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Pharmacological and phytochemical screening of *Ephedra alata* fruit extracts

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Dedication

This project is dedicated to my beloved parents and my family members, as they are my biggest inspiration. I would like to thank my instructors and friends for their care and encouragement during my research work.

Acknowledgement

All praise is for Almighty Allah for all the bounties granted to me and only with His guidance and help this achievement has become possible. I am thankful to my honorable teacher and supervisor, Dr. Nidal Jaradat, Assistant Professor, Department of Pharmacy, for his full cooperation and for his extensive knowledge in research that helped me in all the spheres to perform the research work. I would also like to put forward my most sincere regards and profound gratitude to department of pharmacy instructors and lab techniques who help me during my project application all the time.

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Pharmacological and phytochemical screening of *Ephedra alata* fruit extracts

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

Student Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

DMSO:	dimethyl sulfoxide
DPPH:	2, 2-Diphenyl-1-picrylhydrazyl
IC_{50}:	half maximal inhibitory concentration
E. alata:	*Ephedra alata*
PNPG:	*p*-nitrophenyl glucopyranoside
Trolox:	((s)-(-)-6 hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid)
A_B:	absorbance of the blank solution
A_E:	absorbance of the *E. alata* sample solution
PNPB:	*p*-nitrophenyl butyrate
CAE:	Catechin equivalent
GAE:	Gallic acid equivalent
RUE:	Rutin equivalent
WHO:	World Health Organization
DM:	Diabetic mellitus
DNSA:	3,5-Dinitrosalicylic acid
TPC:	total phenols content
TFC:	total flavonoid content
TTC:	total tannin content

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Abstract

Background: During the latest year's variety of scientific studies focusing all the time on developing new medications to fight diseases that are continuously increasing; in order to maintain the human genus and other living organisms. Discovering and screening for potential anti-obesity, anti-diabetic and antioxidant treatments from natural products still in the recent time the main goal for many of the pharmaceutical scientists.

Methods: Different extract fractions have been prepared by using four solvents with several polarities for extraction of *Ephedra alata* fruits, then determination of natural active ingredients was followed. Finally, assessment protocols for antioxidant, anti-obesity, anti-diabetes and anti-cancer were applied to evaluate the pharmacological effects of these fruits' extracts.

Results: A high content of flavonoids and phenols were observed in the methanol fraction, which reached to 98.95 ± 2.3 mg of RU/g and 33.22 ± 1.56 mg of GAE/g, respectively. As a result, the methanol fraction had significant *in vitro* effects, such as potent antioxidant activity, with an IC_{50} value of 3.09 ± 0.25 µg/mL compared to Trolox (reference compound) that had an IC_{50} value of 3.16 ± 0.74 µg/mL. Also, methanol fraction showed moderate activity against lipase enzyme with an IC_{50} value of 61.65 ± 0.50

µg/mL compared with the IC_{50} of orlistat (reference compound), 12.3 ± 0.35 µg/mL. In addition, the methanol fraction seemed to be the most effective one against α-amylase with an IC_{50} of 16.22 ± 0.6 µg/mL compared to acarbose (reference compound) which had an IC_{50} value of 28.2 ± 1.29 µg/mL. Finally, in the α-glucosidase inhibition assay, the methanol fraction again seemed to be the most potent fraction with an IC_{50} of 32.36 ± 0.63 µg/mL, which was more effective than acarbose its self with an IC_{50} value equal to 37.15 ± 0.33 µg/mL. In addition, the methanol extract derived from *Ephedra alata* fruits induced significant cytotoxicity ($0.0001 \leq p < 0.05$) by approximately 94%.

Conclusion: *Ephedra alata* fruits methanol extract fraction was rich in reducing sugars, flavonoids, phenols, alkaloids. The DPPH assay and digestive enzymes assay showed that this fraction has potent antioxidant, anti-diabetic, and anti-lipase activities, which can be an excellent candidate for biological and chemical analysis and can be further subjected for isolation of the therapeutically active compounds with anticancer potency.

Chapter One

Introduction

1.1 Background

Most of the population still depends on natural remedies, especially in developing countries. In the past, traditional people or ancient civilizations basically depended on local flora and fauna for their survival needs. Despite the fact that some preparations possibly caused harmful effects, or worked by a ceremonial or placebo effect, traditional healing formulations usually had a substantial active pharmacopeia (Bensky, Clavey, & Stõger, 2004).

“Green medicines” have garnered widespread use because they have healthier and less harmful or safer effects than synthetic medicines. Additionally, there are still several reasons why botanical drugs are needed, such as the increase in resistance to antimicrobial and anticancer chemical drugs. Unlike conventional medicines, traditional medicinal systems of the East usually rely on the belief that complex diseases such as diabetes, heart disease, cancer, and psychiatric disorders are best treated with complex combinations of botanical and non-botanical remedies. Medicinal plants have adopted a similar strategy in their biochemical battle against diseases. For example, instead of depending on a single compound to stop pathogens, plants produce families of structurally and functionally diverse

antimicrobial compounds. These act together to prevent resistance development (Pelletier, 2000; Rawat & Uniyal, 2003).

Phenolic compounds in medicinal plants have recently raised considerable interest and have received increasingly more attention due to their bioactive effects. Polyphenols can be retained to the most desirable phytochemical compounds, which have antioxidant activity. These components are classified as secondary plant metabolites and usually exert antimicrobial, anti-inflammatory, and anti-viral effects along with their high antioxidant activity (Arts & Hollman, 2005).

1.2. Description for *Ephedra alata*

Ephedra alata (*E. alata*), also known as Alanda in Arabic, is a member of the *Ephedraceae* family. This subspecies is a perennial genus that can exceed more than one meter in height, has a strong pine odor and an astringent taste, belongs to the Gnetales plant, and is the closest living relative of the angiosperm.

This ephedra subspecies is native to Iran, Algeria, Egypt, Palestine, Lebanon, Jordan, Iraq, Saudi Arabia, Morocco, Libya, and Tunisia (Alqarawi, Hashem, Abd_Allah, Alshahrani, & Huqail, 2014; Caveney, Charlet, Freitag, Maier-Stolte, & Starratt, 2001).

This plant has a light green, densely branched dioecious, small and perennial stiff shrub, about 50–100 cm tall, the twigs appear leafless and the leaves are reduced to small scales, the cones are sessile shaped and

clustered in the axils or at branch tips (M. Nawwar, Barakat, Buddrust, & Linscheidt, 1985).

Figure 1.1. *Ephedra alata* plant red fruits

1.3. The scope of this project

The hypothesis of this project was based on testing the different extract fractions of *Ephedra alata* fruits and some porcine enzymes, such as lipase, α-amylase, and α-glucosidase, using *in vitro* Spectrophotometric analysis to reveal if these plant fruits can be used to form a natural agent for obesity and diabetes management.

In addition, the different extracts of *E. alata* fruits were evaluated for their free radical scavenging capacity to assess their antioxidant activity, and finally, these different fractions were also tested on a select cancer cell lines to estimate their capacities as natural anti-cancer products.

1.4. The proposed objectives

The overall objectives of this project are briefly described in the following steps:

- Fractional serial extraction for separating the active compounds of the *E. alata* fruits using hexane, acetone, methanol, and finally water.
- Application of serial different qualitative and quantitative phytochemical tests to reveal the presence of the most known active phytochemicals like volatile oil, glycosides, alkaloids, flavonoids, phenols, proteins, carbohydrates, and saponin.
- DPPH (2, 2-Diphenyl-1-picrylhydrazyl) inhibitory assay was applied to evaluate the antioxidant activity of the different fruit fractions.
- Each fruit extract fraction was tested *in vitro* on lipase enzyme activity, to assess the anti-obesity action, and each fruit fraction was evaluated on the activity of amylase and glucosidase enzymes to show the fruit's efficacy in treating diabetic patients.
- Assessed the anticancer activity for each extract fraction against HeLa cervical adenocarcinoma cells.

1.5 Related studies on traditional uses of E. alata

E. alata was globally used in folk medicine, especially its stems, as decoction of these stems were used as a stimulant, treatment for kidney health problems, bronchial asthma, circular system disorders, and digestive system disturbances, as well as for treatment of cancer. Also, the plant

stems are chewed to treat bacterial and fungal infections—especially in oral bacterial and fungal infections (Al-Qarawi, Abd Allah, & Hashem, 2012; Freitag & Maier-Stolte, 2003).

The decongestant effect of *E. alata* stems resulted in its widespread use in medicine for the treatment of cough and sinusitis. In Palestine, *E. alata* has been used in folk medicine to treat the common cold, hay fever, asthma, and in the last few years, it was extesivly used for the treatment of cancer (Abourashed, El-Alfy, Khan, & Walker, 2003). Furthermore, the different extracts of *E. alata* were used as depurative, hypotensive, astringent, and anti-asthmatic natural products. The branches of *E. alata* also seemed to be masticated for cephalalgia, used in miscarriage, and as a bronchodilator, antifungal, and antimicrobial natural agents (Ghanem & El-Magly, 2008).

1.5.1 Identification of isolated natural products from the *E. alata* plant in scientific researches

The pharmacological effect of the different *Ephedra* species depends on the phytoconstituents of each one. As shown in general studies, the *Ephedra* species were characterized by the alkaloids and phenolic compounds content, such as trans-cinnamic acid, catechin, epicatechin, symplocoside, and flavonol-3-O- glycosides, and proanthocyanidins (Al-Rimawi et al., 2017).

Furthermore, *E. alata* was shown to contain flavonol glucosides, lucenin III, herbacetin 7-glucoside, herbacetin 7-*O*-(6″-quinylglucoside), herbacetin 8-methyl ether 3-*O*- glucoside-7-*O*-rutinoside, vicenin II, kaempferol 3-

rhamnoside, and quercetin 3-rhamnoside (M. A. Nawwar, El-Sissi, & Barakat, 1984).

1.5.2 Why do some plants have medicinal properties?

Medicinal plants are usually the main source of natural products, especially polyphenolic compounds, which are a group of organic compounds with one or more hydroxyl groups on the aromatic ring structure, varying from simple molecules—such as phenolic acids‘ to highly polymerized compounds like condensed tannins (Liu, 2004).

In addition to polyphenols, flavonoids are considered to be the most common and most widely studied group of plant secondary metabolites, which include several hydroxyl groups within their basic two rings of the carbon skeleton. Commonly, they have a C6-C3-C6 carbon structure consisting of two benzene rings linked by an oxygen containing a heterocyclic structure (Pietta, 2000).

