An-Najah National University
Faculty of Graduate Studies

Synthesis, Formulation and Analytical Method Validation of Rutin Prodrugs

By

Tala Muhammad Ahmad Sarhan

Supervisor

Dr. Murad Abulhasan

Co-supervisor

Dr. Mohyeddin Assali

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This Thesis was defended successfully on 1/11/2017 and approved by:

Defense Committee Members	**Signature**
1. Dr. Murad Abualhasan / Supervisor	**……**
2. Dr. Mohyeddin Assali / Supervisor	**……**
3. Dr. Amjad Abusalha / External Examiner	**……**
4. Dr. Naser Shraim / Internal Examiner	**……..**

Dedication

To my giving father, affectionate mother and my dear uncle whom I always found holding my hands whenever I was about to fall. In my health and in the various days of my sever sickness.

To the Saul of refugees who left their homes to the middle of nowhere, innocent civilians and children who died in recent wares all over the Arab world. I wish I could give more but we are just numbers in this world.

To my everlasting homeland Palestine.

I dedicate this work….

Acknowledgement

My first thank is to the majesty of Allah who is always with us and gives us more than we ever expect. An overwhelming appreciation to the two supervisors of this work Dr. Murad Abualhasan and Dr. Mohyeddin Assali who gave me in the academic and team work level what I will always be grateful for. You have all the privileges and willing to keep up great research work, trust me.

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Finally, all the appreciation to a lovely family and friends who always wished me the best and accepted nothing less than perfect from me.

Tala Muhammad Ahmad Sarhan

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Synthesis, Formulation and Analytical Method Validation of Rutin Prodrugs

تكوين وتركيب مشتقات مركب الروتين والتحقق من طريقة تحليلها

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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.

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AUC	Area under the curve
CMC-XL	Cross- linked sodium carboxymethylcellulose
DCM	Dichloromethane
DMAP	Dimethyl amino pyridine
DPPH	2,2-diphenyl-1-picrylhydrazyl
g	gram
g/mol	gram per mole
GMP	Good manufacturing practice
HPLC	High performance liquid chromatography
IC_{50}	The half maximal inhibitory concentration
ICH	The international conference on harmonization
LOD	Limit of detection
LOQ	Limit of quantification
M	Molarity
MCC	Microcrystalline cellulose
µg/mL	Microgram per milliliter
mg	Milligram
mg/mL	Milligram per milliliter
mL	Milliliter
mmol	Millimoles
nm	Nanometer
NMR	Nuclear magnetic resonance
R_f	Retention factor
rpm	Round per minute
RSD	Relative standard deviation
SD	Standard deviation
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
USP	United states pharmacopeia
UV	Ultraviolet
UV/Vis	Ultraviolet/Visible
V/V	Volume per volume
λ_{max}	Wavelength of maximum absorption

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Abstract

Background: Rutin is a plant extract that belongs to the flavonoid group of compounds. Many studies showed that Rutin has a potential pharmacological uses such as antioxidant, anti-inflammatory and antihypertensive activities. Rutin is widely used as medicinal product and food supplement and marketed in different pharmaceutical dosage formulations. However, rutin suffered from low systemic bioavailability due to its low absorption from the gastrointestinal tract and its low water solubility. In this study, we aimed to improve the water solubility and consequently its dissolution profile by synthesizing a more soluble derivative of rutin.

Method: Decaacetylated ester of rutin was first synthesized. Then selective partial deacetylation was performed to produce the hexaacetylated ester of rutin. Water solubility of the new derivative as well as its dissolution was compared to rutin. An evaluation of the antioxidant activity of the hexaacetylated derivative was tested using 2,2-diphenyl-1-picrylhydrazyl reduction method. Moreover, A Ultraviolet/Visible spectrophotometric method was developed and validated for the analysis of a tablet formulation of the newly synthesized derivative. An ultraviolet spectrophotometric

quantitative analytical method for rutin derivative as active particle ingredient and in a tablet dosage form was developed and validated according to the International Conference on Harmonization and international guidelines.

Results: The hexaacetylated ester derivative of rutin was successfully synthesized and the derivative structure was confirmed by nuclear magnetic resonance. Moreover, the water solubility and the dissolution profile were improved by approximately two fold increase compared to that of the original rutin. Water solubility of the partially acetylated product has been increased from 0.07 to 0.15 mg/mL and its dissolution increased from 22% to 37.5% compared to the original rutin. Moreover, the antioxidant activity results showed that the newly synthesized derivative preserved the antioxidant activity of the original rutin. An easy and feasible analytical method was developed and validated. The developed method was found to be linear, precise, accurate and selective with a lowest limit of detection and lowest limit of quantification of 0.00854 and 0.0259 mg/mL respectively. The formulated tablets of hexaacetylated rutin in our research laboratory were found to be stable under accelerated conditions as well as long term conditions for three months.

Conclusion: An improvement on the solubility of rutin was achieved by selective acetylation of some of the OH groups of rutin. The tablet formulation of the partially acetylated ester derivative of rutin gives a better dissolution over the already marketed rutin tablets.

Chapter One

1. Introduction

1.1 Flavonoids

Flavonoids or (bioflavonoids) definition covers a large group of natural substances that are generally considered plant secondary metabolites, they are found in a wide variety of plants giving them their characteristic pigmentations and colors [1].

Flavonoids are hydroxylated phenolic compounds with a common benzo- γ - pyrone structure. The various compounds of this family differ from each other in the number of OH groups (degree of oxidation), the substitutions and conjugations added to the structure and the polymerization between two or more monomers to different degrees. They are either found in the free form (aglycones) or bounded to a sugar (β-glycoside) [1, 2].

Their basic structure consists of 15 carbon atoms skeleton of three rings (A, B and C), two benzene rings and one heterocyclic pyran ring as shown in **Figure 1.1.**

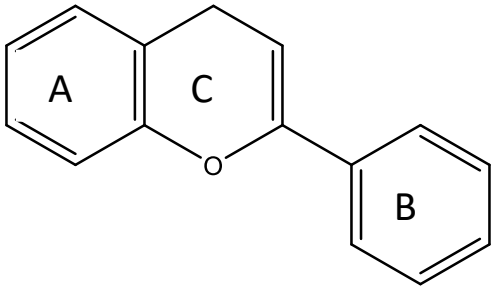


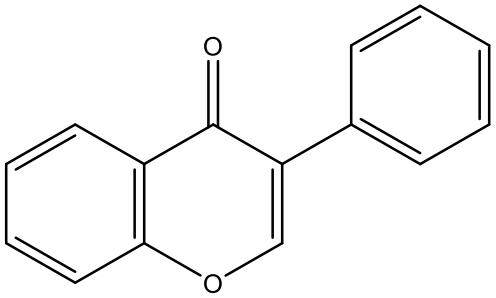
Figure 1.1: Flavonoids basic structure.

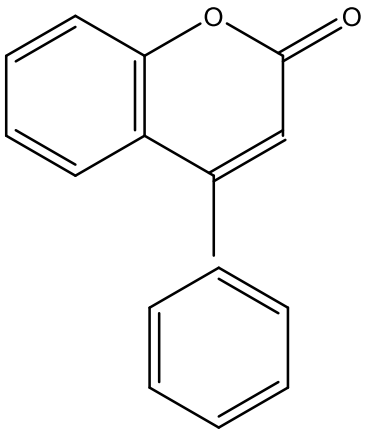
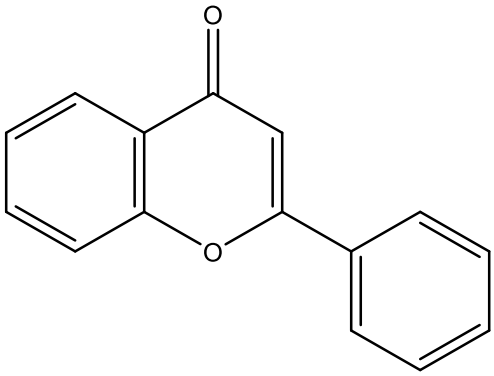
The broad term of flavonoids is further subdivided into groups based on the degree of unsaturation as well as the substitutions on the ring C and their positions.

They can be classified into three groups: isoflavones which are derived from 3-phenyl-1,4-benzopyrone structure having the B ring attached at 3 position of the C ring, neoflavonoids which are derived from 4-phenyl-1,2-benzopyrone structure having the B ring attached to the 4 position of the C ring. Finally, the flavonoids with the B ring linked to the position 2 of the C ring and those can be classified into subgroups which are: flavones, flavonols, flavanones and flavanonols. These classes of compounds all contain a characteristic ketone moiety. Other non ketone containing compounds which are generally classified as flavonoids include flavanols (catechins) and anthocyanins [2, 3].

The basic structure of the flavonoids groups is illustrated in **Table 1.1.**

Table 1.1: Structural classification of flavonoids.

Group	**Structural formula**
Isoflavones	

Neoflavonoids	
Flavones	

Flavonoids group of compounds which are widely available from various plant sources gained much interest for the versatile health benefits that their supplementation in human diets provides.

They proved their role as antioxidants by acting as free radical scavengers for the reactive oxygen species [2-4], which is largely due to their characteristic poly phenolic structure. Their potency and efficacy varies from one flavonoid to another according to the number and position of OH groups in their structure, the degree of oxidation and unsaturation as well as other substitutions and conjugations linked to their basic formula [5-8].

1.2 Rutin

Rutin (quercetin-3-rutinoside; **Figure 1.2**) is one of the major flavonoids which belongs to the isoflavones class and is found in a variety of plants. Buckwheat (Fagopyrum esculentum Moench) plant is known as a major source of rutin [9-11], Tartary buchwheat (Fagopyrum tataricum Gaertn) is a richer source that contains larger amounts of rutin than the common buckwheat plant [12-16]. Other plant sources include rheum species [17] and asparagus [18].

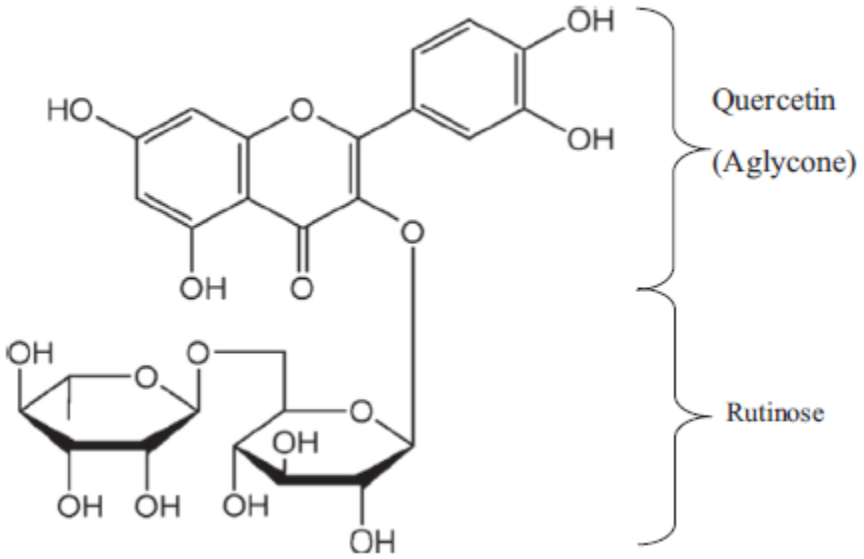


Figure 1.2: Chemical structure of Rutin (quercetin-3-rutinoside) [19].

Rutin is a 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one, it has the chemical formula $C_{27}H_{30}O_{16}$ with a 10 hydrogen bond donor and 16 hydrogen bond accepter. Rutin is a pale yellow crystals with a molecular weight of 610.5175 g/mol, melting point 242 °C (468 °F, 515K) [20].

Rutin is a well- known and studied flavonoid glycoside which has already shown a promising potentials and health benefits if given as a supplement or added to human diets from its natural food sources.

