Deanship of Graduate Studies

Al-Quds University

Favorable attributes and biological effects of Palestinian Botanical Extracts of Punica Granatum

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M.Sc Thesis

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Favorable attributes and biological effects of Palestinian Botanical Extracts of Punica Granatum

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Thesis Approval

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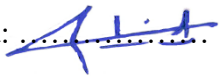
Dedication

I dedicate this thesis, first, to my beloved mother and father, Nahla and shqair, I would not be the person I am today if it was not for them. Second, I would like to dedicate this work to my brothers Ahmad& Mohammad , my lovely sisters Jumana & Amal whom always supported me in all hard times in my work, thirdly to my friends especially Alaa, Tala whom supported me in my journey to achieved degree in master. Third, dedicated to every staff in Al-Quds University whom help me with patience & love. Last but not least I dedicate this thesis to all merciful souls all over the world.

Declaration

I certify that this thesis submitted for the degree of master is my own research, expect where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for higher degree to any other university or institution.

Signed: 

Enas' S. A. shqair

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Abstract

Pomegranate (Punica granatum L.) is an ancient tree in Palestine and the Arabic world that has many benefits in food, cosmetics, health, and medicine to treat some prevalent diseases like diabetes. Non-enzymatic glycation generates a diverse set of compounds known as advanced glycation endproducts (AGEs) that accumulate in the body and cause the development of chronic diseases in humans, such as type-2 diabetes, atherosclerosis, and alzahemir. Therefore, the development of a natural AGEs inhibitor needs extensive research investigation. Pomegranate peel extracts (PPEs) with rich antioxidants such as tannin and phenols show considerable ability as natural inhibitors in treating diabetics. In the present study, the hydrozable tannins (HTs) of pomegranate peel (PP) were extracted by sonication (Extract A) and reflux (Extract B) methods with the same solvents. For the comparison with hydrozable tannins, ethanol (Extract E) and acetone (Extract F) solvents were used to extract tannins and other chemicals as a mixture. The anti-glycation production of end products was assessed with four types of extracts by using an in vitro glucose-bovine serum albumin (BSA) test. The antioxidant effects were evaluated by two methods: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and the ferric reducing antioxidant power assay (FRAP). A Folin-Ciocalteau test was used to determine total phenolic content (TPC), and a colorimetric assay was used to determine total flavonoid content (TFC). The antibacterial effects were evaluated by the disc-diffusion method to investigate the inhibition zone of each extract versus gram positive bacteria (MRSA and S.aureus) and gram negative bacteria (E. coli and pseudomonas). The obtained

results show that concentrations of 30–150 ppm of extracts indicate an inhibition action of AGE-formation. Extract A shows the highest inhibition ranges at 150 ppm by 64.62%, followed by Extract B, F, and E, with inhibitory percent's of 63.92%, 41.60%, and 31.130%, respectively. This potency in suppressing AGE end products is due to the existence of bioactive chemicals in four extracts, which was evidenced by the TPC and TFC assays. 453.675 mg QAE/g dry extract, 28.14 mg QEQ/g extract A, 409.00 mg QAE/g dry extract 22.49 QEQ/g dry extract of extract B, 335.33 mg QAE/g dry extract, 59.05 mg QEQ/gram dry extract F, 208.33 mg QAE/g dry extract, 19.37 QEQ/g dry extract. With the DPPH scavenging method, extract F shows 100% inhibition at 100 ppm, while in the FRAP assay, extract A shows higher potency than other extracts. An antibacterial assay demonstrates the ability of extracts to inhibit the growth of four types of bacteria. PPEs can be a safe and cheap alternative to treating diabetic diseases.

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Abbreviation

- CTs : Nonhydrozable tannins or condense tannins
- HTs : Hydrozable tannins
- PAs : Proanthocyanidins
- GA : Gallic acid
- EA : Ellagic acid
- TA : Tanninc acid
- PGG : Pentagalloylglucose
- TFC : Total Flavonoids Content
- TPC: Total phenolic Content
- AGEs : Advances glycation End products
- ROS : Reactive oxygen species
- MPO: Myeloperoxidase
- DPPH : 1,1-diphenyl 2-picrylhydrazyl (DPPH•) radical
- BSA : Bovine serum albumin
- QEQ: Quercetin equivalent
- GEQ: Gallic acid equivalent
- FRAP : Ferric Reducing Antioxidant Power
- HPLC: High Performance Liquid chromatography
- BHT : Butylated hydroxytoluene
- PP: pomegranate peel
- PPE: pomegranate peel extracts
- PPp: pomegranate peel powder
- S.aureus : Staphylococcus aureus
- MRSA: Methicillin-resistant Staphylococcus aureus
- E.coli: Escherichia co

Chapter one

Introduction

1.1. Pomegranate (Punica granatum L.)

Plant botanical extract is widely widespread in different sectors of industry, such as drug manufacture, food and cosmetics, as an effective, eco-friendly and safe alternative to harsh chemicals for humans or the environment. Which have many nutrition values such as vitamins, polymers, carbohydrates, and more effective secret compounds from nature.

Pomegranate (Punica granatum L.) is one of the oldest fruits in the world from ancient times, a native fruit of Persia and some surrounding countries. It was conceived that it would grow in Iran first and then reach the entire world. (1)

Figure 1: Punica granatum tree

The pomegranate fruit is one of the most economically important fruits for many countries in the production of juice and jam in the food sector. Moreover Pomegranate is a traditional medicine fruit that has been widely used in the treatment of inflammation and as an antibacterial. [2, 3] All parts of the pomegranate (fruits, flowers, peels, seeds, and leaves) are considered an important source of nutrients and are used in cosmetics and medicine. Several studies have proven the effectiveness of pomegranates in cancer treatment. [4]

A fruit consists of three separate parts: the first layer is the rind or peel, which forms the outer layer of the fruit; secondly, a thin layer of mesocarp extends between the peel and the aril; and finally, the arils contain the freshly juiced juice and seeds. [5] The seeds take up 3% of total weight, contain 30% oil, and the juice makes up 30% of the fruit weight, mainly consisting of 85% water, 10% sugars, 1.5% pectin, organic acid, polyphenols, and flavonoids such as anthocyanin and tannins. And the peeled interior membrane bulb takes up around 50% of the weight. [6, 7]

The juice contains many beneficial compounds such as acids, sugars, vitamins, polysaccharides, and polyphenols. [4] Peels are abundantly available as agro-industrial by-products. They contain many nutrient compounds such as tannins, which have many beneficial applications. The chemical composition and quantity of pomegranate fruit vary depending on the country, climate, and storage conditions. [7, 8]

1.2. Tannins

Tannins are the main components in pomegranate peel (PP). They are polyphenols with a high molecular weight with different molecular sizes and complexities that

have potency as natural antioxidants. Tannins with low molecular weight are soluble in water (20–35°C). [10] It is classified as either condensed or nonhydrozable tannins (CTs) and hydrozable tannins (HTs). [11] The two categories are characterized by different structures and molecular weights and the effects of each type on animals during digestion. [10]

Table 1: Some structures of chemical compounds in pomegranates. [9]

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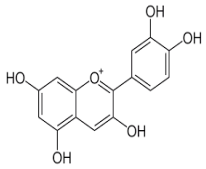
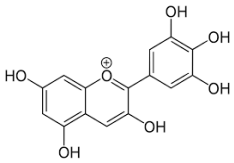
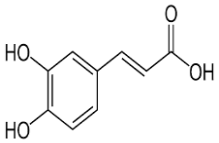
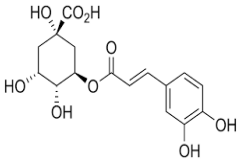
Cyanidin 	Delphinidin 	caffeic acid 	Chlorogenic acid 
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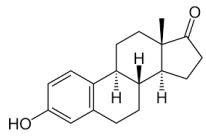
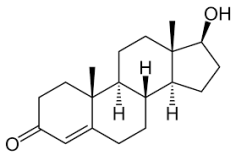
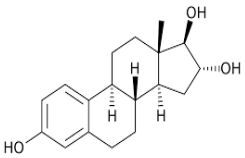
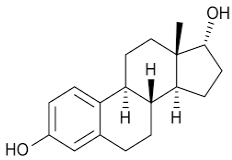
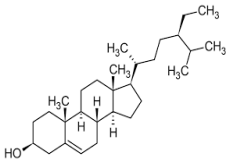
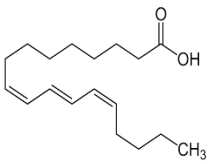
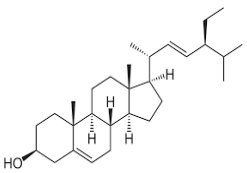
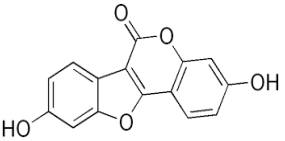
Estrone 	Testosterone 	Estriol 	17α-Estradiol 
Beta-sitosterol 	Punicic acid 	stigmasterol 	Coumestrol 
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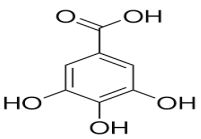
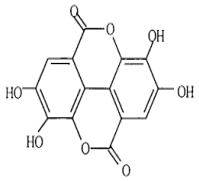
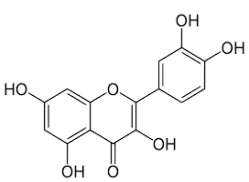
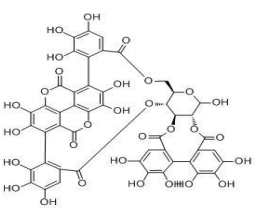
Gallic acid 	Ellagic acid 	Quercetin 	punicalagins 