Furthermore, tannins may be formed by condensations of flavone derivatives, which have been transported to the wood tissue of plants or polymerization of quinone units in these plant tissues. Tannins may be toxic to bacteria, filamentous fungi and yeasts. As an antimicrobial, condensed tannins mostly interact with cell walls, preventing growth and protease activity within cells (Cowan, 1999).

The alkaloids are usually identified as heterocyclic nitrogen compounds. The most well-known member of this group is morphine, which was discovered in 1805 from *Papaver somniferum*, commonly known as opium

poppy. Berberine is a valuable member of the alkaloid group. It is most effective on plasmodia and trypanosomes. Its mechanism of action of is usually attributed to its ability of inclusion within DNA molecules (Villinski et al., 2003).

Volatile oils are isolated from some medicinal plants and are used to produce essential oils that play a very important role in medicinal applications. These oils are almost reveled with complex structures and can be comprised of 100 or more compounds. These oils have several uses (Reeds, 2000). For example, lavender oil has long been used in the production of perfumes and also has estrogenic and anti-androgenic effects in humans (Groom, 1997; Henley, Lipson, Korach, & Bloch, 2007), which may cause some problems for prepubescent boys and pregnant women. Also, lavender essential oil has been used as an insect repellent (Debboun, Frances, & Strickman, 2014).

1.5.3 Estimation of antioxidant activity depending on free radical scavenging assay

Oxidation is a process that occurs within the human body that causes damage to cell membranes and other structures, including cellular proteins, lipids, and DNA molecules. When oxygen is rapidly metabolized, it will produce unstable molecules called free radicals that usually steal electrons from other molecules, resulting in DNA damage and other body cells. However, the damage caused by an excessive upload of free radicals over time may become irreversible and lead to certain dangerous diseases,

including heart disease, liver disease, and some types of cancers, especially oral, stomach, esophageal and bowel cancer (Jadhav, Nimbalkar, Kulkarni, & Madhavi, 1995).

Acceleration of the oxidation process can be achieved by stress, cigarette smoking, sunlight, pollution, alcohol, and many other factors. Despite being unwanted metabolic by-products, free radicals are continuously released by aerobic metabolisms (Mantle, Eddeb, & Pickering, 2000). In addition, free radicals can also be released by light energy, photochemical smog, tobacco by-products, radiation, physical stress, and polyunsaturated fats (as in deep fried foods that lead to the destruction of the immune system and alteration of proteins resulting in gene expression changes) (Pourmorad, Hosseinimehr, & Shahabimajd, 2006).

1.5.4 Anti-obesity evaluation using the lipase inhibition assay

According to data collected from the World Health Organization (WHO) global survey on traditional, complementary/alternative, and herbal medicines, the market for these kinds of medicines is steadily growing worldwide. In actuality, the utilization of phytopharmaceuticals and nutraceutical products is continuously expanding. Nowadays, many people have been using these natural formulations in the treatment or prevention of various diseases and health disorders in different national healthcare centers (Bustanji et al., 2010; Zheng, Duan, Gao, & Ruan, 2010).

The identification and characterization of many genes involved in lipid metabolism have resulted in a rich pool of potential targets for drugs to

cure obesity and other metabolic disorders. Pancreatic lipase, the basic lipid digesting enzyme, removes fatty acids from the α and α′ positions of dietary triglycerides to yield the lipolytic product, β-monoglyceride, and long-chain saturated and polyunsaturated fatty acids. Inhibition of pancreatic lipase is an attractive targeted approach for the discovery of potent anti-obesity agents for obesity management (Thomson, De Pover, Keelan, Jarocka-Cyrta, & Clandinin, 1997; Tsujita, Ninomiya, & Okuda, 1989).

Obesity seems to be a result of an imbalance between energy intake and energy expenditure which usually leads to abnormal weight gain and increases the risk for many chronic illnesses, such as cardiovascular diseases like hypertension, and also diabetes (Lavie et al., 2016).

One of the screening methods used in the discovery of anti-obesity drugs is to search for potent lipase inhibitors derived from plant extracts. Several studies have been conducted on the anti-lipase activity of natural products. For example, in a study on an extract of *Taraxacum officinale*, porcine pancreatic lipase was inhibited by 86.3% at a concentration of 250 μg/ml (J. Zhang et al., 2008).

1.5.5 Anti-diabetic evaluation using α-amylase and α-glucosidase inhibitory effects

Diabetic mellitus (DM) is globally defined as a condition that results from abnormal metabolism of carbohydrates, precipitated by many factors that cause insulin deficiency and/or insulin resistance. This health problem

prevails worldwide with its occurrence rising at an alarming rate all over the world. Different complications encompass all the vital organs of the human body as a consequence of the metabolic derangement in diabetes (Tiwari et al., 2011; Wadkar, Magdum, Patil, & Naikwade, 2008).

Hyperglycemia is still considered as the classical risk in the development of diabetes and the complications related to diabetes. Therefore, control of blood glucose levels is the key in the early treatment of DM and delaying of macro and micro vascular complications. One therapeutic approach is the prevention of carbohydrate absorption after food intake, which is facilitated by the inhibition of carbohydrate digestive enzymes, especially α-glucosidase and α-amylase present in the brush borders of intestine and mouth saliva, respectively (Karthic, Kirthiram, Sadasivam, Thayumanavan, & Palvannan, 2008).

The diversity of medicinal plants has been an exemplary source of drugs that have been derived directly or indirectly from them. It was discovered that nearly 800 plants may possess anti-diabetic activity through hypoglycemic effects, which resulted due to their ability to restore the function of pancreatic tissues by increasing insulin output, blocking the intestinal absorption of glucose, or facilitating the production of metabolites in insulin dependent metabolic processes (Algariri et al., 2013; Malviya, Jain, & Malviya, 2010).

Some previous scientific researchers showed the activity of many medicinal plants in this field, for example, the *Citrus limetta* fruit peel exerted an important, potential hyperglycemic reducing effect in

streptozotocin-induced diabetic rats (KunduSen et al., 2011). Other researchers have conducted studies to investigate the suppression of intestinal glucose absorption from carbohydrates using *Amaranthus viridis* Linn (Kumar et al., 2012), *Andrographis paniculate* (Subramanian, Asmawi, & Sadikun, 2008), and flowers of *Punica granatum* (Yuhao Li et al., 2005).

1.5.6 Anticancer evaluation for medicinal plants extracts

Cancer is a condition that refers to different types of diseases that are typically characterized by rapid and abnormal growth of human cells beyond the usual boundaries—proliferation cannot be controlled and cells acquire anti-apoptosis features giving them the ability to penetrate and destroy the normal body tissue. Also, cancer can spread and affect any organ or part of the human body (Yiwei Li, Kong, Bao, Ahmad, & Sarkar, 2011).

The development process for cancer drugs has relied on natural products where more than 70% of the available anti-cancer drugs are from natural resources, and plants are most often used. However, more than 3000 plants in the world have been reported and known to have anti-cancer properties (Jacobo-Herrera et al., 2016).

Although several plants have provided useful drugs for the treatment of a variety of cancers, the anticancer potential of the Plant Kingdom which contain approximately 400,000 plant species known worldwide, still largely unexplored (Ulloa et al., 2017).

An array of diverse plant bioactive compounds have been tested for their anticancer effects, such as flavonoids, which are part of the human diet and thought to have positive effects on human health, such as reducing the risk of cardiovascular diseases and cancer. Most of the beneficial effects of flavonoids are attributed to their antioxidant and chelating abilities. Globally, several new strategies have been developed to manage and treat cancer. One such approach could be a combination of an effective photochemical with chemotherapeutic agents, as the combination would enhance the efficacy while reducing toxicity to normal tissues (Peterson & Dwyer, 1998).

Chapter Two

Materials and Methods

2.1. General background

The use of bioactive compounds in different commercial products, as seen in pharmaceutical, food, and chemical industries, signifies the need of the most appropriate and standard method to extract these active components from plant materials. In addition to conventional methods, several new methods have been established, but till now, no single method has been adopted as the standard for extracting bioactive compounds from these medicinal plants (Smith, 2003).

Some extraction methods, such as maceration, will be critically influenced by the different type of solvents used during the extraction procedure. Usually, no effect will be observed from the solvent volume used on the biologically active compounds, such as that used in the extraction of propolis at ratio [1:10 w:v], suggesting that the use of solvents at a greater ratio is not necessary. In this research, the fractional extraction method was followed in order to separate bioactive compounds from *E. alata* fruits.

2.2. Collection and preparation of plant fruits before the extraction process

E. alata fruits were collected from different Palestinian sites in June and July of 2018. Identifications were performed by the pharmacognosist

professor, Nidal Jaradat, at the Pharmacognosy Laboratory at An-Najah National University-Nablus Palestine. The fruits were washed and cleaned two times with distilled water and dried in the oven at 40 ºC. Finally, the fruits were grounded into powder by the mechanical grinder to and kept in a well closed container with a suitable label for further use.

2.3. Chemical reagents for *in vitro* tests of plant extracts

The chemicals used in this project included different liquids, solids, and solutions and are listed and summarized in Table 2.1 below.

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

Chemicals and reagents	Source	Source country
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich	Denmark
Orlistat	Sigma-Aldrich	Germany
p-nitrophenyl butyrate (PNPB)	Sigma-Aldrich	Germany
Porcine pancreatic lipase type II	Sigma-Aldrich	USA
p-nitrophenyl glucopyranoside (PNPG)	Sigma-Aldrich	USA
Dimethyl sulfoxide (DMSO)	Riedel-de Haen	Germany
Ferric chloride	Riedel-de Haen	Germany
Sulfuric acid (H_2SO_4)	Alfa-Aesar	England
Methanol, 99.9%	Loba/chemie	India
Iodine I_2	Riedel-de Haen	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 ((s)-(-)-6 hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid	Sigma-Aldrich	Denmark
Acarbose	Sigma-Aldrich	USA
α-glucosidase (Baker's Yeast alpha glucosidase)	Sigma-Aldrich	USA
α-amylase	Sigma	India
3,5-Dinitrosalicylic acid (DNSA)	Sigma-Aldrich	USA
Potassium phosphate	Sigma-Aldrich	USA
Gallic acid	Sigma Chemicals	USA
Sodium carbonate	Merck	India
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
L-glutamine solution	Sigma	United Kingdom (UK)
MTS reagent	Promega	USA
Phosphate-buffered saline	RPMI-1640 medium Sigma-Norwich	United Kingdom (UK)
Pen-Strep solution composed of penicillin and streptomycin	BI	Israel

2.4. Instrumentation

The serial steps including the extraction process, phytochemical analysis and Spectrophotometric analysis, on *E. alata* fruits, were established using the following instruments and tools that are listed in Table 2.2, some of which are shown in Figure 2.1.