It has a strong antioxidant activity [21, 22], anti-inflammatory effect [23], antihypertensive effect [24, 25], strengthens the capillary vessel walls and reduces capillary fragility. Moreover, it improves vascular blood flow in the micro- circulation. It therefore protects from varicous veins, hemorrhoids, diabetes related vascular problems, painful and restless swollen legs and other vascularity problems [26, 27].

Other industrial applications of rutin include its use as a natural pigment, food preservative, stabilizer and UV absorber.

Rutin, its aglycone quercetin as well as other quercetin glycosides and derivatives of these two compounds are currently used as medicinal products and supplements for the health potentials and benefits they provide. However, the absorption of rutin and quercetin from the gastrointestinal tract is slow and unreliable contributing to their law plasma bioavailability. This is most likely due to the low solubility exhibited by these compounds.

Quercetin has a low bioavailability that differs from one food source to another, and according to the type of glycoside form that it is found in.

Some glycoside forms surprisingly showed a better bioavailability than quercetin while others such as rhamnoglucoside (rutinoside) which is known as rutin and has a bioavailability of a proximately 20 % of other

quercetin glucosides and it is absorbed with less efficiency and a longer time from the gastrointestinal tract than the quercetin aglycone [28-30].

From a review of 97 bioavailability studies of polyphenols done by Manach *et al* [28]., the mean AUC for rutin after a dose corresponding to 50 mg quercetin equivalent was 2.9 ± 0.9 µmol h/L and the mean T_{max} and C_{max} where 6.5 ± 0.7 hour and 0.20 ± 0.06 µmol/L respectively.

Rutin is already found as a commercial product and supplement in the market for different companies in the dosage form of tablets with the strength of 250 and 500 mg, it has also been formulated in a topical dosage forms. However, its poor water solubility and hence dissolution is still an issue preventing from obtaining the full health benefits.

In the purpose of resolving the problem of the poor bioavailability of rutin and other related benzopyran glycosides, a variety of procedures have been suggested. They were either a development of new and novel formulations that provide optimum release and dissolution properties, or semi synthetic procedures aiming to synthesis new derivatives of rutin having a better physiochemical properties, most importantly solubility.

One of the formulation approaches used was the complexation of rutin with 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) which had a significant advantage of improving the bioavailability by the increase in solubility, dissolution rate and gastrointestinal stability [31, 32]. Another approach was the synthesis of rutin nanocrystals followed by direct compression into tablets. The nanocrystal formulation showed an improved dissolution behavior over the already marketed tablets suggesting a better

bioavailability in the human body [33]. Other improved rutin oral formulations included loading the drug into hydrophilic polymer carriers such as cross-linked sodium carboxymethylcellulose (CMC-XL) [34].

In order to have a view on the semi synthetic or natural derivatives of rutin that are known so far, we must first have an understanding of the structure activity relationship of rutin and other flavonoid compounds. Apparently, the larger the number of OH groups added to their basic structure, the higher the antioxidant and radical scavenging activity they have. The presence of an ortho 3′, 4′ dihydroxy substituents in the B ring greatly enhances their antioxidant potential. Moreover, the reduction of the double bond between carbon 2 and 3 of the C ring reduces the compounds antioxidant activity. Other structural features that affect the antioxidant and antiradical activity include the presence of a free OH groups at C-3 and C -7 and the conjugation between the rings A and B [5-8].

Examples of semi synthetic derivatives of rutin include the hydroxyethyl rutosides (e.g. monoxerutin, troxerutin and tetrahydroxyethyl rutoside) which are water -soluble derivatives [1]. The commercial product venoruton which contains a mixture of hydroxyethyl rutosides is found in the market and used in the treatment of chronic venous insufficiency and hypertension conditions [35, 36]. However, when one or more phenolic OH groups are blocked in a semi synthetic derivative of such a benzopyran glycoside, it greatly affects the activity of the compound.

Therefore, there is still a need to produce derivatives of benzopyran glycosides having well defined structures and characterized in that only the

sugar portion of the molecule is derivatised, leaving phenolic hydroxyl groups unaffected.

Another known water-soluble derivative of rutin is the 4^{G}-α-D-Glucopyranosyl-rutin or G-rutin, which is enzymatically synthesized and shown to reduce oxidative damage and suppress glycation reaction that is common to diabetes in rodent models [37-39].

Enzymatic polymerization of rutin has been suggested. Rutin polymer with molecular weight of several thousand was synthesized which had a good water solubility. Moreover, it had an improved radical scavenging and antioxidant activity over the rutin monomer [40].

Efforts have also been made to synthesize partially esterified derivatives of rutin, where only the OH groups of the sugar part are masked leaving the phenolic OH groups unmasked in order not to affect the activity of the compound. Rutin hexasuccinate derivative was synthesized and shown to have about 100 fold increases in its water solubility and slightly lower activity in inhibition of lipid peroxidation of brain membranes than the original rutin [41, 42]. The structure of rutin hexasuccinate is shown in **Figure 1.3**.

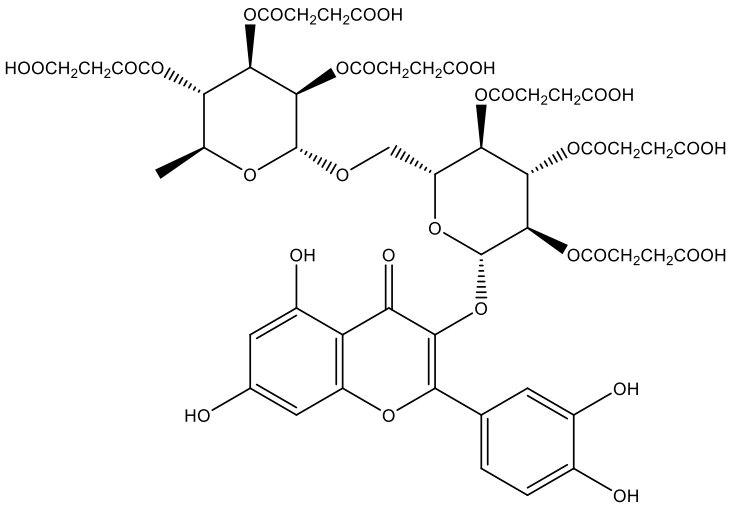


Figure 1.3: Structure of rutin hexasuccinate ester.

A method for the synthesis of rutin hexapropionate ester, which has the structure shown in **Figure 1.4**, was also established by Baldisserotto *et al.* [43]. The derivative rutin propionate has shown improved lipid solubility over rutin, its partition coefficient (LogP) was 1.09 and a lipid formulation was therefore suggested.

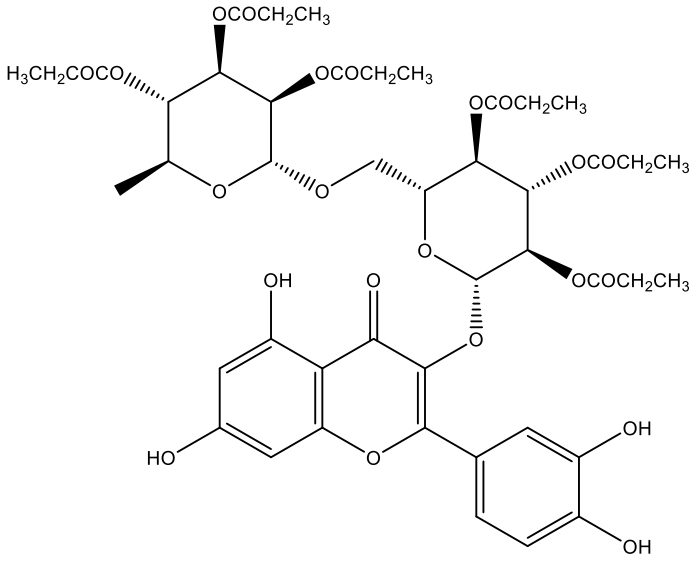
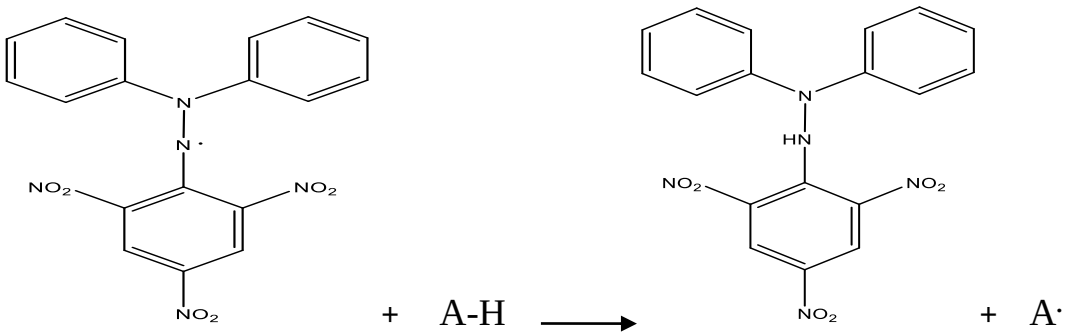


Figure 1.4: Structure of rutin hexapropionate ester**.**

1.3 Establishment of antioxidant activity using DPPH reduction method

The compound quercetin and the various quercetin derived glycosides are, like most of the flavonoid family of compounds, known to have a strong antioxidant activity. Antioxidant compounds have the capability of acting as a free radical scavengers providing a various protective and beneficial health effects. Different methods are available for *in vitro* measurement and comparison of the activity of antioxidant compounds. The 2,2-diphenyl-1-picrylhydrazyl method depends mainly on the reduction reaction that occurs on the compound DPPH when it is reacted with a compound having an antioxidant capacity.



DPPH (Purple color, 517 nm)

DPPH-H Colorless

Scheme 1.1: DPPH reduction reaction.

DPPH is a compound with a purple color having an intense absorption at 517 nm wavelength. The antioxidant capacity of a specific antioxidant compound can be estimated by tracking the reduction in the absorbance

measurements that results when different concentrations of the antioxidant agent are added to the DPPH solution [44].

1.4 Tablet formulation and dissolution testing

1.4.1 Tablet formulation

Tablets are the most widely used dosage form for the oral delivery of most drugs. They generally include the active ingredient to which excipients are added and the powder is then compressed to have the final tablet dosage form.

Various excipients are available for formulation and are used for different purposes. Diluents or fillers are used to give the actual weight of the tablet (e.g. lactose, dextrose, microcrystalline cellulose). Binders give the adhesiveness and binding to the tablet and include synthetic materials like polyvinylpyrrolidone, methyl cellulose, hydroxypropyl methyl cellulose and pregelatinized starch as well as natural acacia and tragacanth gum. Some binders are used in the dry form while others are added in the form of solution.

Disintegrants are added to tablets for the purpose of disturbing the aggregation of the solid dosage form when it comes in contact with water generally by swelling and rupturing or by other mechanisms. Sodium starch glycolate, starch and microcrystalline cellulose are the most widely used disintegrants.

Other common excipients used in low amounts in the tablet formulation include glidants and lubricants. Glidants are added to the powder to

increase adhesiveness and improve flow properties. Colloidal silicon dioxide, starch and talc are commonly used glidants. Lubricants such as magnesium stearate prevent the adhesion of the tablets to the die face during compression.

Different methods are available for the manufacturing of tablets; direct compression of the blended powder is the simplest method. Granulation of the powder before compression is often needed when flowability problems are encountered which can affect the content uniformity of tablets. Wet granulation is the main method used while dry granulation can be used when the compound is not stable to heat or water [45].

1.4.2 Improvement of tablet dissolution

Dissolution of the solid dosage form is critical for a successful drug delivery through the oral route, especially when the drug is poorly soluble in the aqueous medium. Formulation factors play a role in determining the rapid disintegration and hence dissolution of the formulated tablets.

The reduction of particle size in most cases will result in enhanced drug dissolution from tablet dosage forms [46-48], another factor affecting dissolution is the different excipients added to the formulation .Excipients such as disintegrants [49], diluents, binders, lubricants [46-50] and granulating agents have all shown to affect drug dissolution.