1.2.1. Condense tannins

Condensed tannins or proanthocyanidins (PAs) are oligomers or polymers consisting of flavonoid units linked by carbon-carbon bonds. In an ethanol solution with heat, the condensed tannins will decompose and convert to proanthocyanidins. The most frequent structural units of condensed tannins are: (1) (+)-catechin; (2) (-)-epicatechin; (3) (+)-gallocatechin; (4) (-)-epigallocatechin; (5) (-)-epigallocatechin gallate. [12]

1.2.1. Hydrozable tannins

Tannins that are hydrozable (HTs) are compounds with a central core of carbohydrate (e.g. D-glucose). Gallotannins are molecules that are polyol esterified with gallic acid (GA) and ellagitannins are carbohydrate esterified with hexahydroxydiphenic acid as ellagic acid (EA). Generally, HTs have less concentration than CTs in a plant. [10] Pentagalloylglucose (PGG) is a basic unit of the metabolism of hydrolysable tannins and other molecules derived from it. Gallotannins is a gallic acid that surrounds the glucose unit. Ellagitannins is a hexahydroxydiphenic acid or ellagic acid that surrounds glucose. There are many different compounds that form by the formation of oxidative linkages. [12]

1.2.1.1. Gallic acid

Gallic acid (3,4,5 trihydroxybenzoic acid), whose molecular formula is($C_7H_6O_5$), is a major type of hydrozable phenolic compound in pomegranate peel, which is a secondary metabolite, has many beneficial properties in medicine, cosmetic and food as antioxidants, antimicrobial, anti-inflammatory, and anticancer. [13]

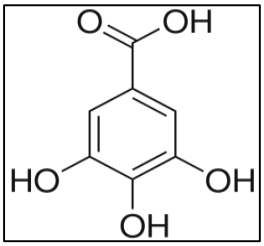


Fig 2: Chemical structure of Gallic acid [9]

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1.2.1.1. Ellagic acid

Ellagic acid (2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione), with a molecular weight of 302 g/mole, is a potent polyphenol compound with numerous applications in the pharmaceutical and cosmetic industries as an antioxidant, anti-mutagenic, anti-microbial, anti-cancer, and anti-inflammatory for chronic diseases. [14]

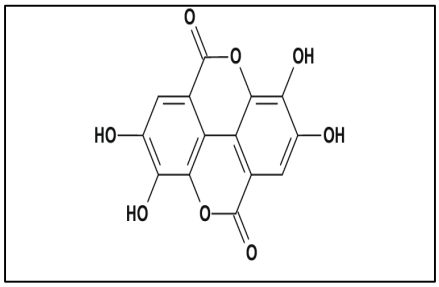


Fig3: Chemical structure of Ellagic acid [9]

1.2. Peel phenolic extraction modeling

Different types of polar and nonpolar solvents were used to extract phenol compounds from pomegranate peel powder (PPp), such as methanol, acetone, ethanol, ether, acetic acid, and ethyl acetate with different concentrations. Polar solvents have a higher tendency to extract phenol than nonpolar solvents and water. Methanol extraction has the highest antioxidant ratio. [15, 16] There are many factors affecting the ratio of antioxidants, including: solvents, pomegranate peel particle size, ratio between solvents and PPp, method of extraction and

temperature. Small particle size means more surface area, so decreasing solvent transfer time across the particles results in increased yield and efficiency of antioxidant extraction. [17] Water extraction needs a higher temperature for extraction of phenols and antioxidant compounds. The combination of water and methanol with 2% acetic acid and ethyl acetate increases the yield of EA and the DPPH radical scavenging effect sequentially by (7.06–13.63%) and (38.21–14.9 1 mg/mL). [18]

1.4. Traditional medicinal uses

Pomegranate is an ancient plant that has been used in the treatment of various diseases and inflammations such as diarrhea, intestinal worms, bleeding noses, and ulcers. The pomegranate peel was widely used as powder for topical treatments such as bleeding and other uses as aqueous extract by boiling the peel for 10–40 minutes. [19]

1.5. Anti-inflammatory and anti-allergic properties

According to a scientific consensus based on the weight of strong scientific data supporting the medicinal effects of pomegranates and their molecules, the methanol extract of pomegranate peel has the ability to reduce inflammation and allergic reactions. [20]

According to a group of researchers, PPEs has anti-inflammatory characteristics following intraperitoneal (25, 50, and 100 mg/kg) and intraventricular (10, 25, and 50 μ g/3μL/rat) administration. Experiments at the same intraperitoneal dosage levels revealed a 52–82 percent reduction in pain index and a significant decrease in albumin causing breech claw. [21]

1.6. Antimicrobial effect

Many studies have shown that pomegranate fruit with rich antioxidant compounds have strong antimicrobial activity. Peel extract, which is rich in tannins, especially punicalagin, has been reported in many studies as an effective antimicrobial compound. Also, Ellagic acid compound, has a significant influence on the antimicrobial and antifungal activity. The pomegranate peel, with its rich antioxidant and phenol compounds, can be a safe alternative to synthetic preservatives, antimicrobials, and antifungals, especially in the food industry. [22]

1.7. Anticancer Activities of Pomegranate Extracts

In previous research, pomegranate extracts have shown anticancer effects in a variety of human cancer cells. [23]Pomegranate extracts have been studied for their pharmacological effects. Oily polyphenols, such as cyclooxygenase and lipoxygenase, inhibit eicosanoid enzymes. [24] In vitro and in vivo, scientific research has proven that flavonoids and tannins suppress the activity of cancer cells. [25]

1.8. Antiglycation and Diabetes complications

Advanced glycation products (AGEs) is a bimolecular result resulting from a non-enzymatic reaction between amino acids of protein, lipid, and nucleic acid with reducing sugars (Millard reaction). AGEs formation has a real impact on health, leads to organ damage and affects negatively the function of some organs such as the heart, kidney, nerves, eyes and blood vessels. Aging with poor glycemic control and increased glucose gives rise to increased formation of AGEs and, as a result, increases the oxidation reaction. In-vitro and in-vivo studies using natural compounds such as flavonoids from different plants were carried out to study the effects of these natural

compounds on AGEs formation. The mechanism of inhibition involves two methods first: Type A inhibitors (sugar competitors), which prevent the sugar attachment by transforming the amino acids and peptides; and Second, type B inhibitors react with ketose or aldose groups of sugars and prevent them from binding to proteins. [26]

1.9. Pomegranate peel extract control sugar in the blood

Hypoglycemic drugs from natural sources are a healthy substitute to synthetic drugs with undesirable side effects and are too expensive for the consumer. One of the natural alternatives that lower blood sugar is pomegranate peel extract (PPE), which contains a high concentration of antioxidants. Mss. Khalil, E. A. et al. in a clinical study on diabetic rats for 4 weeks of treatments to study the effects of PPE on the sugar and insulin levels in the blood, the mechanism of action of PPE as anti-diabetic activity by protection of the pancreas, energizing of ß cells, increasing the number of ß cells, and posterior release of insulin. [27]

1.10. Toxicological levels of PPE

PPE may theoretically cause toxicity if levels of ingestion or exposure exceed the boundary threshold. PPE and its extracts are used in a variety of culinary items; their popularity is increasing for nutritional and functional purposes, where the issue of toxicity and safety receives the most attention. In the past few years, lethal doses or concentrations of PPE and some fractionated components have been investigated in vitro and in vivo. [28]

Vidal et al. (2003) found that a hydroalcoholic pomegranate extract (whole fruit) (introduced in IP to OF-1 mice) had a satisfactory safety profile with an intense LD50 of 731.1 mg/kg body weight, this is considerably greater than the amounts used in

Cuban medicine. When toxicity in experimental animals is indeed a concern, large dosages of pomegranate extract, PPE, and fractionated components (> 2000 mg/kg body weight) are tested. At dosages up to 2000 mg/kg body weight, PPE galactomannan polysaccharide (recognized to have a toxic effect on cancerous cells) demonstrated no notable harm in BALB/c mice. [29]

1.11. Problem

Tannins in pomegranate peel require an efficient and safe extraction method and various solvents that require less effort and are more effective in application. Diabetes is the world's most common and riskiest disease. Researchers are continuously working to create natural anti-glycation drugs that are low-cost and low-risk. This study aimed to investigate the in-vitro anti-glycation activity of the different 4 types of pomegranate peel extracts (an agriculture by-product) with the study of total phenol, antioxidant, total flavonoids, and antibacterial effects.

