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Faculty of Graduate Studies

Nopal Cactus Phyto-Chemical Content and Antidiabetic Effect

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Dedication

To my beloved father and mother, who raised me to be I am today... ….

To my beloved husband and sons ... ….

To my brothers , my sisters and their families who have supported me …..

To the memory of my dearest friends …….

I dedicate this work

Acknowledgement

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My deepest gratitude to my family; who enlightened my academic path with care and support.

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Nopal Cactus Phyto-Chemical Content and Antidiabetic Effect

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: **إسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviation

DMSO :dimethyl sulfoxide
DPPH : 2, 2-Diphenyl-1-picrylhydrazyl
IC_{50} : half maximal inhibitory concentration
pNPG : p-nitrophenyl-α-D-glucopyranoside
DNSA: (3,5--dinitrosalicylic acid)
Trolox : ((s)-(-)-6 hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid)
A_B : absorbance of the blank solution
A_T : absorbance of the tested sample solution

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Abstract

Background: Hyperglycemia and oxidative stress are important factors associated with chronic diseases such type 2 diabetes mellitus so it's a challenge for pharmaceutical industry to develop new formulations especially from natural sources to manage these problems. Therefore, the aim of this study is to evaluate the antioxidant, anti α-amylase and α-glucosidase activities of *Opuntia ficus-indica* cladodes and fruit juice.

Methods: Four solvents differ in their polarities were used for extraction of *Opuntia ficus-indica* cladodes while fruit juice was only freeze dried, then qualitative phytochemical tests were applied to determine the bio active ingredients and finally each extract was *in-vitro* investigated to evaluate their efficacy in free radical scavenging, α-amylase and α-glucosidase inhibitory activities.

Results: *Opuntia ficus-indica* cladodes methanol extract has the highest content in Flavonoid, phenols, alkaloids as it also has a significant antioxidant capacity with IC_{50} value (6.16±0.59 µg/ml) ; compared to

Trolox (reference compound) which had IC_{50} value (2.09±1.7µg/ml). Acarbose was used as reference to detect α-amylase inhibition activity, it seems that aqueous extract was the most potent with IC_{50} value (16.98±0.77µg/ml) followed by acetone extract with IC_{50} value (25.11±0.89µg/ml) .

Also all extracts showed inhibitory activity with different degrees against α-glucosidase enzyme, the aqueous extract showed the most potent activity with IC_{50} value (79.43±1.16□g/ml) followed by acetone extract IC_{50} value (125.89±1.31□g/ml), comparing these results to Acarbose, which presenting IC_{50} value (38.02±0.44µg/ml).

Conclusion: From this study *Opuntia ficus-indica* cladodes and fruits extracts can be considered as sources of bioactive compounds for nutrition, health and disease, so further *in-vivo* studies should be carried out to determine their potentiality against diabetes mellitus and oxidative stress diseases.

Chapter One
Introduction

1.1. Research overview

From ancient times to the present; several plants have been used not only as food, but also as materials for alternative medical therapy prepared by our ancestors learned the using of certain herbs to heal some diseases. Then these natural products have been utilized as medicines for thousands of years . These medicines initially took the form of crude drugs such as tinctures, teas, powders, poultices and other herbal preparations [1, 2]. For example the most well known example to date would be the preparation of the anti-inflammatory agent, acetyl salicylic acid [aspirin] derived from the natural product: salicin isolated from the bark of the willow tree plant (*Salix alba* L) [3]. Another example involves the plant genus Salvia which grows usually throughout southwestern region of United States and north western Mexico; which was used by Indian tribes of southern California in aiding of childbirth as male newborn babies were ‘cooked’ in the hot Salvia ashes as it was believed that these babies consistently grew to be the strongest and healthiest members of their respective tribes and they will acquire immune from all respiratory ailments for life [4].

Now a days the consumption of fruits and vegetables is gaining more attention of worldwide since their health benefits such as cardio protective, anti-cancer, anti-diabetic and anti-obesity activities. The bioactive compounds including polyphenols that has many forms as flavonoids,

tannins, catechins, vitamins C and E, β-carotene and several others confirm these health-protective benefits [5].The antioxidant ability of phenolic compounds in fruits and vegetables could be attributed to their properties such as reducing agents, hydrogen donors, singlet hydrogen quenchers [6].

As usually known antioxidant pathway occurring in human body can scavenge free radicals, and so keeps the balance between oxidation and anti-oxidation process .for example to habits that confuse this balance is the exposure of cigarette smoking, alcohol, radiation and environmental toxins induces the production of certain ROS and RNS, which disrupt the balance between oxidation and anti-oxidation and so resulting in some chronic and degenerative diseases [7].

Recently phytochemicals derived from natural sources have received much attention in the treatment of diabetes for various reasons and several researchers have focused on isolation of hypoglycemic agents from these medicinal plants [8]. Plant polyphenols and flavonoids are some of the naturally occurring anti diabetic agents that showed an inhibitory effect on carbohydrate hydrolyzing enzyme , by their capacity to bind with proteins. This phenomenon contributes to lower postprandial hyperglycemia in diabetes and so these compounds will form a valuable alternatives for chemical anti diabetic medications [9].

Scientists recently focused on cladode and seeds of *Opuntia ficus-indica* which contains major phytochemicals in search of new natural antioxidants. Cactus "*Opuntia* spp", belonging to Cactaceae family is

native to Mexico. However, this plant is widely distributed in arid and semi-arid regions of Africa, Central America, Mediterranean region and South Africa. *Opuntia* genus has more than 200 species . Moreover, its important economic value, fast grows, less water requirement, and adaptation to nutrient-deficient soils made the cactus pear a prominently cultivated crop all over the world [10,11].

1.2. Hypothesis of this work

This work based on a hypothesis that both *Opuntia ficus-indica* cladodes and fruits will contain several bio active compounds when their different extracts are investigated in-vitro on free radical scavenging power to evaluate antioxidant activity and their action on α-amylase and α-glucosidase enzymes inhibition to find the activity of these extracts in diabetes disease management.

Also cladodes and fruits of *Opuntia ficus-indica* may form an efficient natural source for developing some herbal medications in treating diabetic patients with properties of being safe or free from side effects occurred with chemical medications.

1.3. Objectives

The following objectives serve the goal of this research, which are:

- Performing fractional extraction procedure to Prepare different extracts from *Opuntia ficus-indica* cladodes using solvents with different

polarities like n-hexane, acetone, methanol and distilled water. Also preparation of fruit juice extract.

- Qualitative analysis of phytochemical constituents that present in each prepared extract to find if flavonoids, phenols , carbohydrates, glycosides, alkaloids and other active compounds present or not.
- Study the effect of each extract on free radical scavenging capacity to evaluate in-vitro antioxidant power.
- Study the activity of each extract on α-amylase and α-glucosidase to evaluate the ant diabetic capacity of this plant
- Compare which is more bio active part of this plant the cladodes or the fruits following the results of each test.

1.4. *Opuntia ficus-indica* plant description

prickly pear or Opunti is the common name of *Nopal cactus* plant which considered as a member in Cactaceae family, this family includes nearly 1500 species of cactus species . *Nopal cactus* is a tropical and it is also a subtropical example in this plant family . It may grow in arid and semi-arid climates with a geographical distribution encompassing Mexico, Latin America, South Africa and Mediterranean countries [12].

