An-Najah National University

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Pharmacological and Phytochemical Screening of *Rumex Rothschildianus* Extract

By

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This thesis was defended successfully on 16/4/2019 and approved by:

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Dedication

To my dear family, my mother, father, sister and brothers for their patience and encouragement, and my lovely husband, and to my friends and colleagues.

WITH ALL LOVE AND RESPECT

Acknowledgement

Thanks first go to ALLAH. I would then express my sincere thanks and gratitude to my supervisor, Dr. Nidal Jaradat for his guidance, encouragement and help during this study. My special thanks to all who have helped me in this study, including Mr. Abed Arhman Qabaha. My deepest respect and appreciation to the instructors and lab technicians of the Department of Pharmacy, notably Dr. Fatima Hussein and Miss. Linda Arar. In addition, my deepest thanks, respect and appreciation to my dear mother, father, husband, sisters and brothers for their patience and encouragement. To my friends and colleagues and everyone who has wanted me to succeed, progress and develop, and who have shared their emotions with me.

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Pharmacological and phytochemical screening of *Rumex rothschildianus* extract

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

Declaration

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

Student's Name: **اسم الطالب: غادة فواز دعاس**

Signature: **التوقيع:**

Date: **التاريخ: 2019/4/16**

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List of Abbreviations

PNPB	p-nitrophenyl butyrate
DNSA	3,5-dinitrosalicylic acid
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
IC_{50}	half maximal inhibitory concentration
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
A_B	absorbance of the blank solution
A_{ts}	absorbance of the tested sample solution
MCF7 cells	human cervix adenocarcinoma cell line
CAE	Catechin equivalent
GAE	Gallic acid equivalent
RUE	Rutin equivalent
***P*NPG**	p-nitrophenyl glucopyranoside

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Abstract

Background: Herbal products are a rich source of bioactive compounds, making them an important and attractive field for scientific research. This study investigated the pharmacological properties of *Rumex rothschildianus* leaves in obesity, cancer, diabetes and oxidative stress.

Methods: For fraction preparation, four solvents with different polarities were chosen for the extraction of *Rumex rothschildianus* leaves. Several qualitative phytochemical tests were then performed to determine the components of the extracts. I*n-vitro* assays were performed to evaluate the extracts for antioxidant properties and α-amylase, α-glucosidase and lipase inhibitory activities, as well as cytotoxicity.

Results: The acetone fraction of *R. rothschildianus* leaves showed the most significant results. Having the highest content of flavonoids and phenolics, it also had the highest antioxidant capacity with an IC_{50} value of 6.3 ± 0.43 µg/ml, compared to 3.1 ± 0.92µg/ml for trolox, the reference compound. The acetone fraction also special had an IC_{50} value of 26.3 ± 0.57 µg/ml for the lipase inhibition assay. The acetone fraction was also the most potent inhibitor of α-amylase and α-glucosidase, with IC_{50} values of 19.05 ± 0.75 µg/ml and 54.9 ± 0.33 µg /ml, respectively, compared to 37.15 ± 0.33 µg/ml by the reference compound acarbose. The hexane

fraction showed 99% inhibition of HeLa cells and 92.4% inhibition for MCF7 cells, which might have been due to its content of terpenoids and steroids.

Conclusion: The acetone fraction of *R. rothschildianus* leaves might provide a source of bioactive compounds for the treatment of diabetis, obesity and oxidative stress. Similarly, the hexane fraction might yield compounds for cancer managment. Clearly, these initial indications need further purification of potentially active compounds, and ultimately, *in-vivo* studies to determine their effectiveness.

Chapter One

Introduction

1.1 General project overview

Plants have been used as therapies since ancient times. Roots, seeds, bark, leaves, and flowers have all been used for remedial purposes. In the present day, synthetic medicines are available and are effective in the treatment of a wide range of diseases; however, some people still prefer herbal medicines as they are viewed as being less harmful to the human body [1,2].

Medicinal plants are by definition the source of phytochemical compounds that possess therapeutic activities. These properties rely upon the presence of different secondary metabolites, such as phenolics, terpenoids and alkaloids [3].

Harmful free radicals are known to play an important role in many major health problems, such as cancer, cardiovascular disease, rheumatoid arthritis, cataracts, and Alzheimer's disease and other degenerative diseases related to aging. Antioxidants are beneficial components that neutralise these free radicals before they can attack cells, and hence they prevent damage to cell proteins, lipids and carbohydrates. A variety of both natural and synthetic antioxidants have been proposed for the treatment of human diseases. Such interest in the role of antioxidants in human health has prompted research in the fields of food science and medicinal herbs, assessing the function of herbs as antioxidants. Antioxidant action includes

free radical scavenging capacity, inhibition of lipid peroxidation, metal ion chelating ability and also reducing capacity [4].

Inflammation is a normal response to fighting infections, leading to a cascade of events, such as vascular changes and the release of chemicals that help in the destruction of harmful agents at the injury site and the repair of damaged tissue. It is recognised as swelling, redness, pain, and heat; however, severe inflammation must be reduced to avoid excessive destruction of tissues. The action of phospholipase or cyclooxygenase (COX-1/COX-2) can be inhibited by steroidal or non-steroidal anti-inflammatory agents [5].

New anti-microbial agents need to be found due to the increase in resistant micro-organisms, the emergence of new infectious agents and toxicity concerns regarding some of the current drugs. It has been shown that many plants species have potential anti-inflammatory properties, including cloves, which contain eugenol, sage containing carnosic acid and also rosemary containing rosmarinic acid, which provides protection against inflammation by inhibiting COX-2 [6].

Rumex spp. are distributed worldwide and comprise about 200 species of herbs. They belong to family Polygonaceae and are plants that contain a large number of complex and biologically active phytochemicals. The root and aerial parts of members of the Polygonaceae, including *Rumex* spp., have been used as traditional herbal medicines throughout the world for several therapeutic purposes, such as in psychological conditions, as antioxidants, and for their cytotoxic, anti-fertility, anti-inflammatory,

purgative, anti-tumour, astringent, anti-dermatitis, diuretic, antiviral and antimicrobial effects [7-11]. The main chemical constituents founded in *Rumex* are anthraquinones and flavonoids [12].

The genus *Rumex*, which includes many edible plants, has attracted the attention of investigators interested in treating dangerous viral diseases, such as acquired immune deficiency syndrome (AIDS) caused by human immunodeficiency virus-1 (HIV), herpes and influenza, and also sexually transmitted diseases, especially herpes, warts and *Chlamydia* [13,14].

1.2 Hypothesis related to this project

Based on a hypothesis that different fractions extracted from *Rumex rothschildianus* leaves would yield bioactive compounds, we subjected these fractions to different *in-vitro* antioxidant, antibacterial and cytotoxicity evaluation tests. In addition, leaves of *Rumex rothschildianus* might also provide chemicals that have anti-cancer activities, possibly free of the safety and side-effect concerns of those currently used in chemotherapy.

1.3. Overall project objectives

The goals of this study were:

- The extraction of active phytochemical compounds from *R. rothschildianus* leaves using solvents with different polarities, n-hexane, acetone, methanol and distilled water.

- Qualitative and quantitative phytochemical testing of constituents present in the fractions, such as proteins, flavonoids, phenols, carbohydrates, glycosides, alkaloids, saponins, terpenoids and volatile oils.
- Spectrophotometric analysis of fractions from *R. rothschildianus* leaves to evaluate *in-vitro* antioxidant levels by free radical scavenging assay.
- To studying the effect of leaf fractions on lipase, α-amylase and α-glucosidase, as indicators in the treatment of obesity and diabetes.
- To evaluate the anticancer activity of each fraction against human liver cancer (HepG2) and colon cancer cell lines.

1.4 Description of Rumex rothschildianus

Rumex rothschildianus is the sole member of a unique section of the genus *Rumex*, in the family Polygonaceae. This species is a very rare small dioecious annual, endemic in Palestine. It has a mean height of 45 cm, being characterised by erect stems holding radical petiolate leaves, which are short-hastate at the base and short-acuminate at the apex. Flowers have a diameter of 3─4 mm, while pistillate flowers are about 2 mm in diameter with a coriaceous membranous layer [15]. The plant was collected from Costal area/Palestine and deposited in the pharmacognosy Laboratory under the voucher specimen code Pharm-PCT-2066.

1.4.1. Use of *Rumex rothschildianus* in traditional medicine

Rumex spp. are widespread in different regions of Turkey, being represented by 22 species. Some of the most common species are *R. patientia* L., *R. crispus* L., *R. acetosa* L. *R. caucasicus* RECH., and *R. alpinus* L. *R. alpinus* and *R. caucasicus* are perennial plants distributed in middle and eastern Anatolia at an altitude of 1000-3000 m above sea level. The *Rumex* genus has been widely used in traditional medicine in Turkey to treat disorders, such as constipation, diarrhea and eczema [16,17].

The genus also has some laxative, diuretic, antipyretic, wound healing and anti-inflammatory effects. Many people in the eastern part of Turkey use young leaves of Rumex spp. as a preservative in cheese, as well as giving foods aroma [18].

1.4.2. Scientific research on *Rumex* spp.

A variety of research has been carried out on *Rumex* species, such as antimicrobial activities being reported for some species. Some bioactive phytochemicals have previously been found in *Rumex vesicarius* L., such as carotenoids, tocopherols, polyphenols, flavonoids, and ascorbic acid, which have a role as antioxidants and natural detoxifying agents. The dietary intake of antioxidant phytochemicals, like carotenoids, phenolics and flavonoids may protect against non-communicable diseases in humans, especially cancer, cardiovascular disorders and other health problems related to oxidative stress [19,20].