Table 2.2. The instruments and tools used during the overall experimental steps

Instruments and tools	Supplier	Country
Oven	Arilevy	Israel
Balance	Radwag- AS 220/c/2	Poland
Micropipette	Mrc	Israel
Freeze dryer	Mill rock technology-model BT85	China
Rotary evaporator	Heidolph OB2000-VV2000	Germany
Grinding machine	Moulinex model- Uno	China
Filter papers	Macherey-Nagel, MN 617and Whatman no.1	USA
Beakers and conical flasks	Mrc	Israel
Microplate reader	Unilab, 6000, Mandaluyong	USA
Water bath	Lab tech, BPXOP1001040, Namyanghi	South Korea
Vacuum Buchner funnel	Mrc	Israel
CO_2 incubator	(Esco serial number 2012-74317; Changi	Singapore
Inverted microscope	MRC, IX73, Hong Kong	China
Vortex	Heidolph company, 090626691, Schwabach	Germany
Microwave-ultrasonic reactor extractor	LAB-KITS UM2015042801A, Hong Kong	China

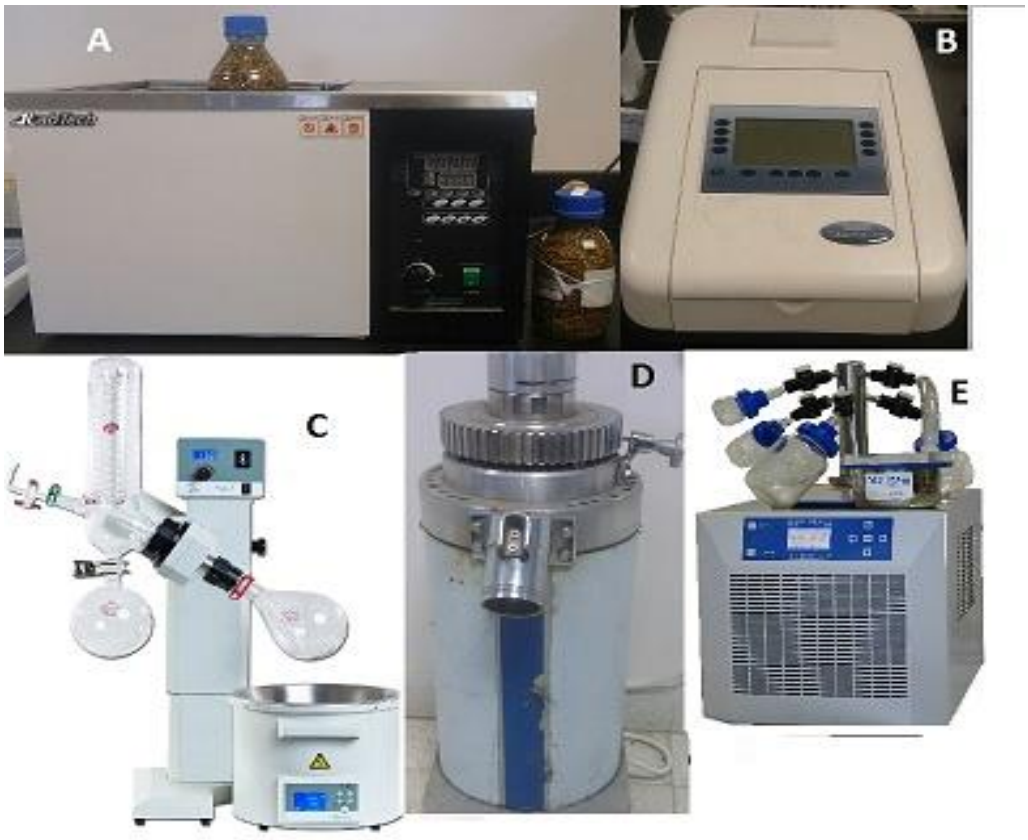

Figure 2.1. Examples of the equipment and instruments used in this study. A) Shaker; B) Spectrophotometer; C) Rotary evaporator; D) Mechanical grinder; E) Freeze dryer.

2.5. Preparation of four different extract fractions from *E. alata* fruits

The extraction process adopted in this research was based on the fractional extraction procedure, which involved adding solvents in a sequential manner based on their polarity, starting with the most nonpolar solvent, hexane, followed by acetone (polar aprotic organic solvent), then methanol—a highly polar, low molecular weight alcohol—and finishing the extraction steps with distilled water (polar inorganic solvent). For the preparation of each extract fraction, 25 g of the dried, grounded fruits were first soaked in 0.5 L of hexane for 72 hr (three days) in a shaker device, with continued shaking at approximately 100 rounds/minute at room

temperature. Then, the hexane was replaced with 0.5 L of acetone at the same conditions mentioned above, followed by methanol and finally distilled water. Each organic fraction was filtered using suction filtration and concentrated under vacuum on a rotary evaporator, while the aqueous fraction was collected as a powder using a freeze dryer. All crude fractions were kept at 4 ˚C for further use (Michel, El-sherei, Islam, Sleem, & Ahmed, 2013).

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

% Yield = (weight of *E. alata* fruits extract / weight of dry fruits) × 100%

2.6. Phytochemical screening by qualitative tests for determination of bioactive ingredients in *E. alata* fruits

It is well-known that plants produce many organic chemical compounds that are biologically active, not just in themselves, but also in other organisms. Some of these chemicals enhance the plants' own survival. Listed below are examples of the most commonly used tests in phytochemical analysis (Harborne, 1998; Iqbal, Salim, & Lim, 2015).

- **Test for Complex polysaccharides (Molisch's Test)**

  About two drops of Molisch's reagent, which is an α-naphthol solution, were added to 10 mg of the extract in 5 mL of an aqueous solution placed in a test tube. 1 mL of concentrated sulfuric acid was allowed to flow down drop by drop in test tube so that the acid formed a layer beneath the aqueous solution without mixing with it. A purple ring was

formed at the interface of the two liquids, which indicated the presence of carbohydrates, specifically complex polysaccharides like cellulose.

- **Test for Alkaloids (Mayer's Test)**

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

- **Test for Saponin (Foam Formation Test)**

  About 0.5 mL of each extract was mixed and well shaken with distilled water in a clean test tube. If a froth (foam formation) was produced, stabilized for 1–2 minutes, and also persisted upon warming, it was taken as evidence for the presence of saponin compounds in these fruits.

- **Test for Reducing Sugars (Fehling's Solution Test)**

  Fehling solutions (A and B), 1 mL from each, was added to 1 mL of plant extract in a test tube, followed by heating using water bath. A brick red precipitate appearance indicated the presence of reducing compounds.

- **Test for Glycosides (Keller-Killani Test)**

  The test to assess the presence of cardiac glycosides was performed by adding 5 mL of each extract to 2 mL of glacial acetic acid, containing one drop of ferric chloride solution. The new mixture was then subjected to 1 mL of concentrated sulfuric acid. A brown ring at the interface indicated the presence of glycosides, especially cardiac glycosides.

- **Test for Phenol (Ferric Chloride Test)**

  About 2–3 drops from a diluted solution of 5% $FeCl_3$ was added directly to each extract fraction, and the formation of a black or blue green coloration indicates the presence of phenols.

- **Test for Terpenoids (Salkowski's Test)**

  Roughly 2 mg of each extract fraction was mixed with chloroform. Then, concentrated sulfuric acid was added from the side, gradually, of a test tube to form a layer. The formation of a reddish-brown precipitate at the interface indicated the presence of Terpenoids.

- **Test for Tannin Identification (Gelatin Formation Test)**

  One milliliter of a 1% w/v solution of gelatin in water, containing 10% sodium chloride, was prepared and added to each extract fraction. If a white precipitate formed, tannins were present.

- **Test for Flavonoids (Alkaline Test)**

  Flavonoids are widely available throughout the plant kingdom and they have several medicinal uses and actions. They usually act as pigments giving a yellow color to flowers and fruits. They are identified in plant extracts by adding a few drops of a strong basic solution, especially NaOH solution, to about 2 mg of extract in a test tube. A brick yellow color will appear, but after adding about 1 mL of concentrated HCl, the mixture will it turn colorless, indicating the presence of flavonoids.

- **Proteins and Amino Acids (Biuret's Test):**

Two to three drops of copper sulphate ($CuSO_4$) solution were added to each extract fraction. The appearance of a red-violet color indicated the presence of proteins, as well as free amino acids.

- **Volatile Oil Identification Test**

  Two to three drops of concentrated potassium hydroxide (KOH) solution were added to each extract fraction in a glass watch and left overnight. The appearance of a needle shaped precipitate indicated the presence of some volatile oils.

2.7. The quantitative analysis for the determination of phytochemical compounds in each extract fraction

- **Procedure for total phenols content (TPC) in *E. alata* fruit extract fractions**

  The content of total phenolic compounds of *E. alata* fruits for four different extracts was assessed using the Folin-Ciocalteu Reagent (FCR). FCR, or Folin's phenol reagent, is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. The general experimental procedure can be summarized by the following steps (Cheung, Cheung, & Ooi, 2003):

  Using a 100 mL volumetric flask, a 7.5% sodium carbonate (Na_2CO_3) solution was prepared by dissolving 7.5 g of Na_2CO_3 in less than 100 mL of distilled water, then, distilled water was used to bring the volume up to 100 mL. in a similar manner of sodium carbonate

solution a stock solution of the standard solution (Gallic acid solution) was prepared by dissolving 100 mg of gallic acid and dissolved up to 100 mL of distilled water.

The reaction mixture was prepared by mixing 0.5 mL of each extract solution, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 mL of 7.5% sodium carbonate (Na_2CO_3) in a test tube for each sample. The sample tubes were incubated for 45 minutes at 45 ºC. The absorbance was determined using a spectrophotometer at wave length 765 nm. The samples were prepared in triplicate for each analytic trial to obtain the mean and standard deviation values.

- **Procedure for total flavonoids content (TFC) in *E. alata* fruit extract fractions**

The total flavonoid content (TFC) was determined according to the procedure adopted by Chang et al. The TFC was calculated from the calibration curve of Rutin (the used standard) and expressed as the milligram of Rutin Equivalent per gram of extract (mg RU/g extract). The experimental steps are summarized as follows (Chang, Yang, Wen, & Chern, 2002):

The calibration curve for Rutin was established using serial dilutions, starting with the preparation of a stock solution of 100 µg/mL—10 mg of Rutin was dissolved in 10 mL of distilled water and diluted to 100 mL. Subsequently, the stock solution was diluted to provide a series of concentrations of 10, 30, 40, 50, 70, and 100 µg/mL.