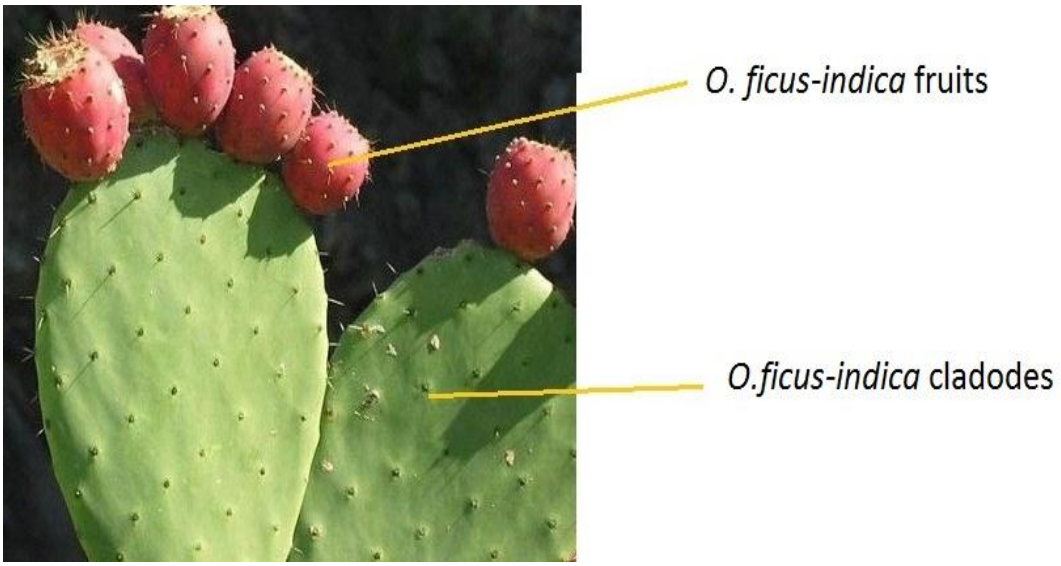

Figure (1.1) : *Opuntia ficus--indica* plant cladodes and fruits.

The cactus pear fruit which usually called prickly pear fruit is an oval elongated berry, with a thick peri-carp and a juicy pulp with a considerable number of seeds and a semi-hard rind with thorns. The peri-carp of this pear and the edible pulp may have different colors like green, greenish white, cherry-red, or purple hues, canary yellow and red [13]. Prickly pears average weight vary usually from 100 to 160 g depending on the origin site and cultivation. Its known that the usable part of the fruit is composed of peel (48%--52%) and pulp (48%--52%). The pulp can be almost subdivided into seeds and strained pulp (44%--45%). The fruits with white pulp and green rind are preferred for human consumption as food, and their domestic production related to almost 95% of the total production [14].

Mexico is the main producer of *Opuntia ficus--indica* (L.) Mill species, and considered for more than 45% of the worldwide production; however, only 1.5% of this percentage production is exported [15].

1.4.1. *Opuntia ficus-indica* cladodes and fruits uses in traditional medicine

Prickly pear fruit has long been known in traditional medicine for treating a number of pathologies such as ulcer, dyspnea, and glaucoma, as well as liver conditions, wounds and fatigue. Different studies using European and Asian varieties of cactus pears have shown notable antioxidant activities that reduce significantly the oxidative stress in patients and may prevent chronic pathologies. In this sense, some preparations of fleshy stems (cladodes) have been tested for the treatment of diabetes symptomatology in humans and animal models [16-18].

Opuntia ficus-indica fruits , similar to other fruits and vegetables, was used in Mexican folk medicine for the treatment of certain diseases including ulcers, dyspnea and liver disease .Recent evidence suggested that they have some anti-inflammatory effects . The mechanisms exercising their health benefits are not fully understood . In the past, studies addressed the presence of dietary fiber, but now, the nutraceuticals benefits are presumed to be established from their antioxidant properties [19,20].

1.4.2. Recent researches on *Opuntia ficus-indica* cladodes and fruits

Different studies using European and Asian researches for cactus pears showed notable antioxidant effects that reduce significantly the oxidative stress in patients and may be important in preventing chronic pathologies. In this view some preparations of fleshy stems (cladodes) have been tested for the treatment of diabetes symptomatology in humans and animal models [21,22].

Some authors have also reported that the fresh stems and nopal are a good source of fiber that also helps to reduce the blood sugar and plasma cholesterol levels . The cactus pear fruit may be considered a functional food; this feature has been attributed to its bioactive compounds such as vitamins especially C E, polyphenols, β-carotene and its derivatives, flavonoid compounds (for examples; hespiridin, quercetin, and naringin), taurine (type of amino acids) and pigments [23-26].

1.5. Medicinal plants as a source of bioactive compounds

Natural products provide several opportunities for new drug discoveries because of the unmatched chemical diversity they may provide. According to the World Health Organization (WHO), above 80% of the world's populations rely on folk medicine for their primary healthcare requirements [28].

This has captured the interest of many scientific researchers to develop local medicinal plants for valuable medicinal traits. A lot of studies indicated that medicinal plants contain in their tissues bio active compounds like peptides, long unsaturated chains of fatty acids, alkaloids, essential oils, phenols. Some of these compounds have significant therapeutic activities against animal pathogens, including bacteria, fungi and viruses [28,29].

Herbs therapeutic use is as old as human civilization and has evolved along with it. Local practitioners basically used indigenous plants and herbs for centuries all over the world to manage several ailments and these have showed clear pharmacological effects, Historically, the majority of new drugs have been generated from natural products {secondary metabolites} and from compounds synthesized later from natural products . Natural products have long been an important source for the discovery of new drugs due to their chemical diversity and also due to their ability to act on various biological systems [30,31].

The biosynthesis and breakdown of proteins, fats, nucleic acids and carbohydrates, which are essential to all living organisms, is known as primary metabolism with the compounds involved in the pathways known as [primary metabolites]. The mechanism by which an organism biosynthesizes compounds called [secondary metabolites] is often found to be unique to an organism or is an expression of the individuality of a species and is referred to as ‘secondary metabolism’. Secondary

metabolites are generally not important for the growth, development or reproduction of an organism and are produced either as a result of the organism adapting to its surrounding environment or to act as a possible defense line against predators to assist in the survival of the plant. The biosynthesis of secondary metabolites is derived from the basic processes of photosynthesis, glycolsis and the Krebs cycle to afford biosynthetic intermediates that finally results in the formation of secondary metabolites also known as natural products [32,33].

1.5.1. Qualitative phytochemical screening tests for identification of active therapeutic compounds in the different cladodes extracts and fruit juice

Plants natural products classified to primary and secondary metabolites with divergent functions [34]. The primary metabolites, amino acids, simple sugars , proteins and lipids are used usually in cellular processes. Secondary metabolites are biochemically active compounds {flavonoids, alkaloids, terpenoids, steroids, saponins, etc.}, they are produced in response to stress with complexity in their structure and more restriction in distribution than observed in the primary metabolites [35].

1.5.2. Estimation of antioxidant activity depending on free radical scavenging assay

Free radicals and reactive oxygen species ‘ROS’ are highly reactive structures that may damage cell structures such as nucleic acids,

carbohydrates, lipids, proteins . Oxidative stress is a state that contributes to many pathologies including cardiovascular diseases, diabetes, neurodegenerative diseases, atherosclerosis and cancer [36].

An antioxidant can be usually defined as any substance which delays or inhibits oxidative damage to a target molecule [37]. The characteristic feature of an antioxidant is its capability to scavenge the free radicals due to their redox hydrogen donators and singlet oxygen quench[38].

Physicochemical changes in oxidatively--modified proteins include alterations in protein hydrophobicity, solubility, proteolytic affinity, and thermal stability . In food systems, mild and limited protein oxidation may be desirable due to the enhancement of functionalities . However, uncontrolled and irreversible protein oxidation always leads to inactivation of functional proteins and enzymes. The accumulation of oxidatively-modified proteins is considered as one factor that contributes to decreased product qualities [39].