Rumex hastatus contains nepalin, nepodin, rumicin, phenolic compounds, hastatusides A and B, resveratrol, rumexoside, torachrysone-8-yl β-D-glucopyranoside, rutin, and orientaloside [21]. In *R. crispus,* some bioactive phytochemicals were obtained from an ethyl acetate fraction, such as kaempferol, quercetin, kaempferol-3-O-Alpha-L-rhamnopyranoside and quercetin-3-O-Alpha-L-rhamnopyranoside [22]. Active natural products have also been isolated and identified from *Rumex dendatus* L., especially six pure flavonols, kaempferol 3-O-*β*-galactoside, kaempferol 3-O-*β*-glucoside, kaempferol 3-O-rutinoside, isorhamnetin 3-O-*β*-galactoside, isorhamnetin 3-O-*β*-glucoside and isorhamnetin 3-O-rutinoside [23].

1.5. Medicinal plants as a source of bioactive compounds

Some plants are major sources of active therapeutic compounds, such as morphine, cocaine, quinine, nicotine and muscarine. Almost all these compounds have been the basis of synthetic drugs, e.g., local anesthetics developed from cocaine. Other clinically important drugs have been extracted from plants, notably the anticancer agent paclitaxel (taxol) from yew trees, and the antimalarial agent artemisinin from *Artemisia annua* [24].

Useful therapeutic effects can come from mixing secondary products present in medicinal plants. These compounds are mostly secondary metabolites, like alkaloids, steroids, tannins, flavonoids and phenolics, which are synthesised and deposited in specific parts of these plants. In general, leaves are a favourable storage organ for such compounds. Fruits also contain a substantial amount of active ingredients, and are often

consumed as juice to obtain their therapeutic benefit. Other plant parts which have been extracted for therapeutic compounds are flowers, roots, stem bark, aerial parts and seeds [25,26].

Secondary metabolites of medicinal plants are usually used as the basis for the production of valuable synthetic preparations, as pharmaceuticals, cosmetics, and recently, nutraceuticals. Secondary metabolites have been revealed as potential new drugs, herbicides, antibiotics and insecticides. Their therapeutic effect stems from their antioxidant, anti-aging, anti-cancer, anti-atherosclerotic, anti-inflammatory and also antibacterial activities [27,28].

1.5.1. Qualitative phytochemical screening tests for the identification of active therapeutic compounds in extracts of *R. rothschildianus*

Traditional medicines and medicinal plants in mainly developing countries are widely used as remedial agents for the maintenance of health. The therapeutic properties of plants could be based on the anti-oxidant, anti-microbial, and antipyretic effects of the phytochemical constituents [29]. Examples of such phytochemical compounds are alkaloids, which tend to be poisonous if consumed in large amounts due to their stimulatory effects, producing excitation associated with cell and nerve disorders [30]. Another example is phenolic compounds, which are some of the most widespread molecules among plant secondary metabolites, and are known to act as natural antioxidants [31].

1.5.2. *In vitro* antioxidant assessment based on a free radical scavenging assay with herbal extracts

Utilization of oxygen allows humans to metabolize fats, proteins, and carbohydrates for energy; however, it does not come without cost. Oxygen is a highly reactive molecule that can form free radicals, which can potentially damage healthy cells, destroying their structure and function. Cell damage caused by free radicals is a major contributor to aging and to degenerative diseases, such as cancer, cataracts, cardiovascular disease, brain dysfunction and those affecting the immune system [32].

An antioxidant is any substance which delays or inhibits this oxidative damage by free radicals [33]. The characteristic property of an antioxidant is its ability to scavenge the radicals due to their redox hydrogen donors and quench singlet oxygen [34].

To protect cells and organ systems against reactive oxygen species, humans have evolved a highly complex antioxidant protection system. This system includes several components, both endogenous and exogenous in origin, that work interactively and synergistically to neutralize free radicals. Increasingly, many plant-derived compounds, often called phytonutrients or phytochemicals, are being defined as antioxidants [35,36].

1.5.3. *In vitro* anti-obesity assessment using a lipase inhibition protocol for plant extracts

In 2014, the World Health Organization (WHO) showed that more than 1.9 billion adults are overweight, with 600 million of them being obese [37].

Obesity is considered to be a global epidemic, which needs urgent prevention. Obesity usually results from an imbalance of energy intake and expenditure. This imbalance leads to abnormal weight gain and an increased risk for chronic health disorders, especially cardiovascular disease, diabetes and some forms of cancer [38,39].

Although physical activity and lifestyle changes can contribute in reducing body weight and elevating average daily metabolic rate, there is a high failure rate for long-term body weight reduction. One possibility is to combine lifestyle changes with inhibitors of fat absorption to provide weight management [40].

The most common fat absorption inhibitor used is Orlistat, which may moderately reduce fat absorption via lipase inhibition; however, it can also cause side effects,such as diarrhea, steatorrhea, oily stools and incontinence [41]. A number of research groups have focused on screening plant extracts for potential lipase inhibition, since medicinal plants have been used as dietary supplements for weight reduction and management [42].

1.5.4. *In vitro* α-amylase and α-glucosidase inhibition assays to evaluate the use of plant extracts in diabetes management:

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by both postprandial and fasting hyperglycemia, with many disturbances in carbohydrate, fat and protein metabolic processes. Hyperglycemia in diabetes results from an absolute deficiency in insulin secretion (type 1 DM), a block in insulin action in the body (type 2 DM), or it can result

from both processes. Diabetes has increased greatly worldwide in recent years. The estimated number of people suffering from diabetes was 30 million in 1985, 150 million in 2000 and 246 million in 2007, according to the International Diabetes Federation, with the number expected to become 380 million by 2025 [43].

Diabetes management includes enhancement of the effect of insulin at target tissues, by using sensitisers like biguanides and thiozolidinediones. Endogenous insulin secretion can also be stimulated with sulfonylureas, especially glibenclamide and glimepiride. Finally demand for insulin can be reduced using enzyme inhibitors, such as acarbose and miglitol [44].

In alternative medicine, diabetes can be treated with a variety of herbal extracts. Herbal drugs have the advantages of effectiveness, safety, and acceptability by most patients. Medicinal herbs and natural products are known to retard the absorption of glucose by inhibiting carbohydrate hydrolyzing enzymes, such as pancreatic amylase [45]. Inhibition of this enzyme delays carbohydrate digestion, lowering the glucose absorption rate and so reducing the postprandial plasma glucose rise. Several indigenous medicinal plants show strong inhibition of α-amylase [46].

Another critical digestive enzyme is pancreatic α-glucosidase, which can also be inhibited by medications, such as acarbose, miglitol and voglibose, decreasing hyperglycemia by reducing the digestion and absorption of glucose in the intestines [47].

1.5.5. *In vitro* anti-cancer evaluation of plant extracts

Globally the need for new sources of pharmacological and physiological active compounds is a major priority for scientists. Medicinal plants are now being used directly as therapeutic agents in the treatment of many diseases, such as cancer, heart disease and diabetes [48]. About 20% of all the deaths in the world are related to cancer, with more than one third of the world's population being affected by this disease. Cancer disease continues to be a primary cause of death due to limited and insufficient means of treatment for a diverse range of cancers [49].

Some of the most common causes of cancer are smoking related. Sometimes it occurs due to a specific genetic background, from radiation, chronic virus inflammation, unhealthy food or environmental factors. Many patients suffering from cancer prefer herbal medicine when standard cancer treatment fails, or they use it in addition to conventional therapies. Although our understanding of the cellular and molecular mechanisms of cancer has advanced rapidly, deaths from this disease are still high [50]. An Ethnopharmacological survey recently estimated 60.9% of cancer patients were taking herbal remedies. This study also revealed that a large percentage of indigenous plants are used by these patients around the world [51].

Chapter Two

Materials and Methods

2.1. General background on experimentation

Extraction was considered a crucial first step in research on natural products, allowing chemical components to be separated from plant material and characterised. Several extraction techniques were available; however, the most common ones used in plant extraction were also the most straightforward. These were methods where plant material was soaked and macerated in mild solvents. Decoction in distilled water was broadly applied in traditional Chinese medicine, and was an effective method, particularly when an organic solvent was undesirable. Other solvents can be used in conventional extraction, such as acetone, petroleum ether and hexane [52,53].

2.2. Collection and preparation of plant material

R. rothschildianus leaves were harvested randomly from different regions of Palestine, between February and March 2018. They were identified by Dr. Nidal Jaradat, from the Pharmacognosy Laboratory at An-Najah National University.

Plant leaves were cleaned and dried in an oven at 45ºC. They were then ground with a mechanical grinder into a fine powder, which was stored in airtight containers at 4 ºC.

2.3. Liquid and solid chemical reagents used in the experimentation

Liquid and solid chemical reagents used in the experimental part of this study are listed in Table 2.1.

Table 2.1: Liquids and solid chemicals used for extraction and phytochemical analysis

Reagents	Supplier	Country of Supplier
Sodium hydroxide (NaOH)	Frutarom, Ltd.	Israel
(Hydrochloric acid) HCl	SDFCL	India
Ninhydrin reagent	Alfa Aesar	England
Magnesium ribbon	Frutarom, Ltd.	Israel
Folin-Ciocalteu's reagent	Sigma-Aldrich	USA
(Dimethyl sulfoxide) DMSO	Riedel-de Haën	Germany
Ferric chloride	Riedel-de Haën	Germany
(Sulphuric acid) H_2SO_4	Alfa Aesar	England
Methanol, 99.9%	Loba Chemie	India
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)	Sigma-Aldrich	USA
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich	USA
Iodine (I_2)	Riedel-de Haën	Germany
Benedict's solution	Alfa Aesar	England
Hexane	Frutarom, Ltd.	Israel
Acetone	Frutarom, Ltd.	Israel
Chloroform	Frutarom, Ltd.	Israel
Molisch's reagent	Alfa Aesar	England
Trolox	Sigma-Aldrich	Denmark
DPPH	Sigma-Aldrich	Denmark
Orlistat	Sigma-Aldrich	Germany
P-nitrophenyl butyrate (PNPB)	Sigma-Aldrich	Germany
Porcine pancreatic	Sigma-Aldrich	USA

lipase, type II		
p-Nitrophenyl α-D-galactopyranoside (PNPG)	Sigma-Aldrich	USA
Acarbose	Sigma-Aldrich	USA
α-Glucosidase (baker's yeast α-glucosidase)	Sigma-Aldrich	USA
α-Amylase	Sigma-Aldrich	India
3,5-Dinitrosalicylic acid (DNSA)	Sigma-Aldrich	USA
Potassium phosphate	Sigma-Aldrich	USA
RPMI-1640 medium	Sigma-Aldrich	United Kingdom
Penicillin streptomycin solution (Pen-Strep)	Biological Industries	Israel
L-Glutamine solution	Sigma-Aldrich	UK
Phosphate buffering saline	Sigma-Aldrich	UK
CellTilter 96® Aqueous One Solution Cell Proliferation (MTS) Assay	Promega	USA

2.4. Instrumentation used in the tests

Instrumentation used for *in vitro* analysis of the fractions is listed in Table 2.2. Figure 2.1illustrated some of these devices.