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

The previous steps were performed for each fruit extract fraction, and finally, the absorbance was recorded at a 415 nm wavelength, and distilled water with methanol, 10% $AlCl_3$ and potassium acetate was used as a blank solution. The samples were prepared in triplicate for each analytic trial to obtain the mean and standard deviation values.

- **Procedure for total tannin content (TTC) in *E. alata* fruit extract fractions**

For total tannin content assessment, according to the method of Sun et al., the four *E. alata* fruit extract fractions were subjected to the following steps (Sun, Ricardo-da-Silva, & Spranger, 1998):

Catechin was used as reference compound to construct the calibration curve for the needed calculations. A 100 µg/mL stock methanolic solution was prepared, and then, serial dilutions were obtained (10, 30, 50, 70 and 100 µg/mL).

A 4% methanolic vanillin solution was prepared freshly. 100 µg/mL stock solution from each fruit extract fraction was prepared using methanol as a solvent. For the working solution, each test tube contained 0.5 mL from each extract mixed with 3 mL of vanillin solution and 1.5 mL of concentrated HCl.

The mixture was allowed to stand for 15 min, and then the absorption was measured at 500 nm against methanolic vanillin as a blank. All working samples were analyzed in triplicate. The total tannin in each fraction was expressed as catechin equivalents (mg of CAE/g of plant fraction).

2.8. Antioxidant activity for *E. alata* fruits using 2;2-diphenylpicrylhydrazyl (DPPH) radical scavenging assay (RSA)

Experimental steps for this assay are listed as follows according to a previously adopted protocol with some modifications (Alali et al., 2007):

The *E. alata* fruit extract stock solutions were serially diluted to achieve concentrations of 100 µg/mL, 50 µg/mL, 30 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL and 2 µg/mL using methanol as solvent.

Each test tube contained 1mL of each concentration and was marked properly. 1mL of 0.002% methanolic DPPH solution was added to each test tube, and 1mL of methanol was added to each test tube to bring the final volume up to 3 mL (caution: DPPH is light sensitive, so preparation of working test tubes should be performed with minimum light exposure).

The samples were incubated for 30 minutes in a dark place, then, their optical densities were determined using the spectrophotometric measurement at a wave length of 517 nm. The equation used in this analytical study in order to calculate the inhibition percentage is shown below:

$$\% \text{ DPPH inhibition} = (A_B - A_E)/A_B \times 100\%$$

A_B is the recorded absorbance of the blank solution

A_E is the recorded absorbance of *Ephedra* sample solution.

2.9. Porcine pancreatic lipase inhibition assay to assess of *E. alata* fruit extracts in obesity treatment

The porcine pancreatic lipase inhibitory method was followed in this study according to protocols from Zheng et al. and Bustanji et al., with some modifications. The steps of this precedure are described, in brief, below (Drent et al., 1995). A stock solution of 500 µg/mL from each plant fraction, in 10% DMSO, was used to prepare five different solutions with the following concentrations: 50, 100, 200, 300 and 400 µg/mL. A 1 mg/mL stock solution of porcine pancreatic lipase enzyme was freshly prepared in tris-HCl buffer before use. The substrate used for this study, *p*-nitrophenyl butyrate (PNPB), was prepared by dissolving 20.9 mg in 2 mL of acetonitrile. For each working test tube, 0.1 mL of porcine pancreatic lipase (1 mg/mL) was mixed with 0.2 mL each diluted solution series for each plant fraction. The resulting mixture was then brought to a total volume of 1 mL, by adding Tri-HCl solution and incubated at 37 °C for 15 minutes. Following the incubation period, 0.1 mL of PNPB solution was added to each test-tube. The mixture was incubated for 30 minutes at 37 °C. Pancreatic lipase activity was determined by measuring the hydrolysis of the PNPB compound into *p*-nitrophenolate ions at 410 nm using a UV spectrophotometer. The same procedure was repeated for Orlistat, which

was used as a standard reference compound. The equation used in this analytical study is shown below:

% Lipase inhibition = $(A_B-A_E)/A_B \times 100\%$

A_B is the recorded absorbance of the blank solution

A_E is the recorded absorbance of the *Ephedra* sample solution.

2.10. *In vitro* evaluation of α-amylase inhibitory activity of each extract fraction of *E. alata* fruits

The α-amylase inhibitory activity of each extract fraction was carried out according to the standard method, with minor modifications (Sudha, Zinjarde, Bhargava, & Kumar, 2011).

Each extract fraction was dissolved in a few milliliters of 10% DMSO and then further dissolved in buffer (0.02 M of Na_2HPO_4/NaH_2PO_4, 0.006 M NaCl, at pH 6.9) to give concentrations of 1000 μg/mL, from which the following dilutions were prepared: 10, 50, 70, 100, 500 μg/mL. The porcine pancreatic α-amylase enzyme solution was freshly prepared at a concentration of 2 units/mL in 10% DMSO.

For working solutions, a volume of 0.2 mL of enzyme solution was mixed with 0.2 mL of each *E. alata* fruit extract fraction and was incubated for 10 min at 30 °C. After the incubation period, 0.2 mL of a freshly prepared 1% starch aqueous solution was added to each working solution, followed by an incubation period of at least 3 min. The reaction was stopped by the addition of 0.2 mL dinitrosalicylic acid (DNSA) yellow color reagent. Each working solution was then diluted with 5 mL of distilled water and then

boiled for 10 min in a water bath at 90 °C. The mixture was cooled to room temperature, and the absorbance was taken at 540 nm. The blank was prepared following the same steps above, but the plant fraction was replaced with 0.2 mL of the previously describe buffer. Acarbose was used as the standard reference following the same steps used for extract fractions.

The α-amylase inhibitory activity was calculated using the following equation:

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

As given in equation:

A_B: is the absorbance of blank

A_E: is the absorbance of *Ephedra* sample

2.11. α-glucosidase inhibition in vitro evaluation for each extract fraction of E. alata fruits:

The α-glucosidase inhibitory activity of each extract fraction was carried out according to the standard protocol, with some modifications (Ademiluyi & Oboh, 2013).

The enzyme, alpha-glucosidase (1 U/ml), and 20 µL of different concentrations of each extract fraction (100, 200, 300, 400 and 500 mg/mL) were added to a test tube. In each working test tube, a reaction mixture contained 0.1 mL of alpha-glucosidase solution was mixed with 0.2 mL from each extract dilution and 0.5 mL of phosphate buffer (100 mM, pH = 6. 8). The samples were incubated at nearly 37°C for 15 min.

After this incubation period, 0.2 mL of 5 mM PNPG (the substrate used for this experiment) was added to the reaction mixture, and the samples were again incubated at 37 °C for 20 min. The reaction was terminated by adding 0.1 M sodium carbonate (Na_2CO_3). The absorbance at the 405 nm wave length was recorded 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 (%) = $(A_B - A_E/A_B) \times 100\%$

A_B is the absorbance without enzyme inhibitor

A_E is the absorbance in the presence of *Ephedra*

2.12. In-vitro evaluation of anticancer activity for each extract fraction of E. alata fruits

HeLa cervical adenocarcinoma cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin antibiotics and 1% L-glutamine. The cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were seeded at 2.6 x 10^4 cells/well in a 96-well plate. After 48 hr, the cells were incubated with various concentrations of the tested compounds for 24 hr. Cell viability was assessed by Cell Tilter 96® Aqueous One Solution Cell Proliferation (MTS) Assay according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Briefly, at the end of the treatment, 20 µL of MTS solution per 100 µL of media was added to each well and incubated at 37 °C for 2 hr. The absorbance was measured at 490 nm (Mosmann, 1983).

Chapter Three

Results

3.1. Quantitative phytochemical screening tests for the determination of bi-active compounds in each *E. alata* fruit fraction

After performing the previously mentioned phytochemical laboratory tests, it was observed that the different *E. alata* fruit extract fractions contained a variety of active phytochemical ingredients, which were summarized in Table 3.1.

It was observed that the alternative fractionation process, adding different solvents in this extraction method, was effective in separating the active phytoconstituents from each other. Proteins, saponin, reducing sugars, and starches were collected intensively in the aqueous fraction, while alkaloids appeared in all extract fractions. Flavonoids, which are always resembled about anti-oxidant activity, appeared in all extract fractions, and they were found in high quantity in the methanol layer. Phenols and tannins were observed in all fractions but seemed to be concentrated in the methanol layer.

Positive results for volatile oil were found in the methanol fraction, confirming that the presence of one of the alcohol-type volatile oils.

Table 3.1. Phytochemical screening tests for different extract fractions of *E. alata* fruits.

Where (+) means low content, (++) mild content, (+++) high content and (-) absent

Phytochemical active constituent	**Hexane extract**	**Acetone extract**	**Methanol extract**	**Aqueous extract**
Protein & amino acids 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	-	++	+++	+
Saponin Foam test	-	-	-	+
Glycosides Keller-Killani Test	-	-	-	-
Steroids	++	-	-	-
Terpenoids Salkowski's test	+	-	-	-
Alkaloids Wagner's test	+	+	+++	+
Volatile oil KOH test	-	-	+	-

As shown in Table 3.2, the percent yield results recorded after calculations of each extract fraction revealed that the highest yield was observed in the methanolic fraction, which was 29%, followed by the acetone and aqueous fractions, which were 15.6% and 15.08%, respectively.

Table 3.2. The yield percentage for four extract fractions of *E. alata* fruits

Extract Fractions	Extract (g)	Yields, %
Hexane	2.15 g	8.6%
Acetone	3.9 g	15.6%
Methanol	7.25 g	29%
Aqueous	3.77 g	15.08%

3.2. Quantitative analysis for the determination of phytochemical compounds—phenols, flavonoids, and tannins—in each extract fraction

- **Quantitative phytochemical assay of the total phenol content in *E. alata* fruit extracts**

  As mentioned above, we followed in the experimental part a protocol for the estimation of the total phenol content in different *E. alata* fruit extract fractions. The total phenolic content in *E. alata* fruits was expressed by (mg of GA/g of each fruit extract fraction). Figure 3.1 shows the adopted equation from the gallic acid standard curve that used for calculations in this assay, which was $y = 0.009x + 0.021$, $R^2 = 0.992$. Additionally, Table 3.3 displays the absorbance values for the gallic acid standard calibration curve.