1.5.3. Anti-diabetic evaluation using α-amylase and α-glucosidase enzymes inhibition assay

Diabetes has become a major health problem in the world. It is a metabolic disease characterized by a high blood glucose level and can cause other health complications, such as cardiovascular disease, neuropathy, high blood pressure, weakness, gangrene, retinopathy, nephropathy and other dysfunctions [40]. One of the therapeutic approaches aimed to suppress

the glucose production from carbohydrates digestion by inhibiting digestive enzymes, mainly α-amylase and α-glucosidase . Acarbose has been used in the clinical trials as an effective inhibitor of carbohydrate hydrolysis, but has some side effects such as abdominal pain, diarrhea and flatulence [41].

Prolonged hyperglycemia state usually leads to the auto-oxidation of glucose and formation of advanced glycated end products which are included in the generation of reactive oxygen species (ROS) that cause lipid per-oxidation and play an important role in the production of secondary complications in T2D. Oxidative stress is considered to be a common pathway linking diverse mechanisms for the pathogenesis of micro-vascular and macro-vascular un wanted complications of diabetes disease [42].

Diabetes is believed to be one of the most deadly diseases and represents one of the biggest problems in the public health issues . The World Health Organization estimated that the number of persons having diabetes will exceed 360 million by 2030. Diabetes mellitus affects millions of people around the world. According to the GBD (Global Burden of Disease), its prevalence increase was about 30.6% from 2005 to 2015. Type 2 diabetes mellitus was first recorded as a part of metabolic syndrome in 1988, it is characterized by insulin resistance that lead to hyperglycemia, it is caused by a variety of factors especially environmental, genetic, and behavioral ones . Obesity contributes about 50% of DM type 2 cases .Management

methods of diabetes include diet therapy, focusing on exercise therapy along with pharmacotherapy, and clinically using of α-glucosidase inhibitors, insulin, sulphonylurea, biguanide, and finally troglitazone [43].

Postprandial hyperglycemia management is an important key in the treatment of diabetes mellitus. α-Glucosidase secreted from intestinal chorionic epithelium is considered to be responsible for the hydrolysis of several carbohydrates. In 1980s, α-glucosidase inhibitors became a new class of the anti diabetic drugs. α-Glucosidase inhibitors slow down the process of degradation and absorption of carbohydrates by competitive blocking the activity of this enzyme. As noticed the peak concentration of postprandial blood glucose is reduced and so the blood sugar level can be well controlled. α-Glucosidase inhibitors can offer variety of advantages and has been recommend by the Third Asia--Pacific Region Diabetes Treatment Guidelines as the first-line in diabetes treatment for controlling postprandial hyperglycemia [44].

These medications also considered to be useful for individuals taking sulfonylureas and metformin medications , which help to maintain their blood-glucose levels within a safe limit. In vitro data is also useful, particularly when a large number of compounds are to be tested, or when compounds are synthesized with minor modifications in functional groups or different percentages of extract//fractions, etc.; then a simple *in vitro* test can be performed to rule out inactive compounds and hence save considerable time and money. The presence of polyphenols [45] and

flavonoids (vitexin, tricin, naringenin, quercetin, and tricin-7-O-beta-D-glucopyranoside) in fenugreek might be responsible for such activity. The results of current works are interesting, still sufficient *in vivo* findings are required to extrapolate its use in humans [46].

Chapter Two

Methodology

2.1.General background

Utilization of natural products in the development and formulation of new effective medications have been increased over the last years . If phenolic and flavonoids compounds present in high content in medicinal plants that exert antioxidant activities this will make these plants play a role in the prevention of the development of oxidative stress disease [47]. Regarding to the bioactive phytochemicals in these medicinal plants and the interest towards the natural products in several pharmaceutical industry, this research will be an important challenge on conventional drugs development. Several methods that use solvents in the procedures for extraction such as maceration will critically be influenced by the different solvents types. Usually no effect will be observed by the solvent volume used on the biologically active compounds in the poplar type propolis at ratio [1 :10 w:v], suggesting that using of solvents at greater ratio is not necessary [48].

2.2. Plant material collection and preparation before extraction

Opuntia ficus-indica cladodes and fruits of were collected randomly from many cactus trees in north Palestine in June and July 2017.. Taxonomical identifications were established by the pharmacognosist Dr. Nidal Jaradat at the Pharmacognosy Laboratory at An-Najah National University.

After washing plant cladodes well, the peel removed after that dried in the oven at 45ºC and finally the cladodes were grounded by the mechanical grinder into a fine powder and kept in an airtight containers with suitable labeling for future use.

On the other hand Cactus fruits were harvested at maturity (sugar12-14 Brix).since all of samples originated from the same geographical location and were harvested at full maturity, the impact of climate or maturity degree should be excluded.

2.3. Chemical reagents for in-vitro investigation tests of plant extracts:

Liquid and solid Chemical reagents used in the experimental part of this research were listed in table (2.1).

Table (2.1): Liquid and solid Chemical reagents.

Chemicals and reagents	Supplier	Supplier country
DMSO	Riedel-de-haen	Germany
Methanol 99.9%	Lobachemie	India
Hexane	Frutarom LTD	Israel
HCl	SDFCL	India
Benedict's reagent	Alfa-Aesar	England
Ninhydrin solution	Alfa-Aesar	England
Molisch's reagent	Alf-aAesar	England
Folin-Ciocalteu's reagent	Sigm-aAldrich	USA
Iodine	Riedel-de-haen	Germany
$FeCL_3$	Riedel-de Haen	Germany
H_2SO_4	Alfa-Aesar	England
Trolox	Sigma/Aldrich	USA
DPPH	Sigma/Aldrich	USA
*p*NPG	Sigma/Aldrich	USA
Acarbose	Sigma/Aldrich	USA
α-glucosidase (Baker's Yeast alpha glucosidase)	Sigma/Aldrich	USA
α-amylase	Sigma	India
DNSA	Sigma/Aldrich	USA
potassium phosphate	Sigma/Aldrich	USA

2.4. Instrumentation

Instruments that were used in this research were listed in table (2.2).

Table (2.2): Instruments used in the experimental part.

Instrument	Supplier	Supplier Country
Rotary evaporator	Heidolph OB2000-VV2000	Germany
Freeze dryer	Mill rock technology-model BT85	China
Grinder	Moulinex model- Uno	China
Balance	Radwag- AS 220/c/2	Poland
Filter papers	Macherey-Nagel, MN 617and Whatman no.1	USA
Micropipette	Mrc	Israel
Oven	Arilevy	Israel

2.5. Preparation of four different extract fractions from *Opuntia ficus-indica* cladodes and fruit juice extract

A. *Opuntia ficus-indica* cladodes extract preparation

The dried powder of *Opuntia ficus-indica* cladodes was extracted by fractionating method by adding solvents in sequent manner depending on their polarities beginning with the first non polar solvent; hexane then acetone (polar a protic organic solvent) after that methanol (polar alcohol) and finally distilled water (polar protic solvent). For the preparation of each extract fraction about 25 g of the grounded dried cladodes has been subjected first to 0.5L hexane for 72 hr in a shaker device at 100 rounds per minute at 25ºC then hexane was replaced by 0.5L acetone in the same manner methanol and water were used . each organic fraction was filtered and concentrated under vacuum on a rotarry

evaporator device , while the aqueous fraction was dried using a freeze dryer. Finally, all crude fractions were stored at 4°C in the refrigerator for further use [49].

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

Yield% = (weight of extract /dry cladode weight) × 100%

B. Fruit juice extract preparation

The collected fruits of nopal cactus were peeled and squeezed by hand and then juices were filtered using gravity filtration and collected in a separate container to be freeze dried and finally the powdered extract was kept in a closed container at 4ºCin the refrigerator for further use .

The yield of juice extract was calculated using the following formula

Yield% = (Juice extract weight /fruit weight) × 100%

2.6. Qualitative phytochemical screening tests for *Opuntia ficus-indica* cladodes and fruit juice extracts

Phytochemical analysis conducted on the nopal cactus cladodes different extracts and fruit juice extract to reveal if any active compounds which exert some medicinal or physiological activity will be present , examples of these active phytochemicals are Phenols, Alkaloids, Flavonoids, Saponins, Tannins, and Resins. While, fruits showed the presence of Phenols, Alkaloids, Flavonoids, Terpenoids, Steroids [50,51].

