prodrug concept was first achieved by Albert in 1951, while in recent days this approach has been attempted to synthesize derivatives of higher ability to penetrate skin. This approach included the chemical alteration and to modify of a known pharmacologically active compound into a bio reversible form as suggested, with the goal of endowing it suitable physicochemical(pharmaceutical) and/ or pharmacokinetic property and thereby modify the efficacy, membrane permeability and some therapeutically uses [36].

Prodrug is bio-reversible type of drug molecules which can go enzymatic and/or chemical transformation to give the parent drug. It has to transformed into the parent drug and the derived group must be eliminated and excreted rapidly [37].

As reported main parameters that promote the effects of prodrug, are pharmacokinetics, bio pharmaceutics, physicochemical as well as its toxicity and the bioactivity. As skin well known as a highly active metabolic organ and include a multitude of various enzymes that can metabolize a wide range of synthetic and xenobiotic compounds that occurred in nature. Skin can change prodrug to the active parent drug back just when they are in the skin layers. Hydrophilic drugs have poor diffusion

through the skin; the structural modification to formulate derivatives with higher lipophilicity usually increase the skin permeation capacity [38].

Some previous researches reported production of many rutin prodrugs such as rutin stearate, rutin butyrate [39], and rutin derivatives containing a 1, 4-pentadien-3-one moiety [40].

Most popular prodrug from rutin was hydroxyethylrutosides (HRs) which synthesized by substituting rutin hydroxyl groups with O-β-hydroxyethyl groups. Standardized mixtures of HRs consist of mono-HRs, diHRs, tri-HRs, and tetra-HRs, which differ in the number of their hydroxyethyl substituents these HRs mixtures available under different brand names such as Venoruton, Paroven, and others [41].

In addition another prodrug by deglycosilation of rutin to quercertin-3-glucoside (Q3G) was developed, this obtained derivative (Q3G) displayed more potent than rutin and quercetin as anti-proliferative agent against many human tumor cell lines including brain, ovary, breast, prostate, kidney, lung and colon [42].

1.5 Analytical Method Development and Validation of prodrug

Improving and developing of new analytical methods and analytical instruments resulted in reduction of the time and cost for analysis. Method development for the semi synthesized prodrug is usually performed using ultraviolet (UV) and HPLC. It seemed that the improvement of scientific and concrete analytical methods has been obtained from the advanced

analytical instruments which have been adopted. Reduction in the time and cost of analysis as well as enhancing of precision and accuracy have been also obtained by the improvements of the analytical method development and analytical instruments. Improving analytical techniques resulted new method development and validation of degradation products, excipients, active pharmaceutical ingredients and related substances. Quality control laboratories has benefitted from these methods to check the efficacy, purity and safety of drugs. Regulatory authorities check analytical methods and hence approval the entire process of drug development require using validated analytical methods [43-45].

Validation of analytical method required testing of parameters recommended by United States Pharmacopeia (USP), Food and Drug Administration (FDA) and International Conference on Harmonization (ICH). These parameters include: Specificity, Precision both Repeatability and Ruggedness, Accuracy, Limit of Detection (LOD) and limit of quantification (LOQ) [46, 47].

The development of various pharmaceutical dosage forms for the same active molecule is very essential in order to make various way drug administration either oral, parenteral and topical administration. The penetration of topical drugs is a challenge. Isolated animal skin was used to estimate percutaneous absorption of the prepared prodrug molecules, this step is important to understand the pathways, and driving forces of various factor across the skin barrier, after a formulation was completed and quality

control test was finished. The study of drug permeation by Franz cells are reproducible method. It is easy to implement in in the laboratory. It is an authenticated method to evaluate an *in vitro* drug permeation and has many advantages including few handling of tissues, no continuous sample collecting and low amount of drug is needed for analysis [48, 49].

1.6 Objectives of the research:

- Synthesis of a novel lipophlic rutin prodrug (acetyl, propyl and buty l etc).
- To obtain a suitable derivative with more liposolubility & improved drug delivery.
- Test the transpermeability of the synthesized prodrugs
- Test the hydrolysablity of the prodrugs by esterase enzyme.
- Formulation of the most suitable synthesized prodrug rutin.
- To develop a new validated method to quantify rutin derivatives as a raw material or in the final dosage form.

1.7 Significant of the research:

1- The Synthesis of a potentially more lipophilic give novel rutin prodrugs

2- Develop a new validated method to quantify prodrug rutin derivatives as a raw material as in the final dosage form

3- This project will provide a synthetic approach for many similar natural products

Chapter Two
Methodology

2.1 Reagents

Rutin trihydrate (USP grade) powder was purchased from (MP Biomedicals, USA). Acetyl chloride 98% (Lot# S4183352 502), N,N Diisopropylethyl amine (DIPEA) (catalog # 496219), Acetone, ethanol (EtOH), dimethylaminopyridinr (DMAP) , dichloromethane (DCM), and n-hexane (Hex) (catalogue # 2355544800024) were purchased from (C.S. Company, Haifa). Triethylamine (Et_3N) (catalog # 40502L05) and diethyl ether (catalog # 38132) were purchased from (Merck Millipore). Sodium sulfate (Na2SO4). Butryl anhydride (Lot # 10190649, Sigma aldrich), benzoic anhydride (Lot # BCBK8739V, Sigma aldrich), isovaleric anhydride (Lot # SHBJ1435, Sigma aldrich), isobutryl anhydride (Lot # SHBJ0529, Sigma aldrich), propionyl chloride (Lot # 10192402, Sigma aldrich), 1- octanol (O- 4500) , benzyl alcohol, Vaseline , paraffin oil.

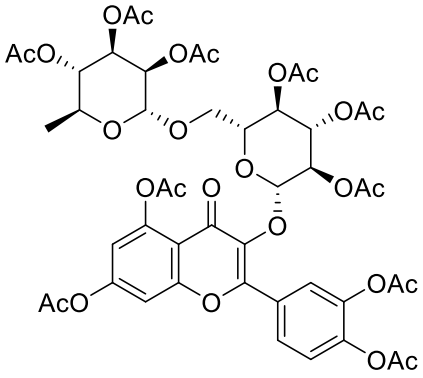
2.2 Equipments

Sensitive weighing balance (Adventurer®, OHAUS Corporation, hotplate stirrer (Lab tech^{R,} daihasn lab tech co,ltd, India), Rotary Evaporator (MRC, ROVA-100, laboratory equipment manufacturer) and NMR spectra performed for the products using Bruker Avance 500 spectrometer at Jordan University. Water bath and Sonicator (Elmasonic S 70 H, Elma®, Germany). Absorption analysis was conducted on (Spectrophotometer-7315, Jenway, UK) using 10-mm quartz cuvettes.

Centrifuge (UNIVERSAL-320, Hettich, Zentrifugen, Germany). Centrifuge-DCS-16-RVT (Prevac, Canada), used for 4 °C, melting point apparatus (GALLENKAMP, UK) and Franz cell diffusion apparatus.

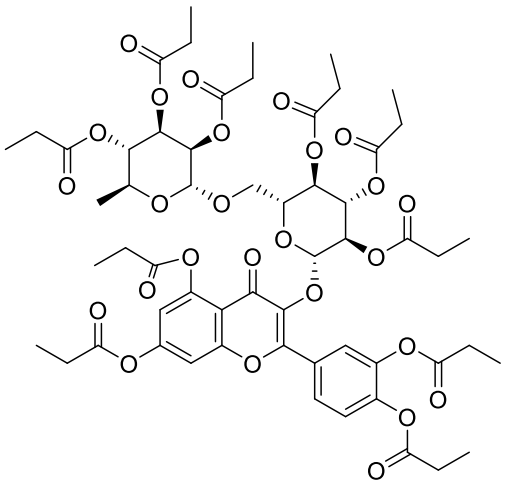
2.3 Chemical synthesis

2.3.1 Synthesis of Decaacetylated rutin (R-10-OAc)



Compound **(R-10-OAc)** was synthesized as published previously by our research groups [50]

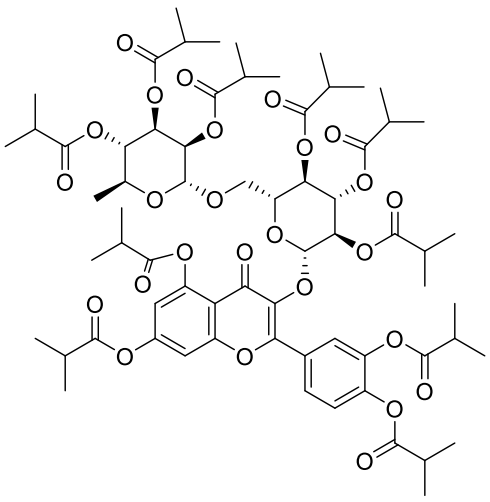
2.3.2 Synthesis of decapropionate rutin (Ru-10-Prop)



The decapropionate rutin was synthesized by dissolving rutin (0.4 g, 0.66 mmol) in 20 mL DCM and then propionyl chloride (1.7 mL, 19.6 mmol) and 4,4-dimethylamino pyridine (2.9 g, 23.7 mmol) were added as catalyst. The reaction was then stirred for 18 hours at room temperature. Then, the reaction was washed with water and brine (100 ml x 3) and the organic layer was extracted. Followed by addition of sodium sulfate as drying agent, filtration and evaporation to obtain the crude product. The product was purified using silica gel column chromatography (eluent: Hexane:Ethyl acetate, 2:1, v/v) giving R-10-O-Propioante (90%) as a white powder (R_f : 0.44, Hex:EtOAC 2:1).