Table 2.2: Instrumentation used in this study

Instrument	Supplier	Country of Supplier
Oven	Ari Levy, Inc.	Israel
Balance - AS 220/C/2	Radwag	Poland
Micropipette	MRC, Ltd.	Israel
Freeze dryer - BT85	Millrock Technology	China
Rotary evaporator - OB2000-VV2000	Heidolph	Germany
Grinder - Uno	Moulinex	China
Micropipettes	Macherey-Nagel	USA
Filter papers – MN 617and Whatman No.1	Macherey-Nagel	USA
CO_2 incubator	(Esco Technologies, Inc.	Singapore
Inverted microscope - IX73	MRC	China
Vortex	Heidolph	Germany
Microwave-ultrasonic reactor extractor	LAB-KITS	China
Microplate reader	Unilab	USA
Water bath - BPXOP1001040	Lab Tech	South Korea

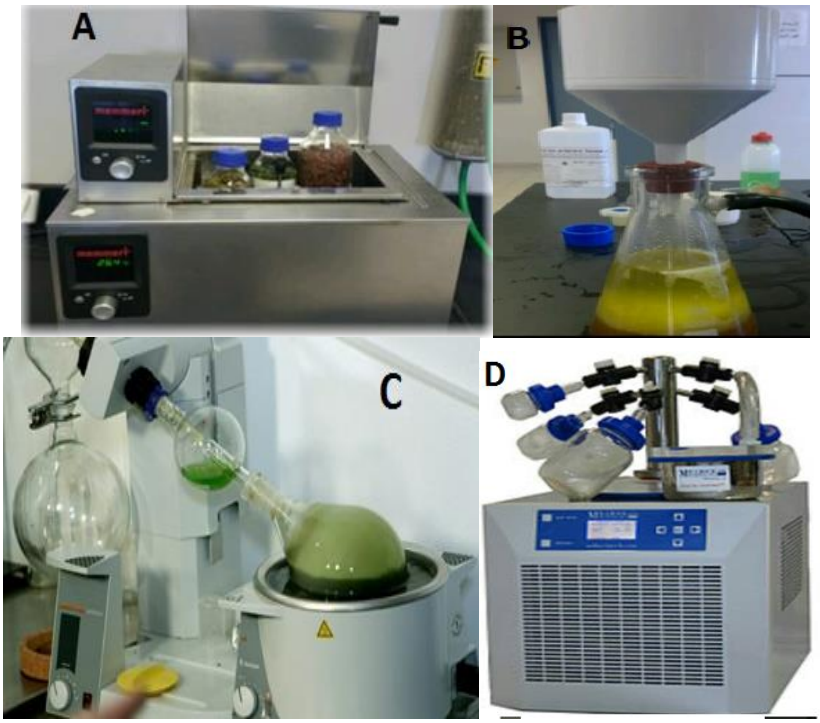

Figure 2.1: Examples of instrumentation used in experimentation: A, shaker; B, suction filtration unit; C, rotary evaporator; D, freeze dryer.

2.5. Extraction of fractions from *R. rothschildianus* leaves

Dried powder of *R. rothschildianus* leaves was extracted by adding solvents sequentially based on their polarity, beginning with the non-polar solvent hexane, and then acetone (a polar aprotic organic solvent), methanol (a polar alcohol), and finally distilled water (a polar protic solvent). For each extraction, about 25 g ground dried leaves was placed in 0.5 l hexane for 72 hr in a shaker device at 100 rotations per minute at 25 ºC. Firstly, the hexane was replaced with 0.5 l acetone, and then subsequently replacement involved equivalent volumes of methanol and water. Incubations in the solvents were as described above for hexane. Each organic fraction was filtered and concentrated under vacuum on a

rotary evaporator, while the aqueous fraction was dried using a freeze dryer. Finally, all crude fractions were stored at 4 ˚C [54].

The yield of each fraction was calculated using the following formula**:**

Yield % = (weight of *Rumex* extract /original dry weight of *Rumex* leaf tissue) × 100%

2.6. Qualitative phytochemical screening tests for *R. rothschildianus* leaves

Phytochemical screening tests of the four fractions were carried out to identify active secondary metabolites. The qualitative results were expressed as (+) for the presence and (−) for the absence of bioactive phytochemicals [55,56].

- **Alkaloids (Mayer's test)**

  Two mg of each fraction was dissolved in 10% v/v hydrochloric acid and filtered. The filtrate was then treated with a few drops of Mayer's reagent (potassium mercuric iodide). A resulting white or pale yellow precipitate indicated the presence of alkaloids in the tested fraction.

- **Carbohydrate (Molisch's test)**

  Two mg of each fraction was dissolved in about 5 ml distilled water and filtered. Two drops of alcoholic α-naphthol solution was added to the filtrate in a small test tube. If a violet ring formed at the interface of the liquids, the presence of carbohydrates was indicated, especially sucrose or complex carbohydrates like cellulose.

- **Reducing sugars (Fehling's solution test)**

  Fehling's solution was prepared previously as follows. Solution A consisted of 34.64 g copper sulphate and 0.5 ml Sulphuric acid adjusted to 500 ml with distilled water. Solution B contained 176 g Sodium potassium tartrate and 77 g sodium hydroxide adjusted to 500 ml with distilled water. Immediately before use, solutions A and B were mixed in equal volumes to create Fehling's solution. About 2 mg of each extract fraction was dissolved in water, and a few ml of Fehling's solution was added to it. This mixture was then warmed. When reducing sugars were present a red precipitate of cuprous oxide was formed.

- **Glycosides (Legal's test)**

  Two mg fraction was hydrolyzed with dilute HCl, and then treated with sodium nitroprusside in pyridine and drops of NaOH solution. The formation of a pink to blood red colour indicated the presence of glycoside.

- **Saponin (foaming test in water)**

  Two mg fraction was placed in a test tube with 2 ml distilled water and shaken. If foam was produced and it persisted for ten minutes, the test was positive for the presence of saponin compounds.

- **Phenols (ferric chloride test)**

  From a 5% $FeCl_3$ solution, 2–3 drops were added directly to each fraction. Production of black or blue-green colouration indicated the presence of phenols.

- **Terpenoids (Salkowski's test)**

  About 2 mg of each fraction was mixed with chloroform. Concentrated sulphuric acid was then gradually poured down the side of the test tubes to form a layer on the mixtures. A reddish brown precipitate at the interface indicated terpenoids were present.

- **Tannins (gelatin test)**

  One millimeter 1% w/v gelatin in 10% sodium chloride was added to each fraction. The presence of tannins was indicated by a white precipitate.

- **Flavonoids (Shinoda test):**

  A small amount of each fraction was dissolved in 5 ml ethanol (95% v/v). A few drops of concentrated hydrochloric acid were added along with 0.5 g magnesium ribbon. The appearance of a pink colour indicated the presence of flavonoid compounds.

- **Volatile oil identification (KOH test)**

  Five mg fraction and 0.5 mL potassium hydroxide (KOH) solution was mixed on a watch glass and left overnight. The formation of a needle shaped precipitate indicated the presence of volatile oils.

- **Proteins (Biuret test)**

  To each fraction, about 2─3 drops of copper sulphate ($CuSO_4$) solution was added. The formation of a red violet colour indicated the presence of proteins.

2.7. Quantitative determination of phenols, flavonoids and tannins in fractions from *R. rothschildianus* leaves

- **Determination of total phenolic content (TPC) in fractions from leaves by colourimetric analysis**

The procedure to determine TPC was based on that of Cheung *et al* [56]. TPC was expressed in milligram of gallic acid equivalents per gram dry weight of leaves (mg GA/g dry weight)

Freshly prepared 7.5% Sodium carbonate solution was made by placing 7.5 g Na_2CO_3 in a volumetric flask and adjusting the volume to 100 ml with distilled water.

A standard reference solution (gallic acid solution) was prepared by dissolving 100mg of gallic acid in distilled water to a final volume of 100 ml. From this, a serial dilution was performed to obtain solutions of gallic acid at 100, 70, 50, 40 and 10 µg/ml).

The stock solutions of the fractions from leaves were prepared by dissolving 100 mg plant extract in distilled water, adjusted to a total volume of 100 ml.

Reaction mixtures were prepared by mixing 0.5 ml of each fraction solution with 2.5 ml 10% Folin-Ciocalteu's reagent, which was dissolved in water with 2.5 ml 7.5% sodium bicarbonate. The sample tubes were incubated for 45 minutes at 45 ºC. Then, the absorbance of each was measured in a spectrophotometer at wavelength 765 nm.

The working samples were prepared in triplicate for each analytic trial, from which the mean and standard deviation values were calculated.

- **Determination of flavonoids content (TFC) in each leaf fraction by colourimetric analysis**

  TFC in the four leaf fractions was assessed using a calibration curve of rutin (standard reference compound). Results were expressed as milligram of rutin equivalent per gram dry weight of leaves extract (mg RU/g dry weight). The TFC experimental procedure was as given in Chang *et al* [57].

  A calibration curve for rutin was established using serial dilutions generated from a stock solution of 100µg/ml. To make the stock solution, 10 mg of rutin was dissolved in 10 ml of distilled water and then diluted to 100 ml. Subsequently, the stock solution was diluted to provide rutin at concentrations of 10, 30, 40, 50, 70 and 100 µg/ml.