Alkaloid identification test

***Wagner's test*:** about 2 mg of the extract was acidified with 1.5 % v/v of diluted hydrochloric acid and adding three drops of Wagner's reagent. If yellow to brown precipitate was appeared; this indicated that alkaloid is present in tested sample.

Glycoside identification test

Keller-Killiani test: about three drops of ferric chloride solution were mixed with tested solution. When concentrated sulphuric acid was added, it will form two layers, if lower layer reddish brown this indicates glycosides.

Tannin identification test

***Gelatin test*:** Test solution was treated with a gelatin solution if gives white precipitate this confirmed that Tannin compound is found in tested sample.

Saponin test

Foam test: about 5 ml of extract was mixed with two to five drop of sodium bicarbonate solution . The tube was vigorously shaken and left for about 3 minutes. Formation of honey comb like froth indicated the presence of saponin.

Phyto Steroid identification test

***Liebermann--Burchard's test*:** about 2 mg of dry extract was dissolved in acetic anhydride then heated to boiling, after that cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. The appearance of green color indicated the presence of phyto-steroids.

Terpenoids identification test

***Salkowaski test*:** add a few drops of concentrated sulphuric acid to the test solution resent in test tube , shaking well and allowed to stand, if lower layer turns yellow this indicated the presence of Terpenoids.

Proteins and amino acids identification test

Ninhydrin test: 2 ml of 0.2% Ninhydrin solution was heated with the 2mg of Crude extract, appearing of violet color indicated the presence of proteins and amino acids.

Reducing sugars identification tests :*Benedict's test*; about 2 ml of Benedict's reagent was boiled with a crude extract, a reddish brown color indicated the presence of the reducing sugars like sucrose.

Starch identification test

***Iodine test*:** about 2 ml of iodine solution was mixed with crude extract. Appearance of Purple or dark blue color confirmed the presence of starch.

Complex polysaccharides (like fibers) test

Molisch's solution test: Shake 2 ml of Molisch's solution with crude plant extract then add 2 ml of H_2SO_4 concentrated and poured carefully along the side of the test tube. a violet ring appeared at the inter phase of the test tube indicated the presence of fibers and other complex polysaccharides.

Phenols identification test

Ferric chloride test: about 2mL of 2% solution of $FeCl_3$ was mixed with crude extract. If Black or blue-green color appeared this indicated the presence of phenols.

2.7. Free radical scavenging assay for Antioxidant activity

Free diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging assay was the used protocol in our project to measure activity of the different extract fractions as antioxidant agents [52].

1000 □g/ml methanolic stock solution was prepared for each plant extract fraction as well as for Trolox (the used standard reference compound with a potent antioxidant activity). serial dilutions were then prepared from the previous stock solution to make the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 and 100 µg/ml) . 1mL of each plant working solution was mixed 1mL of DPPH (0.002 g/mL) methanolic solution (the used free radical) which must be freshly prepared and 1mL methanol was then added to the previous mixture . The

blank solution of the series concentrations was DPPH with methanol only in a ratio of 1:1. The solutions were incubated at room temperature (25ºC) in a dark place for about 30 minutes. Then, their optical densities were determined by using the spectrophotometer at a wave length of 517 nm.

Activity as an antioxidant agent the following equation was used to calculate % DPPH inhibition for each plants extract fraction and the Trolox compound:

DPPH inhibition % = $(A_B-A_T)/A_B \times 100\%$

A_B is the recorded absorbance of the control solution

A_T is the recorded absorbance of the tested sample solution.

2.8. In-vitro evaluation of α-amylase inhibition activity of each extract fraction of *Opuntia ficus-indica* cladodes and fruit juice

The α-amylase inhibitory activity of each extract fraction was carried out according to the standard method with minor modification. Each extract fraction was dissolved in 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). A volume of 0.2mL of porcine pancreatic α-amylase enzyme solution with concentration of (2 units/ml) was mixed with 0.2mL of the plant fraction and was incubated for 10 min at 30 °C. Thereafter, 0.2mL was added then from the 1% starch

aqueous solution which must be freshly prepared to each test tube and after that be incubated for at least 3 min. The reaction was stopped by the addition of 0.2mL di nitro salicylic acid (DNSA) color reagent and was then diluted with 5 ml of distilled water and then boiled for 10 min in a water bath at 90 °C. The mixture must be cooled to reach room temperature, and the absorbance was taken at 540 nm . The blank was prepared following the same quantities but replacing the plant fraction with 0.2mL of previous buffer. [53].

Acarbose was used as standard reference following the same previous steps

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

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

As given in equation:

A_B: is the absorbance of control

A_T: is the absorbance of tested sample

2.9. In-vitro evaluation of α-glucosidase inhibition activity for each extract fraction of *Opuntia ficus-indica* cladodes and fruit juice

The α-glucosidase inhibitory activity of each extract fraction was carried out according to the standard protocol with some modification [54].In each test tube a reaction mixture containing 50 µl phosphate buffer (100 mM, pH = 6. 8), 10 µl alpha-glucosidase (1 U/ml), and 20 µl of varying concentrations of extract and fractions (100,200,300,400 and 500 mg/ml) then incubated at nearly 37°C for 15 min. after this preincubation 20 µl of (5 mM) PNPG was added as a substrate of the reaction and again incubated further at 37°C for 20 min. The reaction was terminated by adding 50 µl Na_2 CO_3 (0.1M). The absorbance of the released p-nitrophenol was recorded at 405 nm wave length . Acarbose at same concentrations as plant extract was used as appositive control

The results were expressed as percentage inhibition, which was calculated using equation below:

Inhibitory activity (%) = $(A_B - A_T/A_B) \times 100\%$

Where,

A_B is the absorbance without enzyme inhibitor

A_T is the absorbance of test sample that contain enzyme inhibitor.

Chapter Three
Results

3.1. Quantitative Phytochemical screening tests for determination of bio-active compounds in each extract fraction

Following the mentioned phytochemical laboratory tests in the methodology part of this thesis ; the different extract fractions of *Opuntia ficus-indica* cladodes and fruit juice contained variety of phytochemical active ingredients which were summarized in (Table 3.1).

It was observed that aqueous extract fraction was rich in polysaccharides which may be fiber , glycosides, proteins , glycosides and phenols while methanol fraction contained flavonoids ,phenols, alkaloids , glycosides and steroids.

On the other hand Tannin compounds appeared in both acetone and hexane extract fractions.

Also fruit juice extract was rich in polysaccharides ,saponin, flavonoids , phenols ,glycosides .

Table (3.1): Phytochemical screening tests for different extract fractions of *Opuntia ficus-indica* cladodes and fruit.

Phytochemical compound	**Aqueous extract**	**methanol extract**	**Acetone extract**	**Hexane extract**	**Fruit juice (freeze drying)**
Protein& amino acids Ninhydrin test	+	-	-	-	+
Reducing sugars Benedicts test	-	-	-	-	+
Complex polysaccharides Molisch's' test	+	-	-	-	+
Starch Iodine test	-	-	-	-	-
Phenols Ferric chloride test	+	+	-	-	+
Tannins Gelatin test	-	-	+	+	+
Flavonoids Alkaline reagent	-	+	+	-	+
Saponin Foam test	+	-	-	-	+
Glycosides Keller-Killiani test	+	+	+	+	+
Steroids Salkowaski test	-	+	+	+	-
Terpenoid Salkowaski test	-	-	+	-	-
Alkaloids (Wagner's test)	-	+	+	+	-

From these observations of phytochemical screening tests we found that each used solvent in the extraction process was suitable for some active compound and not for others as mentioned above polysaccharides and saponin appeared in the aqueous layer while flavonoid and phenols

appeared in methanol layer; this may be explained due to the polarity of each solvent.