For example, a hydrophobic excipient such as the lubricant magnesium stearate has shown to retard tablet dissolution. While the type and quantity of disintegrants used is known as a major factor affecting disintegration of

the tablet dosage form. Addition of surfactants also helps in improving the dissolution of hydrophobic and poorly water soluble drugs [47, 49].

The pressure applied to tablets during compression stage also affects the hardness and hence the disintegration of the formulated tablets to some extent [46].

1.4.3 Dissolution test

The dissolution test for the tablet dosage forms is done in purpose of assuring that the dosage form within the GI turns into solution from which the drug can be absorbed within a reasonable time. Various dissolution testing apparatus have been developed; the most widely used include the basket apparatus and the paddle apparatus. A good knowledge of the drug product characteristics (e.g. solubility over different pH values, pKa and logP) is of great benefit when developing a new dissolution test method.

Suitable dissolution medium is chosen and the use of buffers of different pH values in the range of 1- 6.8 is sometimes required. The apparatus is generally set at speed of 50-100 round per minute for the basket method and 50- 75 rpm for the paddle method. Temperature is kept constant at 37 ± 0.5 °C and samples are withdrawn for analysis after specified time intervals [51, 52].

1.5 Analytical method development and validation

1.5.1 Development of analytical procedures

The development of a method in the pharmaceutical industry for the assay of drugs in their final dosage form and the routine use for quality control purposes is usually performed by either using ultraviolet/visible spectrophotometer or the high performance liquid chromatography (HPLC) instruments. For a certain drug, the analytical instrument is chosen according to the drugs specific characteristics and by searching the literature for previous methods used for the analysis of that drug and whether it has a definite absorption and spectrum on the UV/Vis range and a λ_{max} that enables the use of UV spectrophotometer or if the HPLC was used for the analysis of the drug product with a good separation and a defined peak and the HPLC then will be a good choice.

The UV spectrophotometer is an instrument that measures the reduction in the intensity of a beam of light in the UV/Vis range when it is passed through a solution containing a substance that has absorption potential. The absorbance of the sample is measured using the Beer-Lambert law. The instrument can be used for qualitative as well as quantitative analysis of drug products.

In order to develop an analytical method using the UV spectrophotometer instrument, the full spectrum of a specific drug sample must be first obtained over the different wavelengths and the λ_{max} is generally chosen where the absorption is maximal and the error is therefore minimized.

As the Beer-Lambert law does not apply to high absorbance values (higher than 1), a concentration of the drug is then chosen that gives an absorbance of approximately 0.9 in order to maintain the desired accuracy and precision and a calibration curve is then constructed by using dilutions of the stock solution until a linearity is obtained [53].

1.5.2 Validation of analytical methods

Validation of the developed analytical procedures is done according to the ICH recommendations to assure that the developed method is capable of constantly giving the intended results with appropriate accuracy and precision [54, 55].

The parameters of method validation generally include linearity, range, accuracy, precision, specificity, limit of detection, limit of quantification and robustness [56].

Accuracy is a term that measures how close is the value measured by the instrument to a previously accepted true value. Accuracy can be confirmed by testing over three different known concentrations with three replicate for each concentration and data is represented by percent recovery.

Precision is indications of how close to each other are the measurements of repeated samples taken from the same reference homogenous sample. There are different levels of precision; repeatability is concerned of the precision over the same day during an analytical procedure and it is tested either by analysis of three different concentrations with three replicates for

each concentration, or a by taking 6 measurements for the 100% test concentration.

Intermediate precision which can be inferred by taking repeated measurements over different days and by changing the instrument and operator. Finally, reproducibility which confirms precision over different laboratories and efforts are then made to have a standard method. The data for precision is represented by standard deviation, relative standard deviation and the confident interval.

Linearity of the developed method is the ability to set a linear relationship between the analyte concentration and the test measurements. It can be established within a certain range of concentrations by preparing dilutions of a certain concentration and the linearity is then confirmed statistically from the correlation coefficient and the equation from which the y-intercept and the slope of the regression line can be deduced. The range of analyte concentrations within which the analysis can be applied with acceptable accuracy and precision can generally be taken from the linearity study and is reported during the validation process.

Concerning specificity of the analytical method, it confirms the ability to assess the intended analyte in presence of other components which are expected to be present (e.g.; degradation products, impurities).

Detection limit and quantification limit are two other parameters of validation. Limit of detection is the lowest concentration of the analyte that can be detected by the instrument, while quantification limit is the lowest

concentration of the analyte which can be quantified with an acceptable accuracy and precision.

The last step of the validation step is establishment of robustness, which gives an indication of the stability of the method to small changes in the analysis parameters (e.g. pH and temperature).

1.6 Objectives of the current study

From our point of view, one of the reasons leading to the poor water solubility of rutin is the fact that the compound has a large number of OH groups in its structure, leading to the formation of hydrogen bounding between the rutin molecules themselves thus preventing the bounding with the water molecules. This therefore led to our desire to develop a semi synthetic method for the synthesis of rutin hexaacetylate derivative. Rutin hexaacetylate derivative is a partially acetylated rutin in which the OH groups of the sugar moiety were acetylated leaving the phenolic OH groups unmasked.

Our work was conducted in the purpose of developing a novel partially acetylated derivative of the flavonoid glycoside rutin, with improved water solubility and oral bioavailability characteristics over rutin. Then formulation of the new derivative in an oral tablet formulation that provides optimum release and dissolution properties. Finally, development and validation of an analytical method using UV/Vis spectrophotometry for the assay of the hexaacetylated rutin derivative.

1.7 Significance of the research

1- Synthesis of a potentially more soluble novel rutin Prodrug with increased bioavailability.

2- Development of a new validated method to quantify rutin derivatives as a raw material or in the final dosage form.

3- The newly synthesized rutin Prodrugs will have the potential to be superior to the original rutin and can be registered for patency.

4- The rutin Prodrug which has an improved solubility and bioavailability can be formulated in a reduced dose compared to the original rutin, which will be beneficial to patient and has economical benefit to the pharmaceutical industry.

5- The experience of production of rutin prodrugs is of a great benefit to the pharmaceutical companies providing them with a semi synthetic approach for many similar natural products.

Chapter Two

2. Methodology

2.1 Materials

All the chemical reagents used in this research project including the synthesis, formulation and analytical validation were purchased from reliable sources. The reagents were of analytical grade and were used without any further purification steps while formulation ingredients used were all of pharmaceutical grade.

Triethyl amine was purchased from (SD fine-chem. Limited, India). Glacial acetic acid was obtained from (Frutarom Ltd, Haifa). 2,2-diphenyl-1-picrylhydrazyl(DPPH),6-hydroxy-2,5,7,8-etramethylchroman-2-carboxylic acid (Trolox), pyridine, acetic anhydride, Sodium hydroxide (NaOH), Hydrochloric acid 32%, hydrogen peroxide 30% and Potassium dihydrogen phosphate (KH_2PO_4) were all obtained from (Sigma-Aldrish company, USA). Methanol and acetone > 99% solvents were also purchased from (Sigma-Aldrish, USA). Ethyl acetate, hexane and methylene chloride solvents as well as sodium acetate were purchased from (Chen Samuel Chemicals Ltd, Haifa) and the acetonitrile solvent was purchased from (Bio-lab Ltd, Jerusalem). Rutin trihydrate powder (USP grade) was purchased from (MP Biomedicals, USA). Microcrystalline cellulose, magnesium stearate, aerosil and acdisol formulation components were given as a gift from Jerusalem Pharmaceuticals Company, Palestine.

2.2 Instrumentation and techniques

The instruments used during the various stages of this work include: hotplate stirrer (Lab tech^{R,} daihasn lab tech co,ltd, India), Ultraviolet-Visible Spectrophotometer- JENWAY 7315 (Biobby Scientific ltd, UK), sensitive weighing balance (Adventurer®, OHAUS Corporation, USA), rotavapor (VV2000 OB2000, Heidolph, Germany), centrifuge (UNIVERSAL 320, Hettich Zentrifugen, Germany), ultrasonic cleaner (Elmasonic S 70 H, Elma®, Germany), thermo shaker (BOECO TS-100, Germany), pH meter- JENWAY 3510, oven (Arilevy), Pressure Gauge (Simadzu corporation 5 TON, Japan), dissolution tester BTC -9100 (Hsiang taimachinery industry ltd, Taiwan) and melting point apparatus (GALLENKAMP, UK).

Various techniques were performed during this work included:

1. **Ultraviolet-visible (UV/Vis) spectrophotometry:** A (7315 Spectrophotometer, Jenway, UK), was used to obtain the UV spectrum of the different compounds as well as developing the UV/Vis analytical assay method .A Quarts cuvette was used in taking all measurements.
2. **Nuclear Magnetic Resonance (NMR) spectroscopy**:

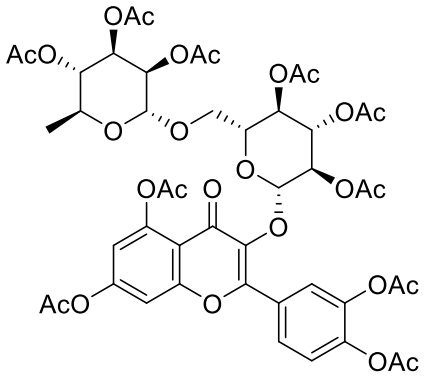
   Proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectra were run either on a JEOL Lambda Delta 400 (400 MHz) or Bruker AMX-400 (400 MHz) spectrometer. The deuterated solvent used for each of the compound is specified in the text, chemical shifts are stated in parts per million (ppm) and multiplicity

indicated as singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), pentet, sextet and multiplet (m). Coupling constants (J) are quoted in hertz (Hz).

3. **Chromatography**: silica Gel (230-400 mesh, Sigma Aldrich, USA) was used to purify the products, TLC (DC-Fertigfolien ALUGERAM®SIL G/UV_{254}, MACHEREY NAGEL Company, Germany) was used to monitor the reactions, chromatographic spots were visualized using UV Lamp (Model UVGL-58, Mineralight® Lamp, USA).

2.3 Chemical synthesis

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

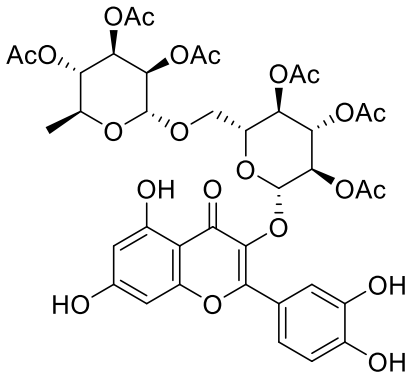


Synthesis method of decaacetylated rutin ester and hexaacetylated ester of rutin prodrugs was similar to the synthesis method of decapropionated and hexapropionated esters developed by Baldisserotto et al [43]. The decaacetylated rutin compound was obtained by addition of 6 mL of acetic anhydride and (40 mg, 0.33 mmol) of 4-Dimethylaminopyridine into a stirred solution of (1.22 g, 1.97 mmol) rutin trihydrate powder in 10 mL pyridine. The reaction was stirred for 18 hours under room temperature.

The mixture was then neutralized by addition of about 50 mL of 1 M HCl slowly until full precipitation occurs, filtered and dried in the oven. After then, the residue was purified by silica gel column chromatography (eluent: Ethyl acetate /Hexane, 2/1, v/v) giving R-10-OAc (84%) as a white powder (R_f : 0.32, EtOAC: Hex 2:1).