1.12. Objective

*The main objective of this study is:

I. Studying the extraction process of peel pomegranate (agro-industrial byproducts) in different solvents and methods.

*The specific aims of this research are to:

I. Extraction of condense & hydrosable tannins from pomegranate peel.
II. Studying in-vitro anti-glycation assay, antimicrobial test, antioxidant activity, phenols, and flavonoids content for four type of PPE.

Chapter two

Literature Review

Tannic acid (TA) has been incorporated into the diet as the feed for rats to in-vivo study its potency on the functional state of the rat intestinal epithelium by measuring the nitrogen and mineral concentration in fecal excretions. TA in diet feed increases the Na ion and k ion in rat feces, and ingestion of tannin increases nitrogen exerted. As a result, oxidation of TA decreases the nitrogen hypersecretion and the quantity of glucosamine excreted, protecting the digestive mucosa by increasing mucus secretion and nitrogen in rat feces. (30)

An in-vitro study was carried out by B. H. Kroes to determine the potency of gallic acid as an anti-inflammatory agent towards zymosan-induced acute food pad swelling in mice. By measuring the scavenging of O_2^- anions by gallic acid, the result was obtained that gallic acid inhibits inflammation mainly by scavenging reactive oxygen species (ROS) and by inhibition of myeloperoxidase (MPO) release by polymorph nuclear leukocytes (PMNs). (31)

In 2004, Keiji Funatogawa's research addressed "Antibacterial Activity of hydrolyzable Tannins Derived from Medicinal Plants against Helicobacter pylori." In this study, the antibacterial efficacy of 40 plant-derived compounds was evaluated against H. pylori in cultured cells. All biodegradable tannins tested had a potent antibacterial impact against H. pylori. The results obtained confirmed the ability of HTs as antibacterial, especially monomeric HTs, to have strong activity against bacteria. (32)

The results of a 1999 study on 26 types of tannins as antimicrobial against gram positive, gram negative bacteria, and yeast showed moderate potency against these microorganisms: gram negative bacteria (Staphylococcus oureus, acillus subtilis), gram-negative bacteria (Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis), and yeasts. [33]

The synergistic effect of ellagic acid and quercetin was studied on cell death and proliferation-related variables in the MOLT-4 human leukemia cell line. The result showed anticarcinogenic potential at low concentration. Ellagic acid considerably enhanced the effects of quercetin (5 and 10 mole/L, respectively) in decreasing growth and autophagy. [34]

In 1997, the experimental part was carried out to measure the activity of purified EA as antitumorigenic and antipromoting. The result of the experimental part showed that feeding the mice with EA purified in pyridine or dimethylformamide reduced the lung multiplicity to 3.9 (43%), 2.9 (57%), and 2.9 (57%) tumors/mouse, respectively. [35]

Tannins have been studied against skin tumor promotion induced by ultraviolet-B radiation. Topical application of Tarapod tannic acid (TA) on mouse skin for 20 min before exposure to UV-B resulted in inhibition of tumor incidence, with 8 mg of TA inhibiting tumor yield by 70% at week 25. [36]

In 2019, Mastrogiovanni, Fabio, et al., research was conducted in vitro on the effectiveness of PPE against inflammation in Human Intestinal Caco-2 Cells and Ex Vivo Porcine Colonic Tissue Explants. In the experimental part, the Caco-2 cells were treated with different conc. of PPE (1.0, 2.5, 5.0, 10, and 25 µg/mL) for 24 hours, with an inhibition of cytotoxic effects as a result of treatment. In an ex vivo study on freshly excised colonic tissues of six 38-day-old pigs, with a range of conc. of PPE (1 µg /ml

to 25 µg/ml) resulted in 5 µg/mL and 25 µg/mL of PPE reducing the CXCL8 concentrations compared to control. As a result, the study showed that PPE at 5µg/ml consistently has a significant anti-inflammatory effect. These results indicate the anti-inflammatory ability of bioactive chemicals derived from the peel of pomegranate waste in cells and tissues of the gastrointestinal tract. [37]

In a study, ellagic acid of pomegranate rind extract showed good results as a whitening agent when taken orally. An in-vivo study showed inhibition of tyrosinase activity and ultraviolet-induced pigmentation. And these findings are consistent with inhibiting tyrosine from melanocyte proliferation and melanin synthesis. [38]

Ellagic acid has been studied to treat breast cancer (by Neng Wang in 2002); the study showed the ability of EA in nontoxic concentration to have anti-angiogenesis effects via the VEGFR-2 signaling pathway in breast cancer. It was discovered that ellagic acid suppressed a number of VEGF-induced angiogenesis activities, including endothelial cell proliferation, migration, and tube formation. In addition, it reduced the activity of VEGFR-2 tyrosine kinase and downstream signaling pathways in endothelial cells, including MAPK and PI3K/Akt. Ellagic acid also reduced the development of new vessels in the placental membrane of chicks and buds in the aorta of chickens. In addition, ellagic acid significantly suppressed MDA-MB-231 cancer progression and P-VEGFR2 expression in breast cancer grafts. According to molecular docking simulations, EA may establish hydrogen bonds and aromatic contacts within the ATP-binding region of the VEGFR-2 kinase subunit. Together, ellagic acid and VEGFR-2 signaling may have antiangiogenic effects in breast cancer. [39]

The antimicrobial effect of PPEs with water as a solvent was studied by Kanatt, Sweetie R., Ramesh Chander, and Arun Sharma. PPE had a minimum inhibitory

concentration (MIC) of 0.01 percent against Staphylococcus aureus and Bacillus cereus, indicating strong antibacterial activity.At a dose of 0.1 percent, Pseudomonas could be suppressed, but it was ineffective against E. coli and S. typhimurium. PE was added to popular chicken meat products to extend their shelf life in cold storage by 2–3 weeks. PE also worked well in preventing oxidative rancidity in these chicken products. The minimal inhibitory concentration was determined using several concentrations of PE (0.01 %), 0.05 %, and 0.1 %). PE was effective against gram-positive bacteria at concentrations of as low as 0.01 percent. [40]

In 2015, Sud & Mahesh conducted research to estimate the influence of the solids/solvent ratio (1:10–1:30), incubation time (15-45 min), and temperature (50-70 °C) on total phenolic extract, which was evaluated by using the response surface method (RSM) to maximize conditions for the extraction of bioactive compounds from pomegranate peel (Punica granatum L.). To extract the polyphenols, each iterative optimization had a solvent concentration of 60% ethanol. As a result, the optimum yield of total phenol content (TPC) , sugar lowering, total flavonoids content (TFC) , and radical scavenging activity (DPPH) was achieved in the condition in which the solids to solvents proportion was 1:30, the temperature was 50 °C, and the extraction duration was 45 minutes at the maximum concentration. (24.54%) radical scavenging activity (DPPH) was obtained, with TPC of 510 mg GAE/g, TFC of 16.4 mg Quercetin/g, and sugar lowering activity of 0.18%.[41]

Pomegranate peel extract investigate potency as anti-glycation agent in Yagi, Masayuki, et al research. The experimental part was evaluated according to method of Vasan et al. with some modification, then High Performance Liquid Chromatography (HPLC) analysis and measuring the AGE-derived crosslink cleaving activity. When six distinct pomegranate extracts were tested for AGE crosslinking activity, pomegranate

ellagitannin (31.39%) and pomegranate leaf extract (31.22%) gave better results than the activity ratio of four different pomegranate extracts. Both AGE crosslink cleaving potential and collagen crosslink cleaving activity were found in two types of pomegranate extracts and nine types of pomegranate-derived compounds. [42]

Liu, Weixi, et al. in 2014 has been study the potency of pomegranate peel extract PPE rich in phenol and tannins as natural inhibitor of glycation end products. The experiment part show the ability of PPE as anti-glycation agent in specific mechanism by scavenging reactive carbonyl species in different stages in glycation endproducts. [43]

El-Hadary, A. E., & Ramadan, M. F. et al. carried out an in-vitro study on the total phenol, total flavonoids, and antioxidant activity, and an in-vivo study of the hypolipidemic, anti-hyperglycemic, and hepatoprotective impacts of hydro-methanol pomegranate (Punica granatum L.) peel extract (MPE). Phenolic & flavonoids compound analyzed by HPLC. In-vivo experiment, 56 Wister albino rats were divided into eight groups as shown in table 2.