For the results related to %yield of each extract fraction it was found that

The highest yield was achieved in aqueous fraction which was 28.8%, also the fruit juice extract was also high with 28% yield

Table (3.2): The yield percentage of *Opuntia ficus-indica* cladodes extract fractions and fruit juice

Extract Fractions	**Extract (g)**	**Plant material (g)**	**Yields, %**
Hexane	1.55g	25 g	6.2%
Acetone	1 g	25 g	4%
Methanol	0.94 g	25 g	3.76%
Aqueous Fruit juice	7.2g 280g	25 g 1000g	28.8 % 28%

3.2. Free radical scavenging assay for antioxidant evaluation

For free radical scavenging activity the four different plant extract fractions and fruit juice was evaluated by DPPH radical protocol using Trolox as a potent antioxidant reference. The results for DPPH percentage inhibition were shown in table (3.3) and figure(3.1).

Table (3.3): The DPPH inhibition percentage of *Opuntia ficus-indica* cladodes extract fractions and fruit juice compared to Trolox (reference compound) and values of IC_{50}.

Conc □□g/mL)	Trolox	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction	Fruit juice
0	0	0	0	0	0	0
1	32.9±1.23	3.18±0.49	6.05±0.83	53.5±0	28.98±0.3	6.05±0
2	58.12±1.75	10.19±0.75	17.51±1.33	53.82±0.79	28.98±0.52	17.51±1.14
3	69.85±2.23	10.19±0.62	21.65±0	54.14±0.63	35.03±0	25.9±2.22
5	75.8±1.62	10.19±1.77	24.52±0	54.14±0.83	35.98±1.03	35.12±0
7	80.12±2.1	10.9±0.62	28.6±1.03	54.46±0.3	39.8±1.03	37±03
10	83.22±1.52	11.14±1.15	29.93±1.7	56.05±0	43.3±0	40.9±0
20	87.25±1.85	11.14±0	30.25±1.24	59.55±0	43.3±1.24	44±1.14
30	88.5±1.3	13.05±0	37.89±0.75	59.55±1.15	46.17±0.59	44.8±2.22
40	88.65±1.82	13.05±0.57	45.22±1.24	59.55±0.75	49.68±1.7	58.12±0
50	92.3±1.34	21.33±0.63	46.81±0.21	59.55±0.75	53.18±0.49	58.12±1.52
80	96.31±2.25	21.33±0.78	52.22±0.3	62.42±0.49	57.96±0	76.34±2.2
100	97.12±1.4	21.33±0.41	52.22±0	65.92±0.62	60.5±0.21	83.95±1.44
IC_{50} (µg/mL)	2.09±1.7	316227±0.72	74.13±0.53	6.16±0.59	31.62±0.65	19.49±1.08

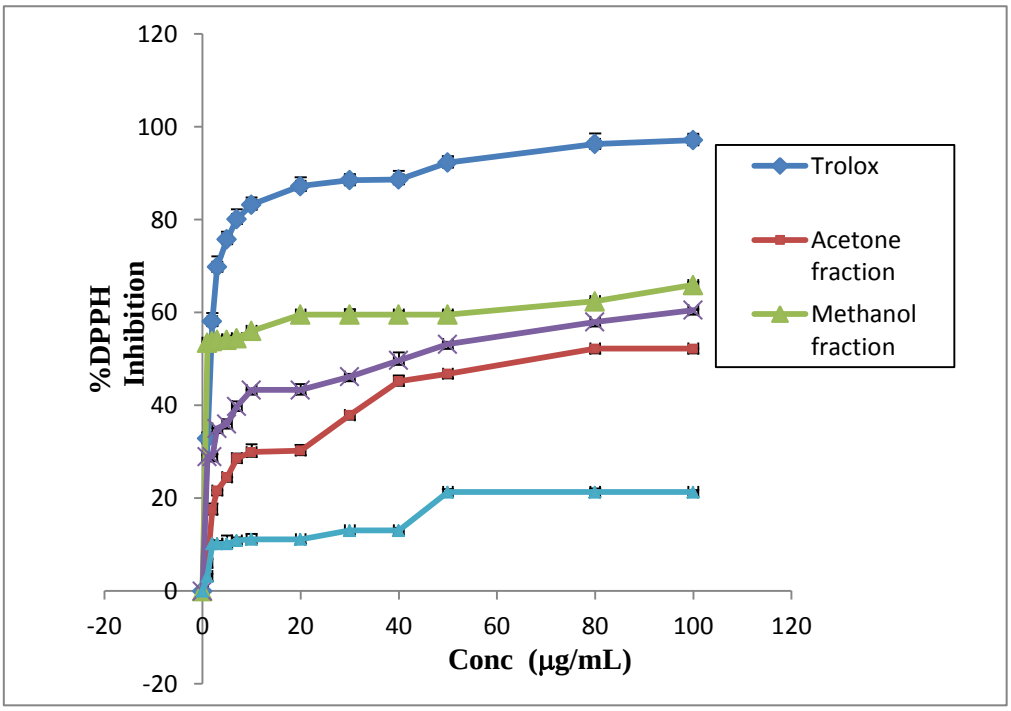

Figure (3.1) : % Inhibition of DPPH by standard reference; Trolox standard and cladodes different extract fractions.

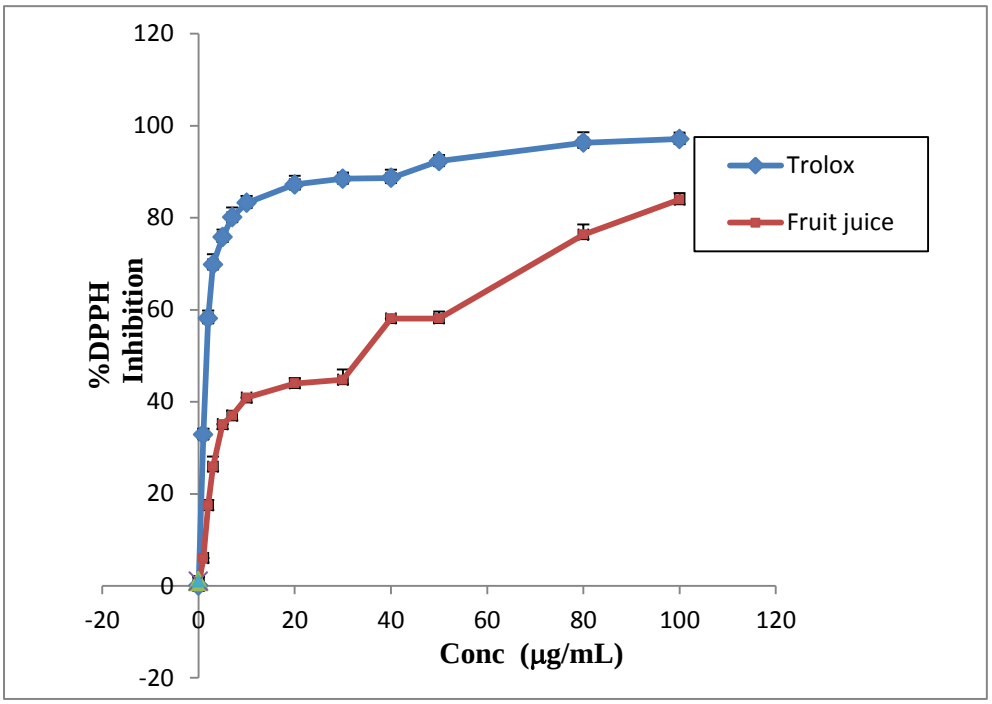

Figure (3.2) : % Inhibition of DPPH by standard reference; Trolox standard and fruit juice extract**.**

3.3. α- amylase inhibitory activity of *Opuntia ficus-indica* cladodes and fruits extracts

The different *nopal cactus* cladodes extract fractions and the fruit juice were evaluated by in-vitro assessment method on α-amylase enzyme and the results were summarized in figures (3.3) and (3.4) as well as in table (3.4) .