^{1}H NMR (500 MHz, $CDCl_3$) : δ 7.90-7.86 (m, 2H, Ar), 7.27 (s, 1H, Ar), 7.24 (dd, *J* = 3.3 Hz, *J* = 1.2 Hz, 1H, Ar), 7.20 (d, *J* = 1.0 Hz, 1H, Ar), 6.67 (dd, *J* = 2.2 Hz, *J* = 1Hz, 1H, OCHO), 5.35 (d, *J* = 7.9 Hz, 1H, OCH‘O), 5.24-5.11 (m, 3H, CHO, 2CHO), 5.05-4.99 (m, 3H, OC<u>H</u>O, 2CHO), 4.89 (t, *J* = 9.6 Hz, 2H, 2CHO), 3.61-3.55 (m, 2H, C<u>H</u>CH_2O, C<u>H</u>CH_3), 3.51-3.43 (m, 2H, CHC<u>H_2</u>O), 2.56-2.49 (m, 8H, 4ArOCOCH_2), 2.23-2.09 (m, 12H, 6OCOCH_2), 1.08-0.95 (m, 33H, OCOCH_2C<u>H_3</u>, CH_3). ^{13}C NMR (125 MHz, DMSO): δ 173.4, 173.3, 173.1, 172.9, 172.5, 172.2, 171.9, 171.8, 171.7, 156.6, 154.6, 154.5, 150.1, 142.2, 136.4, 128.5, 127.6, 124.7, 124.0, 114.9, 98.6, 97.5, 70.1, 69.1, 69.0, 68.9, 66.2, 27.4, 27.3, 27.2, 27.0, 17.1, 9.5, 9.4, 9.3, 9.1, 9.0.

2.3.3 Synthesis of the isobutyrate rutin (Ru-10-iBu)

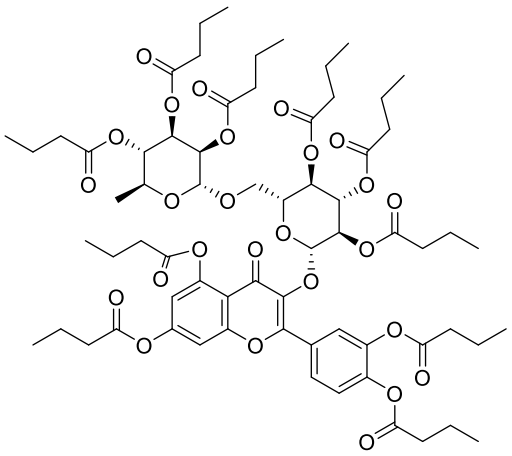


The synthesis of the isobutyrate rutin, the rutin (0.4 g, 0.66 mmol) was dissolved in 15 mL of DCM, then DMAP (0.6 g, 4.91 mmol), triethylamine (0.69 mL, 4.91 mmol) and isobutyric anhydride (3.26 mL, 19.7 mmol) were added. The reaction was stirred at room temperature overnight. Then the reaction was washed with DCM (200 mL x 3) and HCl 1M (100 mL x 3). The organic layers were collected and dried using anhydrous Na_2SO_4. The crude product was purified using silica gel column chromatography (eluent: Hexane/Ethyl acetate, 4/1, v/v) giving R-10-O-*i*Buterate (98%) as a brown powder (R_f : 0.48, Hex:EtOAC 4:1).

^{1}H NMR (500 MHz, DMSO): δ 7.99-7.97 (m, 2H, Ar), 7.50 (d, *J* = 2.4 Hz, 1H, Ar), 7.38 (d, *J* = 9.2 Hz, 1H, Ar), 7.08 (d, *J* = 2.1 Hz, 1H, Ar), 5.69 (d, 1H, *J* = 7.9 Hz, OCHO), 5.49 (t, 1H, *J* = 9.6 Hz, OCH‘O), 5.01-4.94 (m, 3H, CHO, 2CHO), 4.89-4.84 (m, 3H, OC<u>H</u>O, 2CHO), 4.76 (t, 1H, *J* = 10.1 Hz, CHO), 4.48 (s, 1H, CHO), 3.98-2.94 (m, 2H, C<u>H</u>CH_2O, C<u>H</u>CH_3), 3.57-3.54 (m, 2H, CHC<u>H_2</u>O), 2.89-2.76 (m, 5H, 5C<u>H</u>$(CH_3)_2$),

2.40-2.21 (m, 5H, 5C<u>H</u>$(CH_3)_2$), 1.06-0.88 (m, 63H, 10CH($C\underline{H_3}$)$_2$, CH_3). ^{13}C NMR (125 MHz, DMSO): δ 175.7, 175.6, 175.4, 175.3, 175.2, 174.8, 174.5, 174.2, 174.0, 171.7, 156.6, 154.6, 150.2, 144.6, 142.2, 136.3, 128.6, 127.4, 124.9, 123.9, 115.1, 114.7, 110.1, 98.7, 97.6, 72.2, 72.0, 71.9, 70.0, 69.2, 68.8, 66.3, 33.9, 33.8, 33.7, 33.6, 33.5, 19.4, 19.1, 19.0, 18.9, 18.8, 18.7, 17.2.

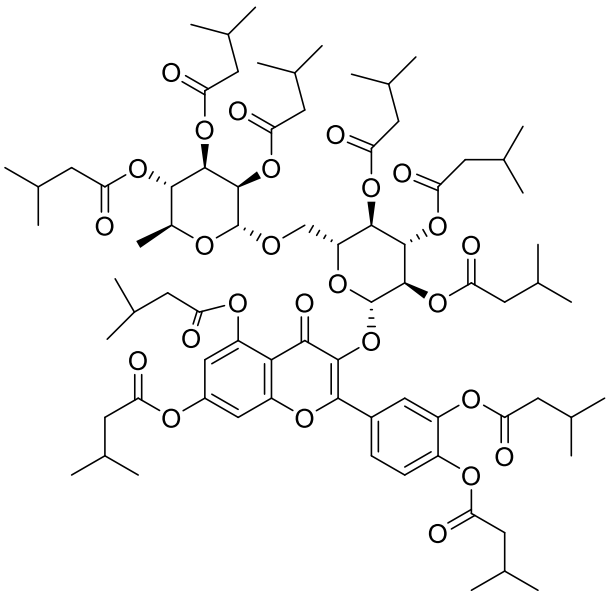
2.3.4 Synthesis of the butyrate rutin (Ru-10-Bu)



The synthesis of the butyrate rutin, the rutin (0.4 g, 0.66 mmol) was dissolved in 15 mL of DCM, then DMAP (0.52 g, 4.27 mmol), triethylamine (0.6 mL, 4.27 mmol) and butyric anhydride (3.21 mL, 20.3 mmol) were added. The reaction was stirred at room temperature for 24 hours. Then the reaction was washed with DCM (200 mL x 3) and HCl 1M (100 mL x 3). The organic layers were collected and dried with anhydrous Na_2SO_4. The crude product was purified using silica gel column chromatography (eluent: Hexane/Ethyl acetate, 1/1, v/v) giving R-10-O-Buterate (93%) as a brown powder (R_f : 0.19, Hex:EtOAC 1:1).

^{1}H NMR (500 MHz, DMSO): δ 8.03-7.99 (m, 2H, Ar), 7.55 (d, 1H, *J* = 2.1 Hz, Ar), 7.43 (d, 1H, *J* = 8.5 Hz, Ar), 7.11 (d, 1H, J = 2.1 Hz, Ar), 5.73 (d, 1H, *J* = 7.6 Hz, OCHO), 5.48 (t, 1H, *J* = 9.6 Hz, OCH‘O), 5.05-4.99 (m, 3H, CHO, 2CHO), 4.93-4.88 (m, 3H, OC<u>H</u>O, 2CHO), 4.77 (t, 2H, *J* = 9.7 Hz, 2CHO), 3.98-3.94 (m, 2H, C<u>H</u>CH_2O, C<u>H</u>CH_3), 3.57-3.53 (m, 2H, CHC<u>H_2</u>O), 2.69-2.58 (m, 8H, 4ArOCOCH_2), 2.32-2.05 (m, 12H, 6OCOCH_2), 1.74-1.41 (m, 20H, 10OCOCH_2C<u>H_2</u>), 1.04-0.80 (m, 33H, OCOCH_2CH_2C<u>H_3</u>, CH_3). ^{13}C NMR (125 MHz, DMSO): δ 174.8, 172.4, 172.2, 172.1, 172.0, 171.8, 171.6, 171.2, 170.9, 170.7, 156.6, 154.5, 150.0, 144.4, 142.1, 136.4, 128.5, 127.5, 124.8, 124.0, 115.0, 114.7, 110.1, 97.5, 72.1, 71.9, 71.8, 70.0, 69.0, 68.9, 66.5, 66.2, 36.0, 35.8, 35.7, 35.6, 35.5, 35.4, 18.5, 18.4, 18.3, 18.2, 18.1, 18.0, 17.2, 14.0, 13.8, 13.7, 13.6.