Table 2: 8 groups of albino rats with different treatment as anti-hyperglycemic

Group number	Type of treatment
Group 1	Control (normal)
Group 2	PPE (200 mg/kg)
Group 3	Diabetic
Group 4	Diabetic + MPE (200 mg/kg)
Group 5	Diabetic + glibenclamide (10 mg/kg)

Group number	Type of treatment
Group 6	Hyperlipidemia
Group7	Hyperlipidemia + MPE (200 mg/kg)
Group 8	Hyperlipidemia + Atorvastatin (10 mg/kg)

The result was obtained with (188.9 mg GAE/g extract) of TPC, (13.95 mg QE/g extract) of TFC, 93.97% of antioxidant activity (DPPH) and 90.92% of antioxidant activity (ABTS•+). Tables 12 and 13 show that 23 phenolic compounds and 20 flavonoids compounds were detected in HPLC analysis. Demonstrated antihyperglycemic and antihyperlipidemic actions by acting as a strong reactive oxygen scavenger via its antioxidant components in diabetic and hypolipidemic rats. MPE improved liver and kidney function compared to conventional drugs. [44]

Table 3: Phenolic compounds in MPE analyzed by HPLC

No	Compound	Retention time (min)	Content (mg g−1)
1	Gallic acid	7.43	2.5000
2	Pyrogallol	7.74	45.358
3	4-Amino-benzoic acid	8.95	0.084
4	Protocatchuic	9.07	1.987
5	Catechein	9.2	3.275
6	Chlorogenic acid	9.4	1.562
7	Catechol	9.74	5.965
8	Caffeine	10.01	1.339

No	Compound	Retention time (min)	Content (mg g−1)
9	P-hydroxybenzoic	10.18	7.017
10	Caffeic acid	10.49	0.458
11	Vanillic acid	10.57	0.805
12	p-coumaric acid	11.74	0.086
13	Ferulic acid	11.99	0.492
14	Iso-ferulic acid	12.43	0.117
15	Rosmarinic acid	12.75	1.173
16	Punicalagin	12.92	98.02
17	Ellagic acid	13	12.561
18	Benzoic acid	13.3	0.666
19	α-coumaric acid	13.45	0.268
20	3,4,5-methoxy-cinnamic acid	13.73	0.164
21	Coumarin acid	13.96	0.912
22	Salycilic acid	14.21	0.108
23	Cinnamic acid	14.81	2.5

Table 4: Flavonoids in MPE analyzed by HPLC

No	Compound	Retention time (min)	Content (mg g−1)
1	Apigenin-6-arbinose 8-glactoside	11.46	0.353
2	Apigenin-6-rhamnose 8-glactoside	12.14	0.719

No	Compound	Retention time (min)	Content (mg g−1)
3	Naringin	12.39	0.944
4	Luteo-7-glucoside	12.46	0.622
5	Rutin	12.49	0.265
6	Hesperidine	12.52	5.047
7	Quercetrin-3-O-glucoside	12.58	0.189
8	Kamp.3,7-di rhamoside	12.75	0.323
9	Apigenin.7-O-neohespiroside	12.96	0.329
10	Quercetrin	13.27	3.519
11	Apigenin-7-glucoside	13.48	0.780
12	Kaemp-3-(2-p-comaroyl) glucoside	14.25	1.024
13	Quercetin	14.30	0.215
14	Acacetin7 neo hesperside	14.41	0.342
15	Naringenin	14.57	0.089
16	Hesperetin	14.86	0.523
17	Acacetin 7-O-rutinoside	14.91	0.121
18	Rhamentin	15.51	0.351
19	Apegnin	15.58	0.107
20	Kampferol	15.78	0.106

Table 5: Effect of treatment with MPE on HbA1c, and blood glucose in normal, diabetic, and hyperlipidemic rats

Group number	Type of treatment	HbA_{1c} (%)	Glucose (mg dl−1)
Group 1	Control (normal)	84.67 ± 6.28	5.26 ± 0.23
Group 2	MPE (200 mg/kg)	5.04 ± 0.23	76.62 ± 6.28
Group 3	Diabetic	11.7 ± 0.23	296.6 ± 6.28
Group 4	Diabetic + MPE (200 mg/kg)	6.56 ± 0.23	121.6 ± 6.28
Group 5	Diabetic + glibenclamide (10 mg/kg)	5.98 ± 0.23	101.6 ± 6.28
Group 6	Hyperlipidemia	7.02 ± 0.23	138.3 ± 6.28
Group7	Hyperlipidemia + MPE (200 mg/kg)	6.47 ± 0.23	105.0 ± 6.28
Group 8	Hyperlipidemia + Atorvastatin (10 mg/kg)	6.27 ± 0.23	115.0 ± 6.28

Chapter three

Experimental Part

3.1. Materials and Reagents

Pomegranate fruits were collected from Jerusalem city, Palestine, ethanol 60% , acetic acid , hydrochloric acid 5% (HCl), monopotassium phosphate (KH_2PO_4) , Disodium phosphate (Na_2HPO_4), distilled water, fructose, glucose, DPPH, methanol, trichloroacetic acid, potassium ferricyanide, Ferric chloride, Butylated hydroxytoluene (BHT), sodium bicarbonate, sodium nitrite, aluminum chloride, sodium hydroxide were purchased from sigma Aldrich. .

Quercetin standard with CAS No. (117-39-5) Q4951 and Gallic Acid standard (with CAS No. 149-91-7) G7384, Albumin from human serum (BSA) CAS No. (70024-90-7) A9511, Folin & Ciocalteu′s phenol reagent F9252, were purchased from sigma Aldrich.

3.2. Instruments

PERKIN-ElMER Lambda 5 UV/VIS Spectrophotometer, FLUROSKAN ASCENT FL, Analytical balance SHIMADZU ATx324 320g in Balances (S-841),), Rockyvac 300 Vacuum Pump, ONiLAB Magnetic Hotplate Stirrer (MS-H-S-Pro), Stuart Rotary Evaporator (RE 400) with Digital Water Bath (RE 400 DB), Ultrasonic Bath (Sonicator) IKON INDUTRIES (170VAC – 270VAC).

3.3. Sample preparation of punica granatum

Pomegranate fruit was collected from Palestinian original plantation, then peels and bulb was separated and washing with acetic acid and water, then drying in dark place from 3-4 weeks. The dried peels collected and grinding to fine particle.

3.3.1. Extraction of pomegranate peels

3.3.1.1. Ethanol extraction (Extract E):

15 gram of pomegranate peels powder extracted twice with 52.5 ml 99.9% ethanol for 1.5 hour by sonication method at 37 ºC, then left for 24 hours before filtration, then evaporated at 50-57 C by rotary evaporation at al-Quds University, chemistry lab. The extract then collect and stand to drying for 2 weeks at room temperature.

3.3.1.2. Acetone extraction (Extract F)

15 gram of pomegranate peels powder extracted twice with 52.5 ml 99% acetone for 1.5 hour by sonication method at 37 ºC, then left for 24 hours before filtration, then evaporated at 50-57 ºC by rotary evaporation at al-Quds University, chemistry lab. The extract then collect and stand to drying for 2 weeks at room temperature.

3.3.1.3. Extraction of hydrozable tannins from pomegranate peel extract

Extraction process of HTs was carried out according to Lu, J., & Yuan, Q. (2008) method with some modification. [45] Two different methods were used:

3.3.1.3.1. Reflux method (Extract A):

100 g pomegranate peel powder with 350 ml solutions of ethanol 60%, water, and acetic acid in ratio of (18:5:1) was mixed and reflux in water bath at range of temperatures (70-80 ºC) with stirring for 1.5 hour then decant the extract, re-extracted the residue with the same condition as mentioned above, the combined extracted stand

for 24 hour then centrifuge at 4000 rpm for 15 min three time, then the solution will evaporated by rotary under vacuum.

3.3.1.3.2. Sonication method (Extract B):

100 g pomegranate peel powder with 350 ml solutions of ethanol 60%, water, and acetic acid in the ratio of (18:5:1) was mixed and extracted by sonication at 24 ºC for 1 hour, then decanted the extract, re-extracted the residue with the same conditions as mentioned above, and the combined extracted stood for 24 hours, then centrifuged at 4000 rpm for 15 min, and evaporated by rotary under vacuum.

3.3.1.3.3. Hydrolysis of tannins

Hydrolysis the crude extract with 5% HCl, adding deionized water to crude until the volume reach 300 ml and reflux with 5% HCl for 4 hours at 100 ºC in water bath. Then filtrate the solution with buchner funnel, wash filter with distilled water then dried it for week.

3.4. Measurement of the Extraction Yield

The dry extract from each plant sample was weighed, and the percent yield was determined using the equation:

Extract yield = weight of dried extract/ weight of dried plant *100%

3.5. Fluorescence-based assay of the inhibition of AGEs formation

Assay of the inhibition of AGEs formation of each samples was carried by fluorescence and was performed as previously described (Harris, Cory S., et al., 2011). [46] With some modifications as follows:

a. Preparation of reagents:

1. (100mM) sodium phosphate monobasic monohydrate buffer (pH 7.4) was prepared.
2. Bovine Serum Albumin (BSA) stock solution 1mg/ml was prepared by dissolve 1mg albumin in 100 ml phosphate buffer (pH 7.4).
3. Stock solution (1mg/ml) of mixed glucose & fructose (1:1) was prepared in phosphate buffer pH 7.4.

b. **Preparation of samples and standard:**

Each sample was dissolved in 2 ml DMSO then filtrated and dilute with 99% ethanol to prepare range of concentrations (150, 120, 90 and 30 ppm). Quercetin standard with same conc. of samples was prepared in 99% ethanol.

c. Test samples:

1. Preparing extracts sample to test:

0.5 ml of each concentration samples was taken and mix with 0.3 ml phosphate buffer pH 7.4, 0.1 ml solution sugar and 0.1 ml BSA solution.