Table (3.4): α-amylase inhibitory activity of *Opuntia ficus-indica* cladodes fou fractions and fruit juice extract compared with Acarbose (reference compound)

Conc (µg/mL)	Acarbose	Hexane Fraction	Acetone Fraction	Methanol fraction	Aqueous fraction	Fruit Juice
0	0	0	0	0	0	0
10	53.22±1.2	49.7±0.3	54.94±0.83	25±0.75	65.69±1.15	36.37±0.63
50	54.91±0.58	63.66±0.67	65.4±0.79	25±0.75	65.69±1.15	36.37±0.63
70	66.1±1.34	63.95±0.52	65.4±0.79	35.75±0.62	68.31±0.59	55.77±0
100	66.1±1.62	63.95±0.52	67.44±1.03	35.75±0.62	73.54±0.48	55.77±0.83
500	72.54±1.37	64.53±0.74	67.44±1.03	61.43±0.78	77.03±0.49	55.77±0.83
IC_{50} (µg/mL)	**28.18±1.22**	**31.62±0.55**	**25.11±0.89**	**309±0.7**	**16.98±0.77**	**97.72±0.58**

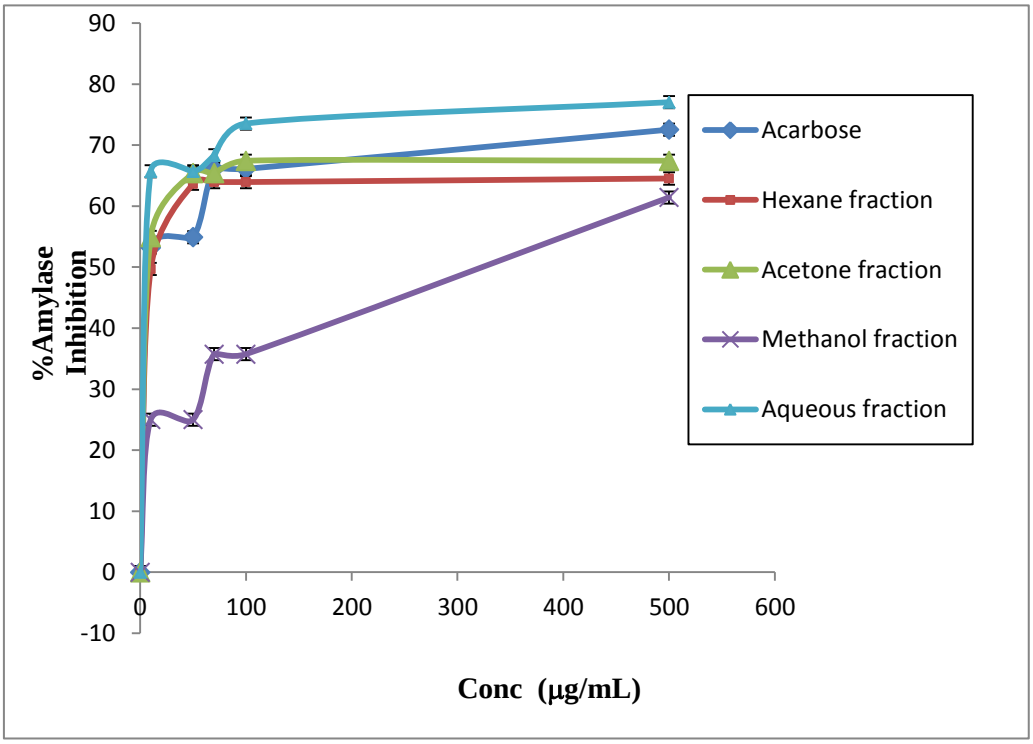

Fig (3.3) : % Amylase inhibition by each extract fraction of *Opuntia ficus-indica* cladodes and Acarbose on the activity of porcine pancreatic α-amylase.

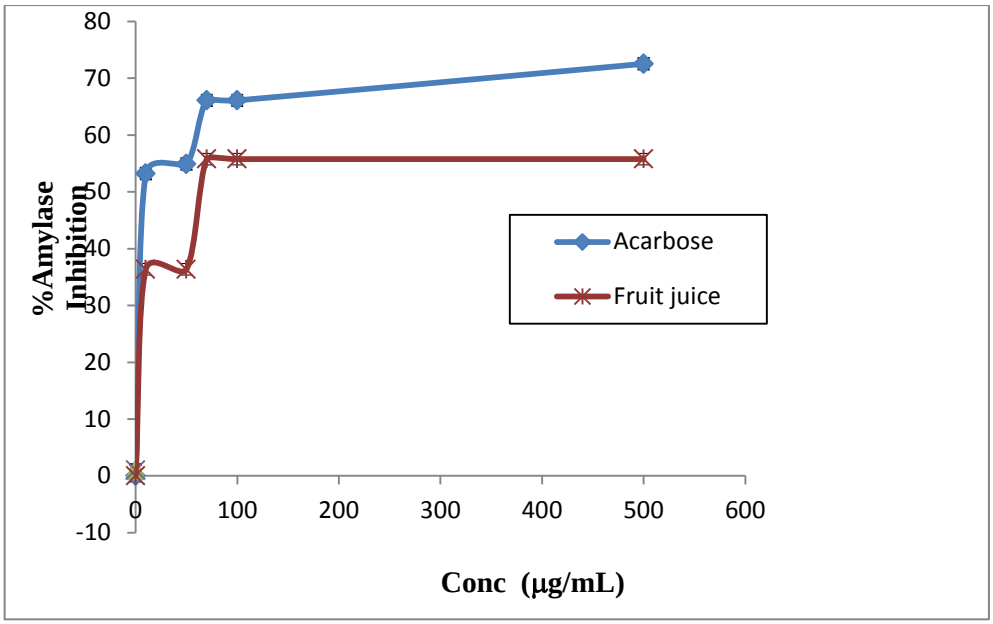

Fig (3.4) : % Amylase inhibition by each extract fraction of *Opuntia ficus-indica* fruit juice and Acarbose on the activity of porcine pancreatic α-amylase.

3.4. α-Glucosidase inhibitory activity of *Opuntia ficus-indica* cladodes and fruits extracts

The results of inhibitory effects of cladodes extract and fruit juice extract was established in figures (3.5) and (3.6) as well as in table (3.5).

Table (3.5): α-glucosidase inhibitory activity of *Opuntia ficus-indica cladodes* four fractions and fruit juice compared with Acarbose.