Table 3.3. Recorded absorbance values for each concentration of Gallic acid (standard compound)

Concentration of Gallic acid (μg/mL)	Absorption at λmax = 765 nm
0	0
10	0.142
40	0.496
50	0.557
70	0.798

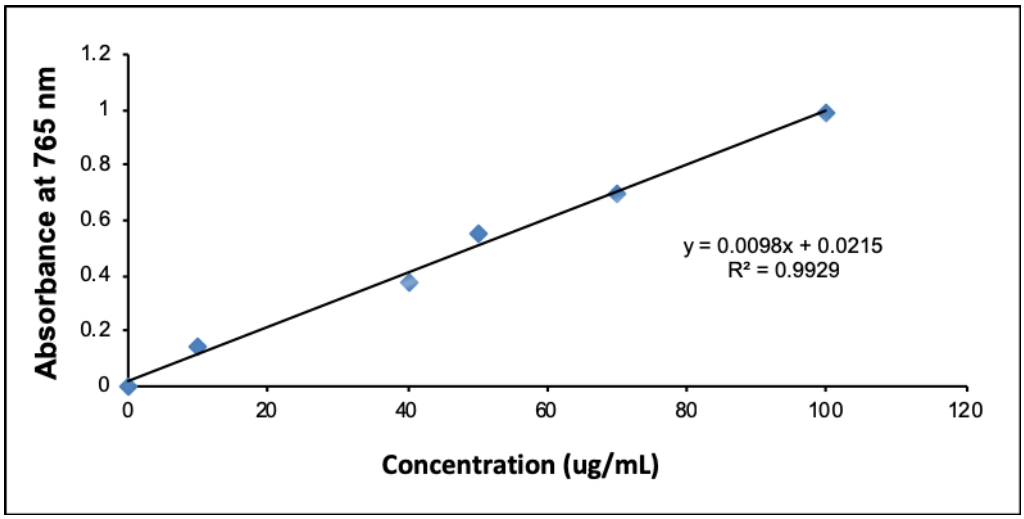

Figure 3.1. Standard calibration curve of Gallic acid (reference phenol compound)

- **Quantitative phytochemical assay of the total flavonoids content in *E. alata* fruit extracts**

  The collected data from the standard calibration curve for Rutin is shown in Table 3.4 and Figure 3.2. The equation used to calculate the estimated total flavonoid contents (TFC) in the four *E. alata* extract fractions was y = 0.003x + 0.008, R^2 = 0.994.

Table 3.4. Recorded absorbance values for each concentration of Rutin (standard flavonoid compound)

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

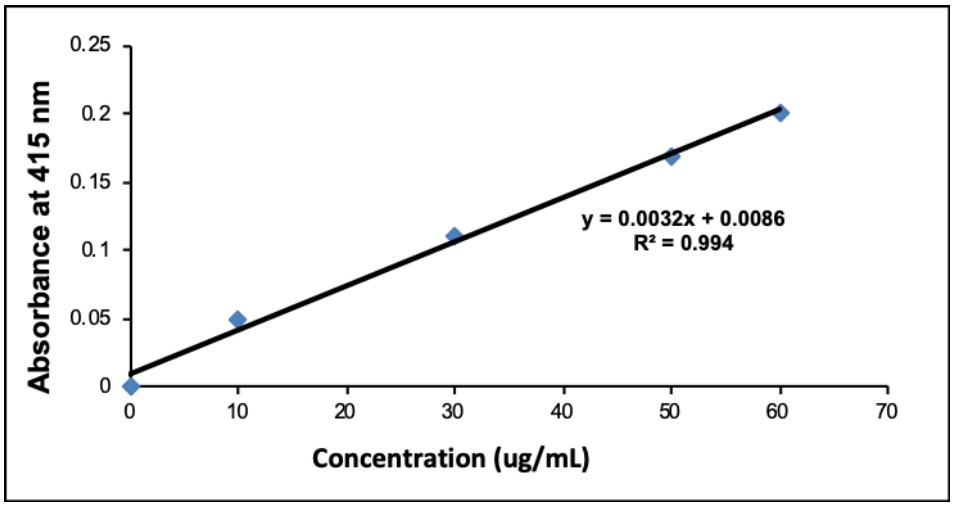

Figure 3.2. Standard calibration curve of Rutin (reference flavonoid compound).

- **Quantitative phytochemical assay of the total tanninl content in *E. alata* fruit extracts**

  The evaluation of total tannin content, the absorption values of several concentrations of the standard Catechin are listed in Table 3.5.

  The data collected from the standard calibration curve of Catechin is shown in Figure 3.3. The equation used to calculate the estimated the total condensed tannins content (TTC) in the four *E. alata* fractions was $y = 0.001x + 0.002$, $R^2 = 0.991$—where y is the absorbance at 500 nm and x is the total tannins content in the four extract fractions.

Table 3.5. Absorption values of several concentrations of the standard Catechin

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

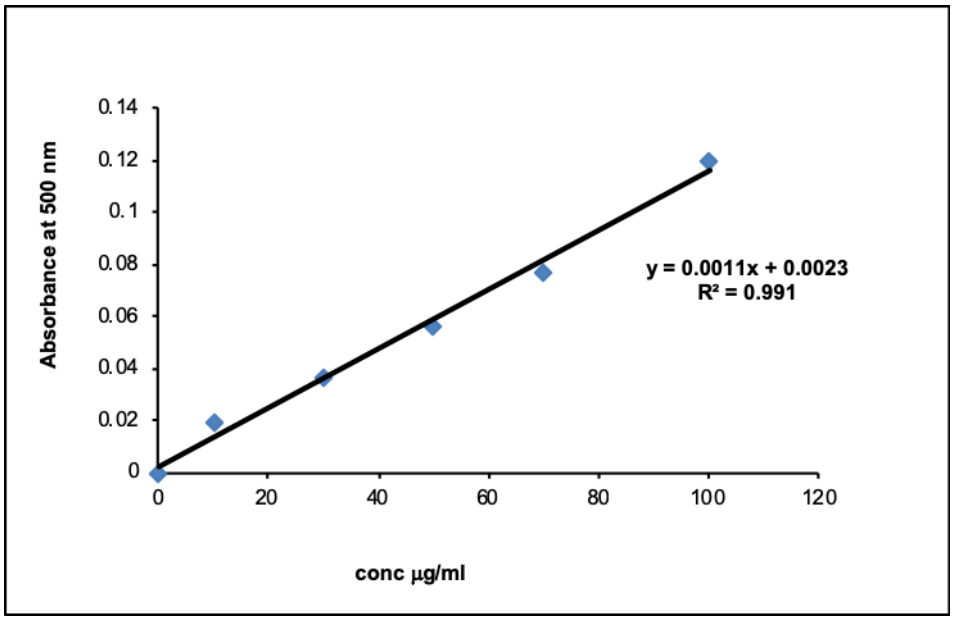

Figure 3.3. Standard calibration curve of Catechin (reference tannin compound)

The calculated results for the total phenol content (TPC), the total tannins content (TTC), and the total flavonoids content (TFC) are listed in Table 3.6.

Table 3.6. Quantitative phenols, tannins and flavonoids contents of the hexane, acetone, methanol and aqueous fractions of *E. alata* fruits

***E. alata* Fruit Extract Fractions**	**Total flavonoids contents, mg of RU/g of dry extract ± SD**	**Total phenol contents, mg of GAE/g of dry extract ± SD**	**Total tannin contents, mg of CAE/g of dry extract ± SD**
Hexane	-	5.72 ± 0.39	2.5 ± 0.70
Acetone	58.95 ± 2.33	19.85 ± 1.62	10.5 ± 0.70
Methanol	98.95 ± 2.3	33.22 ± 1.56	17.5 ± 0.70
Aqueous	32.3 ± 2.4	25.9 ± 0.78	1.5 ± 0.70

3.3. Free radical scavenging assay for antioxidant evaluation

For free radical scavenging activity, the four different plant extract fractions and fruit juice were evaluated with the DPPH radical protocol,

using Trolox as a potent antioxidant reference. The results for DPPH percentage inhibition are shown in Table 3.7 and Figure 3.4.

Table 3.7. The DPPH inhibition percentage of the different *E. alata* fruit extract fractions compared to Trolox (standard compound) and the IC_{50} values.

Conc. (µg/mL)	Trolox (standard) % inhibition	Hexane Fraction % inhibition	Acetone fraction % inhibition	Methanol fraction % inhibition	Aqueous fraction % inhibition
2	53.43 ± 3.46	19.25 ± 0	53.37 ± 0.46	57.05 ± 0.49	14.8 ± 0
5	61.51 ± 0.34	19.59 ± 0.48	53.54 ± 0.71	63.99 ± 0.26	28.71 ± 0.48
10	91.41 ± 0.34	30.4 ± 0	57.76 ± 0.47	91.5 ± 0.41	38.34 ± 0.72
20	97.29 ± 0.34	31.58 ± 0.71	58.1 ± 0.95	94.37 ± 0.17	55.5 ± 0.21
30	97.29 ± 0	60.25 ± 0.78	65.7 ± 0.23	96.6 ± 0	81.75 ± 0
50	97.29 ± 0	60.25 ± 0.78	65.7 ± 0.23	97.05 ± 0.21	81.75 ± 0
100	97.54 ± 0.69	60.81 ± 0	66.8 ± 0	97.05 ± 0.21	92.7 ± 0.7
IC_{50}	3.16 ± 0.74	45.7 ± 0.39	9.03 ± 0.51	3.09 ± 0.25	11.7 ± 0.3