2. Preparing negative control:

0.3 ml phosphate buffer, 0.1 ml solution sugar, 0.1 ml BSA solution & 0.5 ml 99% ethanol.

4. Preparing of positive control :

0.5 ml standard solution of each concentration was mixed with 0.3 ml phosphate buffer pH7.4, 0.1 ml solution sugar & 0.1 ml BSA solution.

All test samples were prepared in glass test tube & cover then incubated in incubator shaker at al-Quds university lab at 37 **ºC** for 7 days.

d. Fluorescence-based assay of the inhibition of AGEs formation:

After 7 days of incubation, quantitative analysis was carried of fluorescent advanced glycation End products (AGEs) were formed in each sample by using fluorometer (Nutrition and Health Research institute, Al-Quds University) at excitation and emission wavelengths of 455 nm and 375 nm, respectively.

The percentage of inhibition of AGEs formation was determined by following equation:

$$\% \text{ inhibition} = \frac{(\text{F negative control} - \text{F experimentalcorrected})}{\text{F negative control}} * 100\ \%$$

Where F negative control: the fluorescence reading of negative control against blank as base line & F experimental corrected: The fluorescence reading of samples control against blank as base line

3.6. Total phenolic content (Folin-Ciocalteau assay)

The total phenol of four different extracts (hydozable tannins :sonication method extract A and Reflux method extract B and crude extracts: ethanol (E) and acetone (F)) were obtained by Folin-Ciocalteau reagents according to (Uddin, Md, et al) .[47] The conc. extracts (A, B, E, and F) were prepared by dissolving in 2 ml 99% DMSO and diluted with distilled water to prepare sequentially (100, 100, 200 & 150 ppm), 0.5 ml of each extract with 2.5 ml of Follin reagent (10 %, v\v), 2.5 ml sodium carbonate (7.5 %, w\v) then incubated for 30 min in the dark at room temperature, the absorbance was measured at 760 nm. Different concentration of gallic acid standard (20-110 ppm) for calibration curve. Results were expressed as mg Gallic acid equivalents (GAE)\g sample.

3.7. Total flavonoid content (colorimetric assay)

Total flavonoid content of extracts was evaluated with method of (Chang, Chia-Chi, et al**)**. [48] 2000 ppm of A, B, E & F extracts were prepared, 0.5 ml sample of each extract was taken and mixed with 1.5 of 95% ethanol, 0.1 ml aluminium chloride (10% w/v), and 0.1 ml of 1M sodium acetate, and 2.8 ml distilled water, then incubated for 30 min in dark at room temperature. The absorbance was measured at wave length 415 nm. Different concentrations of querctin (2.5-200 ppm) were prepared for calibration curve .

3.8. Antioxidants assay

3.8.1. Free radical scavenging activity (DPPH reagent)

Antioxidant power of extracts was carried out using the method of (Jothy, Subramanion L., et al.).[49] with some modification, different conc. of each samples A,B,E&F (300,150,100,90,80 ,70 & 50 ppm), from each conc. extracts was taken 0.5 ml , mix with 1.5 ml DPPH(0.04g/100 ml 80% methanol) , 3ml 96% ethanol, the negative control was prepared of 1.5 DPPH & 3.5 80% ethanol), then incubated in dark for 30 min at room temperature. Ascorbic acid standard of different conc. as positive control, the absorbance was measured at 517 nm. The results were obtained as a percentage of inhibition the control.

The percentage inhibition of DPPH of the samples and known solutions of ascorbic acid were calculated by the following equation:

$$\%\ \text{of inhibition} = \frac{A^{\circ} - A}{A^{\circ}} * 100\ ,$$

Where A˚ is the absorbance of a solution of negative control of DPPH at 515 nm and A is the absorbance of the sample extract at 515 nm.

3.8.2. Ferric reducing antioxidant power assay (FRAP)

The antioxidant power of extracts was determined by the ability of the antioxidant reduction process of ferric ion (Fe^{3+}) to blue ferrous (Fe^{2+}) complex, by the VongsaK method with some modification (Vongsak, Boonyadist, et al.). [50] 0.5 ml of each sample extract (100, 80, 60, and 40 ppm) was mixed with 0.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of potassium ferricyanide (1% w/v) and incubated at 50°C for 20 minutes before adding 2 ml of 10% trichloroacetic acid and then centrifuged at 40 rpm for 10 minutes. The absorbance was measured at 700 nm. A solution of BHT with a range of concentrations (100, 80, 60, and 40 ppm) was prepared as a positive control.

3.9. Determination Antibacterial Activities

The antibacterial activities were studied by the disc-diffusion method mentioned by (Essawi, T., & Srour, M., 2000). [51] Two species of gram negative bacteria (E. coli and Pseudomonas) and two species of gram positive bacteria (S. aureus and MRSA) were activated. Agar media was prepared by mixing the powder with distilled water and then sterilized. The media was poured into sterilized petri dishes and let stand for 2 hours before use. 0.01 g of sample A, 0.01 g of sample B, 0.04 of sample E and 0.04 of sample F. All of the samples were prepared with 0.5 mL of DMSO. The slime solution with four types of bacteria suspension compared to the 0.5 McFarland standard was prepared and distributed on a marked petri dish. A sterilized disk of filter pepper was placed on a petri dish, and the extract was distributed on the disk by micro pipette, using the solvent DMSO as a negative control. Plates were then incubated at 37°C for 24 h. The

clear zone around the holes indicates the inhibition power of the extract as an antibacterial.

3.10. Statistical analysis

Extraction methods, Antiglycation Assay, DPPH assay, FRAP antioxidants assay, total phenolic and flavonoid contents were studied in triplicate of each type and concentration of PPE. The data is presented as means ± standard deviations.

Chapter four

Result and discussion

4.1. Extraction yield

The weight of dried extracts was obtained after evaporation of the solvent for 5 weeks at room temperature. For the reflux method extract, the result was 2.6993 g per 100.1 pomegranate peel powder (PPp) , the sonication method extract was 2.1106 g per 100.24g PPp, the acetone extract 1.08g per 15.01g PPp and for the ethanol extract 1.77g per 15.02 g PPp, the extract yield of the reflux method, sonication method, acetone and ethanol (2.697%, 2.106%, 7.195% & 11.784%) respectively**.**

Figure 4: HTs powder by two method (sonication method& reflux method) after drying for 4 weeks

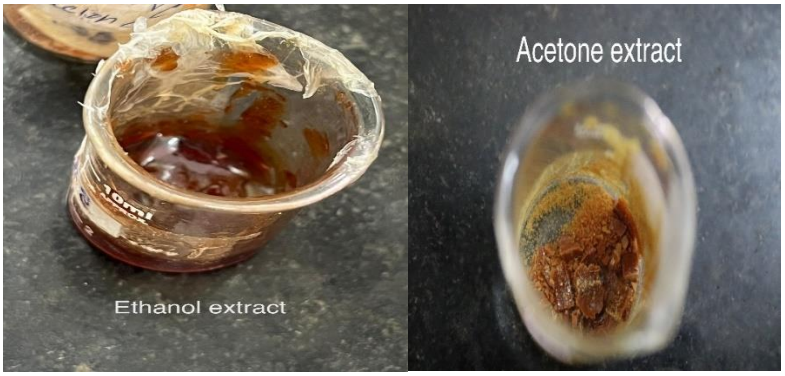


Figure 5: Acetone & ethanol extracts after 6 weeks of drying

4.2. Anti-glycation End Products formation (AGEs) Assay

By using a fluorometer to measure the fluorescence or light emitted by fluorescing glycation products with a specific wavelength of excitation and emission, the test showed that pomegranate peel extracts from four different extraction methods were found to have positive effects on the reduction of AGEs. In this simulation, higher concentrations of each extract and a positive control imply a higher inhibition percent of glycation products between glucose and fructose as mixed with albumin serum in the simulation as in the human body. As shown in figures 6 and 7, the hydrozable tannins in each sample for sonication (A) extract and reflux (B) extract have a potency against glycation. The percentage of inhibition of AGE started at 23% and 20.88%, respectively, for the lower concentration of 30 ppm and linearly increased with increasing concentration to 64.26% and 63.92, respectively, for the higher concentration of 150 ppm. For the whole extracts (acetones and ethanol), the percentage of inhibition of AGE started at 13.38% and 10.09%, respectively, for the lower concentration of 30 ppm and linearly increased with increasing concentration to 41.60%, 31.13%, and 6%, respectively, for the higher concentration of 150 ppm.