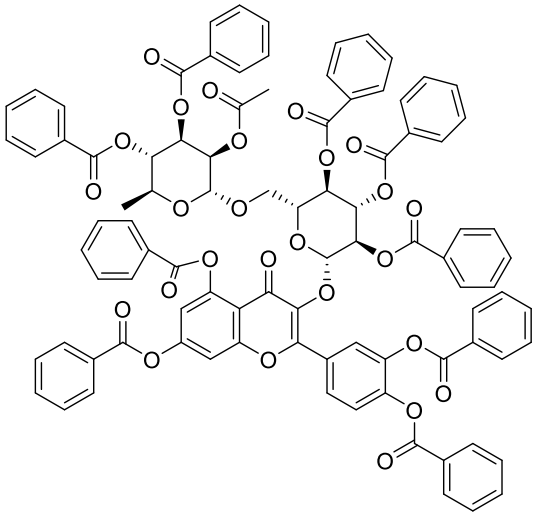
2.3.5: Synthesis of the isovaleric rutin (Ru-10-O-*i*Valerate)



The synthesis of the isovalerate rutin, the rutin (0.4 g, 0.66 mmol) was dissolved in 15 mL of DCM, then DMAP (0.6 g, 4.91 mmol), triethylamine (0.91 mL, 6.45 mmol) and isovaleric anhydride (4.00 mL, 20.01 mmol) were added. The reaction was stirred at room temperature for 18 hours. Then the reaction was washed with DCM (200 mL x 3) and HCl 1M (100 mL x 3). The organic layers were collected and dried with anhydrous Na_2SO_4. The crude product was purified using silica gel column chromatography (eluent: Hexane/Ethyl acetate, 4/1, v/v) giving R-10-O-*i*Valerate (91%) as a brownish oil (R_f : 0.55, Hex:EtOAC 4:1).

^{1}H NMR (500 MHz, DMSO): δ 8.02-8.00 (m, 2H, Ar), 7.52 (d, *J* = 2.4 Hz, 1H, Ar), 7.42 (d, *J* = 8.9 Hz, 1H, Ar), 7.10 (d, *J* = 2.1 Hz, 1H, Ar), 5.70 (d, 1H, *J* = 7.9 Hz, OCHO), 5.49 (t, 1H, *J* = 9.5 Hz, OCH‘O), 5.06-4.98 (m, 3H, CHO, 2CHO), 4.92-4.88 (m, 3H, OC<u>H</u>O, 2CHO), 4.79 (t, 1H, *J* = 10.1 Hz, CHO), 4.50 (s, 1H, CHO), 3.97-2.93 (m, 2H, C<u>H</u>CH_2O, C<u>H</u>CH_3), 3.57-3.52 (m, 2H, CHC<u>H_2</u>O), 2.23-1.84 (m, 30H, 10 C<u>H_2</u>$CH(CH_3)_2$,10CH_2C<u>H</u>$(CH_3)_2$), 1.05-0.80 (m, 63H, 10CH(C<u>H_3</u>$)_2$, CH_3). ^{13}C NMR (125 MHz, DMSO): δ 176.2, 175.9, 175.6, 175.4, 175.3, 174.9, 174.7, 174.6, 174.5, 172.2, 157.1, 155.6, 150.5, 144.4, 142.2, 136.3, 128.6, 127.4, 124.9, 123.9, 115.1, 114.7, 110.5, 98.6, 97.9, 72.2, 72.1, 71.7, 70.0, 69.0, 67.8, 66.5, 44.9, 44.8, 44.7, 44.6, 44.5, 33.9, 33.9, 33.7, 33.6, 33.4, 19.5, 19.2, 19.1, 19.0, 18.8, 18.7, 17.2.

2.3.6 Synthesis of the benzoate rutin (Ru-10-O-Benzoate)



The rutin (0.4 g, 0.66 mmol) was dissolved in 15 mL of DCM, then DMAP (0.6 g, 4.91 mmol), triethylamine (0.91 mL, 6.45 mmol) and benzoic anhydride (4.45 g, 19.7 mmol) were added. The reaction was stirred at room temperature for 18 hours. Then the reaction was washed with DCM (200 mL x 3) and HCl 1M (100 mL x 3). The organic layers were collected and dried with anhydrous Na_2SO_4. The crude product was purified using silica gel column chromatography (eluent: Hexane/Ethyl acetate, 4/1, v/v) giving R-10-O-benzoate (90%) as a white powder (R_f : 0.2, Hex:EtOAC 2:1).

^{1}H NMR (500 MHz, DMSO): δ 8.45-8.42 (m, 2H, Ar), 8.28 (d, *J* = 7.9 Hz, 2H, Ar), 8.20 (d, *J* = 7.9 Hz, 2H, Ar), 8.11 (d, *J* = 8.2 Hz, 2H, Ar), 8.01 (d, *J* = 7.9 Hz, 2H, Ar), 7.96-7.23 (m, 45H, Ar), 6.20-6.13 (m, 2H, 2OCHO), 5.66 (t, 1H, *J* = 8.5 Hz, CHO), 5.59-5.51 (m, 3H, 3CHO), 5.40 (t, 1H, *J* = 9.5 Hz, CHO), 4.97 (s, 1H, CHO), 4.58 (bs, 1H, CHO), 4.12 (m, 1H, CHC$\underline{H_2}$O), 3.92 (d, 1H, *J* = 10.4 Hz, CHO), 3.72-3.68 (m, 1H,

CHC$\underline{H_2}$O), 1.09 (d, *J* = 6.1 Hz, CH_3). ^{13}C NMR (125 MHz, DMSO): δ 171.8, 167.8, 165.6, 165.5, 165.3, 165.0, 164.9, 164.8, 164.1, 164.0, 163.8, 156.6, 155.0, 154.5, 150.0, 144.7, 142.7, 136.8, 135.0, 134.8, 134.5, 134.3, 134.2, 134.0, 133.3, 131.3, 130.6, 130.5, 130.2, 130.1, 129.8, 129.7, 129.6, 129.5, 129.4, 129.3, 129.2, 129.1, 129.0, 128.9, 128.6, .1284, 128.3, 127.6, 124.4, 115.5, 110.4, 99.1, 98.1, 73.3, 72.9, 72.8, 71.7, 70.3, 70.2, 66.6, 36.7, 24.8, 24.2, 21.2, 17.6.

2.4 Determination of Octanol-water partition coefficient (LogP)

The wavelength of maximal absorption (λ_{max}) of each synthesized compound using UV/Vis spectrophotometer device was determined by performing UV-Vis scan in the range of (200-600 nm). Octanol-water partition coefficient (log P) was determined using a constructed calibration curve of a serial diluted standard of the rutin derivative. The calibration curve was prepared from a stock solutions of 1 mg/ml of each synthesized rutin derivatives. Five serial dilutions were then prepared form the above stock solution and were used to construct a calibration curve. Calibration curves were constructed by using Microsoft Excel 2007. The display regression equation was used to calculate the concentration.

Estimation of the octanol-water partition coefficient (P) was determined for the decaacetylated rutin, decabuterate rutin, decabenzoate rutin, decaisobuteric rutin, decaisovaleric rutin, decapropionate rutin and underivatized rutin. The synthesized rutin derivatives (10 mg) was added to 20 ml 1-octanol and 20 ml water in a round bottom flask. The mixture was

then stirred for 30 minutes at 25 ^{0}C. The mixture was centrifuged for 10 minutes at 4000 rpm and the two layers were separated. The absorbance of the upper layer (1-octanol layer) was measured. The amount of partitioned synthesized rutin ester in the organic layer was calculated by applying the absorbance reading to the calibration curve regression line equation. Octanol-water partition coefficient (LogP) was calculated according to the following equation:

$$\text{Log P} = log \frac{\text{amount of material partitioned into 1-octanol layer}}{\text{amount of material partitioned into water layer}}.$$

2.5 Topical ointment Formulation:

The ointment was prepared by weighing 8.2 g vaseline as base, 1.25 g paraffin oil as base and were mixed together at 75 C°. The active ingredient (0.050 g) and 0.5 g benzyl alcohol were dissolved and added to the melted vaseline and paraffin oil mixture. The reaction mixture was then left to cool down to room temperature. Two more ointment formulation were prepared in the laboratory in which the amount of benzyl alcohol (penetration enhancer) was varied (0.25 g and 0.75g) while keeping the other compositions the same. The amount change of benzyl alcohol was adjusted by changing vaseline amount in order to have a net weight of 10 g. The intentional variation in new two formulations was done in order to optimize the best diffusion through the skin.