Conc (µg/mL)	Acarbose	Hexane Fraction	Acetone Fraction	Methanol fraction	Aqueous fraction	Fruit Juice
0	0	0	0	0	0	0
100	65.5±0.5	25.5±0.5	25.75±0.75	16±1	50±0.3	32.25±1.06
200	67.5±0	40.5±2.5	54±2	33±0	54±2	40.5±0
300	73.5±0.33	40.5±2.5	62.21±0.78	44.5±1.5	66.5±1.5	49.75±0.77
400	85.1±0.79	64.33±0.52	66.5±1.5	44.5±1.5	66.5±1.5	62.55±0.63
500	92.15±0.6	65.26±0.74	66.5±1.5	68.47±1.03	77.51±0.49	65.26±0.74
IC_{50} (µg/mL)	**38.02±0.44**	**251.18±1.3**	**125.89±1.31**	**501.18±1.01**	**79.43±1.16**	**199.52±0.64**

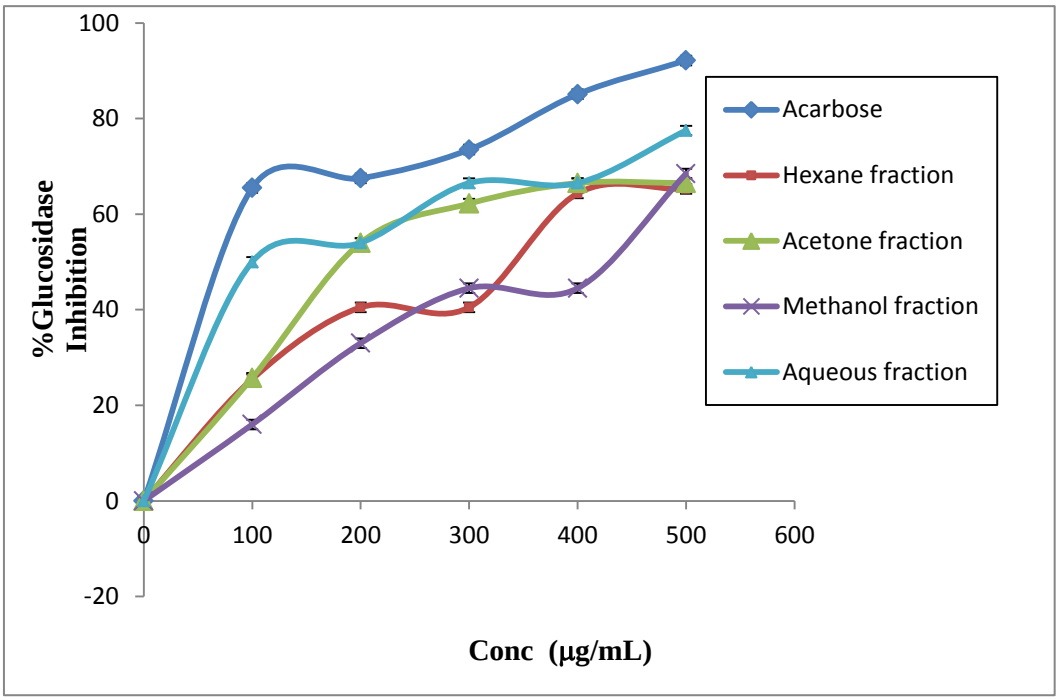

Fig (3.5) : % Glucosidase inhibition by each extract fraction of *Opuntia ficus-indica* cladodes and Acarbose on the activity of α-glucosidase.

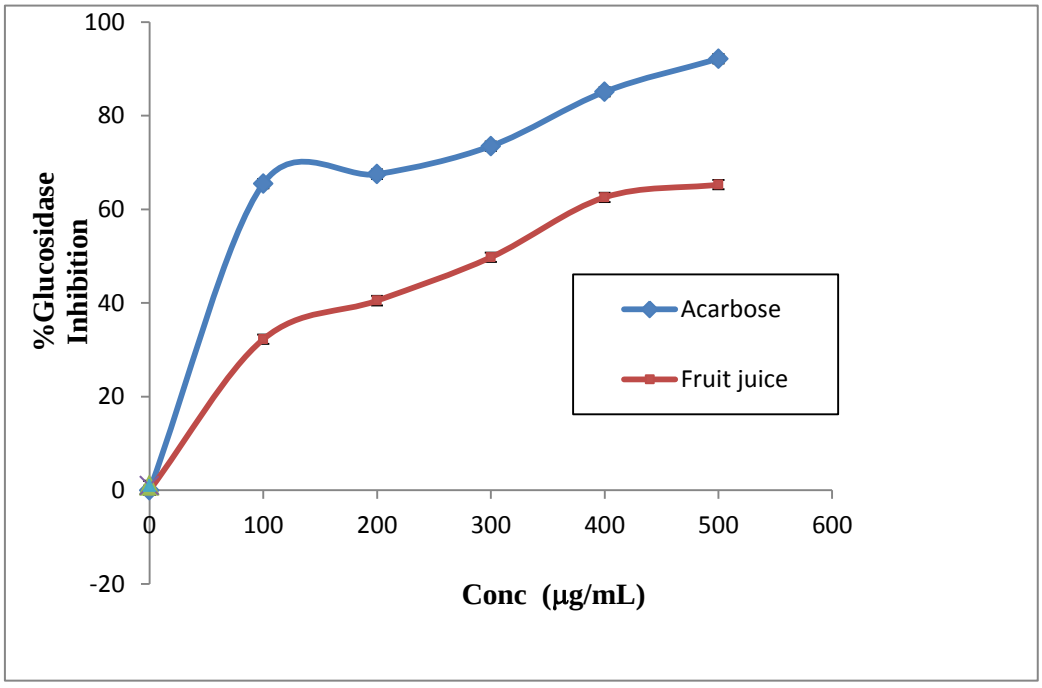

Fig (3.6) : % Glucosidase inhibition by each extract fraction of *Opuntia ficus-indica* fruit juice and Acarbose on the activity of porcine α-glucosidase.

Chapter Four

Discussion and Conclusion

4.2. Free radical scavenging assay for antioxidant evaluation of *Opuntia ficus-indica* cladodes and fruits extracts

As proposed about foods and raw plant extracts with IC_{50} values <50 µg/mL they will exert a high antioxidant capacity [55]. Therefore, *Opuntia ficus-indica* cladodes could be considered with high antioxidant capacity especially for methanolic extract which recorded IC_{50} value equal to 6.16±0.59 µg/mL and aqueous extract with IC_{50} value equal to 31.62±0.65µg/mL (Table 3.3). The same result was observed for fruit juice extract that showed potent antioxidant activity with IC_{50}value equal to 19.49±1.08 µg/mL these results were compared to Trolox which a reference compound that exerted potent antioxidant effect with IC_{50}value equal to 2.09±1.7µg/mL . Interestingly, as observed cladodes showed a higher capacity to donate electrons than hydrogen atoms in the DPPH scavenging assay as lower IC_{50} was recorded comparing with fruits.

On the other hand acetone extract showed weak antioxidant activity with higher (IC_{50}) value which was 74.13±0.53 µg/ml and hexane extract was totally in active in antioxidant assay. From these results we can confirm that the presence of phenols , flavonoids in a plant will form a powerful scavenger for free radicals like DPPH that used in this study; these two phytochemicals were appeared in methanol, and fruit juice extracts while

hexane and acetone extracts did not contained these phytochemicals as given above in screening tests.

As usually known Phenolic compounds are classified as secondary metabolites produced by plants; they form one of the most common and wide spread substances in medicinal plants that act as anti-oxidants, as they protect cells and body chemicals from oxidative damage, which caused by free radicals and reactive atoms that cause our bodies. It has been observed that these phytochemicals deactivate the substances that promote the formation of tumors in human bodies [56, 57].

In addition flavonoids are a large class of natural phenolic compounds usually present in fruits, vegetables, grains, bark, roots, stems, flowers, tea, and wine . These compounds exhibited antioxidant activity and ensure healthy circulation, flavonoids also help to strengthen capillary walls. These natural compounds, at times, are referred to as phyto-estrogens.

Phyto-estrogens are considered to be associated with reduction of menopausal symptoms, curing some osteoporosis cases , improvement of cholesterol levels in the blood and reducing the risk of certain factors related to coronary heart diseases and cancer [58,59].