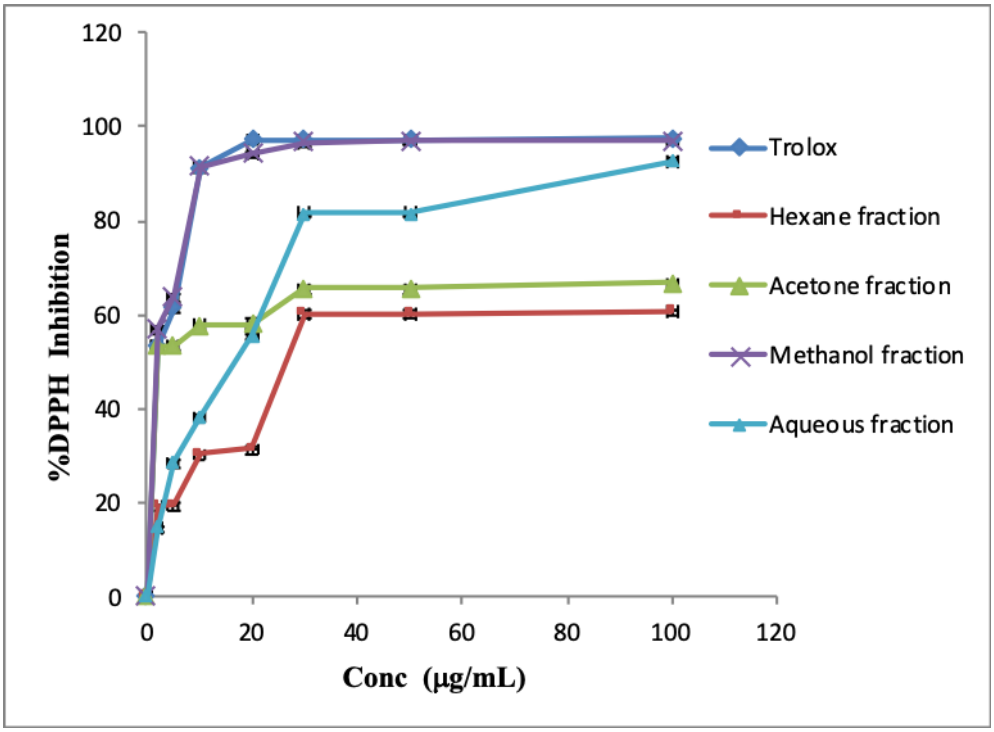

Figure 3.4. The DPPH inhibition percentage of the different *E. alata* fruit extract fractions compared to Trolox (standard compound).

3.4. Assessment of *in vitro* porcine pancreatic lipase enzyme inhibition activity

The hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol was used to measure the influence of the four *E. alata* fruit fractions on the porcine pancreatic lipase enzyme. The assay detected the inhibitory activity of the four extract fractions by comparing to Orlistat, a strong lipase inhibitory agent. The results of the lipase enzyme inhibitory activity and the lipase inhibition IC_{50} values for the four fractions and Orlistat are shown in Table 3.8 and Figure 3.5.

Table 3.8. The lipase inhibition percentage of the different *E. alata* fruit extract fractions compared to Orlistat (standard compound) and the IC_{50} values

Conc. (µg/mL)	Orlistat (standard) % inhibition	Hexane fraction % inhibition	Acetone fraction % inhibition	Methanol fraction % inhibition	Aqueous fraction % inhibition
50	91.05 ± 0.77	3.8 ± 0.28	52.4 ± 0	48.2 ± 0.28	3.6 ± 0.56
100	93.1 ± 0.42	10.8 ± 1.69	52.4 ± 0	48.6 ± 0.84	10.4 ± 0.56
200	94.3 ± 0.42	15.6 ± 0.56	55.2 ± 0.56	55.6 ± 0.56	17 ± 0.28
300	97.4 ± 0.12	53 ± 0.84	55.2 ± 0.56	59.4 ± 0.28	53.2 ± 0.56
400	97.5 ± 0	55.6 ± 0.56	55.2 ± 0.56	98.8 ± 0.56	53.2 ± 0.56
IC_{50} (µg/mL)	12.3 ± 0.35	1995.3 ± 0.79	114.81 ± 0.34	61.65 ± 0.50	2137.96 ± 0.49

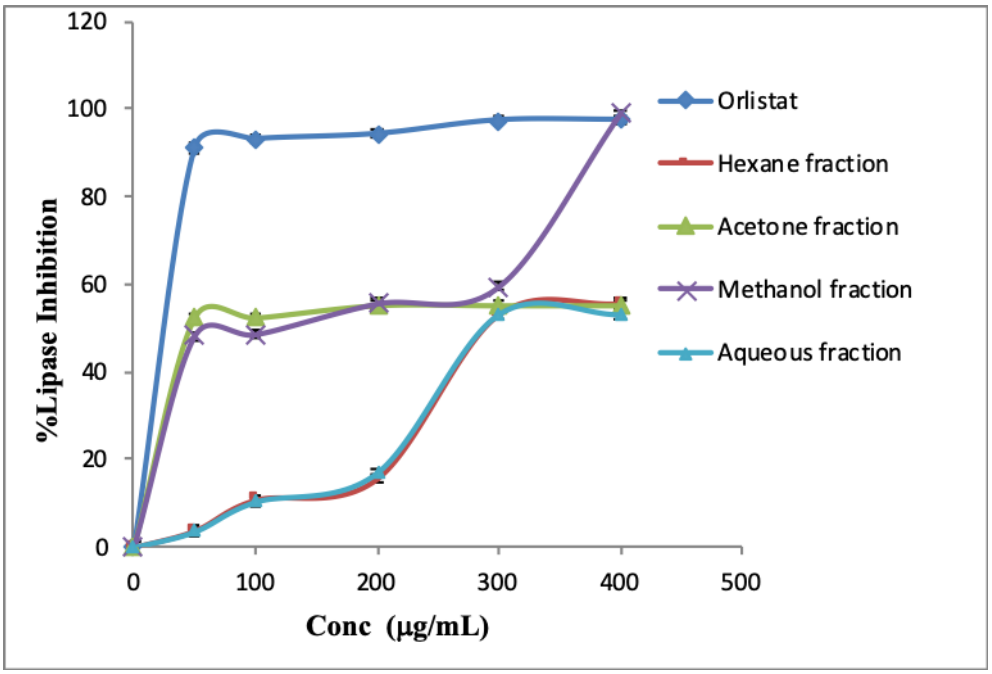

Figure 3.5. The lipase inhibition percentage of the different *E. alata* fruit extract fractions compared to Orlistat (standard compound).

3.5. Assessment of *in vitro* α-amylase inhibition activity

Inhibition of α-amylase by the four different *E. alata* fruit fractions was detected by the previous experimental protocol and compared to acarbose, a strong α-amylase inhibitory agent. The α-amylase inhibitory activity and IC_{50} values for the four fractions and acarbose results are shown in Table 3.9 and Figure 3.6.

Table 3.9. The α-amylase inhibition percentage of the different *E. alata* fruit extract fractions compared to Acarbose (standard compound) and the IC_{50} values.

Conc. (µg/mL)	Acarbose (standard) % inhibition	Hexane fraction % inhibition	Acetone fraction % inhibition	Methanol fraction % inhibition	Aqueous fraction % inhibition
10	53.22 ± 1.2	23.65 ± 2.12	14.67 ± 2.12	64.52 ± 0.21	38.61 ± 2.1
50	54.91 ± 0.58	31.13 ± 4.23	23.65 ± 2.12	66.76 ± 0.42	64.37 ± 0
70	66.1 ± 1.34	64.52 ± 0.21	31.13 ± 4.23	66.76 ± 0.42	65.23 ± 0.37
100	66.1 ± 1.62	64.52 ± 0.21	31.13 ± 4.23	66.76 ± 0.42	65.23 ± 0.37
500	72.54 ± 1.37	68.25 ± 0.49	66.45 ± 0.91	97.75 ± 0.21	67.23 ± 0.24
IC_{50} (µg/mL)	28.2 ± 1.29	66.07 ± 1.23	316.22 ± 2.32	16.22 ± 0.6	33.88 ± 0.58

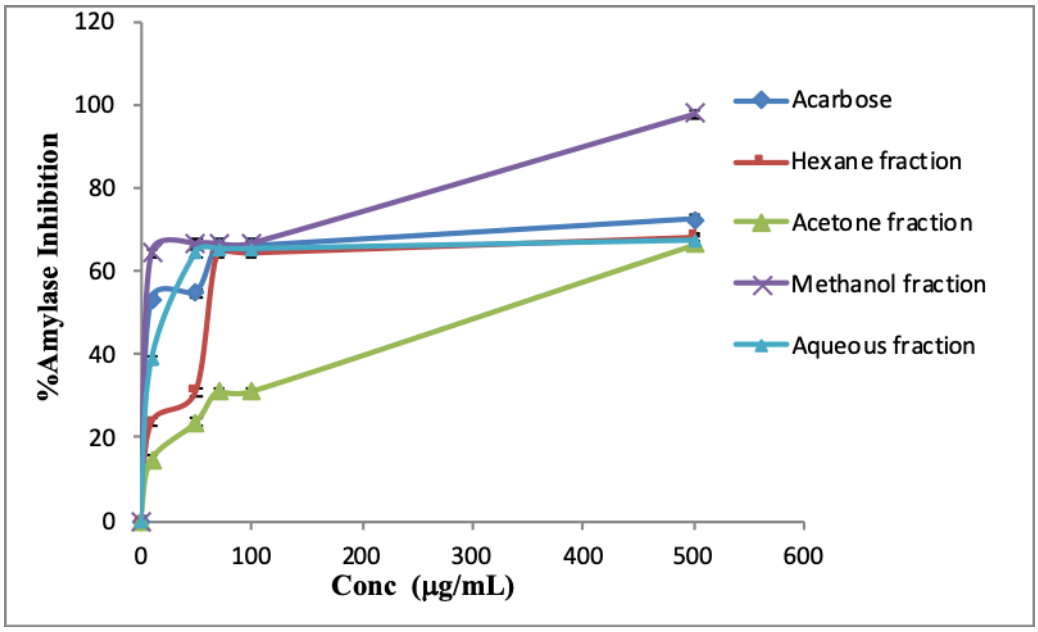

Figure 3.6. α-amylase inhibition percentage of the different *E. alata* fruit extract fractions compared to Acarbose (standard compound).

3.6. Assessment of *in vitro* α-glucosidase enzyme inhibition activity

Inhibition of α-glucosidase by the four different *E. alata* fruit fractions was detected by the previous experimental protocol and compared to acarbose, a strong α-glucosidase inhibitory agent. The α-glucosidase inhibitory

activity and IC_{50} values for the four fractions and acarbose results are shown in Table 3.10 and Figure 3.7

Table 3.10. The α-glucosidase inhibition percentage of the different *E. alata* fruit extract fractions compared to Acarbose (standard compound) and the IC_{50} values.