Four different active ingredients were formulated into ointment. These ingredients were: decaacetylated rutin, decaisobuterate rutin, decapropionate rutin and decabuterate rutin.

2.6 Determination of diffusion of Rutin and synthesized rutin esters through Franz diffusion cell:

At First the solubility of the synthesized rutin prodrugs in ethanol was examined. One of the synthesized ester (Decapropionate) was used to examine its solubility in ethanol. The solubility of this produg will be an indicator of the solubility of the other synthesized esters in ethanol. Moreover, the exact solubility of the underivatized rutin were determined. Two separate stock solutions of 2 mg/ml of decapropionate rutin and underivatized rutin were prepared. Five serial dilutions were then prepared form the above stock solutions and were used to construct a calibration curve at λ_{max} 320nm and 360nm for decapropionate rutin ester and rutin respectively. A calibration curve was plotted using Microsoft Excel 2007. The displayed regression line equation was used to calculate the solubility. The solubility in ethyl alcohol for rutin and decapropionate rutin ester derivative were determined by adding of 1 mL of ethyl alcohol to an excess amount of rutin or decapropionate rutin derivative in sterile 2 mL centrifuge Tube (Eppendorf). Centrifugation was then performed at 10000rpm for 10 minutes. The absorbance of the supernatant solution of the samples was measured after centrifugation. The concentration of the dissolved rutin or the decapropionate rutin compound was then calculated

from the calibration curve.

The diffusion through a skin of the rutin derivative in the ointment formulation of a dissected mouse was examined using Franz diffusion cell shown in figure 2.6 .

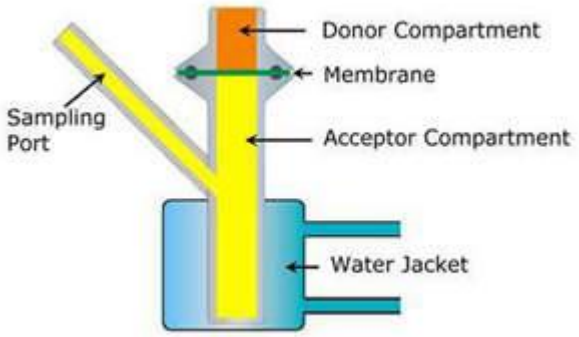


Figure 2.6: Franz diffusion cell**.**

A fresh skin from either the abdomen or the back of mouse was installed on Franz diffusion cell apparatus in our research Laboratory. A definite amount of the ointment were weighed and applied to the skin of the mouse after filling the receptor chamber with ethyl alcohol **(Table 2.1).** The donor chamber is placed over the membrane and sealed with a clamp. Samples were then withdrawn every half hour for six hours. The same experiment was repeated three times for each formulation and the average reading was taken for calculation.

The concentration of the released active ingredient was calculated using the absorbance at maximal absorption (λ_{max}) determined previously for each active ingredient and applying the regression line equation generated for the calibration curve

Table 2.1: Weights applied on the skin of the Franz diffusion

Ointment	Weight (gm)
Decaacetylate rutin	0.2149gm
Decabuterate rutin	0.226gm
Decaisobuterate rutin	0.135gm
Decapropionate rutin	0.123gm
Original rutin	0.235gm

2.7 Analytical Method development

Analytical method development using UV spectrophotometer was done for the Decaacetylated rutin ester in an ointment dosage form. The method development steps and tested validation parameters are illustrated in this section.

2.7.1 Determination of a selective wavelength of maximum absorbance

The Decaacetylated rutin ester was dissolved in ethyl alcohol and was diluted to a sufficient quantity with ethanol, the UV absorbance in the range of 200-600nm was scanned and recorded to determine the wavelength of maximal absorption (λ_{max}).

The Selectivity of the wavelength (λ_{max}) absorption to the active ingrdeint in the ointment was performed by scaning Decaacetylated rutin UV absorbance spectrum in the range (200-600nm) in presence of benzyl alcohol. The synthesized rutin prodrug 0.1 mg/ml was dissolved in ethyl alcohol and was placed in a 25 ml volumetric flask. Two drops of benzyl alcohol was added to the solution and the volume was then completed to 25 ml. The mixture was allowed to shake for 1 minute. The solution mixture was then scanned in the range (200-600nm) to determine the (λ_{max}).

2.7.2 Method of analysis of formulated ointment

Decaacetylated rutin ester ointment (2 g) was weighed and heated until the petroleum jelly and paraffin oil was dissolved. The dissolved ointment was mixed with ethanol (15 mL) in a clean Falcon Tube and put it in centrifuge 4000 rpm for 10 minute to separate it. The sample after being separated was filtered and then the volume was completed to 20 ml. The absorption of the filter solution was measured using UV/Vis device at λmax (320 nm). The actual amount of deccaacetylated rutin was calculated using the established regression line equation of the calibration curve.

2.7.3 Analytical method validation:

Linearity and range;

The linearity and range of method was performed by preparing a stock solution (0.2 mg/ml) which was prepared by weighing 20 mg of decaacetylated rutin ester powder and dissolved it in 100 ml ethyl alcohol solvent. The stock solution (0.2mg/ml) was further diluted to prepare five solutions (0.06, 0.08, 0.1, 0.12, 0.14 mg/ml). A Calibration curve was constructed using the predetermined maximal absorption (λ_{max}) of the compound. The regression line equation and the R^2 of the diagram were generated using Microsoft excel 2007.

Accuracy and precision:

Decaacetylated rutin ester ointment (2 g) was weighed and heated until the petroleum jelly and paraffin oil were dissolved. The mixture was then put it in a sterile Falcon Tube having ethanol. The mixture was then put in centrifuge to separate it. The sample mixture after being separated was filtered and the volume was completed to 20 ml with ethanol. The absorption of the filter solution was measured using UV/Vis device at λmax (320 nm). The actual amount of deccaacetylated rutin was calculated using the established regression line equation of the calibration curve.

Robustness of the analytical method:

Robustness of the developed analytical method was tested by applying small intentional variations to the method analytical test conditions. These variations involved testing on different days, using different analysts, measuring at different UV/Vis spectrophotometer instrument wavelengths (320 ±2 nm). The experiment was carried out with a concentration of 0.1 mg/ ml.

2.8 Stability indicating study of decaacetylated rutin under different stress conditions:

A stability indicating study was done using forced degradation which was done by subjecting the active ingredient along with the excipients to stress conditions of acid/base hydrolysis, heat, light and oxidation. A solution having a concentration of 0.1 mg/mL was subjected to different

stress condition and samples were analyzed frequently; stress testing is stopped if 5-20% degradation is obtained, or when no degradation is observed after the maximum recommended time. Three separate sample solution (9 ml) were taken. To the first samples 1 ml of 1N HCl was added, to the second sample 1N NaOH and to the third 3% H_2O_2 were added. Other sample solutions were subjected to heat (70 ^{0}C) and UV (254 nm) light. Thus all the samples were examined under stress conditions including: thermal, photolytic, oxidizing, acidic and basic stress conditions.

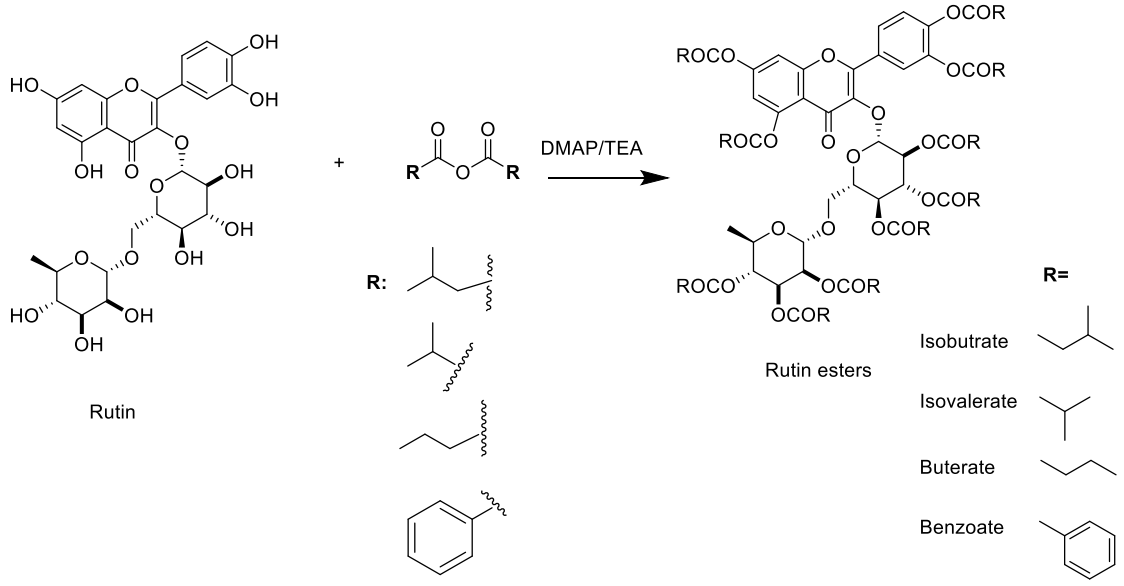
2.9 *In vitro* hydrolysis test

The synthesized rutin prodrugs (R-10-OAc, R-10-Prop, R-10-Bu, R-10-iBu) were exposed to esterase enzyme to be hydrolyzed. It was achieved by incubating 1 mg of rutin prodrug within 10 mL ethyl alcohol containing 1 mg of esterase enzyme. Samples were taken from the solution over a period of eight hours. Each time an aliquot was taken and replaced with ethyl alcohol to maintain the sink condition. The concentration of the hydrolyzed prodrug was determined using the developed Spectrophotometric method.