^{1}H NMR (500 MHz, CDCl3) : δ 7.90-7.84 (m, 3H, Ar), 7.28-7.24 (m, 2H, Ar), 5.36 (d, 1H, J = 7.3 Hz, OCHO), 5.22 (t, 1H, J = 8.3 Hz, OCH‘O), 5.13 (d, 1H, J = 9.3 Hz, CHOAc), 5.02 (d, 2H, J = 7.3 Hz, 2CHOAc), 4.87 (t, 3H, J = 9.3 Hz, 3CHOAc), 3.59-3.44 (m, 3H, OCH2, OCHCH3), 3.20 (dd, 1H, J = 10.8 Hz, J = 5.9 Hz, OCHCH2), 2.37-1.87 (m, 30H, 10OAc), 0.99 (d, 3H, J = 6.4 Hz, CH3). ^{13}C NMR (100 MHz, $CDCl_3$) : δ 172.1, 170.3, 170.2, 170.0, 169.9, 169.7, 169.4, 168.3, 168.2, 168.0, 167.9, 161.9, 156.8, 156.3, 155.9, 154.8, 154.1, 150.3, 144.3, 141.9, 137.1, 127.4, 124.8, 123.6, 115.2, 113.6, 109.2, 105.3, 101.3, 100.1, 99.7, 97.7, 73.3, 73.0, 72.7, 72.6, 71.6, 71.0, 69.7, 69.5, 69.2, 67.1, 66.5, 60.5, 21.3, 21.2, 21.0, 20.8, 17.3.

2.3.2 Synthesis of Hexaacetylated rutin (R-6-OAc)



R-10-OAc (500 mg, 0.49 mmol) was stirred with anhydrous MeOH (25 mL), triethyl amine (2 mL) was added to the solution. The mixture was

then stirred at room temperature under argon atmosphere for 1 hour. Addition amount of the triethyl amine (0.5 mL) was added and the reaction was continued under the same conditions for another hour.

The reaction mixture was then neutralized with 2 M HCl by addition slowly under 0 ^{0}C until the pH turns acidic. Methanol solvent was removed from the solution by vacuum evaporation and the crude mixture was washed with 90 mL dichloromethane and 40 mL 1 M HCl. The DCM organic phase was then dried (Na_2SO_4) and vacuum evaporated.

The residue was then purified by silica gel chromatography (eluent: DCM/MeOH, 15/1, V/V). The partially acetylated rutin product R-6-OAc was obtained (83%) as a pale yellow powder (R_f: 0.35 DCM:MeOH 15:1).

^{1}H NMR (400 MHz, $CDCl_3$) : δ 7.59 (d, 1H, *J* = 2.4 Hz, Ar), 7.45 (dd, *J* = 8.5 Hz, *J* = 2.1 Hz, Ar), 6.83 (d, 1H, *J* = 8.2 Hz, Ar), 6.78 (d, 1H, *J* = 2.1 Hz, Ar), 6.53 (d, 1H, *J* = 1.8 Hz, Ar), 5.64 (d, 1H, *J* = 7.9 Hz, OCHO), 5.39 (t, 1H, *J* = 9.6 Hz, OCH‘O), 5.11 (dd, 1H, *J* = 9.8 Hz, *J* = 7.9 Hz, CHOAc), 4.98-4.88 (m, 3H, 3CHOAc), 4.72 (t, *J* = 9.9 Hz, CHOAc), 4.50 (t, 1H, *J* = 1.5 Hz, CHOAc), 3.96-3.56 (m, 4H, OCH_2, $OC\underline{H}CH_3$, $OC\underline{H}CH_2$), 2.04-1.88 (m, 18H, 6OAc), 0.87 (d, 3H, *J* = 6.4 Hz, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) : δ 171.1, 170.9, 170.7, 170.5, 169.2, 169.1, 162.3, 157.8, 155.9, 154.3, 154.1, 150.3, 143.3, 142.2, 138.1, 126.4, 124.3, 123.1, 115.8, 112.2, 108.3, 106.1, 101.1, 100.3, 98.7, 96.3, 73.7, 72.5, 71.3, 69.7, 69.2, 67.4, 66.2, 60.5, 21.5, 20.8, 17.7.

2.4 Physicochemical properties determination of hexacetylated rutin

2.4.1 Melting point determination

Melting point (Mpt) was determined using a melting point apparatus and the results were represented in degrees Celsius (°C). In the purpose of determination of the melting point characteristic of the synthesized hexaacetylated ester of rutin, a small amount of the powder was inserted into a capillary tube and the tube was thereafter subjected to gradual heating through a variable heater device and the powder was observed through a lens in order to record the melting point on the first sign of turning into liquid.

2.4.2 Determination of water solubility of hexaacetylated rutin derivative

The UV absorbance spectrum (200-600 nm) of the hexaacetylated rutin ester was first obtained using a UV/Vis spectrophotometer and the wavelength of maximal absorption (λ_{max}) of hexaacetylated rutin ester compound was determined. The blank used to set the reading on zero was methanol. The sample was prepared using methanol as a solvent and a suitable dilution of the sample was carried out to optimize the absorbance readings. The wavelength of maximal absorption was then determined.

A calibration curve for the hexaacetylated rutin derivative was constructed using methanol as a solvent to determine the solubility and dissolution of the newly synthesized hexaacetylated rutin compared with the underivatised rutin.

A stock solution of 0.1 mg/mL of rutin and hexaacetylated rutin ester compounds was prepared. Five serial dilutions were then prepared form the above stock solution (0.015, 0.02, 0.03, 0.04 and 0.05 mg/mL) and were used to construct a calibration curve at λ_{max} 335 nm and 360 nm for hexaacetylated rutin ester derivative and rutin respectively using Microsoft Excel 2007. The equation and R^2 value were also displayed.

The solubility in water and in pH 4.5 acetate buffer for rutin and hexaacetylated rutin ester derivative was determined by addition of 1 mL of water or 4.5 pH acetate buffer to an excess of 5 mg of either rutin or the hexaacetylated rutin derivative. The tubes were placed in the thermo shaker under 37 ^{0}C temperature and speed of 300 rpm. Samples solubility was checked after 24 hours and 48 hours time intervals. The absorbance of the supernatant solution of the samples was taken after centrifugation. The concentration of the dissolved rutin or the hexaacetylated rutin compound was then calculated from the calibration curve that was constructed for each individual compound using methanol as solvent, taking into consideration any dilution factor made on the sample.

2.4.3 Estimation of the octanol-water partition coefficient (LogP)

In order to determine the experimental partition coefficient between 1-octanol and water layers (LogP) for the partial acetylated rutin product, 20 mL water and 20 mL 1-octanol were added to 5 mg of hexaacetylated rutin product. The mixture was then stirred for 30 minutes at 25 ^{0}C temperature. Centrifugation was then performed at 4000 rpm for 10 minutes and the two

layers were separated. The absorbance of the 1-octanol layer (the upper layer) was measured. The absorbance reading was fitted to the calibration curve regression equation in order to estimate the amount of partitioned hexaacetylated rutin ester in the organic 1-octanol layer. LogP was calculated according to the equation:

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

Note: Amount of material partitioned into water layer in mg = 5 – amount of material partitioned into 1-octanol layer.

2.5 Antioxidant activity

2.5.1 DPPH reduction method

The antioxidant capacity of rutin, hexaacetylated rutin derivative and deacaacetylated rutin ester was compared to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); a water soluble vitamin E analogue which is generally used as antioxidant standard. The test was performed using DPPH reduction method. The method depends on the measurement of the decay of the absorbance that results from the reduction of 2.2-diphenylpicrylhydrazyl (DPPH), which is purple colored and has a maximal absorption at 517 nm wavelength, into DPPH-H. The color therefore disappears and turns into yellow [44].

2.5.2 Preparation of stock and working solutions

For each of the compounds that is desired to test its antioxidant activity; a stock solution (one) of 10 mg/mL was prepared by weighting 100 mg of the

antioxidant in 10 mL methanol solvent. 5 mL of the above stock solution was diluted up to 50 mL with methanol to prepare stock solution (two) of 1 mg/mL. Serial solutions of different concentrations were then prepared by making suitable dilutions from the two stock solutions (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/mL).

A solution of DPPH of concentration 0.002% w/v was freshly prepared. Samples were prepared by mixing DPPH, methanol and the previously prepared working solutions in a ratio of 1:1:1 respectively. Methanol was used as the blank and the first sample of each series of dilutions which is antioxidant free was prepared by adding DPPH and methanol in a ratio of 1:2. All samples were then kept in the dark for 30 minutes and the absorbance was thereafter measured at 517 nm wavelength. The percentage of antioxidant activity of rutin, decaacetylated and hexaacetylated rutin esters as well as Trolox in each sample was calculated according to the equation:

$$\text{Percent inhibition of DPPH activity} = \frac{(\mathrm{A} - \mathrm{B})}{\mathrm{A}} * 100\%$$

Where:

A= absorbance of the antioxidant free sample.

B = absorbance of the sample.

An average of two readings for each of the antioxidants tested was taken and the percentage inhibition curve was set by plotting concentration of the antioxidant versus percentage of the inhibition activity.

2.6 UV/Vis spectrophotometric method development and validation

2.6.1 Choice of the suitable solvent

Solubility of the hexaacetylated rutin ester was tested using different solvents in order to select the most suitable solvent for the analytical assay method.

The solubility testing in different solvent combinations was performed using solution concentration of 0.1 mg/mL, prepared by weighting 10 mg in 100 mL of each solvent, at 25 ^{0}C. The solvents used in the test were as following: methanol only, acetonitrile only, water only, acetonitrile: water (9:1) and methanol: water (9:1).

2.6.2 Determination of wavelength of maximum absorbance

The full UV/Vis absorbance spectrum (200-600 nm) of the hexaacetylated rutin ester was obtained using a UV spectrophotometer. The UV spectrophotometer was set to zero using acetonitrile as a blank. The hexaacetylated rutin ester sample was dissolved in acetonitrile and was diluted to optimize the absorbance readings and determine the wavelength of maximal absorption.

2.6.3 Linearity and range

The linearity and range of the method was established by preparing a stock solution (0.5 mg/mL) which was prepared by weighting 50 mg of hexaacetylated rutin ester derivative powder and dissolving it with 100 mL acetonitrile solvent. The previous stock solution was further diluted 5 times

to prepare a stock solution of 0.1 mg/mL which was used to prepare five working standard solutions in the range of (0.015 - 0.05 mg/mL). The concentrations of working solutions as well as their dilution factors are illustrated in **Table 2.1.**

Table 2.1: Acetonitrile calibration curve test solutions.

Concentration of the solution (mg/mL)	Volume taken from 0.1 mg/mL stock solution (mL)	Final volume completed with acetonitrile (mL)	Dilution factor
0.015	1.5	10	6.67
0.02	2	10	5
0.03	3	10	3.33
0.04	4	10	2.5
0.05	5	10	2

The UV/Vis spectrophotometer instrument was set to zero using the acetonitrile as blank. Absorbance measurements of the test solutions were then taken at λ_{max} 335 nm. The calibration curve was constructed using Microsoft Excel 2007 where x-axis was the concentration of the solutions in mg/mL and the y-axis represented the absorbance readings. R^2 value was then displayed to confirm linearity of the method.

2.6.4 Accuracy and precision.

Accuracy and precision of the developed method were tested using three concentrations around the test concentration of 0.04 mg/mL (0.05, 0.04 and 0.032 mg/mL).

A stock solution of concentration 0.1 mg/mL was first prepared by dissolving 10 mg of hexaacetylated rutin ester powder in 100 mL

acetonitrile. Serial solutions of 0.05, 0.04 and 0.032 mg/mL were prepared by diluting the stock solution with acetonitrile. Absorbance measurements of the 0.04 mg/mL test concentration were taken at λ_{max} 335 nm on three different consecutive days and by using two different UV/Vis spectrophotometer instruments in order to confirm the analytical methods precision. Each measurement of the prepared solutions was read in triplicates.