Conc. (µg/mL)	Acarbose (standard) % inhibition	Hexane fraction % inhibition	Acetone fraction % inhibition	Methanol fraction % inhibition	Aqueous fraction % inhibition
100	65.8 ± 0.42	28.71 ± 0.48	19.59 ± 0.48	55.46 ± 0.53	24.33 ± 0.26
200	67.75 ± 0.35	55.5 ± 0.21	19.59 ± 0.48	59.62 ± 0.53	55.28 ± 0.26
300	73.2 ± 0.42	81.75 ± 0	31.58 ± 0.71	97.15 ± 0.77	55.84 ± 0.53
400	85.35 ± 0.35	92.7 ± 0.7	60.81 ± 0	97.15 ± 0.77	58.1 ± 0.56
500	92.22 ± 0.106	96.26 ± 0.5	60.81 ± 0	97.3 ± 0.56	59.75 ± 0.77
IC_{50} (µg/mL)	37.15 ±0.33	53.7 ± 0.38	794 ± 0.33	32.36 ± 0.63	199.5 ± 0.48

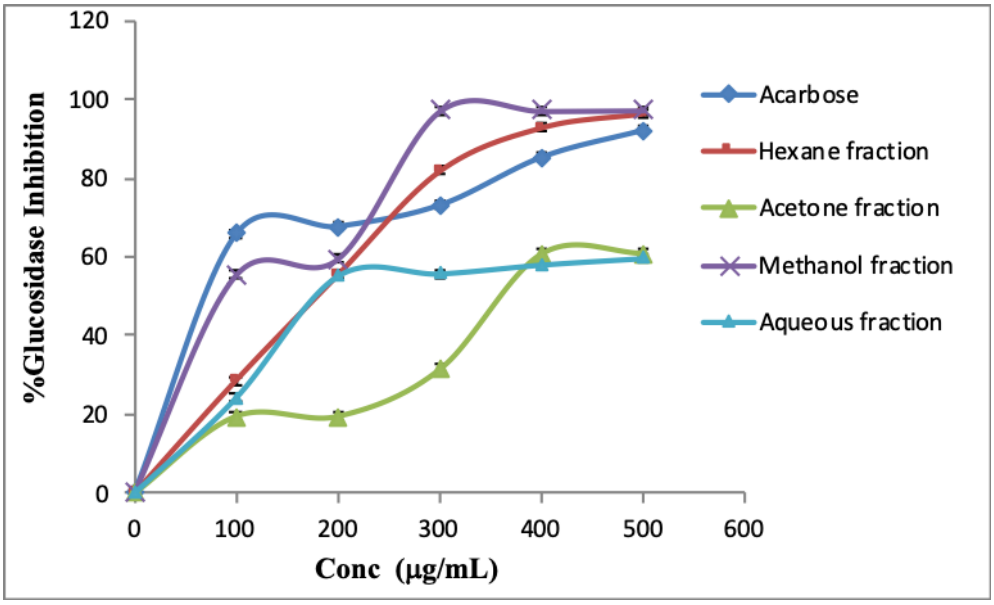

Figure 3.7. α-glucosidase inhibition percentage of the different *E. alata* fruit extract fractions compared to Acarbose (standard compound) and the IC_{50} values.

3.7. *In vitro* cell proliferation assay of each extract fraction of *E. alata* fruit

Cell viability was assessed by Cell Tilter 96® Aqueous One Solution Cell Proliferation (MTS) Assay. The MTS tetrazolium compound is bioreduced by active cells into a colored formazan product. The quantity of formazan product as measured by absorbance at 490nm is directly proportional to the number of living cells in culture. The cytotoxixity of HeLa cells results are shown in Figure 3.8.

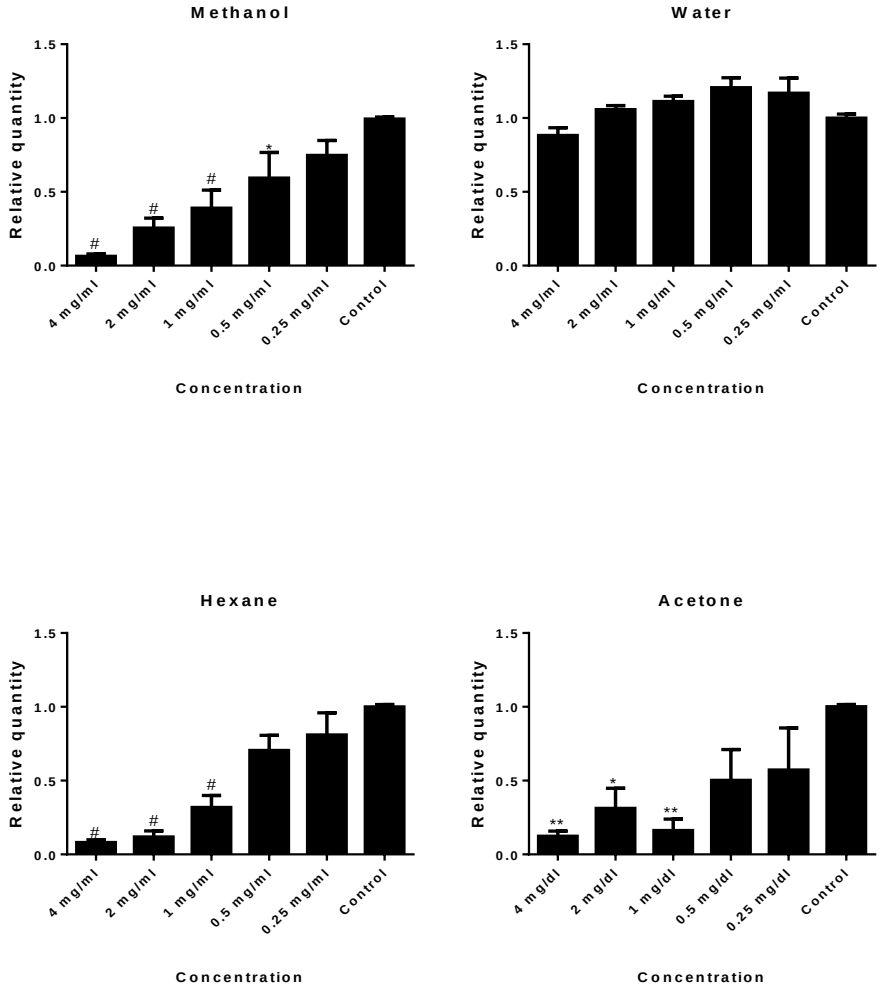

Figure 3.8. The effect of various extracts, derived from *E. alata*, on the cytotoxicity of HeLa cells. HeLa cells were treated with 4, 2, 1, 0.5 and 0.25 mg/mL of acetone, hexane, water and methanol extracts obtained from *E. alata*.

Chapter Four

Discussion and Conclusion

4.1 Free radical scavenging protocol for antioxidant activity of *E. alata* fruit extracts

In humans, free radicals contribute to more than one hundred disorders especially, specifically atherosclerosis, arthritis, ischemia, and reperfusion injury of many body tissues, such as central nervous system injury, gastritis, cancer, and AIDS. Thus, the demand is still present to find information concerning the antioxidant potential of more plant species (Kumpulainen & Salonen, 1999; Pourmorad, et al., 2006).

DPPH accepts electrons from antioxidant molecules, when they are present, and changes color stoichiometrically. This color change usually depends on the number of electrons received from the available antioxidants, which can be determined also by measuring the changes in absorbance (Rice-Evans, Sampson, Bramley, & Holloway, 1997).

The results obtained from the DPPH assay on *E. alata* fruits showed that the methanol extract fraction, with an IC_{50} value of 3.09 ± 0.25 µg/mL, was the most potent fraction in free radical scavenging when compared to Trolox (the standard reference), which had an IC_{50} equal to 3.16 ± 0.74 µg/mL. The acetone and aqueous fractions also appeared to be potent in this assay, with IC_{50} values equal to 9.03 ± 0.51 µg/mL and 11.7 ± 0.3 µg/mL, respectively. The hexane fraction showed weaker free radical scavenging ability with an IC_{50} value equal to 45.7 ± 0.39 µg/mL.

These observations confirmed the results of the quantitative analyses of phenols, flavonoids, and tannins, which showed a high flavonoid and phenol content in the methanol extract fraction, which was equal to 98.95 ± 2.3 mg of RU/g of dry extract and 33.22 ± 1.56 mg of GAE/g of dry extract, respectively. Tannins also showed the highest content in the methanol fraction with a value equal to 17.5 ± 0.70 mg of CAE/g of dry extract.

In general, the phenolic content of all the extracts was considerably high, which could be a major contributor to the strong antioxidant effect of *E. alata* fruit extracts. Therefore, the high phenolic content in fruit extracts will explain these results of high antioxidant activity (Afolayan, Jimoh, Sofidiya, Koduru, & Lewu, 2007).

The study also revealed that all the extracts were rich in flavonoids, which have been observed previously to have strong antioxidant properties. Flavonoids may reduce the risk of cardiovascular disease, cancer, and protect against urinary tract infections (Howell, 2002; Steinberg, Bearden, & Keen, 2003). Flavonoids, as one of the most diverse and widespread groups of natural compounds, are probably the most important natural phenolic compounds. These compounds possess a broad spectrum of chemical and biological activities, including radical scavenging properties, and they are also believed to have an inhibitory effect on carcinogenesis as shown in previous studies (Agrawal, 2013).

4.2 Lipase inhibitory assay for the four *E. alata* extract fractions

In general, lipase is involved in fat metabolism, and it is an important target for inhibitors, since its inhibition limits triacylglycerol absorption in small intestine—leading to a decrease in caloric yield and subsequent weight loss (Friedman & Brandon, 2001).

It was observed from the calculated results from the lipase inhibition assay that *E. alata* fruit extracts did not show potent activity against this enzyme, as the methanol extract fraction showed moderate activity, with IC_{50} equal to 61.65 ± 0.50 µg/mL, when compared with orlistat (the reference compound) that had an IC_{50} value equal to 12.3 ± 0.35 µg/mL. On the other hand, the acetone extract fraction showed a weak inhibitory effect on lipase, with an IC_{50} value equal to 114.81 ± 0.34µg/mL, while the hexane and aqueous extract fractions were totally inactive against this enzyme.

These observations confirmed the results of the quantitative analyses of phenols, flavonoids and tannins that showed a high content of flavonoids and phenols in the methanol extract fraction, which was equal to 98.95 ± 2.3 mg of RU/g of dry extract and 33.22 ± 1.56 mg of GAE/g of dry extract, respectively. Tannins also showed the highest content in the methanol extract fraction, with value equal to 17.5 ± 0.70 mg of CAE/g of dry extract.