Chapter Three
Result and discussion

3.1 Chemical synthesis

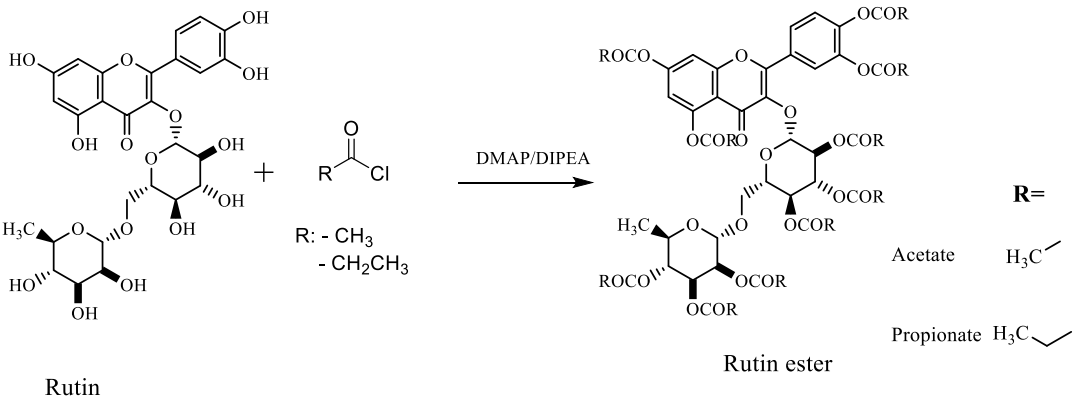
The synthesis of the fully buterated, benzoated, isobuterated and isovalerated rutin ester were successfully achieved. The synthesis using various anhydrides, DMAP and triethylamine as catalysts. The method is summarized as shown **Scheme 3.1.1**. The produced rutin esters were obtained in good yields ≥ 90%. The structures of the products were confirmed by nuclear magnetic resonance.



Scheme 3.1: synthesis of buterated, benzoated, isobuterated and isovalerated rutin ester.

The synthesis of the fully acetylated and propionate rutin esters were achieved in As shown in **Scheme 3.1.2**. The synthesis involved using acyl chlorides and DMAP/ DIPEA order to obtain the fully esterified rutins in good yield ≥ 84%. The structures of the products was confirmed by nuclear

magnetic resonance



Scheme 3.2: Synthesis of acetylated and propionate rutin esters.

3.2 logP of synthesized rutin ester

The λ_{max} for decaacetylated rutin ester derivative and rutin was found to be 330 nm and 360 nm respectively. However, the calibration curves for decabenzoate rutin, decaisobuteric rutin, decaisovaleric rutin, decapropionate rutin, decabuterate rutin were constructed at the predetermined λmax of 300 nm.

The experimentally determined partition coefficient (logP) of the synthesized rutin ester for decaacetylated rutin, decabuterate rutin, decabenzoate rutin, decaisobuteric rutin, decapropionate rutin and decaisovaleric rutin were found to be 0.61, 1.06, 0.81, 0.70, 0.97and -0.60 respectively. The above results indicate lipophilic characteristics of the synthesized rutin derivative compared which has a logP value of -0.64 for rutin except decaisovaleric rutin ester. The logP values indicate a better lipid solubility of the mentioned compounds compared to rutin and the

results indicate that the structural modification done on rutin will improve its transdermal bioavailability of the newly synthesized derivative rutin ester.

3.3 Diffusion of Rutin and a newly synthesis rutin derivative through Franz diffusion cell:

Four rutin ester derivatives were selected for ointment formulation. The selection of the synthesized compounds was based on the lipohilicity and solubility of the synthesized compounds in ethyl alcohol which was used as a solvent in Franz diffusion experiment. Tow rutin ester derivatives were excluded from ointment formulation , The decaisovalerate rutin compound was excluded because it was an low value of log p and the decabenzoate rutin compound was excluded due to its low solubility in ethanol.

Decapropionate ester calculated solubility in ethyl alcohol was (10 mg/ml). The solubility of the rutin derivatives in ethyl alcohol was good for all the synthesized compound. Moreover, the underivatized rutin showed good solubility (0.8mg/ml)

Based on the log P value and the synthetic yield, four derivatives were selected for ointment formulation and their permeability through the skin was tested using Franz diffusion.

The Franz diffusion results of the ointment formulated rutin derivatives of decaacetylated, decapropionate, decabuterate, decaisobuterate rutin ointments are shown in Figure 3.1.

The results indicate that the diffusion was best for decaacetylated rutin compared to the other esterified rutin compounds. While as expected the rutin transdermal diffusion was almost zero.

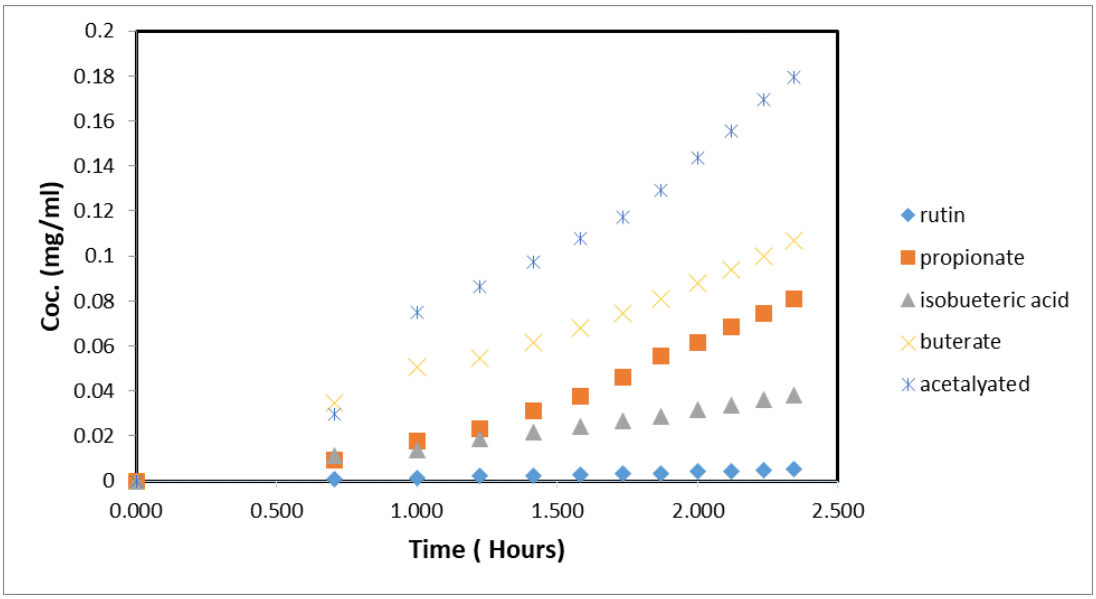


Figure 3.1: The Franz diffusion results of rutin derivatives in ointment formulation.

3.4 Analytical method development

As the decacetylated rutin ester formulated ointment showed the highest transdermal diffusion; a simple and validated analytical method was developed.

The UV absorbance of decaacetylated rutin in the range of (200-600 nm) showed two wavelength of maximum absorption at 245 and 320 nm (**Figure 3.2**). It was decided to consider 320 nm as a measuring (λ_{max}) and

not 245 nm for specificity and accuracy reasons.

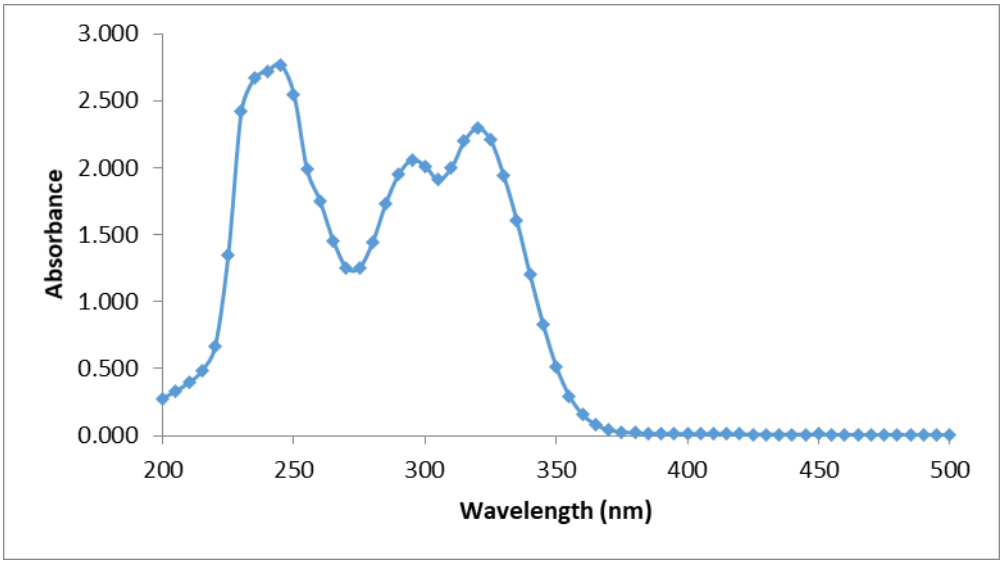


Figure 3.2: UV spectrum of decaacetylated rutin sample**.**

The results showed that there is selectivity of the specified absorbance wavelength with formulation excipients. The UV absorption scan for Decaacetylated rutin in presence benzyl benzoate excipient in the range (200-600 nm) showed no interference with the measuring (λ_{max}). **Figure 3.3** Showed the resulted absorption scan in the range of 200-600 nm with a maximum absorption wavelength of 325 nm.