Accuracy of the method was established using concentrations representing (80%, 100%, and 125%) of the test concentration of hexaacetylated rutin ester along with the formulation excipients. A stock (0.5 mg/mL) solution was first prepared by weighting 50 mg of the hexaacetylated rutin ester powder, 36.4 mg microcrystalline cellulose, 1 mg magnesium stearate, 1 mg colloidal silicon dioxide, 1.6 mg crosscarmellose sodium and the mixture was dissolved in 100 ml acetonitrile. The stock solution was further diluted 5 times with acetonitrile. This solution was used to prepare 0.032 mg/mL solution (80% test concentration), 0.04 mg/mLsolution (100% test concentration) and 0.05 mg/mL solution (125% test concentration). Absorbance measurements of the three concentrations were then taken at λ_{max} 335 nm. The measurements for each solution were taken in triplicates. The Accuracy and precision was performed according to ICH guidelines and data was examined by calculated percent recovery and RSD % [54, 55].

2.6.5 Limit of detection (LOD) and Limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) are parameters usually included when validating an analytical method, they represent the lowest concentration which can be detected by the analytical method (LOD) and the lowest concentration which can be quantified with acceptable accuracy and precision (LOQ). The two values are calculated from the following equations as defined in ICH guidelines [54, 55].

$$LOD = 3.3 * \frac{\sigma}{Slope\ of\ the\ calibration\ curve}$$

$$LOQ = 10 * \frac{\sigma}{Slope\ of\ the\ calibration\ curve}$$

Where σ is the standard deviation of residuals from the calibration curve.

2.6.6 Robustness of the analytical method

Robustness of the developed analytical method was tested by applying small variations in the test conditions. These variations included testing on different days (day two and day three), using different UV/Vis spectrophotometer instrument and making small variations on the measurement wavelength of (335 ±2 nm). The robustness of the method to these variations was judged form the results of % recovery and RSD values.

2.7 Tablet formulation

Tablet formulation was performed in our research laboratory to have an optimum dissolution and release of the hexaacetylated rutin ester and the accuracy of the developed analytical method on the assay of the formulated

hexaacetylated rutin tablets along with the formulation components was further tested. The components of the formulation and their amounts per tablet are illustrated in **Table 2.2**.

Table 2.2: Composition of the formulated tablets.

Component	**Weight per tablet (mg)**
Rutin or the hexaacetylated ester derivative	250
Microcrystalline cellulose (Avicel pH 101)	182
Magnesium stearate	5
Colloidal silicon dioxide (Aerosil)	5
Crosscarmellose sodium (Acdisol)	8
Total weight	450

The tablet was prepared by weighing each component separately. The active ingredient powder was added to microcrystalline cellulose (MCC) and was mixed for 5 minutes. Acdisol was added to the mixture by geometric dilution and mixing was continued for 10 minutes. Aerosil was then added and mixed for 5 minutes. Finally magnesium stearate lubricant was added and mixed for 2 minutes. The resultant mixture was compressed into tablets using a gauge pressure of 5 tons to get tablets with a final weight of 450 mg ± 5%.

2.8 Dissolution profile of the hexaacetylated rutin ester tablets

Dissolution testing of the formulated hexaacetylated ester of rutin as well as rutin tablets was performed according to USP and ICH guidelines [51].

The dissolution tests were done using USP apparatus 2 (shaft and paddle) in 4.5 pH acetate buffer which was prepared according to USP [57]. The dissolution apparatus was set at 50 rpm and 37 ^{0}C for 210 minutes. Dissolution medium (900 mL) was used for each dissolution vessel and one tablet was placed on each trial. Samples were then withdrawn using a syringe and were filtered at each time intervals of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210 minutes. The concentration of the dissolved rutin or hexaacetylated rutin derivative was measured using UV/Vis spectrophotometer. The absorbance readings were applied to the regression equation and the percentage of dissolution were calculated using the following equation:

$$\%\ \text{Dissolved material} = \frac{\text{Actual amount released}}{\text{Theoretical amount in the tablet}} * 100\ \%$$

The average readings of the dissolution data was used to establish and compare the dissolution profiles of rutin and hexaacetylated rutin derivative tablets. Similarity factor (f_2) and dissimilarity factor (f_1) between two dissolution profiles were calculated according to the equations:

$$f_2 = 50 \log\{[1+ (1\backslash n)S_{t=1}^{n} (R_t-T_t)^2]^{-0.5} \times 100\} \ldots\ldots (1)$$

$$f_1 = \{[S_{t=1}^{n} |R_t-T_t|^{t=1}] [S_{t=1}^{n} R_t]\} x100 \ldots\ldots\ldots\ldots\ldots (2)$$

2.9 Forced degradation study

Forced degradation study was performed on the hexaacetylated ester derivative of rutin in order to have an indication on its stability properties

as well as the selectivity of the developed method in the presence of degradation components. The degradation study was performed on the 0.1 mg/mL solution concentration of the hexaacetylated ester derivative of rutin and by using 5 different stress conditions (0.3% H_2O_2, 0.1 N NaOH, 0.1 N HCl, UV light of 254 nm and temperature of 70 ^{0}C).

Samples from each solution of the 5 stress conditions were then diluted by a factor of 2.5 in order to have the absorbance measurements taken at the test concentration of 0.04 mg/mL and the UV absorbance measurements were then used to calculate the percentage of the non degraded hexaacetylated ester of rutin. Time intervals of sampling and testing from each stress condition are illustrated in **Table 2.3**.

Table 2.3: Forced degradation study.

0.3% H_2O_2 Time (hours)	**0.1 N NaOH Time(hours)**	**0.1 N HCl Time (hours)**	**UV light 254 nm Time (hours)**	**Temperature 70 ^{0}C Time (hours)**
0	0	0	0	0
24	24	24	3	24
48	48	48	*NA	*NA
72	*NA	168 (1 week)	*NA	*NA

*NA; Not performed.

2.10 Shelf life and accelerated stability study of the formulated hexaacetylated rutin tablets

Shelf life and accelerated stability testing were performed on the formulated tablets of hexaacetylated rutin ester for 3 months. Tablets were kept at room temperature and at temperature of 40 ^{0}C and the percentage assay was measured at time intervals of (0, 1 month, 2 months, 2.5 months

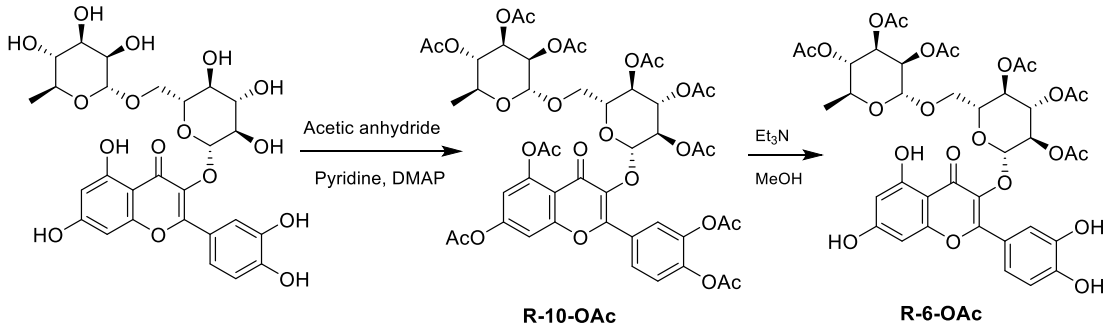
and 3 months). Each tablet containing 250 mg hexaacetylated ester of rutin was grinded and dissolved in 250 mL acetonitrile to prepare stock solution (1 mg/mL). Further dilution was done to prepare a solution of the test concentration (0.04 mg/mL) using acetonitrile as a diluent. The UV absorbance of this solution was measured to calculate the percentage assay of each tablet [58].

Chapter Three

3. Results and discussion

3.1 Chemical synthesis

The synthesis of the fully acetylated and partially acetylated rutin was achieved in two steps reactions. As shown in **Scheme 3.1**, the full acetylation of rutin was synthesized by using acetic acid anhydride, DMAP and pyridine in order to obtain R-10-OAC in good yield. In second step, a partial deacetylation was conducted using triethylamine in methanol in order to hydrolyze specifically the acetylated groups to obtain R-6-OAc in a yield of 83%. The structures of both products were confirmed by nuclear magnetic resonance.



Scheme 3.1: Synthesis of decaacetylated and hexaacetylated rutin derivatives.

3.2 Physicochemical properties results

3.2.1 Melting point of the hexaacetylated rutin ester derivative

The melting point of hexaacetylated rutin ester derivative was determined using a manual melting point apparatus. It was measured to be 155 ± 2 ^{0}C

which is a characteristic that can be used in the identification of the newly synthesized derivative.

3.2.2 Water solubility of rutin and hexaacetylated rutin ester

Solubility test was performed on the newly synthesized hexaacetylated ester and was compared with the solubility of rutin to examine any improvement in the aqueous solubility of the rutin derivative. The test was done in accordance with the USP guidelines and was performed in water and acetate buffer (pH = 4.5). Samples were tested based on the UV absorbance measurement after 24 and 48 hours. The results showed at least two fold increase in solubility for the synthesized hexaacetylated ester compared to the original rutin. The main reason leading to this improvement in the water solubility of rutin is the fact that the original rutin compound has a large number of OH groups in its structure, leading to the formation of intramolecular hydrogen bounding between the rutin molecules themselves thus preventing the bounding with the water molecules, while the number of the available OH groups was reduced in the synthesized hexaacetylated derivative by selective acetylation of some OH groups on the molecule. The detailed calculated solubility of rutin and the hexaacetylated rutin ester are shown in **Table 3.1**.

Table 3.1: Solubility of rutin and hexaacetylated rutin ester.

	Rutin solubility (mg/mL)		Hexaacetylated rutin ester solubility (mg/mL)	
	24 hours	**48 hours**	**24 hours**	**48 hours**
Water	0.06	0.07	0.15	0.16
acetate buffer (pH 4.5)	0.07	0.07	0.13	0.14

3.2.3 logP of hexaacetylated rutin ester

The experimentally determined partition coefficient (logP) of the synthesized hexaacetylated rutin ester was found to be 0.91 compared to logP value of -0.64 for rutin [43]. The above results indicate lipophilic characteristics of the hexaacetylated rutin derivative. However, water solubility of the hexaacetylated rutin was found to be better compared to rutin and the results showed that the structural modification done on rutin will improve the bioavailability of the newly synthesized hexaacetylated rutin ester.

3.3 Antioxidant activity

The percentage inhibition of DPPH activity was calculated by taking an average of duplicate readings for each concentration of the tested antioxidants. The detailed percentage inhibition is shown in **Table 3.2 and Figure 3.1.**

The IC_{50} was calculated using BioData Fit fitting program were the sigmoidal fitting model was the adapted model. The IC_{50} was found to be 2.07, 3.27, 7.32 and 29.88 µg/mL for Trolox , rutin , hexaacetylated rutin ester and decaacetylated rutin ester respectively**.**

Table 3.2: Percentage inhibition of DPPH activity.