4.2. α- amylase inhibitory activity of *Opuntia ficus-indica* cladodes and fruits extracts

The activity of the reference compound Acarbose against this enzyme was used for comparative purposes with its IC_{50} value equal 28.18±1.22 µg/mL

, it seems that aqueous extract was the most potent fraction in α-amylase inhibitory effect with IC_{50} value equal to 16.98±0.77 µg/mL followed by acetone extract with IC_{50} value equal to 25.11±0.89 µg/mL, the third extract fraction with high potency against this enzyme was hexane fraction with IC_{50} value equal 31.62±0.55 µg/mL, while methanol fraction recorded the least value of the half maximal inhibitory concentration IC_{50} which was 309±0.7µg/mL . on the other the fruit juice showed moderate activity on α-amylase enzyme as its IC_{50} value was 309±0.7µg/mL .The previous results confirmed that the potent inhibitory effect on α-amylase by aqueous extract fraction may be referred to the presence of Saponin compounds which are heterogeneous group of natural products found in many plant-derived foods and medicinal plants; which exerted some biological and pharmacological activities including anti-inflammatory, tonic for liver , wound healing, expectorant and hypoglycemic effects [60]. As Known Traditionally saponin compounds have been widely used as detergents, pesticides and molluscicides, in addition to these industrial applications as foaming and surface active agents they have also some beneficial effects on human health [61].

While other extract fractions did not contain saponin as the previous phytochemical screening tests showed. Also this potent effect of aqueous extract may be due to the presence of polysaccharides which may be fibers that noticed in some researches to have hypoglycemic effect; for instance, as observed in previous researches ;crude polysaccharide from *purslane* had hypoglycemic effect in mice [62].

It was observed also that both acetone and hexane extract fractions recorded significant inhibitory activity against α-amylase but less potent than aqueous fraction this result may be due to the presence of tannin compounds in these fractions which have some hypoglycemic effects. Since tannins have been identified in other previous researches as an active anti diabetic and anti-adipogenic components in *Lagerstroemia speciosa*, a marine member of Family *Lythraceae* , which is rich in tannin that may show hypoglycemic property [63,64].

4.3. α-Glucosidase inhibitory activity of *Opuntia ficus-indica* cladodes and fruits extracts

α-Glucosidase; carbohydrate digesting enzyme inhibitors are classified as a third category of oral hypoglycemic agents. Several inhibitors of α-glucosidase, such as acarbose and voglibose are found in natural plant sources, they show effective stabilization of blood glucose levels after food intake and have been now used clinically in the treatment of diabetes mellitus . Only a few α-glucosidase inhibitors are commercially available. All of them contain sugar moieties and their synthesis involves tedious multistep procedures. In addition, clinically they have been related to variety of serious side effects especially for gastrointestinal system [65,66].

The pathway of α-Glucosidase in human bodies based on that this enzyme exhibited a central role in controlling postprandial hyperglycemia, as it breaks down α-1,4-glucosidic linkages of disaccharides, to produce finally simple easily absorbed sugars [67].

All nopal cladode extract fractions showed inhibitory activity against α-glucosidase which is a carbohydrate digestive enzymes, presenting different % inhibition activity against α-glucosidase (Data shown in table 3.5), after calculating IC_{50} values the aqueous fraction showed the most potent activity in enzyme inhibition with IC_{50} value equal to 79.43±1.16□g/mL followed by acetone fraction IC_{50} equal to 125.89±1.31 □g/mL while both hexane and methanol fractions showed less activity against this carbohydrate digestive enzyme with IC_{50} value equal to 251.18±1.35 □g/mL and 501.18±1.01 □g/mL respectively.

Comparing these results to Acarbose; a drug widely used to inhibit α-glucosidase digestive enzyme, which presenting IC_{50} values of 38.02±0.44 μg/ml, nopal cladodes exhibit a moderate inhibitory activity against these enzymes as compared to Acarbose as for aqueous fraction and weak activity as appeared in hexane and methanol fractions.

On the other hand fruit juice extract also showed moderate activity against α-glucosidase enzyme with IC_{50} equal to 199.52±0.64□g/mL compared to Acarbose value. Again the presence of both saponin compounds and polysaccharides that may be fibers in the aqueous extract fraction confirmed the activity of these two phytochemicals as hypoglycemic agents.

However, the chemical synthetic medications which acts as potent inhibitors for α-amylase and α-glucosidase frequently exert some gastrointestinal tract adverse effects , such as flatulence, diarrhea, and abdominal cramps Therefore, valuable trials have been interested on developing safe and effective α-amylase and α-glucosidase inhibitors as functional anti-diabetic medication from natural sources [68,69]. So finally it can be confirmed as in the last decade that the nutritional and health benefits capacity of this cactus has been reported by many academic scientists and some private companies. As observed in previous researches that Several clinical studies on the species *Opunita* have been performed; supporting its use as an anti-hyperglycemic medication. However, its mechanism of action in hypoglycemic activity has been partially understood [70].

3.5. Conclusion

The obtained data from this project on both *Opuntia ficus-indica* cladodes and fruit juice imply that the aqueous fraction from cladodes and the extract of fruit juice exert a significant inhibitory activity on α-amylase and α-glucosidase enzymes. Also for methanol fractions of cladodes extract a potent free radical inhibitory activity was observed.

These obtained results are attractive data to be in vivo studied in the further and to be applied in natural pharmaceutical dosage forms .In order to establish a valuable treatment for diabetes mellitus and oxidative stress cases.

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جامعة النجاح الوطنية

كلية الدراسات العليا

المحتوى الكيماوي لنبات الصبار وتأثيره المضاد لمرض السكري

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الملخص

من تحديات الصناعات الدوائية تطوير العديد من العقاقير ولا سيما الطبيعية منها في محاولة لإنهاء بعض المشاكل الصحية كالسكري والإجهاد التأكسدي اللذان يعتبران المحور الأساسي في هذا البحث لتحضير مستخلصات متنوعة من ألواح وثمار نبات الصبار باستخدام مذيبات مختلفة القطبية مثل الهكسان والأسيتون والميثانول والماء المقطر ومن ثم تطبيق مجموعة من الفحوصات الكيميائية النوعية لمعرفة التأثيرات الطبية لهذه المستخلصات. وقد تم ملاحظة أن المستخلص المائي كان غنيا بمركبات الصابونيين ومتعددات التسكر(كالألياف النباتية) بينما ظهرت مركبات الفينول والفلافونيد في مستخلص الميثانول وهي من المركبات الحيوية التي لها دور فعال كمضادات أكسدة طبيعية.

وتم تدعيم هذه النتيجة من خلال تطبيق هذه المستخلصات المتنوعة على الأنزيمات الهاضمة للنشويات وأهمها أنزيم α-amylase وأنزيم α-glucosidase حيث أظهر المستخلص المائي لألواح نبات الصبار وكذلك مستخلص عصير ثمار الصبار نتائج لافتة في تثبيط هذين الأنزيمين وبالتالي يمكن لهذا المستخلص أن يشكل بالمستقبل بديلا طبيعيا للأدوية الكيميائية المستخدمة في ضبط مستويات السكر في الدم لمرضى السكري. وقد ترجع فاعلية هذه المستخلصات لاحتوائها على مركبات الصابونيين والألياف التي تبطئ عملية الهضم وتقلل من سرعة تحول النشويات الى سكريات بسيطة يمكن امتصاصها بسرعة .وقد تم استخدام مركب Acarbose لغرض المقارنة في الفحوصات الكيميائية على الأنزيمين المذكورين.

من جهة أخرى أظهر مستخلص الميثانول لألواح نبات الصبار تأثيرا واضحا على مركب DPPH

الذي يعتبر من الشواذر الحرة المسببة للإجهاد التأكسدي في أجسام الكائنات الحية وتعزى هذه النتيجة الى احتواء هذا المستخلص على مركبات الفينول والفلافونيد المضادة للأكسدة وقد تم استخدام مركب Trolox لغرض المقارنة في الفحص المضاد للأكسدة.

وبهذه النتائج التي تم التوصل إليها في هذا البحث يمكن لألواح وثمار نبات الصبار أن تشكل تحديا بالمستقبل للصناعات الدوائية الطبيعية التي قد تشكل الخيار الأفضل للعديد من المرضى مقارنة بالعقاقير الكيميائية التي تسبب العديد من الآثار الجانبية على الجسم.