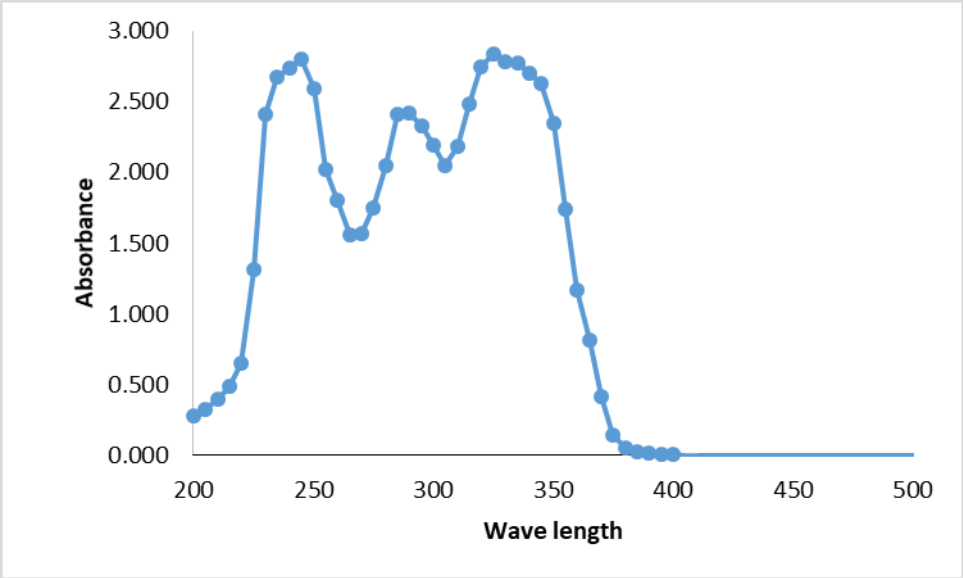


Figure 3.3: Absorption scan of Decaacetylated rutin in presence of benzyl alcohol.

3.5 Method validation

Linearity and range of decaacetylated rutin ester:

The linearity and range of method was done by measuring the absorbance of five prepared test solutions in the range of (0.06-0.14 mg/ml). The concentration (mg/ml) and absorbance these concentrations are shown in Table 3.1

Table 3.1: Ethyl alcohol calibration curve test solution.

Concentration (mg/ml)	Absorbance (nm)
0.06	0.871
0.08	1.058
0.1	1.35
0.12	1.58
0.14	1.803

The calibration curve was constructed using excel 2007 were Y-axis represented the absorbance and X-axis represented the concentration. The calibration curve is shown in **Figure 3.4.** The linearity relationship

between UV absorbance and concentration was examined and it showed a linear relationship with R^2 value of 0.996. The regression line equation was: y = 11.93x + 0.139.

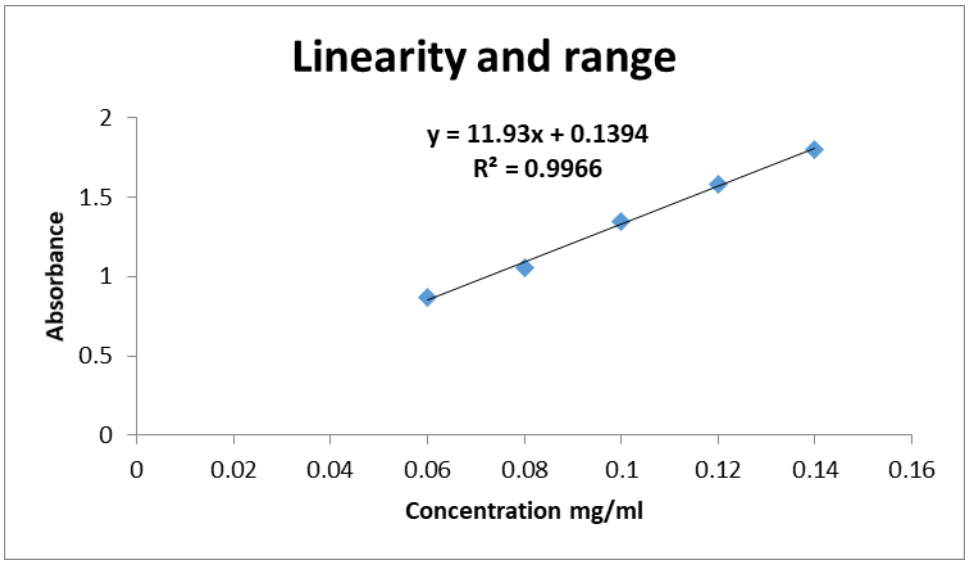


Figure 3.4: linearity curve of decaacetylated rutin ester.

Accuracy and precision:

Decaacetylated rutin ester ointment was analyzed by the developed analytical method using UV/Vis at λ_{max} of 320 nm. The analysis was repeated four times. The concentration of decaacetylated rutin ester in ointment was then calculated and compared with actual concentration; the calculated percentage accuracy and the % RSD are shown in **Table 3.2**

Table 3.2: Accuracy and precision:

Concentration (mg/ml)	**% Accuracy**
0.099627	99.6
0.099031	99
0.099404	99.4
0.099255	99.2
%RSD	**0.25%**

The percentage accuracy and the %RSD results indicate good accuracy and precision of the developed analytical method.

Robustness:

Small variations were applied on a number of the analytical method parameters to confirm robustness of the method. Results shown in Table 3.3 indicate stability of the test when the test was done in different days, different analyst, different instruments, and when applying small variations in the measurement wavelength (± 2 nm). The experiment was carried out with a concentration of 0.01 mg /ml.

Table 3.3: Robustness of the developed UV analytical method

Day to day variations	
Day	**Concentration mg/ml**
Day 1	0.097392
Day 2	0.097541
Day 3	0.097243
RSD%	0.15%
Different analyst	
Analyst 1	0.097392
Analyst 2	0.097168
RSD%	0.16%
Different instrument	
Instrument 1	0.097392
Instrument 2 RSD	0.097765 0.26%
Wavelength variation	
320nm	0.097392
322nm	0.094411
318nm	0.097541
%RSD	1.77%

3.6 Stability indicating study of decaacetylated rutin under different stress conditions:

Decaacetylated rutin solutions were subjected to different stress conditions including 1N NaOH, 1 N HCl, UV light (254 nm), 3% H_2O_2 and 70 ^{0}C temperature. The results under each condition are shown separately in Table 3.4.

Table 3.4: Stability of decaacetylated rutin under different stress conditions:

Stress conditions;	Time (hour)	Concentration	Degradation %
Temperature (70^{0}C)	1	0.063115	63%
	6	0.005365	5.3%
UV (254 nm)	0.5	0.076826	77%
	24	0.014978	15%
1N NaOH	0.5	0.075261	75%
	24	0.049627	50%
1 N HCl	1	0.096647	96%
	25	0.039344	39%
3% H_2O_2	1	0.098137	98%
	26	0.042027	42%

The stability indicating results showed that the compound was unstable under all the subjected stress condition. The main reason explaining the instability of the acetylated rutin ester compound under these stress conditions is the fact that ester bonds are generally unstable bond. Moreover, the results showed decay in the absorbance of all the measured samples under all the above stress conditions which indicate specificity and selectivity of the analytical method.

3.7 *In vitro* esterase hydrolysis

In vitro esterase hydrolysis of the synthesized rutin prodrugs were investigated. Since various prodrugs of the rutin were synthesized by ester bond formation, these prodrugs were incubated with 1 mg of esterase enzyme in order to study the percentage of hydrolysis of the ester bond upon time. As can be observed in **Figure 3.5**, all the synthesized prodrugs have achieved almost complete hydrolysis after seven hours with a half-life of 3.5-4 hours. These results confirm that after the systematic absorption of the prodrugs, they will almost completely convert to the active rutin. Moreover, these data prove that the synthesized ester derivatives didn't impair the activity of the esterase enzyme.