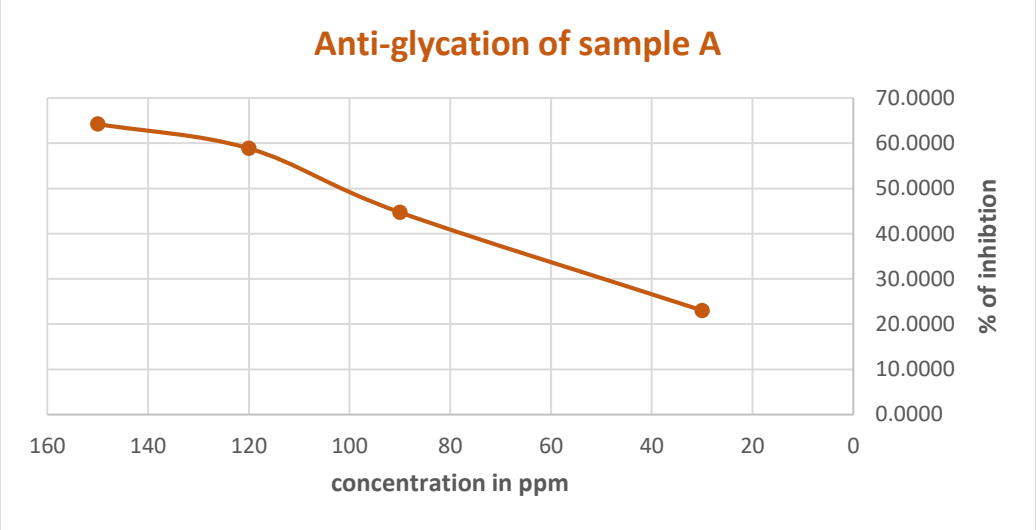


Figure 6: Concentration dependent effects of Sonication method extract (A) on in vitro formation of fluorescent AGEs.

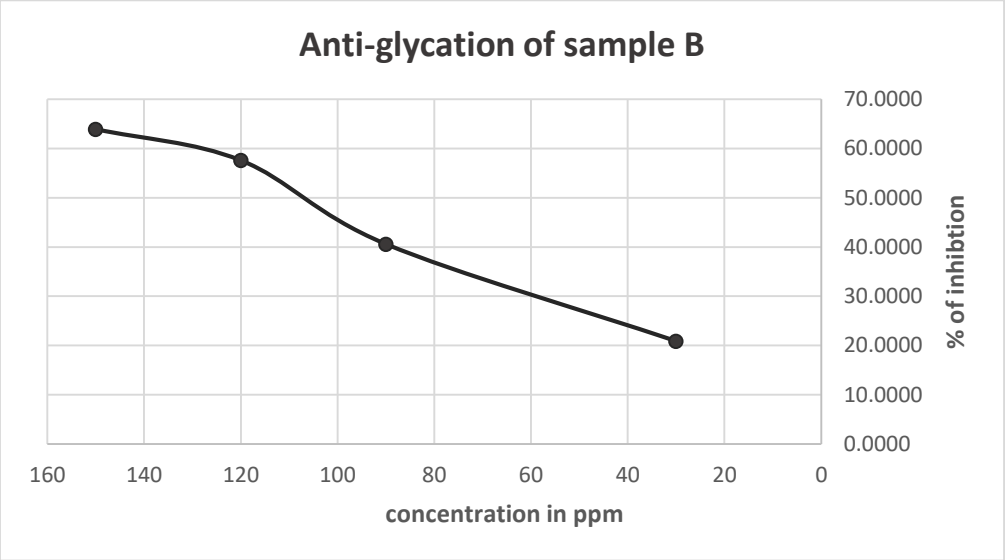


Figure 7: Concentration dependent effects of Reflux method extract (B) on in vitro formation of fluorescent AGEs.

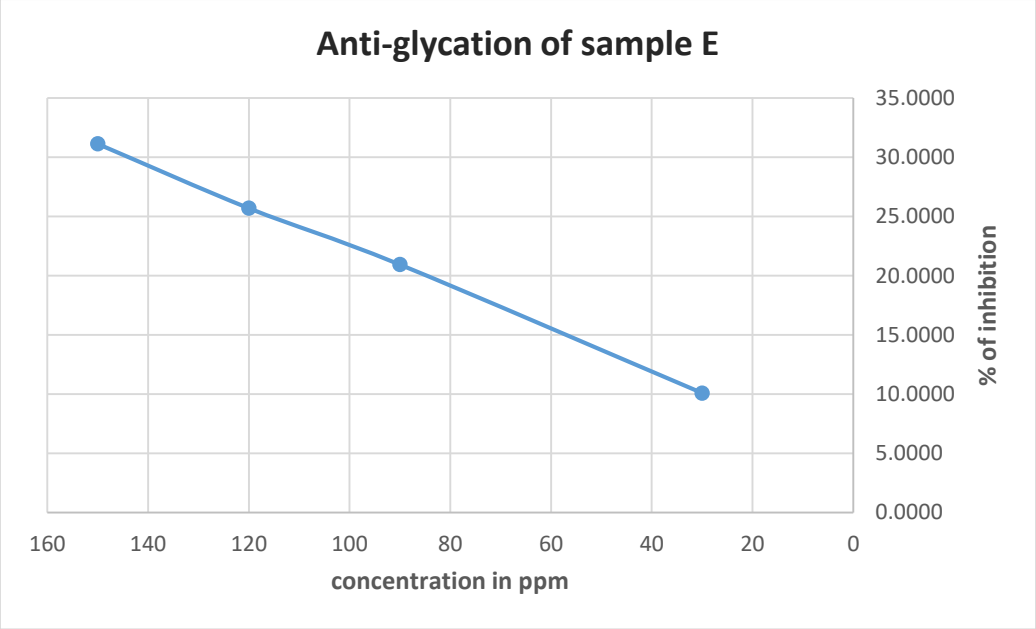


Figure 8: Concentration dependent effects of Ethanol extract (E) on in vitro formation of fluorescent AGEs.

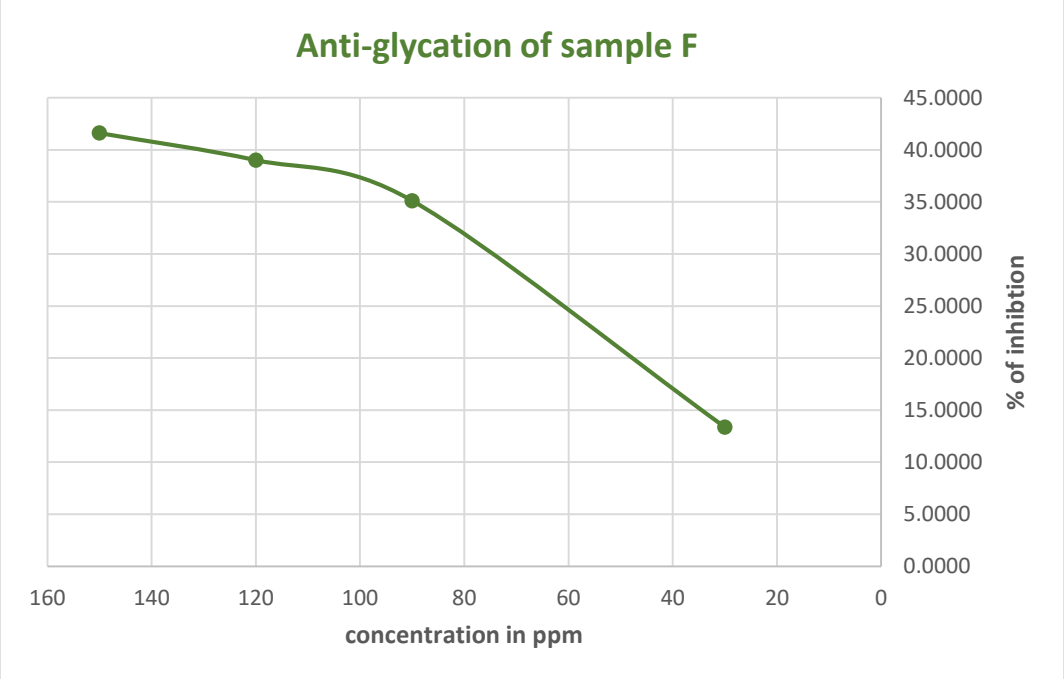


Figure 9: Concentration dependent effects of Acetone extract (F) on in vitro formation of fluorescent AGEs.