Antioxidant concentration (µg/mL)	Percentage inhibition of DPPH activity %			
	Trolox	Rutin	Hexaacetylated rutin ester	Decaacetylated rutin ester
0	0	0	0	0
1	22.14	21.55	4.42	1.60
2	47.74	37.91	8.70	5.16
3	73.66	41.29	11.34	7.47
5	81.92	56.70	19.71	9.29
7	87.13	75.25	33.28	12.10
10	94.65	92.90	70.92	19.24
20	95.33	93.80	82.51	25.34
30	95.33	94.40	84.41	30.46
40	95.55	94.60	85.47	36.18
50	95.82	94.67	87.87	42.24
80	96.31	94.98	92.09	48.05
100	96.80	94.98	93.68	58.42
IC50 (µg/mL)	2.07	3.27	7.32	29.88

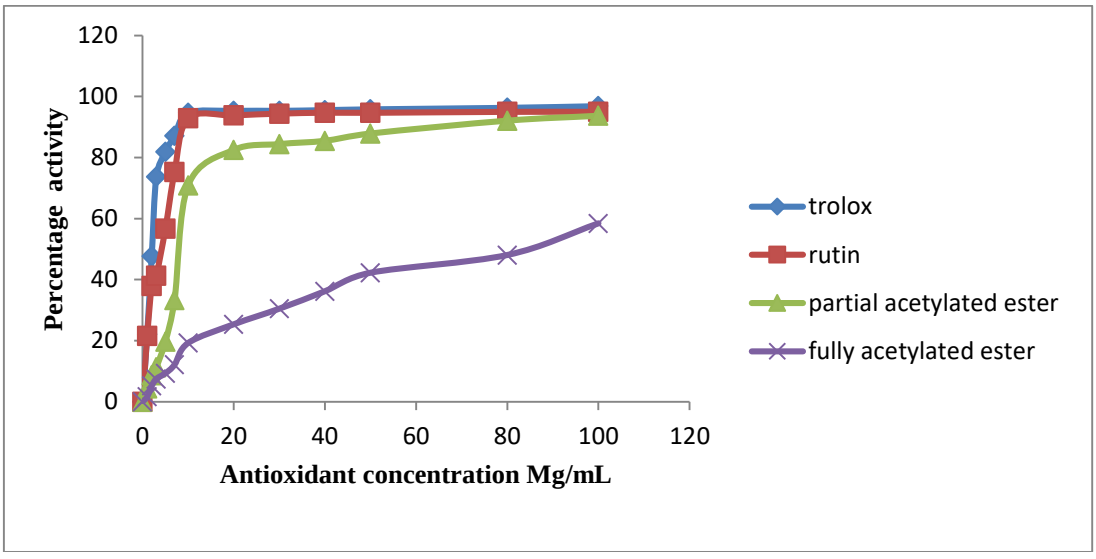


Figure 3.1: Antioxidant activity of Trolox, rutin and rutin acetyl esters.

The antioxidant activity testing using DPPH reduction method showed no significant difference in the antioxidant activity of rutin, hexaacetylated rutin ester and trolox and this clearly demonstrates that derivatisation of rutin to hexaacetylated rutin did not lower its antioxidant activity. However, the decaacetylated rutin ester had a significant drop in its antioxidant activity which is expected as all the phenolic OH groups which are responsible to a large extent for the compounds antiradical and antioxidant activity had been acetylated; while in hexaacetylated rutin ester the phenolic OH groups are active and this may explain why it maintained its antioxidant activity.

3.4 UV spectrophotometric analytical method development and validation parameters

3.4.1 Choice of the suitable solvent

Solubility of 10 mg hexaacetylated rutin ester in 100 mL (0.1 mg/mL) in different solvents was examined. The results showed that the best solubility was obtained from using absolute methanol and absolute acetonitrile solvents. Water solubility was lower compared to the above mentioned solvents. A good solubility was also obtained using combinations of water: methanol and water: acetonitrile in a ratio of 9:1. Absolute acetonitrile was the chosen as the diluents solvent in the development of the spectrophotometric analytical method for the assay of hexaacetylated rutin ester in the tablet formulation. The reason for choosing acetonitrile is mainly due to selectivity of this solvent to hexaacetylated ester of rutin.

Acetonitrile has low solubility for rutin and thus it precipitates out and can be filtered from the sample as rutin is a possible degradation product and hence does not interfere with absorbance readings of the main analyte hexaacetylated rutin ester.

Calibration curve of serial standard solutions of different concentrations of rutin and the hexaacetylated ester of rutin in methanol were plotted against their absorption. The regression line equation was also generated to quantify our targeted analytes of rutin and hexaacetylated rutin ester derivative in different tests such as solubility and dissolution (**Figure 3.2 and 3.3**).

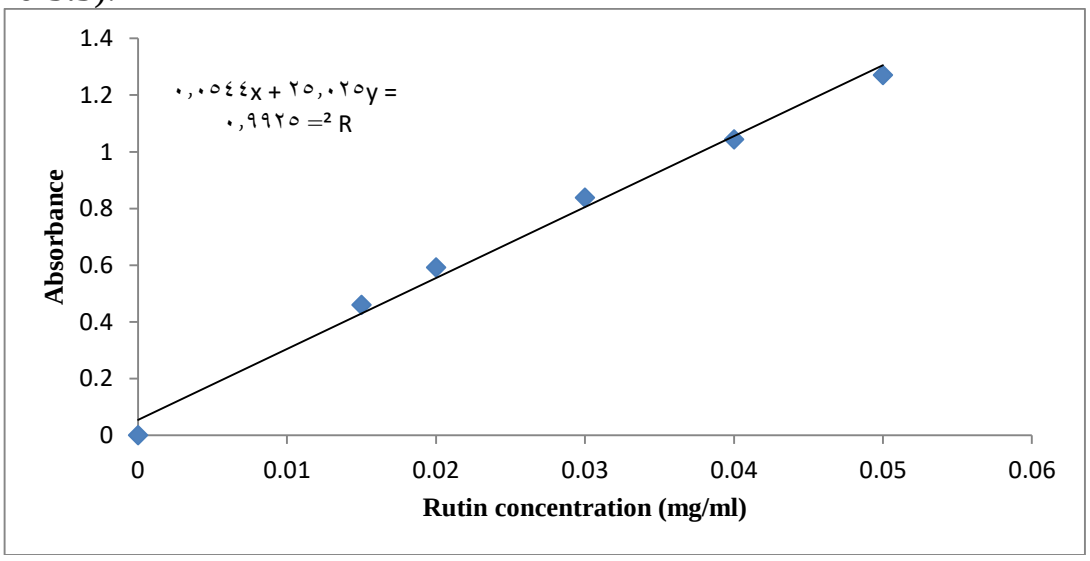


Figure 3.2: Rutin calibration curve in methanol solvent.

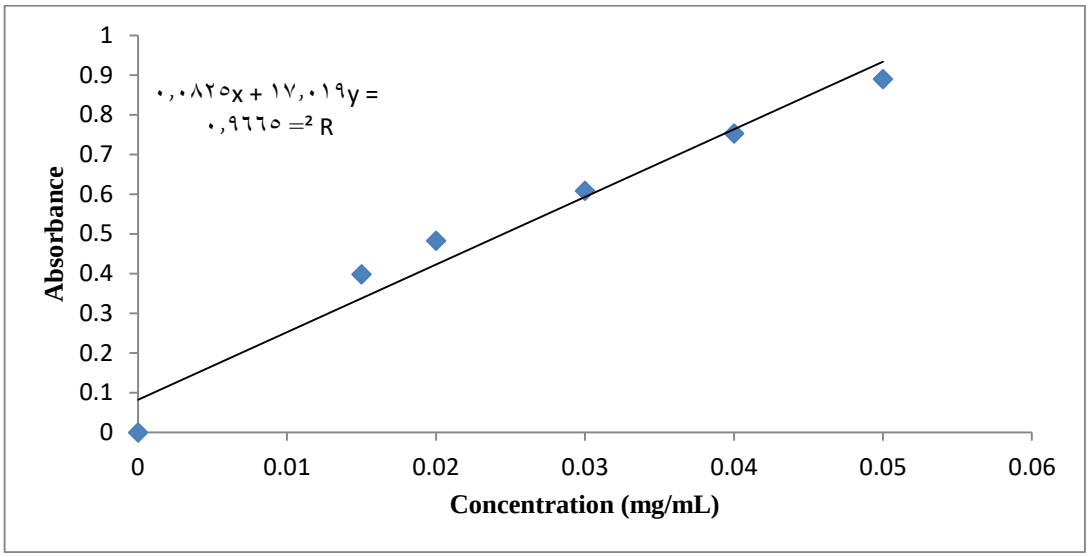


Figure 3.3: Hexaacetylated rutin ester calibration curve in methanol solvent.

3.4.2 Determination of wavelength of maximum absorbance

The UV/Vis scan in the range of 200-600 nm for hexaacetylated rutin ester was performed to determine the maximum absorption. The resulted scan is shown in **Figure 3.5**. The graph showed more than one wavelength of maximum absorption of 335, 225 and 245 nm. Wavelength of maximum absorption of 335 nm was chosen as a wavelength for the analysis of hexaacetylated rutin ester. Other wavelengths of maximum absorption (225 and 245 nm) were excluded for reasons concerning the accuracy and selectivity of the analytical method.

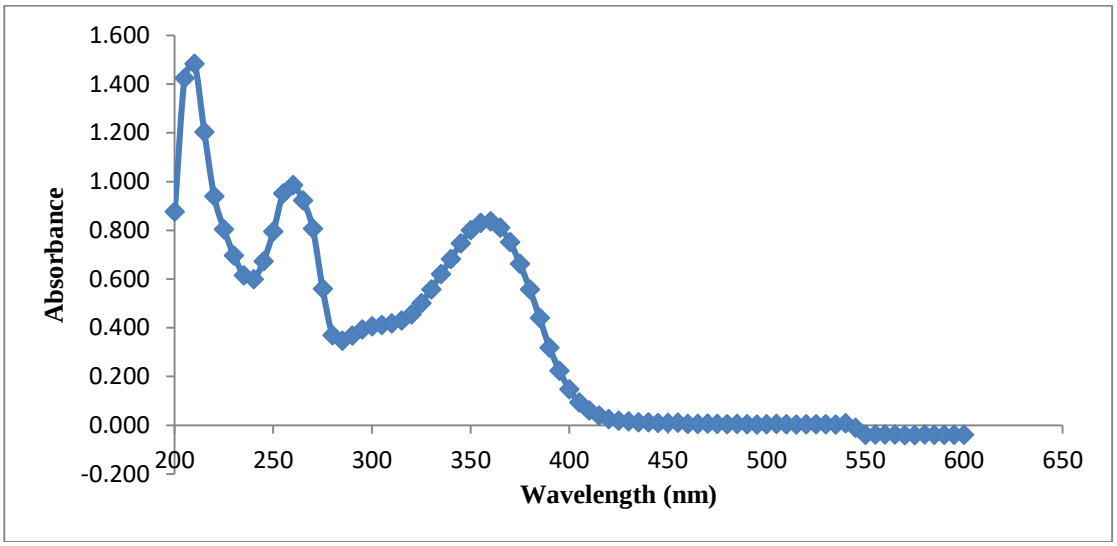


Figure 3.4: UV spectrum of 0.03 mg/mL rutin sample.

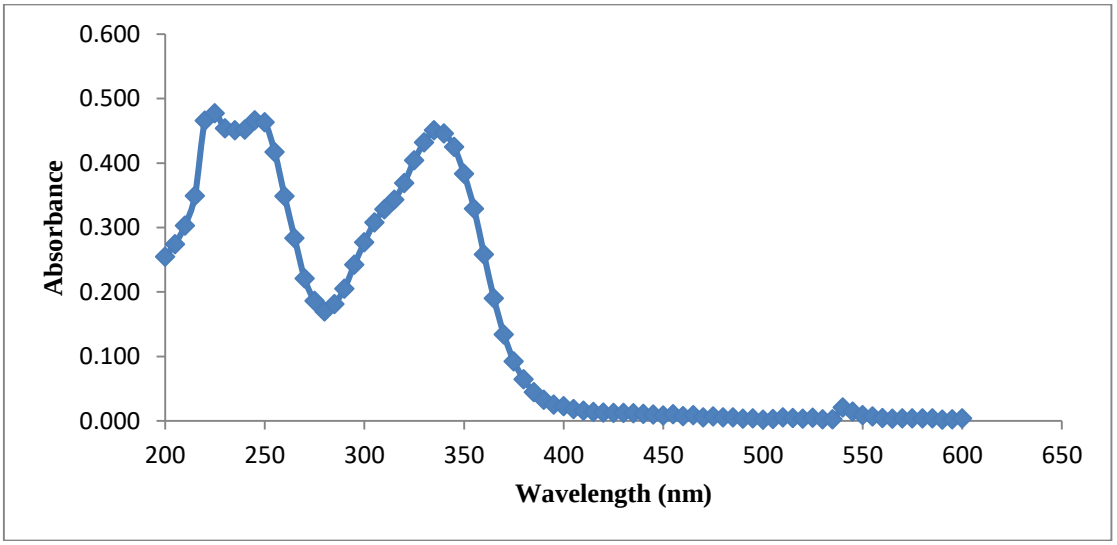


Figure 3.5: UV spectrum of 0.02 mg/mL hexaacetylated rutin ester sample.