  For working solution preparation, 0.5 ml of each fraction solution was mixed with 3 ml methanol, 0.2 ml 10% $AlCl_3$, 0.2 ml 1M potassium acetate and 5 ml distilled water, and then incubated at room temperature for 30 min.

  The previous steps were repeated for each of the fractions, after which, absorbance were measured at a wavelength of 415 nm. For a blank control, a working solution was set up with distilled water in place of the sample extract.

The samples were prepared in triplicate for each analytic trial, from which the mean and standard deviation values were calculated.

- **Determination of total Tannin content (TTC) in leaf fractions by colourimetric analysis:**

The protocol of Sun *et al*. [58] was used to determine TTC in the four leaf fraction, being the most commonly used procedure.

Catechin was used as a reference compound to construct a calibration curve. A 100µg/ml stock in methanol was prepared, from which a dilution series was generated to give catechin concentrations of 10, 30, 50, 70 and 100 µg/ml.

A 4% solution of vanillin in methanol was prepared freshly. Stock solutions of the fractions at 100µg/mL were prepared using methanol as a solvent.

For the working solution 0.5 mL of each fraction solution was mixed with 3 ml vanillin solution and 1.5 ml of concentrated HCL. The mixture was allowed to stand for 15 min, and then the absorbance at 500 nm was measured, using a working solution set up with methanol in place of the sample extract as a blank.

All working samples were analyzed in triplicate, from which the mean and standard deviation values were calculated. Total tannin in each fraction was expressed in terms of catechin equivalents (mg of CAE/g dry weight of leaves).

2.8. Free radical scavenging assay for antioxidant activity in fractions from *R. rothschildianus* leaves

The free 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used to measure antioxidant activity in the different fractions [59].

A 1000 μg/ml stock solution of each plant fraction was prepared in methanol. In addition, a 1000 μg/ml solution of trolox was also prepared (the reference standard). A dilution series was prepared from the stock solutions for each fraction, giving six serial dilutions at 2, 5, 10, 20, 50, and 100 μg/ml. One ml of each extract dilution was mixed with 1ml 0.002 g/ml DPPH in methanol. One ml methanol was added to give a final working volume of 3 ml. The DPPH solution was freshly prepared, as it was very sensitive to light. The blank control of the series concentrations was DPPH in methanol in a ratio of 1:2, without the addition of an extract. All working solutions were incubated at room temperature (25 ºC) in the dark for about 30 minutes. Optical densities were then measured with a spectrophotometer at a wavelength of 517 nm.

The following equation was used to calculate % DPPH inhibition for each plant fraction, with trolox as the standard compound:

DPPH inhibition % = $(A_B - A_{ts})/A_B \times 100\%$

where, A_B is the recorded absorbance of the blank solution, and A_{ts} is the recorded absorbance of the tested sample solution.

2.9. Inhibition assay for porcine pancreatic lipase to evaluate fractions from *R. rothschildianus* leaves for anti-obesity properties

The porcine pancreatic lipase inhibitory method was as described by Bustanji et al. [60], with some modifications.

Stock solutions of 500µg/ml were made from each plant fraction in 10% DMSO. From these, a dilution series of five concentrations of 50, 100, 200, 300 and 400 µg/ml were made. A 1mg/ml stock solution of porcine pancreatic lipase in Tris-HCl buffer was prepared freshly just before use. The substrate, p-nitrophenyl butyrate (PNPB) was prepared by dissolving 20.9 mg in 2 ml acetonitrile.

For each working solution, 0.1ml 1 mg/ml porcine pancreatic lipase was mixed with 0.2 ml plant fraction from each member of the dilution series. Tris-HCL was added to make the final volume of the working solutions 1ml, and they were incubated at 37 °C for 15 minutes. After incubation, 0.1ml p-nitrophenyl butyrate solution was added to each test-tube. The mixture was then incubated for a further 30 minutes at 37 °C.

Pancreatic lipase activity was determined by measuring the hydrolysis of PNPB into p-nitrophenolate at 410 nm, using a UV spectrophotometer. The same procedure was repeated using orlistat as a standard reference compound. Percentage lipase inhibition by plant fractions was calculated with the following equation:

$$\text{Lipase inhibition } \% = (A_B - A_{ts})/A_B \times 100\%$$

where, A_B is the recorded absorbance of the blank solution, and A_{ts} is the recorded absorbance of the tested sample solution.

2.10. *In-vitro* evaluation of α-amylase inhibition by fractions from *R. rothschildianus* leaves

α-amylase inhibitory activity of each fraction was assessed by a standard method with minor modifications [61].

100mg of each fraction was dissolved in a few millilitres of 10% DMSO, and then further dissolved up to 1ooml in 0.02 M Na_2HPO_4/NaH_2PO_4, 0.006 M NaCl, pH 6.9 to give finally stock solutions with concentrations of 1000 μg/ml. From these, the following dilutions were prepared of 10, 50, 70, 100 and 500 μg/ml, using 10% DMSO as the diluent.

A 0.2 ml volume of 2 units/ml porcine pancreatic α-amylase was mixed with 0.2ml plant fraction, and was incubated for 10 min at 30 °C. After incubation, 0.2ml of a freshly prepared 1% starch solution in water was added, and the tubes were then incubated for at least three more minutes. At this point, the reaction was stopped by the addition of 0.2mL 3,5-dinitro salicylic acid (DNSA) colour reagent, and was diluted with 5 ml of distilled water, before being heated at 90 °C for 10 min in a water bath. The mixture was then cooled to room temperature, and the absorbance was measured at 540 nm. The blank control was prepared using the same quantities described above, but replacing the plant fraction with 0.2ml buffer. Acarbose was used as a standard reference following the procedure described above. α-amylase inhibitory activity was calculated using the following equation:

% of α-amylase inhibition = $(A_B - A_T)/ A_B \times 100\%$

where, A_B: is the absorbance of the blank sample, and A_T is the absorbance of the test sample.

2.11. In-vitro evaluation of α-glucosidase inhibitory activity by fractions from R. rothschildianus leaves

Assessment of α-glucosidase inhibitory activity of each fraction was carried out according to the standard protocol, with some modifications [62].

A dilution series of fractions was made, to yield concentrations of 100, 200, 300, 400 and 500 mg/ml. The reaction mixtures contained 0.1 ml 1 U/ml α-glucosidase solution mixed with 0.2 ml of an extract dilution and 0.5 ml 100 mM phosphate buffer, pH 6. 8. The mixtures were incubated at 37 °C for 15 min. Then, 0.2 mL 5 mM *p*-nitrophenyl α-D-galactopyranoside PNPG was added to the reaction mixture and incubated was extended for a further 20 min at 37 °C.

The reaction was terminated by adding 0.1M $Na_2 CO_3$. The absorbance was recorded at a wavelength 405 nm for all samples. Acarbose was used as a positive control at the same concentrations as the plant extracts. The results were expressed as percentage inhibition according to the following equation:

α– glucosidase Inhibition (%) = $(A_B - As/A_B) \times 100\%$

where, A_B is the absorbance without enzyme inhibitor, and A_S is the absorbance in the presence of the enzyme inhibitor.

2.12. *In vitro* evaluation of cell proliferation with fractions from *R. rothschildianus* leaves

HeLa cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin antibiotics and 1% l-glutamine. Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were seeded at 2.6 x 10^4 cells/well in a 96-well plate. After 48 h, cells were confluent, the media was changed. Cells were then incubated with different leaf fraction concentrations ranging from 0.25─10 mg/ml of *R. rothschildianus* for 24 h. Cell viability was assessed with the CellTilter 96® Aqueous One Solution Cell Proliferation (MTS) Assay according to the manufacturer's instructions (Promega Corporation, Madison, WI). Briefly, at the end of the treatment, 20 µl MTS solution per 100 µl media was added to each well and incubated at 37 °C for 2 h. Absorbances were measured at 490 nm [63].

Chapter Three

Results

3.1. Qualitative phytochemical analysis of bio-active compounds in fractions from *R. rothschildianus* leaves

The different fractions of *R. rothschildianus* leaves contained a variety of active phytochemical ingredients (Table 3.1). It was also clear that specific phytochemicals were partition by different solvents with regard to the four fractions.

Table 3.1: Phytochemical screening tests for fractions from *R. rothschildianus* leaves. (+), low content; (++), moderate content; (+++), high content; (-) absent

Phytochemically active constituent	Hexane extract	Acetone extract	Methanol extract	Aqueous extract
Protein Biuret test	-	-	+	++
Reducing sugars Fehling's test	-	-	-	-
Complex polysaccharides Molisch's test	-	-	+	-
Starch Iodine test	-	-	-	+++
Phenols Ferric chloride test	++	+++	+	+
Tannins Gelatin test	+	+	-	-
Flavonoids Shinoda reagent	+++	+++	++	+
Saponins Foam test	-	-	-	++
Glycosides Legal's test	+	-	-	-
Terpenoids and steroids Salkowaski test	+	-	-	-
Alkaloids Wagner's test	-	-	-	-
Volatile oil KOH test	-	-	-	-

The role of each solvent in the fractional extraction process was observed from the partitioning of the active phytoconstituents, which appeared in different fractions. Saponins, starch and protein were only collected in the aqueous fraction. By contrast, alkaloids, volatile oils and cardiac glycosides were absent from all fractions. Phenols and tannins appeared in all fractions except the aqueous extraction, which might be explained by their lack of polarity, so that they only dissolved in organic solvents. Flavonoids were concentrated in the acetone fraction, though other fractions had moderate levels of flavonoids. The hexane fraction was rich in steroids and terpenoids, consistent with their non-polarity.

For the extraction process, methanol showed the highest percentage yield at 29.4%, followed by the acetone fraction with 16.48%. The aqueous extraction yielded 10.64%, while hexane generated the lowest yield at 7.28% (Table 3.2).