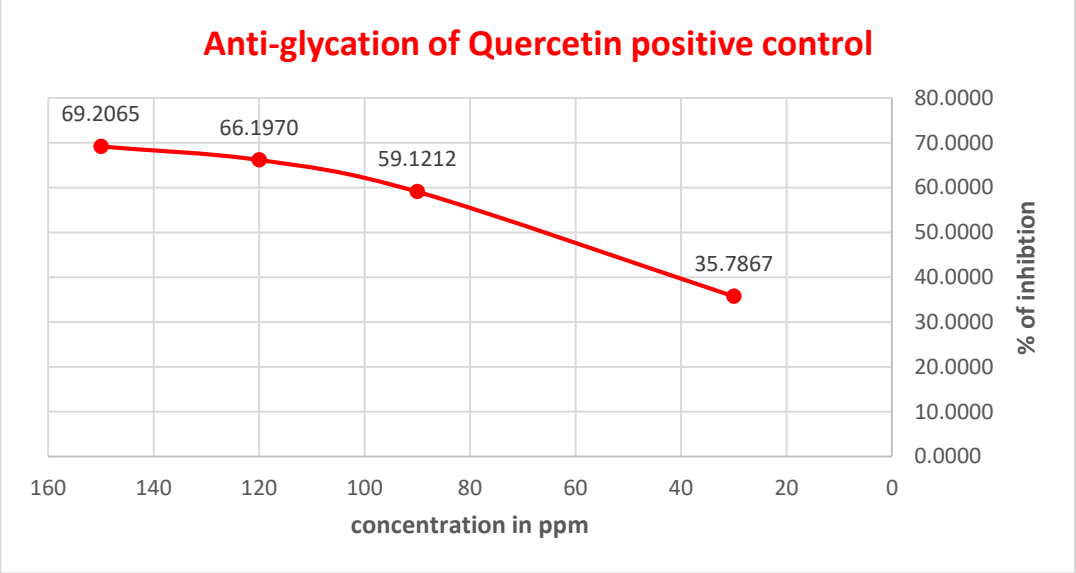


Figure 10: Concentration dependent effects of Quercetin positive control on in vitro formation of fluorescent AGEs.

Figure 10 shows that the formation of fluorescent AGEs was significantly reduced at the higher concentration of positive control Quarctein (150 ppm), a suppression yield of glycation of 69.21% was detected, while at the lower concentration (30 ppm), a suppression yield of 35.79% was detected. In relation to the influence of different concentrations of extracts and a positive control on the production of fluorescent AGEs, four concentrations (30 ppm -150 ppm) of each extract of A, B, E, and F were shown to inhibit the formation of fluorescent AGEs, as shown in figures 6, 7, 8, and 9.

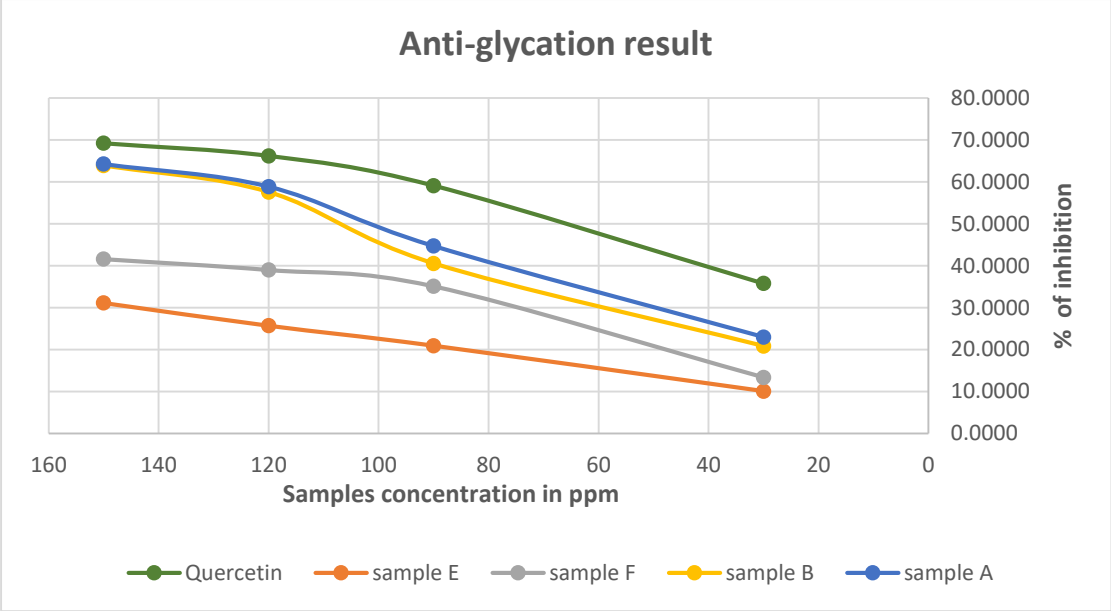


Figure 11: The comparison of concentration dependent effects Sonication method extract (A), Reflux method extract (B), Ethanol extract (E) , Acetone extract (F) and the positive control Querctein on in vitro formation of fluorescent AGEs.

In the comparison, hydrozable tannins in extracts A and B have more potency in inhibition of glycation end products than E and F extracts. The effectiveness of suppressing AGE was assorted as follows: A>B>F>E. In the inhibition of glycation end products, Quarectin positive control has a higher tendency than extracts as mention in figure 11.

4.3. Total phenol content

The total phenol content of four types of PPE is presented in Figure 12. The higher content of total phenol of PPE with different types of extraction methods was, respectively: The sonication extract: 453.675 mg QAE\ g dry extract, followed by a water bath method extract of 409.00 mg QAE/ g dry extract, an acetone extract of 335.33 mg QAEg /dry extract, and an ethanol extract of 208.33 mg QAE/g dry extract.

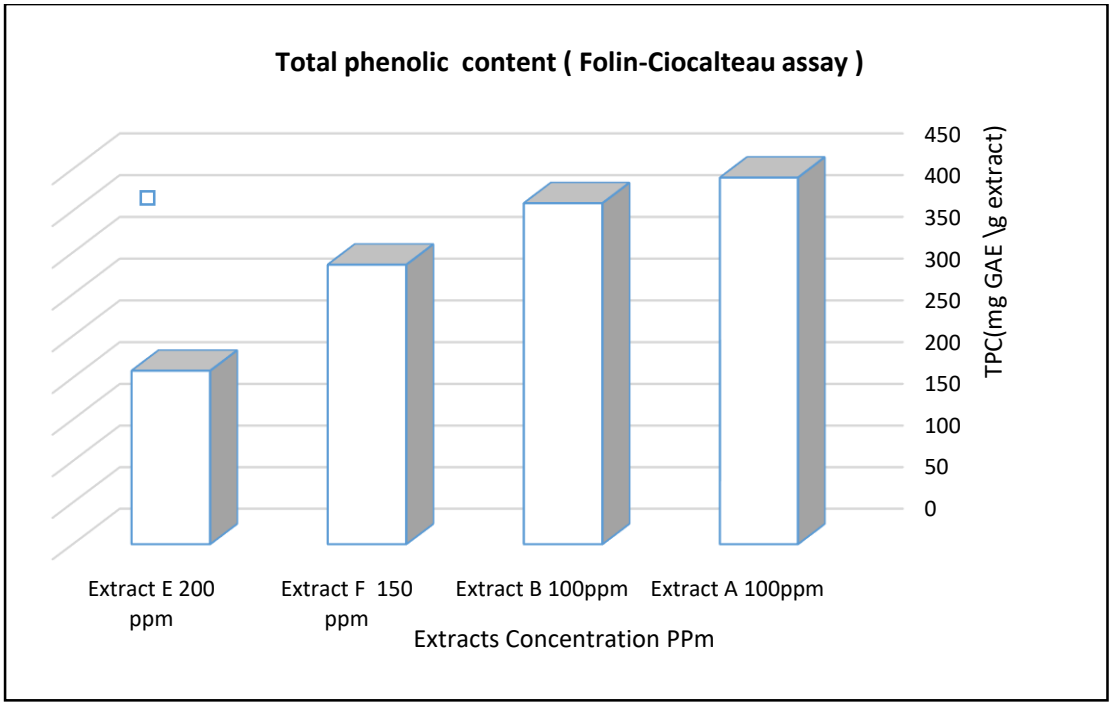


Figure 12: Total Phenolic Content of extracts (Folin-Ciocalteau assay). Extract A: Sonication method extract, Extract B: Reflux method extract, Extract E: Ethanol extract and Extract F: Acetone extract

4.4. Total flavonoids content

Figure 13 depicts the total flavonoids content of four types of PPE. The higher the total flavonoids content of PPE with different types of extraction methods was, respectively: acetone extract: 59.05 mg of QEQ per gram of dry extract, then sonication method extract: 28.14 mg of QEQ per g of dry extract, water bath method 22.49 QEQ g dry extract & 19.37 QEQ g dry extract ethanol extract