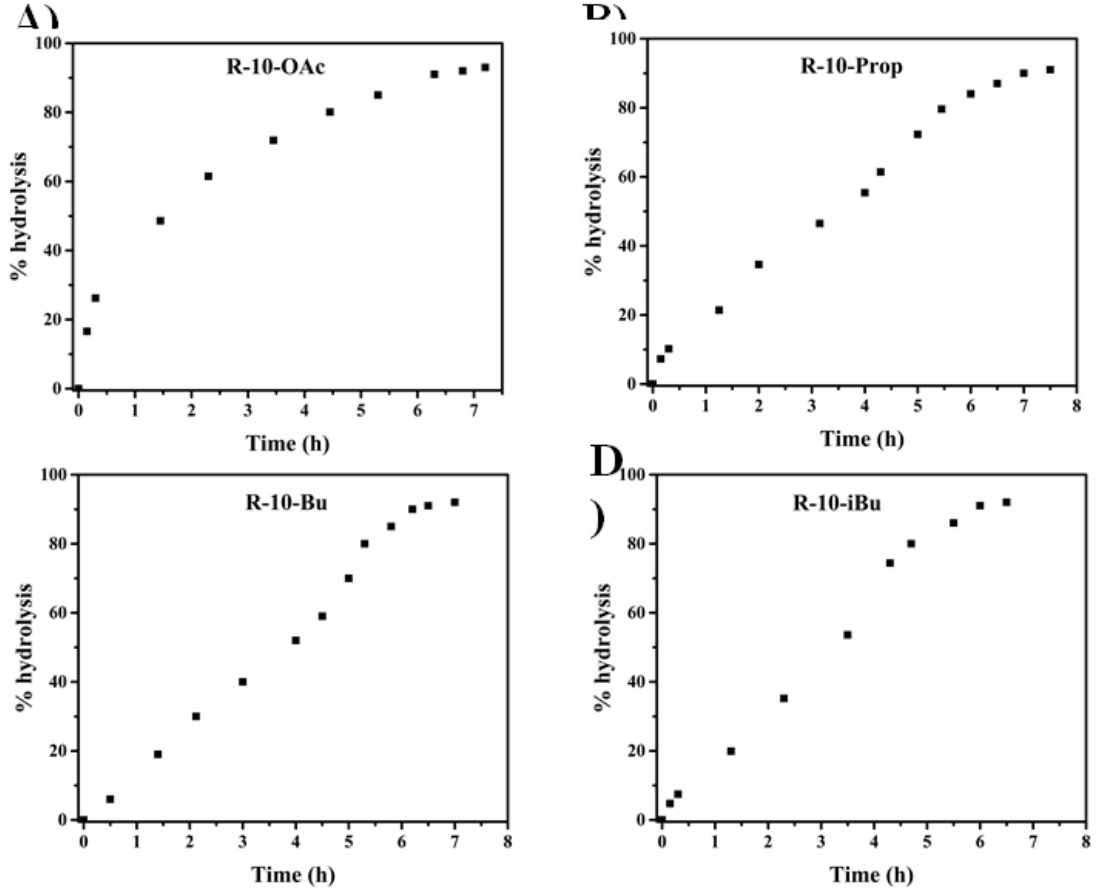


Figure 3.5: *In vitro* esterase hydrolysis of A) R-10-OAc; B) R-10-Prop; C) R-10-Bu; D) R-10-iBu.

Conclusion

In conclusion; A fully esterified decaacetylated rutin, decabuterate rutin, decabenzoate rutin, decaisobuteric rutin, decaisovaleric rutin, decapropionate rutin were synthesized. Decaacetylated (R-10-OAc) showed to have an increased lipid solubility when compared with underivized rutin. The decaacetylated derivative of rutin was synthesized by acetylation of OH groups of the original rutin and thus reduced the intermolecular hydrogen bonding which resulted in marked increase in its lipid solubility. The diffusion was best for decaacetylated rutin compared to the other esterified rutin compounds. As expected rutin trans dermal diffusion was almost zero. The developed UV/Vis analytical method was successfully used in the assay of decaacetylated rutin ointment and the formulated ointment have shown to be unstable when tested. The ointment formula as well as the developed analytical method can be readily used by pharmaceutical companies in the formulation and routine quality control of the newly developed rutin derivative.

References

1. Lahlou, M., **Screening of natural products for drug discovery**. Expert Opinion on Drug Discovery, 2007. **2**(5): p. 697-705.

2. Brodowska, K.M., ***Natural flavonoids: classification, potential role, and application of flavonoid analogues***. **European Journal of Biological Research**, 30/6/2017(Vol 7 No 2 (2017): April-June 2017): p. 108-123.

3. Vinson, J.A. and Y.A. Dabbagh, **Tea phenols: Antioxidant effectiveness of teas, tea components, tea fractions and their binding with lipoproteins.** Nutrition Research, 1998. **18**(6): p. 1067-1075.

4. Sunada, S., et al., **Monoglucosyl-rutin as a potential radioprotector in mammalian cells**. Molecular Medicine Reports, 2014. **10**(1): p. 10-14.

5. Picq, M., et al., **Selective inhibition of separated forms of cyclic nucleotide phosphodiesterase from rat heart by some pentasubstituted quercetin analogs**. Biochem Pharmacol, 1982. **31**(17): p. 2777-82.

6. Biasutto, L. and M. Zoratti, **Prodrugs of quercetin and resveratrol: a strategy under development**. Curr Drug Metab, 2014. **15**(1): p. 77-95.

7. Lauro, M.R., et al., **Fast- and slow-release tablets for oral administration of flavonoids: rutin and quercetin**. Drug Dev Ind Pharm, 2002. **28** (4): p. 371-9.

8. N'Da, D.D., **Prodrug strategies for enhancing the percutaneous absorption of drugs**. Molecules, 2014. **19**(12): p. 20780-807.

9. Panche, A.N., A.D. Diwan, and S.R. Chandra, ***Flavonoids: an overview***. **J Nutr Sci**, 2016. **5**: p. e47.

10. Kumar, S. and A.K. Pandey, ***Chemistry and Biological Activities of Flavonoids: An Overview.*** **The Scientific World Journal**, 2013. 2013: p. 1-16.

11. Duthie, G. and A. Crozier, **Plant-derived phenolic antioxidants**. Current Opinion in Lipidology, 2000. **11**(1): p. 43-47.

12. Heim, K.E., A.R. Tagliaferro, and D.J. Bobilya, ***Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships***. **The Journal of Nutritional Biochemistry**, 2002. **13**(10): p. 572-584.

13. Kumar, S., A. Mishra, and A.K. Pandey, *A***ntioxidant mediated protective effect of Parthenium hysterophorus against oxidative damage using in vitro models**. BMC Complementary and Alternative Medicine, 2013. **13**(1).

14. Karak, P. and B.C.C. Department of Physiology, Bankura - 722101, West Bengal, India., ***BIOLOGICAL ACTIVITIES OF FLAVONOIDS: AN OVERVIEW***. I**nternational Journal of Pharmaceutical Sciences and Research**, 2019. **10**(4).

15. Strissel, P.L. and R. Strick, **Multiple effects of Bioflavonoids on gene regulation, cell proliferation and apoptosis: Natural compounds move into the lime light of cancer research**. Leukemia Research, 2005. **29**(8): p. 859-861.

16. Shen, J.G. and D.Y. Zhou, **Efficiency of Ginkgo biloba extract (EGb 761) in antioxidant protection against myocardial ischemia and reperfusion injury**. Biochem Mol Biol Int, 1995. **35**(1): p. 125-34.

17. Liu, X., et al., **Pycnogenol®, French maritime pine bark extract, improves endothelial function of hypertensive patients**. Life Sciences, 2004. **74**(7): p. 855-862.

18. Siess, M.-H., et al., ***Flavonoids of Honey and Propolis: Characterization and Effects on Hepatic Drug-Metabolizing Enzymes and Benzo[a]pyrene−DNA Binding in Rats***. **Journal of Agricultural and Food Chemistry**, 1996. **44**(8): p. 2297-2301.

19. Russo, A., et al., **Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors**. Cell Biology and Toxicology, 2000. **16**(2): p. 91-98.

20. Pietta, P.-G., ***Flavonoids as Antioxidants***. J**ournal of Natural Products,** 2000. **63**(7): p. 1035-1042.

21. Hosseinzadeh, H. and M. Nassiri-Asl, ***Review of the protective effects of rutin on the metabolic function as an important dietary flavonoid***. **Journal of Endocrinological Investigation**, 2014. **37**(9): p. 783-788.

22. Kreft, S., M. Knapp, and I. Kreft, ***Extraction of Rutin from Buckwheat (FagopyrumesculentumMoench) Seeds and Determination by Capillary Electrophoresis.*** **Journal of Agricultural and Food Chemistry**, 1999. **47**(11): p. 4649-4652.

23. Huang, W.-y., et al., ***Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing***. J**ournal of Zhejiang University SCIENCE B**, 2012. **13**(2): p. 94-102.

24. Abuajah, C.I., A.C. Ogbonna, and C.M. Osuji, ***Functional components and medicinal properties of food: a review.*** **J Food Sci Technol,** 2015. **52**(5): p. 2522-9.