Table 3.2: The percentage yield of fractions from *R. rothschildianus* leaves

Fraction	Extract (g)	Plant material (g)	Yield
Hexane	1.82 g	25 g	7.28%
Acetone	4.12 g	25 g	16.48%
Methanol	7.35 g	25 g	29.4%
Aqueous	2.66 g	25 g	10.64%

3.2. Quantitation of phenols, flavonoids and tannins in fractions from *R. rothschildianus* leaves

- **Quantitative determination of TPC in *Rumex***

Absorbance values were expressed in terms of mg of gallic acid equivalents (GAE)/g dry weight of leaf tissue for each plant fraction. Absorbance values and the calibration curve for gallic acid are shown in Figure 3.1 and Table 3.3.

Table 3.3: Absorbance values of the standard gallic acid at different concentrations

Concentration of gallic acid (μg/ml)	Absorbance at λmax = 765 nm
0	0
10	0.142
40	0.375
50	0.557
70	0.702
100	0.988

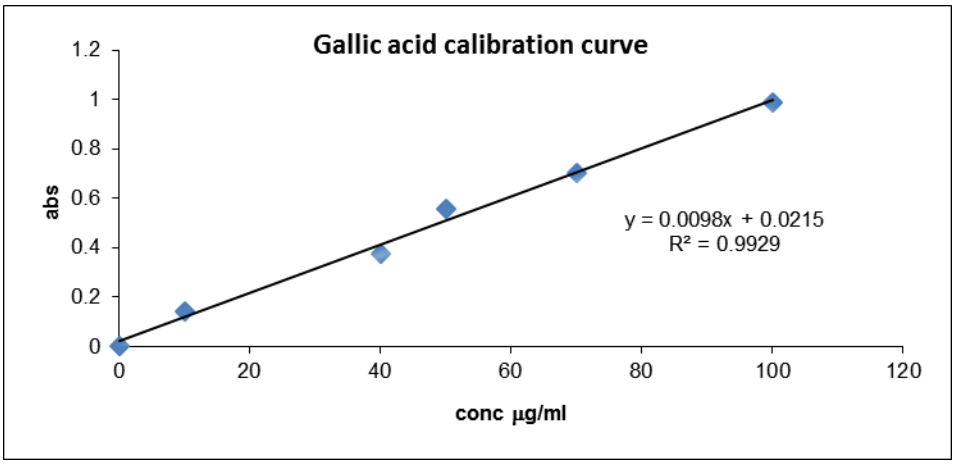

Figure 3.1. Standard calibration curve of gallic acid (standard phenol compound)

TPC results after using the following equation in the calculations are given in Table 3.6.

$y = 0.0098x + 0.0215$, $R^2 = 0.9929$

- **Quantitative determination of TFC in fractions from *R. rothschildianus* leaves**

The collected results for TFC, after using the following equation obtained from Figure 3.2, are given in Table 3.6.

$y = 0.003x + 0.008$; $R^2 = 0.994$

Absorbance values were obtained for rutin (standard flavonoid compound) at different concentrations (Table 3.4).

Table 3.4: Absorbance values for rutin (standard flavonoid compound) at different concentrations

Concentration of rutin acid (µg/ml)	Absorption at λmax = 415 nm
0	0
10	0.049
30	0.11
50	0.17
60	0.2

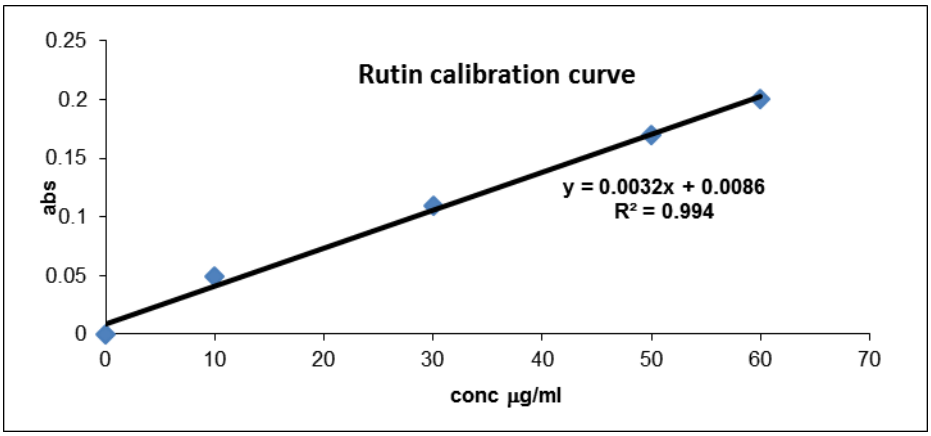

Figure 3.2: Standard calibration curve of rutin (flavonoid standard compound)

- **Quantitative determination of TTC in fractions from *R. rothschildianus* leaves**

The absorption values of several concentrations of the standard catechin were determined (Table 3.5) and a calibration curve of catechin was generated (Figure 3.3).

Table 3.5: Absorption values of the standard catechin at different concentrations

Concentration of catechin (µg/ml)	Absorption at λ_{max} = 500 nm
0	0
10	0.028
30	0.041
50	0.056
70	0.077
100	0.095

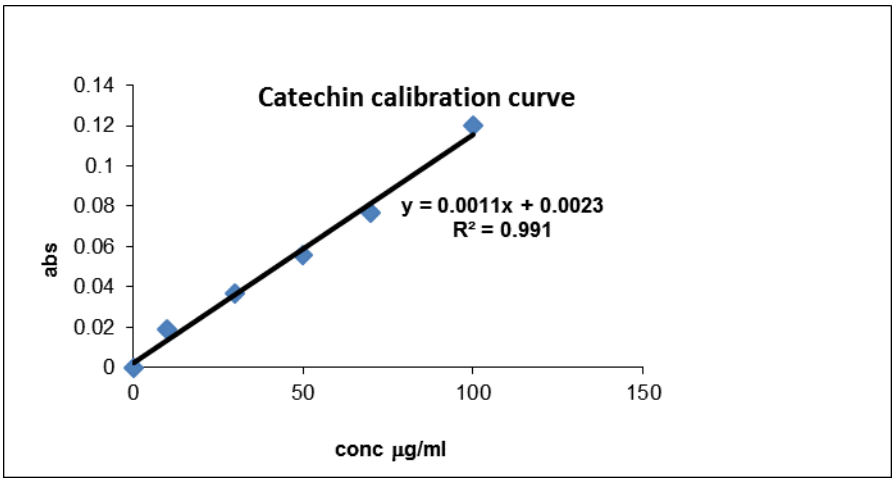

Figure 3.3: Standard calibration curve of catechin (tannin standard compound)

From the calibration curve, the equation of catechin (standard tannin compound) was:

$y = 0.001x + 0.002$, $R^2 = 0.991$

Based on this the TTC values were calculated for the different fractions (Table 3.6).

Table 3.6: Quantitation of phenols, tannins and flavonoids in hexane, acetone, methanol and aqueous fractions of *R. rothschildianus* leaves

Leaf Fractions	**Total flavonoids content (TFC), mg of RUE/g leaf dry weight, ± SD**	**Total phenol content (TPC), mg of GAE/g leaf dry weight, ± SD**	**Total Tannin content (TTC), mg of CAE/g leaf dry weight, ± SD**
Hexane	92.35 ± 2.33	17.66 ± 1.56	2.21 ± 0.014
Acetone	107.3 ± 4.6	28.2 ± 0.78	4.95 ± 0.77
Methanol	55.65 ± 2.33	5.44 ± 1.56	-
Aqueous	32.3 ± 2.35	1.89 ± 1.25	-

3.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH; free radical) scavenging assay for antioxidant evaluation of fractions from *R. rothschildianus* leaves

The results of assessing free radical scavenging activity of four fractions from *R. rothschildianus* leaves, using trolox as a potent antioxidant standard reference, were expressed as percentage DPPH inhibition (Table 3.7 and Figure 3.4).

Table 3.7: The percentage DPPH inhibition of fractions from *R. rothschildianus* leaves compared to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, reference compound) and half maximal inhibitory concentration (IC_{50}) values

Conc (µg/mL)	Trolox	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction
0	0	0	0	0	0
2	53.43 ± 3.46	9.31 ± 3.46	52.27 ± 0	18.86 ± 0.96	41.22 ± 0.45
5	61.51 ± 0.34	39.94 ± 1.7	52.27 ± 0	19.55 ± 0	47.07 ± 1.37
10	91.41 ± 0.34	45.58 ± 0.69	53.4 ± 1.6	18.86 ± 0.96	47.37 ± 0.95
20	97.29 ± 0.34	94.6 ± 0.69	77.04 ± 0.32	23.58 ± 1.21	54.15 ± 0.49
50	97.29 ± 0.34	94.6 ± 0.69	77.04 ± 0.32	27.58 ± 1.72	54.15 ± 0.49
100	97.54 ± 0.69	94.6 ± 0.69	81.58 ± 0.31	32.46 ± 1.18	54.15 ± 0.49
IC_{50} (µg/mL)	**3.1 ± 0.92**	**7.9 ± 1.32**	**6.3 ± 0.43**	**1995 ± 1.01**	**19.95 ± 0.71**

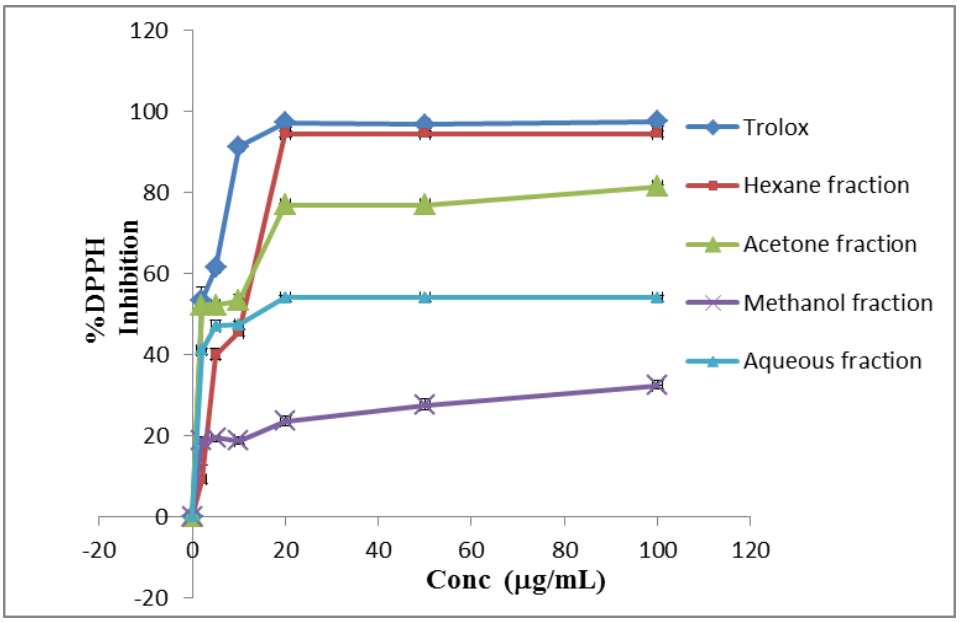

Figure 3.4: % Inhibition of DPPH by standard trolox and fractions from *R. rothschildianus* leaves

3.4. Inhibition assay for porcine pancreatic lipase to evaluate fractions from *R. rothschildianus* leaves for anti-obesity activity

In this assay, anti-obesity activity of fractions from *R. rothschildianus* leaves extract were compared to that of orlistat, a potent lipase inhibitory agent (Table 3.8 and Figure 3.5).