The UV scan clearly showed that at our selected λ_{max} for the hexaacetylated rutin derivative has at least double the absorption compared to the wavelength (360 nm) of rutin (**Figure 3.4**). The selection of this specific wavelength for the analysis of hexaacetylated rutin will improve accuracy and the selectivity of the developed analytical method.

3.4.3 Specificity and selectivity

The Method is considered to be selective when there is no interference in the measurements between the analyte and any degradative or formulation material. To check for any possible interference of added formulation excipients of the tablet formula on the absorbance measurements of the hexaacetylated rutin ester at the selected λ_{max} of 335 nm, a solution of 0.05 mg/mL was prepared along with the formulation components in the same ratios as in the tablet formula. The UV spectrum was measured at 200-600 nm wavelengths (**Figure 3.6**). Concentration of each of the formulation components in this solution are illustrated in **Table 3.3**.

Table 3.3: Concentrations of the formulation excipients added to a 0.05 mg/mL hexaacetylated rutin ester solution.

Excipient	Concentration (mg/mL)
MCC	0.0364
Mg stearate	0.001
Aerosil	0.001
Acdisol	0.0016

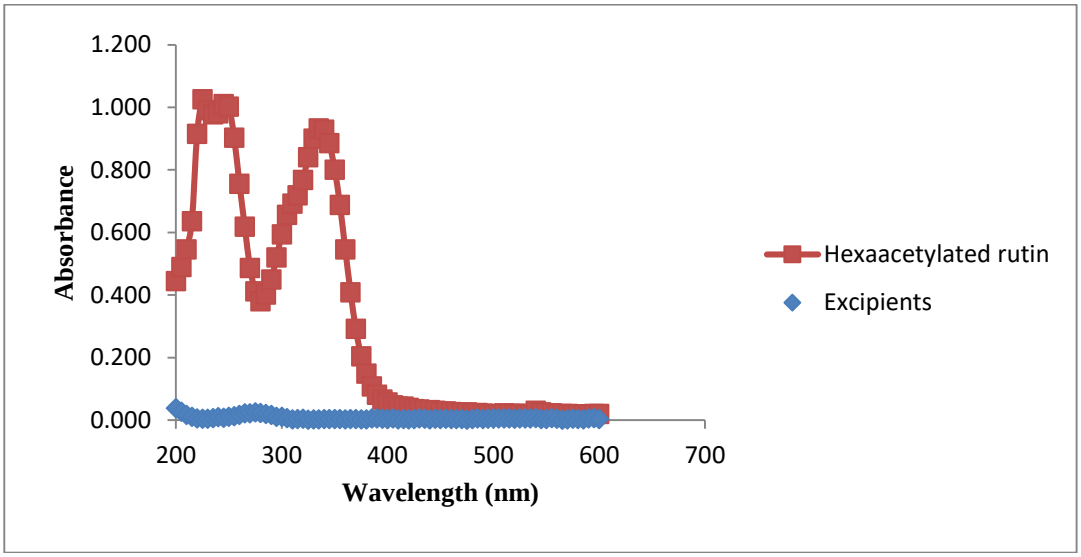


Figure 3.6: UV absorbance spectrum of hexaacetylated rutin ester 0.05 mg/mL solution with formulation excipients.

Results showed no interference in the absorbance of any of the added excipients and hexaacetylated rutin in the scanned range of wavelengths. The absorbance measurements of all the suggested excipients were almost zero and none of them specifically absorbs at the selected measurement λ_{max} (335 nm). The above result demonstrates the selectivity of the method for the quantification of hexaacetylated ester of rutin.

3.4.4 Linearity and range

Linearity and range of the analytical method was performed by measuring the absorbance of five serial standards prepared form stock solution (0.1 mg/mL). The concentrations of the dilutions and their absorbance measurements are mentioned in **Table 3.4**.

Table 3.4: UV absorbance of serial standards of hexaacetylated rutin ester.

Concentration (mg/mL)	Absorbance
0.015	0.381
0.02	0.456
0.03	0.604
0.04	0.775
0.05	0.927

The calibration curve of the hexaacetylated ester of rutin in acetonitrile solvent (**Figure 3.7**) was then plotted and examined for linearity. A linear relationship between concentration and UV absorbance measurements was obtained with a correlation (R^2) value of 0.983 and a regression line equation of y = 17.95x+0.06. The linearity was confirmed in the range of 0.016-0.056 mg/mL which represents 40%-140% of the chosen 0.04 mg/mL test concentration.

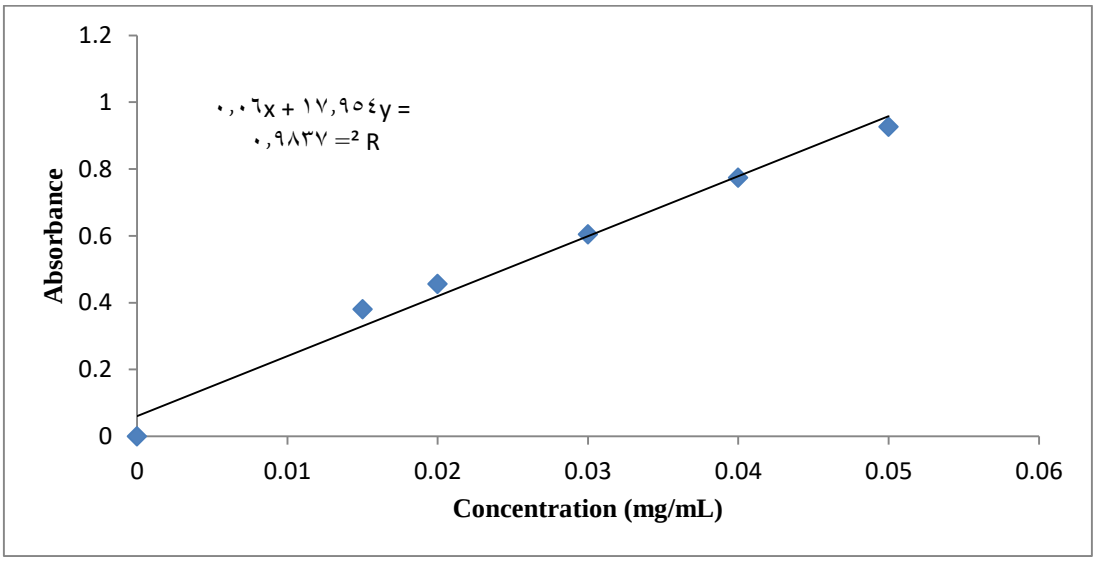


Figure 3.7: Linearity curve of hexaacetylated rutin ester.

3.4.5 Accuracy and precision

Accuracy and precision were performed on three different concentrations representing (80%, 100%, and 125%) of the test concentration (0.04 mg/mL). The formulation excipients were added to all the tested concentrations and all the tests were done nine times.

The accuracy and precision results are shown in **Table 3.5**. The percentage recovery and relative standard deviation results indicate good accuracy and precision of the analytical method. All the percentage recoveries were in the range of (100%± 5%) and the RSD was not higher than 2. Moreover, Statistical t-test and ANOVA to test the variation of the results within the sample or between different samples showed no significant difference.

Table 3.5: Accuracy and repeatability results of hexaacetylated rutin ester.

		Concentration (mg/mL) % of the test concentration		
		(0.032) 80%	**(0.04) 100%**	**(0.05) 125%**
Percentage recovery	**Sample 1**	100.10	97.49	97.15
		99.75	98.18	96.93
		99.23	98.60	96.71
	Sample 2	100.97	98.88	98.05
		100.80	99.16	98.38
		101.32	99.58	97.82
	Sample 3	99.05	100.41	98.16
		99.40	100.27	98.60
		99.23	100.13	98.49
Mean		99.98	99.19	97.81
RSD		0.80	1.01	0.720

3.4.6 Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) of the analytical method were calculated according to the equations stated in the methodology section using the standard deviation of the regression line of the calibration curve. The calculated LOD and LOQ were found to be 0.00854 and 0.0259 mg/mL respectively. The chosen test concentration of 0.04 mg/mL was above the limit of quantification.

The results indicate high sensitivity as the developed method is capable to detect and quantify very low concentration compared the test concentration.

3.4.7 Robustness

Small variations were carried out on a number of the analytical method parameters in order to confirm ruggedness and robustness of the method. Results shown in **Table 3.6** indicate stability and validity of the test result data when the test was done in different days, using different instruments and when applying small variations in the measurement wavelength (± 5 nm). All the results remained within the acceptable recovery range of 100% ± 5. According to the data obtained we concluded that the method is robust enough to reproduce accurate and precise results under different method conditions.

Table 3.6: Robustness of the developed UV analytical method.

Day to day variations		
Day	**Absorbance**	**%Recovery**
Day one	0.775	99.52
Day two	0.768	98.62
Day three	0798	102.74
Mean	**0.780**	**100.29**
RSD:	**1.850**	
Different instruments		
Instrument	**Absorbance**	**%Recovery**
Instrument one	0.775	99.52
Instrument two	0.780	100.28
Mean	**0.778**	**99.90**
RSD:	**0.681**	
Wavelength variations		
Wave length	**Absorbance**	**%Recovery**
333 nm	0.769	98.75
335nm	0.773	99.26
337 nm	0.751	96.44
Mean	**0.764**	**98.15**
RSD	**1.530**	

3.5 Dissolution profile of the hexaacetylated rutin compared to rutin

Dissolution is a common test generally performed on tablets during drug development to simulate its *in vivo* release profile. The test was performed on our formulated rutin and hexaacetylated rutin ester tablets to compare the amount of active ingredient released from each different tablet. An acetate buffer of pH 4.5 was chosen as the dissolution medium, and the dissolution results represent the average dissolution release of 3 tablets for each tablet formula. The percentage of dissolution of rutin and hexaacetylated rutin ester for 210 minutes at each specific time interval is shown in **Table 3.7 and Figure 3.8.**

Table 3.7: Percentage dissolution of rutin and hexaacetylated rutin ester tablets.