25. Serban, T.H.G., ***The isolation and identification of rutin from pharmaceutical products***. Researchgate, December 2016: p. 109-114.

26. Khalifa, T.I., F.J. Muhtadi, and M.M.A. Hassan, **Rutin, in Analytical Profiles of Drug Substances.** 1983. p. 623-681.

27. Yildizo??lu-Ari, N., et al., **Pharmacological effects of rutin**. Phytotherapy Research, 1991. **5**(1): p. 19-23.

28. Luo, H., et al., **Inhibition of cell growth and VEGF expression in ovarian cancer cells by flavonoids**. Nutr Cancer, 2008. **60**(6): p. 800-9.

29. Ratnam, D.V., et al., ***Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective***. **J Control Release**, 2006. **113**(3): p. 189-207.

30. Boudina, S. and E.D. Abel, **Diabetic cardiomyopathy revisited**. Circulation, 2007. **115**(25): p. 3213-23.

31. Das, S. and J.P.N. Rosazza, ***Microbial and Enzymatic Transformations of Flavonoids⊥.*** **Journal of Natural Products**, 2006. **69**(3): p. 499-508.

32. Biely, P., et al., **Enzymatic acylation of flavonoid glycosides by a carbohydrate esterase of family 16**. Biotechnol Lett, 2014. **36**(11): p. 2249-55.

33. Baldisserotto, A., et al., **Design, synthesis and biological activity of a novel Rutin analogue with improved lipid soluble properties**. Bioorganic & Medicinal Chemistry, 2015. **23**(1): p. 264-271.

34. Abualhasan, M., et al., **Synthesis, Formulation and Analytical Method Validation of Rutin Derivative.** Letters in Drug Design & Discovery, 2019. **16**(6): p. 685-695.

35. Di, L. and E.H. Kerns, **Prodrugs, in Drug-Like Properties**. 2016. p. 471-485.

36. Ita, K.B., ***Prodrugs for transdermal drug delivery – trends and challenges.*** **Journal of Drug Targeting**, 2016. **24**(8): p. 671-678.

37. Rautio, J., et al. **Prodrugs: design and clinical applications**. Nature Reviews Drug Discovery 2008 [cited 7 3]; 255-270].

38. Sloan, K.B., **Prodrugs: Topical and Ocular Drug Delivery (Drugs and the Pharmaceutical Sciences) 1st Edition.** 1 ed.

39. Viskupicova, J., et al., **Lipophilic rutin derivatives for antioxidant protection of oil-based foods.** Food Chemistry, 2010. **123**(1): p. 45-50.

40. Han, Y., et al., ***Design, synthesis, and antiviral activity of novel rutin derivatives containing 1, 4-pentadien-3-one moiety***. **European Journal of Medicinal Chemistry**, 2015. **92**: p. 732-737.

41. Stuard, S., et al., ***Five-year treatment of chronic venous insufficiency with O-(ß-hydroxyethyl)-rutosides: Safety aspects***. **International Journal of Angiology**, 2011. **17**(03): p. 143-148.

42. You, H.J., H.J. Ahn, and G.E. Ji, ***Transformation of Rutin to Antiproliferative Quercetin-3-glucoside byAspergillus niger***. **Journal of Agricultural and Food Chemistry,** 2010. **58**(20): p. 10886-10892.

43. R. Lloyd Snyder, J.J.K.a.L.J.G., **Practical HPLC method development,1997.** (2nd Ed) 2007. 179-184.

44. Br. Jay, J.K., and B. Pierre. **Understanding and Implementing Efficient Analytical Methods Development and Validation**. 2003; Available from:

https://www.pharmaceuticalonline.com/doc/understanding-and-implementing-efficient-anal-0001.

45. WHO. **Guidelines for Stability Testing of Pharmaceutical Products Containing Well,Established Drug Substances in Conventional Dosage Forms.** WHO Expert Committee on Specifications for Pharmaceutical Preparations, Technical Report Series 863 1996,; 65–79.].

46. ICH. **Good Manufacturing Practices for Active Pharmaceutical Ingredients.** 2000.

47. Alsante KM, F.R., Hatajik TD, Lohr LL, Sharp TR, Snyder KD, Szczesny EJ. **Degradation and impurity analysis for pharmaceutical drug candidates Handbook of Modern Pharmaceutical Analysis**,. 2001; pp.85-172.].

48. Simon, A., et al., *C**omparative evaluation of rivastigmine permeation from a transdermal system in the Franz cell using synthetic membranes and pig ear skin with in vivo-in vitro correlation*. **International Journal of Pharmaceutics**, 2016. **512**(1): p. 234-241.

49. Kulkarni, K.N. and A. Datta-Gupta, ***Estimating Relative Permeability From Production Data: A Streamline Approach***. **SPE Journal**, 2013. **5**(04): p. 402-411.

50. Murad, A., et al., **Synthesis, Formulation and Analytical Method Validation of Rutin Derivative**. Letters in Drug Design & Discovery, 2019. **16**(6): p. 685-695.

جامعة النجاح الوطنية

كلية الدراسات العليا

تصنيع مشتقات روتين لصياغتها كمستحضر موضعي وتطوير طريقة تحليل مثبتة لها

إعداد

عبير يوسف محمود محمود

إشراف

د. مراد أبو الحسن

د. محيي الدين العسالي

قدمت هذه الأطروحة إستكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

2020

تصنيع مشتقات روتين لصياغتها كمستحضر موضعي وتطوير طريقة تحليل مثبتة لها

إعداد

عبير يوسف محمود محمود

إشراف

د. مراد أبو الحسن

د. محي الدين العسالي

الملخص

الخلفية: زاد الاهتمام بمركبات الفلافونويد بشكل ملحوظ بمرور الوقت بسبب فعاليتها الدوائية والبيولوجية الهامة. يحتوي روتين على ميزات تجعله يستخدم في العديد من الفوائد مثل مضادات الأكسدة، ومضادات الالتهابات، ونشاط العلاج الكيميائي، وحالات المرض المختلفة مثل البواسير، والدوالي، والتهاب الجلد التحسسي. يتوفر روتين في الأسواق كمستحضر موضعي لعلاج العديد من الأمراض مثل النزيف الداخلي والبواسير والدوالي. ومع ذلك، فإن الروتين له قابلية منخفضة للذوبان وعليه فان توافره حيوي محدود بسبب مجموعات الهيدروكسيل الموجودة على الروتين والتي تقلل من قابليته للذوبان في الدهون، وبالتالي فان هذا يحد من استخدامه. في هذه الدراسة ان هدفنا هو تصنيع مشتقات جديده من الروتين قابلة الذوبان بالدهون. المجموعة المقترحة للصنيع كمشتقات للروتين يهدف الى تغيير الذائبية من أجل تسهيل انتقال الروتين عبر الحاجز البيولوجي وبالتالي تحسين توصيل الدواء من خلال الإستخدام الموضعي.

الطريقة: في البداية تم تصنيع ستة مشتقات من استرات الروتين، ثم حساب معامل التوزيع بين الأوكتانول والماء لهذه المركبات. تمت صياغة للمركبات المصنعة في مرهم موضعي وحساب نسبة الانتشار للروتين ومشتقات الروتين من خلال خلية انتشار فرانز. تم تطوير طريقة تحليل مطوره محليًا في مختبراتنا لمشتقات الروتين كمواد خام وعلى شكل جرعة نهائية. ثم تم التحقق من صحة الطريقة التحليلية. وتم إجراء التحقق وفقًا لـ ICH وذلك باختبار والتحقق من: المدى، والخطي، والدقة، والحد الكمي، وحد الاكتشاف.

النتائج: تم التحقق من تصنيع الإيسترات المشتقة من روتين بنجاح. تشير قيم تسجيل هذه المشتقات إلى قابلية ذوبان أفضل لها بالدهون مقارنةً مع الروتين وتشير النتائج إلى أن التعديل الهيكلي الذي تم إجراؤه على الروتين يحسن التوافر البيولوجي عبر الجلد لمشتقات الروتين. أظهرت نتائج الانتشار عبر خلية فرانز أن النفاذية كانت أفضل بالنسبة لاسيتايل روتين مقارنة بمشتقات الروتين الأخرى المؤسترة.

تم تطوير طريقة تحليل بسيطة لتحليل مركب استر الروتين والتحقق من صحتها. وجد أن الطريقة المطورة خطية ودقيقة ومحكمة ومستقرة وانتقائية. مركب اسيتايل روتن تم تصنيعه في مختبر البحث الخاص بنا ليكون مستقرًا في ظل ظروف متسارعة.

الخلاصة: تم الحصول على تحسن في نفاذية الروتين عبر الجلد بإضافة مجموعة على مجموعات الهيدروكسيل في روتين. أظهر استر اسيتايل أفضل انتشار عن طريق الجلد. تم تطوير طريقة تحليلية مصدقة لمركب ديكا اسيتيل روتن. المرهم المصاغ من المركبات المصنعة مستقرًا تحت كل من حالة الإجهاد وفترة الصلاحية.