Table 3.8. Pancreatic lipase inhibition IC_{50} values for fractions from *R. rothschildianus* leaves, with orlistat as the reference compound

Conc. (µg/mL)	Orlistat (standard)	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction
50	91.05 ± 0.77	59.5 ± 0	64.52 ± 0.47	58.55 ± 1.2	21.6 ± 0.98
100	93.1 ± 0.42	59.96 ± 0.23	64.69 ± 0.72	59.96 ± 0.23	23.1 ± 0.7
200	94.3 ± 0.42	63.48 ± 0.44	64.69 ± 0.72	59.96 ± 0.23	23.1 ± 0.7
300	97.4 ± 0.12	65.87 ± 0.46	97.96 ± 0.47	64.18 ± 0	25.48 ± 0.26
400	97.5 ± 0	97.46 ± 0.24	98.95 ± 0.49	65.87 ± 0.46	30.05 ± 0.49
IC_{50} (µg/mL)	**12.3 ± 0.33**	**39.81 ± 0.27**	**26.3 ± 0.57**	**60.26 ± 0.42**	**50118 ± 0.63**

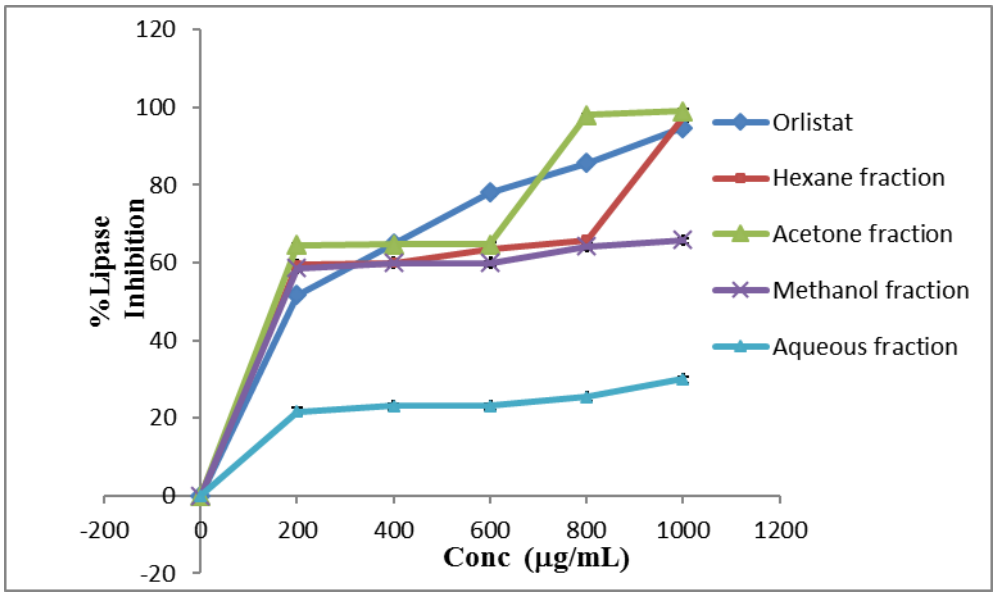

Figure 3.5: % Inhibition of lipase by standard orlistat and fractions from *R. rothschildianus* leaves

3.5. Assessment of *in vitro* α-amylase inhibition by fractions from *R. rothschildianus* leaves

The hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol was used to measure the inhibitory activity of *R. rothschildianus* leaf fractions on porcine pancreatic lipase. Assays were compared to orlistat, a strong lipase inhibitory agent, and IC_{50} values were calculated for the four fractions (Table 3.9 and Figure 3.6).

Table 3.9: Percentage α-amylase inhibition by fractions of *R. rothschildianus* leaves compared to acarbose (standard compound), and values of IC_{50}

Conc. (µg/mL)	Acarbose (standard)	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction
10	53.22 ± 1.2	10.45 ± 0.43	41.22 ± 0.43	12.91 ± 0.86	20.3 ± 0.007
50	54.91 ± 0.58	12.76 ± 0.65	59.84 ± 0.21	21.22 ± 0.41	63.53 ± 0.21
70	66.1 ± 1.34	36.92 ± 2.17	63.84 ± 0.65	21.36 ± 0.2	63.53 ± 0.21
100	66.1 ± 1.62	36.92 ± 2.17	96.15 ± 1.08	39.69 ± 0.22	65.21 ± 0.41
500	72.54 ± 1.37	64.3 ± 0.42	96.92 ± 1.36	47.38 ± 0.41	65.8 ± 0.45
IC_{50}(µg/mL)	**28.84 ± 1.22**	**354.8 ± 1.17**	**19.05 ± 0.75**	**1096 ± 0.42**	**45.7 ± 0.26**

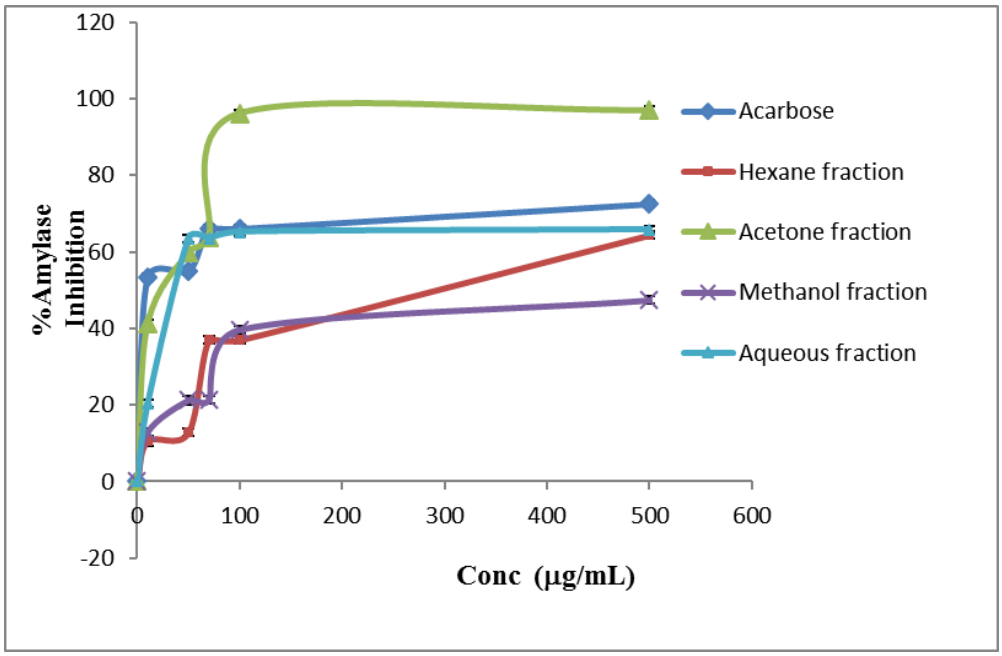

Figure 3.6: α-amylase inhibition percentage of fractions from *R. rothschildianus* leaves compared to acarbose (standard compound)

3.6. Assessment of *in vitro* α-glucosidase inhibition by fractions from *R. rothschildianus* leaves

Results for α-glucosidase were compared with those for acarbose, a strong enzyme inhibitory agent, and IC_{50} values were calculated for the four fractions (Table 3.10 and Figure 3.7).

Table 3.10: Percentage inhibition of α-glucosidase by fractions from *R. rothschildianus* leaves compared to acarbose (standard compound), and values of IC_{50}

Conc. (µg/mL)	Acarbose (standard)	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction
100	65.8 ± 0.42	8.67 ± 2.11	38.34 ± 0.72	33.16 ± 0.19	23.9 ± 0.28
200	67.75 ± 0.35	8.67 ± 2.11	55.5 ± 0.21	37 ± 0.42	24.65 ± 0.21
300	73.2 ± 0.42	33.35 ± 0.63	81.75 ± 0	37 ± 0.42	27.24 ± 0.48
400	85.35 ± 0.35	39.35 ± 0.63	81.75 ± 0	65.15 ± 0.49	29.15 ± 1.2
500	92.22 ± 0.106	66.03 ± 0.6	92.7 ± 0.7	67.41 ± 0.64	60.51 ± 0.72
IC_{50} (µg/mL)	**37.15 ± 0.33**	**2511.8 ± 1.22**	**54.9 ± 0.33**	**251.18 ± 0.43**	**2511.8 ± 0.58**