As reported in previous studies, pancreatic lipase inhibition has been observed by different polyphenolic compound-rich extracts from herbs and spices such as tea (*Camellia sinensis*) (He, Lv, & Yao, 2007), anise myrtle (*Syzygium anisatum*) (Sakulnarmrat & Konczak, 2012), and Chinese

liquorice (*Glycyrrhiza uralensis*) (Won et al., 2007). Pancreatic lipase inhibition by some of the individual polyphenolic compounds has also been reported, mainly as the inhibition percentage and inhibition saturation kinetics, showing IC_{50} values (concentration of compound/sample required to inhibit enzyme activity by 50%). Examples of these polyphenolic compounds include quercetin, which has been observed as one of the best pancreatic lipase inhibitors, while phenolic acids have shown low inhibitory activity (Costamagna et al., 2016; You, Chen, Wang, Jiang, & Lin, 2012).

4.3 α-amylase inhibitory assay for the four *E. alata* extract fractions

From table 3.9, which shows the α-amylase inhibition percentage of the four different *E. alata fruit* fractions compared to Acarbose (standard compound), the IC_{50} values revealed significant activities of almost all collected extract fractions from the *E. alata* fruits. The methanolic fraction seemed to be the most effective, with an IC_{50} equal to 16.22 ± 0.6 µg/mL, followed by the aqueous fraction with an IC_{50} of 33.88 ± 0.58 µg/mL. With an IC_{50} value equal to 66.07 ± 1.23 µg/mL, the hexane fraction also showed a moderate effect. On the other hand, the acetone fraction was the weakest, but still active against the amylase enzyme (IC_{50} = 316.22±2.32 µg/mL). All previous results were compared to acarbose, the reference compound used for this analysis, which had an IC_{50} equal to 28.2 ± 1.29 µg/mL.

The results obtained in this research, especially for the methanol fraction which possessed the highest phenolic content, provide additional evidence

that is in line with previous studies—that natural polyphenols have the ability to inhibit the activity of carbohydrate hydrolyzing enzymes like α-amylase and α-glucosidase (Tundis, Loizzo, & Menichini, 2010).

Also, the efficacy of the hexane fraction against the amylase enzyme confirmed that the previously reported data about Terpenoids that represents a promising source for biologically active, natural compounds, which have potential for the research and development of new substances with pharmacologic activity. The α-amylase inhibitory activity was related only to oleanane-, ursane-, and lupine-type Terpenoids (Sales, Souza, Simeoni, Magalhães, & Silveira, 2012).

The methanol extract fraction of *E. alata* fruits showed a high anti-amylase potency. Thus, this fraction can potentially be offered in some dietary supplements for diabetic patients. This anti-amylase activity may be due to the presence of phenolic compounds and flavonoids, which have been shown to inhibit the amylase enzyme (Rohn, Rawel, & Kroll, 2002).

4.4 α-Glucosidase inhibitory activity of the four E. alata extract fractions

Table 3.9 shows the α-glucosidase inhibition percentage of the different *E. alata fruit* extract fractions compared to Acarbose (standard compound) and the values of IC_{50}. In general, a significant effect of these fruits extract fractions was revealed. Methanol was the most potent fraction with an IC_{50} equal to 32.36 ± 0.63 µg/mL, which was more effective against the enzyme than acarbose with an IC_{50} value equal to 37.15 ± 0.33 µg/mL. The hexane

fraction also was effective against α-glucosidase with an IC_{50} value equal to 53.7 ± 0.38 µg/mL, while the aqueous and acetone extract fractions were weak against this enzyme with IC_{50} values of 199.5 ± 0.48 and 794 ± 0.33 µg/mL, respectively.

The results obtained in this study confirmed previously observed results that revealed the activity of phenolic compounds like *p*-hydroxybenzoic acid, as well as *trans-p*-coumaric acid and epicatechin gallate, and flavonoids, like quercetin that are present in lentil extracts, to be effective inhibitors of some digestive enzymes—lipase and α-glycosidase—contributing to controlling glucose levels in blood, as well as the management of obesity (B. Zhang et al., 2015).

Additionally, different studies have shown that phenolic compounds present in some medicinal plants and fruits are significant inhibitors of digestive enzymes. For example, red fruit extracts, which are rich in phenolic compounds, have been shown to inhibit α-amylase and α-glycosidase, and also exhibit anti-obesity properties by exerting different mechanisms of action on digestive enzymes (McDougall, Fyffe, Dobson, & Stewart, 2005).

4.5 *In vitro* evaluation of cell proliferation for each extract fraction of *E. alata fruits*

As shown in Figure 3.8, treatment of HeLa cells with 4, 2, 1 and 0.5 mg/mL of methanol extract derived from *E. alata* induced significant cytotoxicity ($0.0001 \leq p < 0.05$) by approximately 94%, 75%, 61%, and

41% respectively, while 0.25 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 aqueous extract derived from *E. alata* had no significant effect. Treatment of HeLa cells with 4, 2, and 1 mg/mL of the hexane extract derived from *E. alata* induced significant cytotoxicity ($p \leq 0.0001$) by approximately 92%, 88%, and 68%, respectively, while using the same concentrations of the acetone extract induced cytotoxicity by approximately 80% ($p < 0.01$). However, 0.5 and 0.25 mg/mL of both the hexane and acetone extracts had no significant effect on the cytotoxicity of the cells.

In previous studies on cytotoxicity of the hydroalcoholic extract of the aerial part of *Ephedra alata* Decne, the results showed that the hydroalcoholic extract contained polyphenolic phytocompounds and had anti-proliferative, pro-apoptotic, and cytotoxic potential against the MCF-7 human breast cancer cell line (Corina et al., 2019).

4.6 Conclusion

The *E. alata* fruit extract analysis showed that it contains a mixture of different phytochemicals such as protein, amino acid, reducing sugars and saponin, in addition to phenols, tannins and flavonoids which seems to be in high quantity in particular in the methanol extract. The methanol extract also has potent anti-oxidant, a-amylase and a-glucosidase inhibition and moderate lipase inhibition. Therefore, the methanol extract fraction of *E. alata* provide scientific rationale for the use in the pharmaceutical industry as a low-cost nutrient useful in reducing chronic pathologies such as

diabetes mellitus, obesity, and oxidative stress. The methanol extract fraction was shown to be a potent inhibitor of starch digestive enzymes due to the high content of phenolic compounds and flavonoids, and it was found to be effective in free radical scavenging and lipase enzyme inhibition. In addition, the methanol extract derived from *Ephedra alata* fruits induced significant cytotoxicity ($0.0001 \leq p < 0.05$) by approximately 94%, and therefore, it may represent a good choice for some of the health beneficial herbal supplements and natural medications used in cancer management and can be further subjected for the isolation of the therapeutically active compounds.

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جامعة النجاح الوطنية

كلية الدراسات العليا

الفحوصات الكيميائية للكشف عن المركبات الفعالة والنشاط الدوائي لمستخلصات ثمار نبات العلندة

إعداد

هناء حسين مصطفى دقة

إشراف

د. نضال جرادات

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

2019

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الملخص

في مجال العقاقير الطبية أصبحت العلاجات النباتية تحتل اهتماما ملحوظا لفاعليتها في علاج الكثير من المشكلات الصحية دون إحداث ذلك الضرر للأدوية الكيميائية على الجسم البشري ولذلك يتم حاليا العمل على تطوير بدائل طبيعية فعالة لعلاج العديد من المشاكل الصحية مثل السكري والسمنة والإجهاد التأكسدي وفي هذه الدراسة تمت الفحوصات المخبرية على ثمار نبتة العلندة لتحضير مستخلصات متنوعة بواسطة مذيبات مختلفة في القطبية عن بعضها لعزل المركبات الفعالية كل على حدا مثل الهكسان والأسيتون والميثانول والماء المقطر ومن ثم تطبيق مجموعة من الاختبارات الكيميائية لتحديد نوعية وكمية هذه المركبات الفعالة . وقد تم التوصل الى أن مستخلص الميثانول كان غنيا بمركبات الفلافونيد ومركبات الفينول المضادة للأكسدة بينما ظهرت مركبات التيربينويد عديمة القطبية في مستخلص الهكسان.

بعد الحصول على النتائج المخبرية تم ملاحظة أن مستخلص الميثانول لثمار نبتة العلندة كان له تأثيرا واضحا على مركب DPPH الذي يعتبر من الشواذر الحرة المسببة للإجهاد التأكسدي وقد يعود سبب هذه الفاعلية أن مستخلص الميثانول كان غنيا بمركبات الفينول والفلافونيد المضادة للأكسدة وقد تم استخدام مركب Trolox لغرض المقارنة في هذا الفحص المخبري.

بعد ذلك تم إخضاع هذه المستخلصات لمجموعة اختبارات بغرض معرفة تأثيرها على الأنزيمات الهاضمة للنشويات والسكريات حيث تم اختبار أنزيم α-amylase وأنزيم α-glucosidase بالإضافة الى إنزيم lipase المسئول عن هضم الدهنيات. حيث أظهرت النتائجأن مستخلص الميثانولكان فعالا في تثبيط هذه الإنزيمات الثلاثة وبالتالي يمكن لهذا المستخلص أن يصبح

بالمستقبل بديلا طبيعيا للعقاقير الكيميائية المستخدمة في ضبط مستويات السكر في الدم لمرضى السكري وكبديل طبيعيأخر لعقار الاورليستات لضبط مشكلة السمنة.

حيث أن فاعلية هذا المستخلص قد تكون نتيجة لاحتوائه على مركبات الفينول والفلافونيد بكميات أكثر من المستخلصات الأخرى. وقد تم استخدام مركب Acarbose لغرض المقارنة في الفحوصات الكيميائية على الأنزيمات الهاضمة للنشويات والسكريات بينما استعمل orlistat لغرض المقارنة في فحص أنزيم اللايبز لهاضم للدهنيات.

وبالتالي فقد تم التوصل في نهاية هذه الدراسة الى أن ثمار نبتة العلندة المتوفرة في الأراضي الفلسطينية قد أظهرت نتائج ملحوظة في تثبيط الإنزيمات الهاضمة للنشويات والدهنيات كما أثبتت جدارتها في تثبيط الشواذر الحرة المسببة للإجهاد التأكسدي، وبهذا قد تشكل هذه الثمار مصدرا طبيعيا فعالا في ضبط مشاكل السمنة والسكري والسرطان ولكن هذا يتطلب من الباحثين أجراء المزيد من الفحوصات السريرية للتأكد من وجود هذه الفاعلية داخل الأجسام البشرية. وهكذا توصي هذه الدراسة بالمزيد من الأبحاث العلمية المتقدمة لتطوير صناعات دوائية فعالية على ثمار نبتة العلندة لتحضير بدائل طبيعية بالمستقبل.