Time (min)	**% Dissolution Rutin**	**SD**	**%Dissolution Hexaacetylated rutin ester**	SD
5	9.89	0.132636	1.21	0.314078
10	12.51	0.816221	4.70	1.300416
15	14.50	0.204055	7.16	0.614137
20	16.21	0.336691	10.02	0.99577
25	17.39	0.010203	12.51	0.894495
30	18.19	0.071419	14.76	1.098984
35	18.89	0.010203	15.88	1.475348
40	19.15	0.255069	17.88	0.871541
45	19.33	0.142839	19.40	0.82717
50	19.76	0.051014	20.59	0.87618
55	19.83	0.020406	21.77	0.918839
60	20.15	0.010203	23.01	1.067942
70	20.29	0.102028	25.15	1.069417
80	20.90	0.132636	26.70	1.069839
90	21.02	0.214258	28.13	1.008132
100	21.28	0.020406	29.56	0.857735
110	21.36	0.010203	30.42	0.453871
120	21.44	0.020406	32.13	0.947796
130	21.77	0.469327	32.89	1.040238
140	22.02	0.163244	33.44	1.140221
150	22.04	0.132636	34.38	1.456708
160	22.13	0.061217	34.98	0.765909
170	22.16	0.040811	35.52	0.557629
180	22.17	0.030608	35.97	0.515079
190	22.22	0.020406	36.40	0.530586
200	22.24	0.010203	37.11	0.430963
210	22.26	0.010203	37.41	0.331747

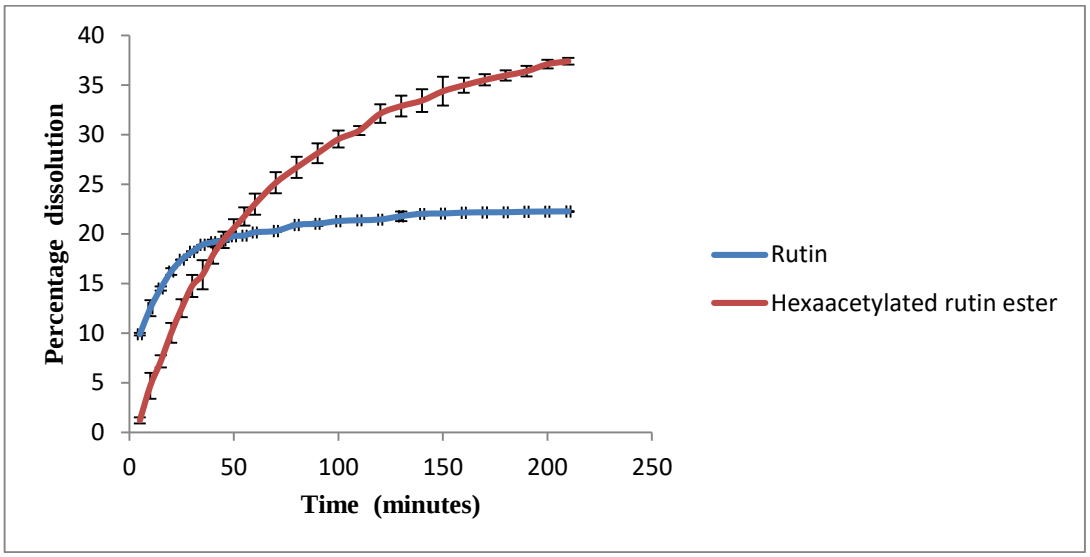


Figure 3.8: Dissolution profile of rutin and hexaacetylated rutin ester tablets.

The above results clearly demonstrate that hexaacetylated rutin ester has a significantly higher dissolution rate reaching a maximum of 37.5% compared the original rutin (22 %). The tablets were formulated using the same excipients for rutin and the derivatised rutin to exclude any variability due to a formulation effect. The result clearly proves the success of achieving our main objective of increasing the dissolution of rutin by derivatisation without affecting its antioxidant activity. Moreover, the statistical test for independent sample T test showed significant difference between the dissolution profile of the rutin and hexaactylated rutin ester $P > 0.05$.

The primary regulations for the comparison of two in vitro dissolution profiles are the similarity factor (f_2) and dissimilarity factor (f_1) as emphasized by the US FDA. The two factors are calculated using two listed formulas and a similarity factor (f_2) of 50-100 indicates similarity of two

tested dissolution profiles. However, a difference factor (f_1) of 0-15 ensures minor difference between two dissolution profiles[59].

The similarity and dissimilarity factors between the two dissolution profiles of rutin and hexaacetylated rutin ester were calculated from the average mean of the dissolution data. The results revealed a similarity factor (f_2) of 53 and dissimilarity (f_1) of 43. The dissimilarity factor that is higher than 15 with significant statistical T test results indicate dissimilarity between the two tested profiles.

3.6 Forced degradation study

A Stability indicating study was done by forced degradation which was conducted by submitting the active ingredient along with the excipients to stress conditions of light, heat, acid/base hydrolysis and oxidation. Solutions of stressed samples were analyzed occasionally; stress testing is terminated if 5-20% degradation is obtained, or after the maximum recommended time if no degradation is observed.

Hexaacetylated rutin solutions were subjected to different stress conditions including 0.1 N NaOH, 0.1 N HCl, UV light (254 nm), 0.3% H_2O_2 and 70 ^{0}C temperature. The results under each condition are shown separately in **Table 3.8**.

Table 3.8: Stability of hexaacetylated rutin under different stress conditions.

Time (hours)	Stress condition	% Recovery
0	**0.1 N HCl.**	90.20%
24		86.76%
48		61.93%
168		50.10%
0	**0.1 N NaOH**	17.27%
24		6.40%
48		3.40%
0	**0.3 % H_2O_2**	98.10%
24		98.60%
48		98.60%
72		99.44%
0	**Temperature 70 ^{0}C**	100.40%
24		100.50%
0	**UV (254 nm)**	100.40%
3		98.80%

The stability indicating results of the hexaacetylated rutin ester showed that the compound was stable when subjected to UV light (254 nm), 0.3% H_2O_2 and 70 ^{0}C temperature. However, stress basic conditions (0.1 N NaOH) caused the most degradation with a fast drop in the percentage recovery to 3.4% in 48 hours. The compound was also unstable in stress acidic conditions (0.1 N HCl) but to a less extent than basic conditions as the percentage recovery dropped to 50.10% in 168 hours. The main reason explaining the instability of the hexaacetylated rutin ester compound in these two stress conditions is the fact that ester bonds are generally unstable to strong basic and acidic conditions. 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 Shelf life and accelerated stability study

The formulated hexaacetylated rutin ester tablets were tested for stability under room temperature and under accelerated conditions of high temperature (40 ^{0}C) within a period of 3 months. Tablets were tested periodically and the percentage assay was calculated. The results clearly demonstrate the stability of the hexaacetylated rutin formulated tablets at both conditions. The stability at accelerated conditions predicts long shelf life of the formulated product at normal storage conditions. The detailed percentage assay results are shown in **Table 3.9.**

Table 3.9: Percentage assay of hexaacetylated rutin tablets after different storage temperature conditions.

Storage period (Days)	Percentage assay of tablets	
	At room temperature	At 40 ^{0}C temperature
0	100.8%	100.8%
30	*NA	102.12%
45	*NA	99.23 %
60	104.17%	100.1%
90	103.38%	102.19%

*NA: Not performed.

Chapter Four

4. Conclusion and future work

Our work was conducted in attempts to synthesis a hexaacetylated ester derivative of the compound rutin. Water solubility of the newly synthesized compound was tested and compared to that of the original rutin. The antioxidant activity of the hexaacetylated rutin compound was evaluated to make sure that the ester derivatisation of the OH groups on the sugar moiety has not affected the compounds antiradical and antioxidant activity.

Hexaacetylated rutin compound was then formulated in a specially designed tablet formula, UV/Vis spectrophotometric method was also developed for the assay of hexaacetylated rutin in their tablet formula at λ_{max} of 335 nm. The method was validated according to ICH guidelines which is important as there are usually less regulations on the GMP practice of herbal products and food supplements. Dissolution test was performed on the formulated hexaacetylated rutin ester tablets and the dissolution profile was compared to that of rutin compound formulated in the same tablet formula. A stress stability study on the hexaacetylated rutin compound was performed under various stress conditions (0.1 N NaOH, 0.1 N HCl, UV light (254 nm), 0.3% H_2O_2 and 70 ^{0}C temperature) as well as an accelerated stability study for 3 months on the formulated tablets under 40 ^{0}C.

In conclusion, the partially esterified hexaacetylated derivative of rutin was successfully synthesized, the R-6-OAc had a twice fold increase in the

water solubility when compared with rutin. The hexaacetylated derivative of rutin was synthesized by selective acetylation of some OH groups of the rutin original and thus reduced the intermolecular hydrogen bonding which resulted in marked increase in its water solubility. The dissolution of the locally formulated tablets was improved from 22% for rutin to about 37.5% for the hexaacetylated rutin. The derivatisation of rutin into hexaacetylated ester apparently didn't have a negative effect on the compounds antioxidant activity as shown in the results of the antioxidant testing.

The developed UV/Vis analytical method was successfully used in the assay of hexaacetylated rutin tablets and the formulated tablets have shown to be stable over a long shelf life when tested over a an accelerated stability study. The tablet 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.

Recommendations for any future work can be illustrated as following:

1- Considering the chemical synthesis of other derivatives of the active ingredient rutin for further enhancement of its oral bioavailability.

2- Conducting a permeability study on the synthesized hexaacetylated rutin and clinical studies in order to have a clear overview of the compounds oral bioavailability.

3- Developing a more reliable HPLC analysis method for use in the analysis of hexaacetylated rutin tablets.

4- Making further modifications on the tablet formula for optimum dissolution and release of the active ingredient.

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جامعة النجاح الوطنية
كلية الدراسات العليا

تكوين وتركيب مشتقات مركب الروتين والتحقق من طريقة تحليلها

إعداد
تالا محمد أحمد سرحان

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 في نابلس- فلسطين.
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الملخص

الروتين هو مركب مستخلص من النباتات وينتمي لعائلة الفلافونات من المركبات. دراسات عديدة أثبتت أن الروتين له إستخدامات علاجية كمضاد للأكسدة ومضاد للإلتهاب ومضاد لارتفاع الضغط. الروتين متوفر بشكل واسع كمنتج طبي ومدعم غذائي ويتم تسويقه بأشكال صيدلانية متعددة. ولكن ، امتصاص هذا المركب من الجهاز الهضمي قليل وبهذا فإن التوافر الحيوي لهذا المركب قليل بسبب ضعف ذائبيته بالماء. بهذه الدراسة نهدف لتحسين ذائبية المركب بالماء وبالتالي توافره الحيوي عن طريق تصنيع مركب مشتق من الروتين.

الطريقة:

في البداية تم تصنيع مركب مشتق من الروتين بعشر روابط من مجموعات الأسيتيل، ثم تمت عملية إزالة متخصصة لبعض روابط الإيستر للحصول على مركب مشتق من الروتين بست روابط من مجموعات الأسيتيل. تم فحص ذائبية المركب الجديد بالماء وكذلك تحلل الأقراص المصنعة منه وتم مقارنتها بمركب الروتين. تم تقييم فعالية المركب المشتق من الروتين بست روابط من مجموعات الأسيتيل كمضاد للأكسدة باستخدام طريقة إختزال مركب ال (DPPH)، كذلك تم تطوير طريقة لتحليل الأقراص المصنعة من المركب الجديد والتثبت منها باستخدام جهاز الأطياف المرئية/ الفوق بنفسجية وفق معايير (ICH).

النتائج:

المركب المشتق من الروتين بست روابط من مجموعات الأسيتيل تم تصنيعه بنجاح كما تم تأكيده من خلال تحليل الرنين النووي المغناطيسي. ذائبية المركب الجديد بالماء وكذلك تحلل الأقراص

المصنعة تم تحسينه بمقدار الضعف تقريباً بالمقارنة مع الروتين. ذائبية المركب المشتق من الروتين بست روابط من مجموعات الأسيتيل تمت زيادتها من 0.07 مغ/مل إلى 0.15 مغ/مل وتحلل الأقراص من 22% إلى 37.5% مقارنة مع مركب الروتين. بالإضافة إلى ذلك، نتائج الدراسة على الفعالية كمضادات للأكسدة أثبتت أن المركب الجديد المشتق من الروتين احتفظ بفعاليته كمركب مضاد للأكسدة. تم تطوير طريقة تحليل سهلة وعملية وكذلك تم التثبت منها. الطريقة التي تم تطويرها وجد بأنها خطية ودقيقة ومتخصصة. أقل تركيز يمكن كشفه وأقل تركيز يمكن قياسه بدقة مقبولة وجد بأنهم 0.00854 و 0.0259 مغ/مل على الترتيب. الأقراص المصنعة من المركب المشتق من الروتين بست روابط من مجموعات الأسيتيل في مختبرنا البحثي وجد بأنها مستقرة على الظروف الصعبة وكذلك العادية لمدة ثلاثة أشهر.

الخلاصة:

تم تحسين الذائبية الضعيفة لمركب الروتين بالماء عن طريق إضافة إيستر من مجموعات الأسيتيل بطريقة متخصصة لبعض مجموعات الكحول (OH) الموجودة بمركب الروتين. الأقراص المصنعة من المركب المشتق من الروتين والمضاف له جزئياً مجموعات الأسيتيل أعطت نتائج تحلل أفضل من أقراص الروتين الموجودة حالياً بالسوق.