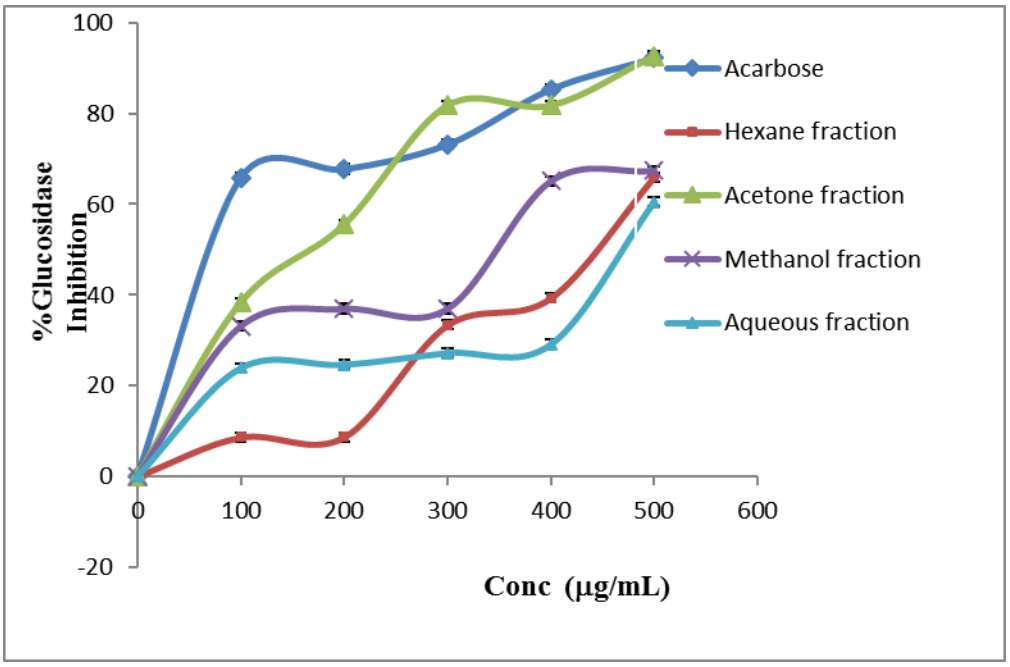

Figure 3.7: α-glucosidase inhibition percentage of fractions from *R. rothschildianus* leaves compared to acarbose (standard compound)

3.7. *In vitro* evaluation of cell proliferation with fractions of *R. rothschildianus* leaves

HeLa cell percentage inhibition on exposure to fractions from *R. rothschildianus* leaves was documented, compared to the control (Figure 3.8).

MCF7 cells percentage inhibition was similarly determined for the four fractions from *R. rothschildianus* leaves, compared to the control (Figure 3.9).

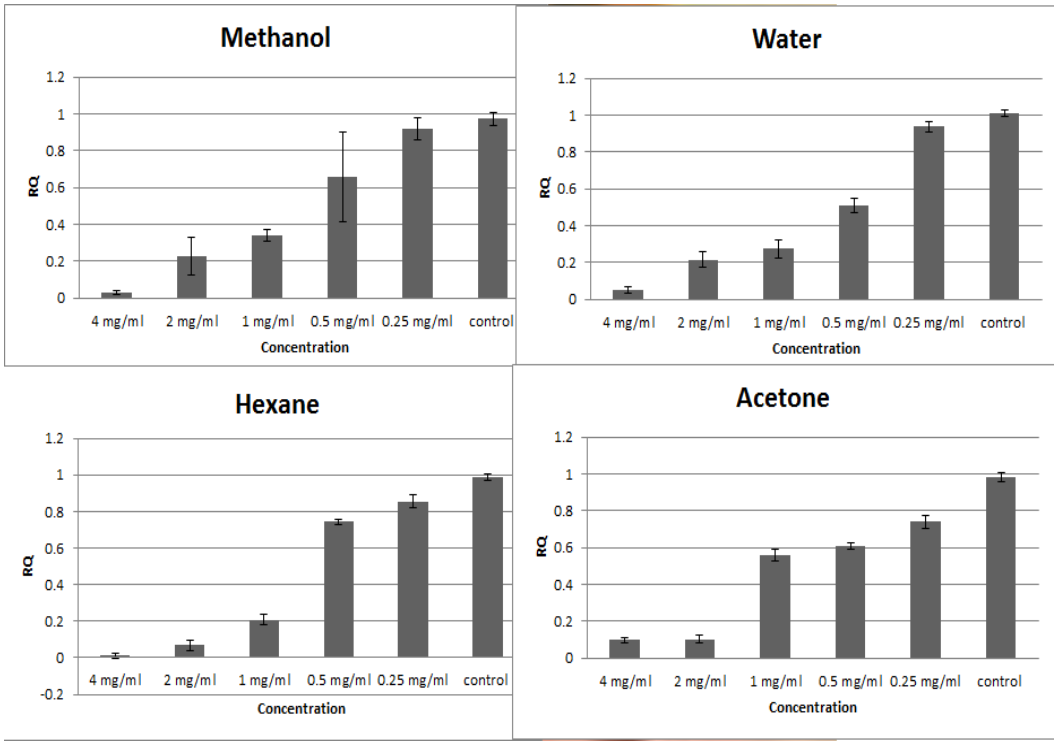

Figure 3.8: HeLa cells percentage inhibition by fractions from *R. rothschildianus*, compared to the control

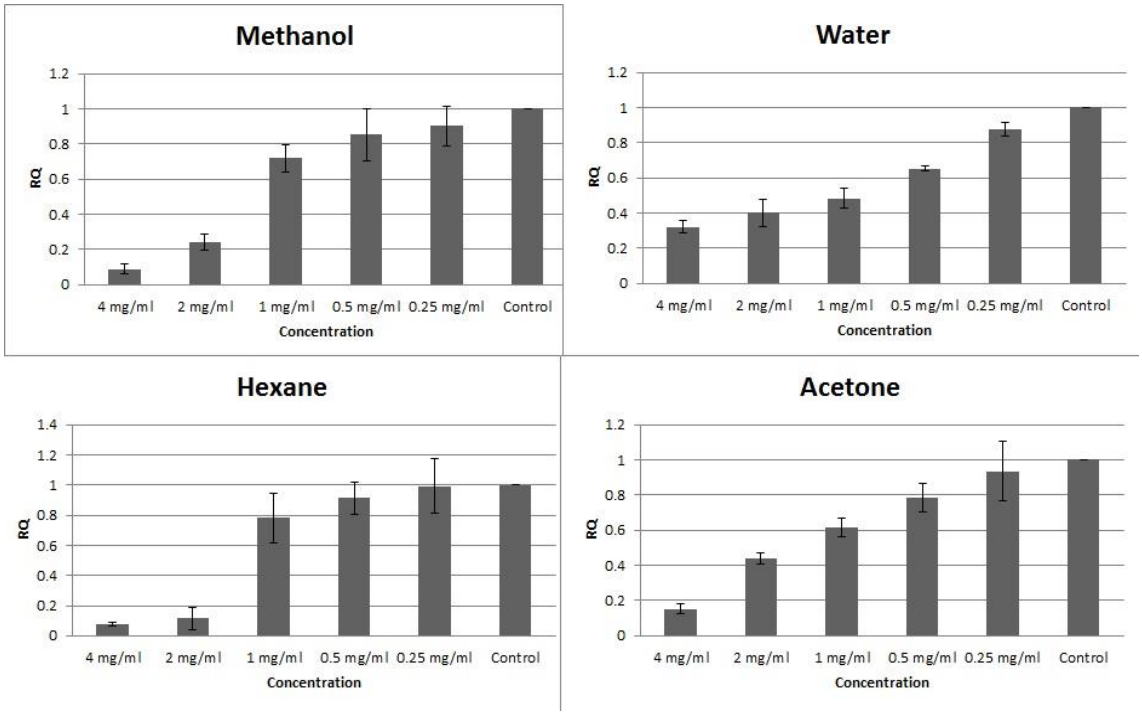

Figure 3.9: MCF7 cells percentage inhibition by fractions from *R. rothschildianus* leaves, compared to the control

Chapter Four

Discussion and Conclusion

4.1. Free radical scavenging assay to evaluate antioxidant activity of fractions from *R. rothschildianus* leaves

The DPPH radical scavenging assay is well known as a simple method for detecting antioxidant capacity in compounds. DPPH is a stable free radical that gives a purple color in alcohol solutions, and on reduction in the presence of hydrogen donating antioxidants, turns the solution colorless [64]. Thus, the characteristic absorption shown by DPPH reduction with increasing concentrations of an antioxidant indicated the DPPH scavenging potential of the compound [65].

Therefore, *R. rothschildianus* leaves could be considered an herbal source for antioxidants, especially for the acetone fraction, which showed an IC_{50} value of 6.3 ± 0.43 µg/ml. Similar results were also obtained for the methanol fraction, which had an IC_{50} value of 7.9 ± 1.32 µg/ml (Table 3.3). The results were compared to trolox, a potent antioxidant compound, with an IC_{50} equal to 3.1 ± 0.92µg/ml.

By contrast, the aqueous fraction only showed moderate antioxidant activity, with a higher IC_{50} value of 19.95 ± 0.71 µg/ml, while the hexane extract was totally inactive in this assay. These results were consistent with the presence of phenols and flavonoids in the plant, a powerful scavenger source for free radicals as shown with DPPH in this study. These results were in line with the diversity of phenolic compounds in plants, simple

phenols like gallic acid and more sophisticated phenolic acids like anthocyanins, hydroxyl cinnamic acid derivatives and flavonoids. All these classes of compounds have received extensive attention due to their multiple physiological functions, especially free radical scavenging, anti-mutagenic, anti-inflammatory and anti-carcinogenic activities [66]. As listed in Table 3.6, the acetone extract recorded the highest content of both phenolic compounds and flavonoids, 28.2 ± 0.78 mg of GAE/g and 107.3 ± 4.6 mg of RU/g, respectively.

A previous report on some *Rumex* species found phenolic compounds in an ethanol extract from leaves of *Rumex vesicarius* L, which were possibly involved in free radical reactions, reducing the stable used DPPH radical to a yellowish coloured diphenylpicrylhydrazine derivative from its original violet colour [67].

4.2. Porcine lipase inhibition assay to assess anti-obesity activity of fractions from *R. rothschildianus* leaves

R. rothschildianus leaves were an excellent alternative natural source of lipase inhibitory agents. The acetone fraction showed an IC_{50} value of 26.3 ± 0.57µg/ml, which was very close to that of the reference compound orlistat (12.3±0.33µg/ml). Hexane and methanol fractions only recorded moderate IC_{50} values, equal to 39.81 ± 0.27µg/ml and 60.26 ± 0.42 µg/ml, respectively; while the aqueous fraction was inactive.

Pancreatic lipase is a major enzyme involved in enterocyte triglyceride absorption. Therefore, its inhibition represents an important strategy in the management of obesity [68].

Plants rich in phenolic compounds have been screened in several reports for anti-lipase activity. Lipase inhibitory activity ranging from 40% to >70% has been found by *in vitro* tests in many different families, including Solanaceae (*Solanum tuberosum*), Brassicaceae (*Brassica nigra* and *Raphanus sativus*), Rosaceae (*Malus domestica* and *Filipendula ulmaria*), Ericaceae (*Arctostaphylos uva-ursi* and *Vaccinium myrtillus*), and Fabaceae (*Pisum sativum* and *Phaseolus vulgaris*) [69,70].

4.3. α-amylase inhibition assay to assess anti-diabetic activity of fractions from *R. rothschildianus* leaves

The acetone fraction was the most potent inhibitor of α-amylase, with an IC_{50} of 19.05 ± 0.75 µg/ml, compared to 28.84 ± 1.22µg/ml for acarbose, the reference compound. This suggested that *R. rothschildianus* might be a powerful herbal remedy for diabetes.

The aqueous fraction only showed moderate activity in this assay with an IC_{50} value of 45.7 ± 0.26 µg/ml, while hexane had an IC_{50} value of 354.8 ± 1.17 µg/ml. The methanol fraction was inactive against α-amylase.

A possible explanation for the aqueous fraction being a good enzyme inhibitor was the presence of saponins. Earlier scientific investigations found that saponins were bioactive against diabetes [71]. The potent effect of the acetone extract fraction against amylase might be due to the high

content of both phenolic compounds and flavonoids. *Corchorus olitorius* exerts α-amylase and α-glucosidase inhibitory effects due to constituents, especially phenolic compounds like caffeic acid [72]. This effect may also be related to flavonoids, which are hydroxylated phenolic compounds, having a benzo-*γ*-pyrone structure [73].

4.4. α-Glucosidase inhibitory activity of fractions from *R. rothschildianus* leaves to evaluate their utility in diabetes management:

The acetone fraction exerted the greatest inhibitory action on α-glucosidase with an IC_{50} of 54.9 ± 0.33µg/ml, compared with that of acarbose, the reference compound, at 37.15 ± 0.33 µg/ml. By contrast, the methanol extract fraction showed only a moderate inhibition of α-glucosidase, with an IC_{50} of 251.18 ± 0.43µg/ml, while the hexane and aqueous fractions were inactive in this assay.

The third category of oral hypoglycemic agents includes α-Glucosidase inhibitors. There are variety of α-glucosidase inhibitors, such as acarbose and voglibose, which usually are found in plant sources. They show valuable stabilization of blood glucose levels after a meal and have been used clinically in the management of diabetes mellitus [74,75].

4.5. *In vitro* evaluation of cell proliferation assays with fractions from *R. rothschildianus* leaves

Treatment of HeLa cells with 4, 2, 1 and 0.5 mg/ml of the methanol extract derived from *R. rothschildianus*, induced significant cytotoxicity ($p \leq 0.0001$) of approximately 97%, 77%, 66% and 35%, respectively (Figure 3.8). The lower concentration of 0.25 mg/ml did not have a significant effect. Treatment of HeLa cells with 10, 5, 2.5 and 1.25 mg/ml of the water extract derived from *R. rothschildianus*, also induced significant cytotoxicity ($p \leq 0.0001$) at approximately 95%, 78%, 72% and 48%, respectively, though the lower concentration of 0.625 mg/ml did not have a significant effect. Treatment of HeLa cells with 4, 2, 1, 0.5 and 0.25 mg/ml of the hexane extract derived from *R. rothschildianus* also induced significant cytotoxicity ($p \leq 0.0001$) at approximately 99%, 93%, 79%, 25% and 15%, respectively. In addition, treatment of HeLa cells with 4, 2, 1, 0.5 and 0.25 mg/ml of the acetone extract derived from *R. rothschildianus* induced significant cytotoxicity ($p \leq 0.0001$) at approximately 91%, 90%, 44%, 39% and 26%, respectively.

Treatment of MCF7 cells with 4, 2, 1 and 0.5 mg/ml of the methanol extract derived from *R. rothschildianus* induced significant cytotoxicity ($p \leq 0.0001$) at approximately 91.12%, 75.92%, 28.13% and 14.8%, respectively, while the lower concentration of 0.25 mg/ml did not have a significant effect (Figure 3.9). Treatment of MCF7 cells with 10, 5, 2.5 and 1.25 mg/ml of the water extract derived from *R. rothschildianus* induced significant cytotoxicity ($p \leq 0.0001$) at approximately 67.7%, 59.8%,

51.58% and 34.6%, respectively, while the lower concentration of 0.625 mg/ml did not have a significant effect. Treatment of MCF7 cells with 4, 2, 1, 0.5 and 0.25 mg/ml of the hexane extract derived from *R. rothschildianus* induced significant cytotoxicity ($p \leq 0.0001$) at approximately 92.4%, 88.56%, 21.8%, 8.8% and 0.7%, respectively. Finally, treatment of MCF7 cells with 4, 2, 1, 0.5 and 0.25 mg/ml of the acetone extract derived from *R. rothschildianus* induced significant cytotoxicity ($p \leq 0.0001$) at approximately 84.7%, 56%, 38.5%, 21.6% and 6.3%, respectively.

From the previous results, the hexane fraction exerted a significant cytoxic effect on both HeLa and MCF7 cell, with inhibition percentages reaching 99% and 92.4% at 4mg/ml of hexane fraction concentration, respectively. This was consistent with the cytoxicity effects of both terpenoids and steroids, which were found in the hexane fraction. Diosgenin, a naturally occurring steroid and triterpenoids found in a number of plants, has been shown to inhibit breast cancer [76,77].

Conclusion

The results from this study on *R. rothschildianus* leaves indicated that the acetone extract fraction had significant potential in providing phytotherapies for diabetes and obesity, based on its potent inhibition of lipase, α-amylase and α-glucosidase. In addition, the acetone fraction also showed a significant free radical scavenging activity. On the other hand, the hexane fraction showed significant inhibition of both HeLa and MCF7 cell lines, which might be related to its high content of terpenes and steroids.

These observations in this study might lead to further *in vivo* studies to develop new natural pharmaceutical formulations effective in the treatment of obesity, diabetes mellitus and some cancers.

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إعداد

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قدمت هذه الأطروحة استكمالا لمتطلبات نيل درجة الماجستير في العلوم الصيدلانية بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.

2019

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الملخص

تشكل النباتات الطبية نافذة مهمة لدى الباحثين في مجال العقاقير الطبية للعمل على تطوير بدائل طبيعية فعالة لعلاج العديد من المشاكل الصحية مثل السكري والسمنة والإجهاد التأكسدي دون أن تحدث هذه البدائل الأعراض الجانبية التي تظهر خلال استعمال الأدوية الكيميائية وفي هذا البحث تم تسليط الضوء على نبتة الحميض لتحضير مستخلصات متنوعة من أوراقها المجففة باستخدام مذيبات مختلفة القطبية مثل الهكسان والأسيتون والميثانول والماء المقطر ومن ثم إتباع مجموعة من الفحوصات الكيميائية لتحديد نوعية وكمية المركبات الفعالة في هذه المستخلصات . وقد تم ملاحظة أن مستخلص الأسيتون كان غنيا بمركبات الفلافونيد ومركبات الفينول المضادة للأكسدة بينما ظهرت مركبات الصابونيين في المستخلص المائي.

ومن ثم تم تطبيق مجموعة من الفحوصات المخبرية على هذه المستخلصات وبالتحديد على الأنزيمات الهاضمة للنشويات وأهمها أنزيم α-amylase وأنزيم α-glucosidase بالإضافة الى إنزيم lipase المسئول عن هضم الدهنيات. حيث أظهر مستخلص الأسيتون نتائج قوية في تثبيط هذه الإنزيمات الثلاثة وبالتالي يمكن لهذا المستخلص أن يشكل بالمستقبل بديلا طبيعيا للأدوية الكيميائية المستخدمة في ضبط مستويات السكر في الدم لمرضى السكري وكبديل طبيعي لعقار الاورليستات للتخلص من السمنة . وقد ترجع فاعلية هذه المستخلصات لاحتوائها على مركبات الفينول والفلافونيد.وقد تم استخدام مركب Acarbose لغرض المقارنة في الفحوصات الكيميائية على الأنزيمات الهاضمة للنشويات بينما استعمل orlistat لغرض المقارنة في فحص أنزيم اللايبز لهاضم للدهنيات.

بالإضافة الى الفحوصات السابقة أظهر مستخلص الأسيتون لأوراق الحميض تأثيرا واضحا على مركب DPPH الذي يعتبر من الشواذر الحرة المسببة للإجهاد التأكسدي في الأجسام الحية وقد يكون السبب أن مستخلص الأسيتون غني بمركبات الفينول و الفلافونيد المضادة للأكسدة وقد تم استخدام مركب Trolox لغرض المقارنة في الفحص المضاد للأكسدة.أما بالنسبة لمستخلص الهكسان فقد أظهر فاعلية واضحة في تثبيط الخلايا السرطانية من نوعي HeLa and MCF7 وقد تعزى هذه الفاعلية لاحتواء هذا المستخلص على مركبات ستيرويدية وتيربين نباتي والذي كان له في ابحاث سابقة تاثير واضح في تثبيط الخلايا السرطانية.

وهكذا يمكن اعتبار هذا البحث على أوراق نبات الحميض تحديا بالمستقبل للصناعات الدوائية الطبيعية التي قد تصبح الخيار الأفضل للعديد من المرضى مقارنة بالعقاقير الكيميائية المرتبطة بالكثير من الآثار الجانبية على الجسم.