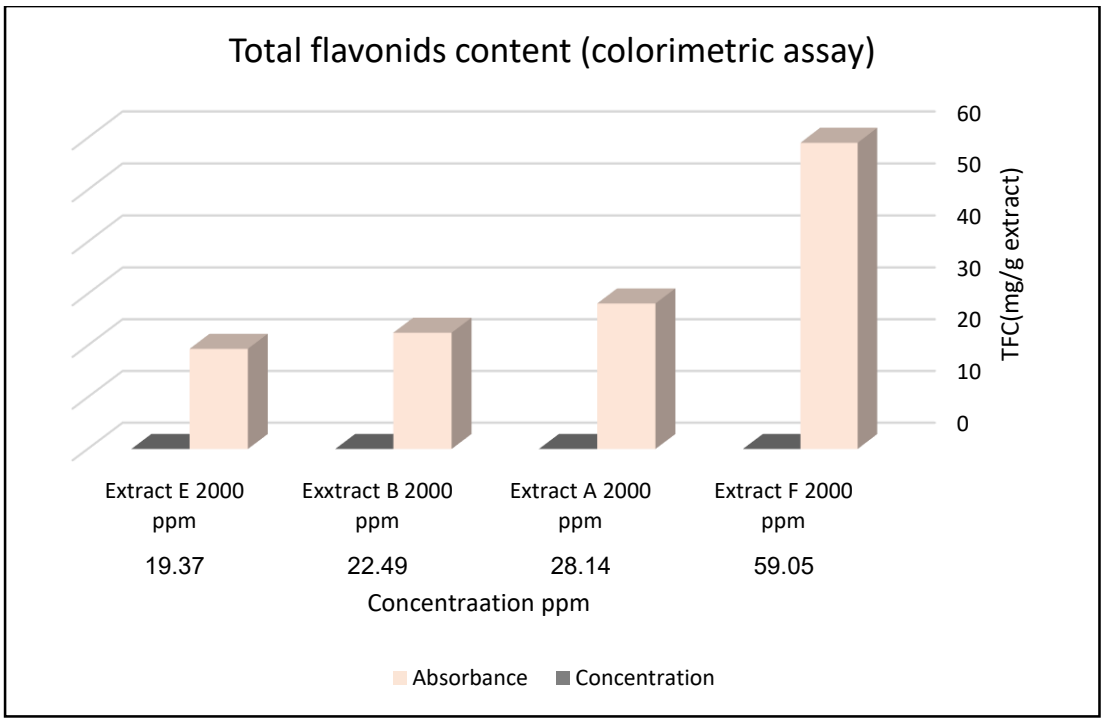


Figure 13: Total Flavonoids Content of extracts, Extract A: Sonication method extract, Extract B: Reflux method extract, Extract E: Ethanol extract and Extract F: Acetone extract (Colorimetric Assay).

4.5. Antioxidant assay

4.5.1. Free radical scavenging activity

In DPPH free radical scavenging, the four different extracts (A, B, F&E) of PPE appear potency as antioxidants, in the manner of dose-dependent of scavenging effect as shown in figures 14,15,16 &17 for Extract A, B, F & E, respectively was 0.9762, 0.9014, 0.9695& 0.9861. Extract F show the most powerful extract of antioxidants against free radical by 100% inhibition at 100 ppm, the other extracts expose inhibition percent less than 100% at concentration level 100 ppm of each extracts A, E & E (63.895, 63.396 & 40.80) respectively as show in figure18.

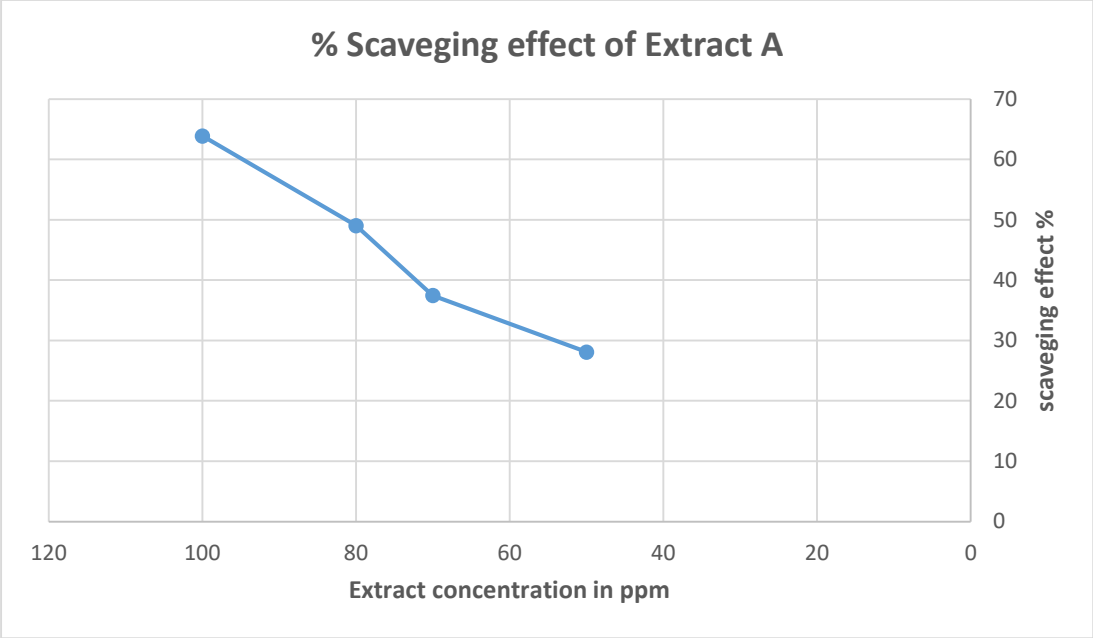


Figure 14: DPPH scavenging activity of Extract A (Sonication method extract) in different concentration.

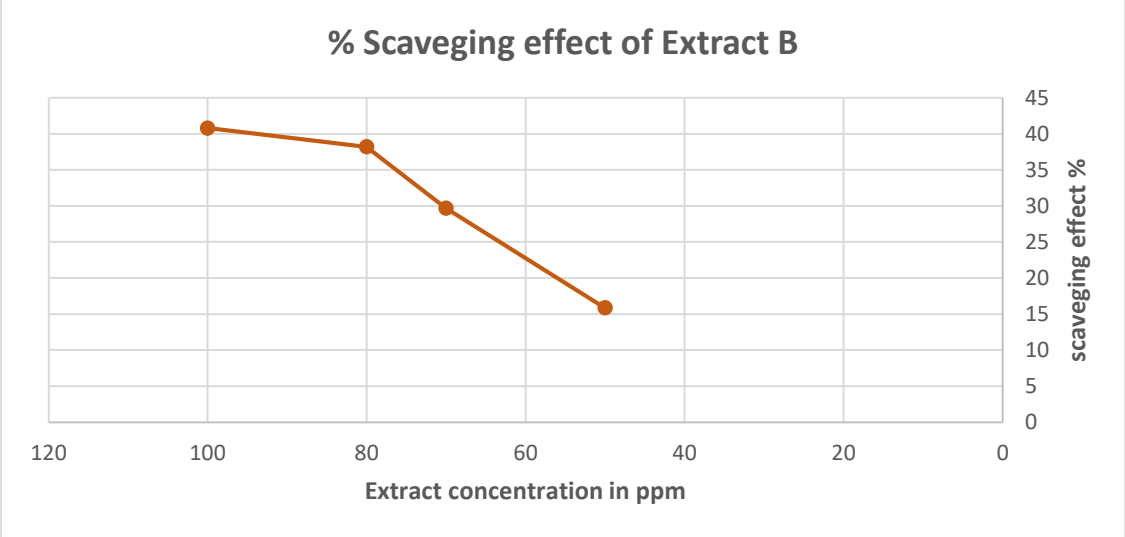


Figure 15: DPPH scavenging activity of Extract B (Reflux method extract) in different concentration.

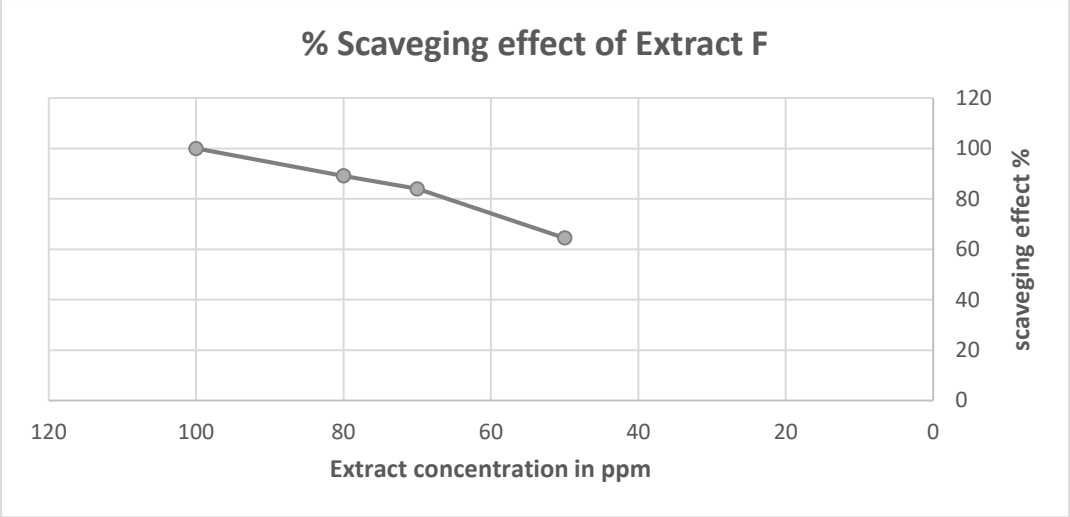


Figure 16: DPPH scavenging activity of Extract F (Acetone extract) in different concentration.

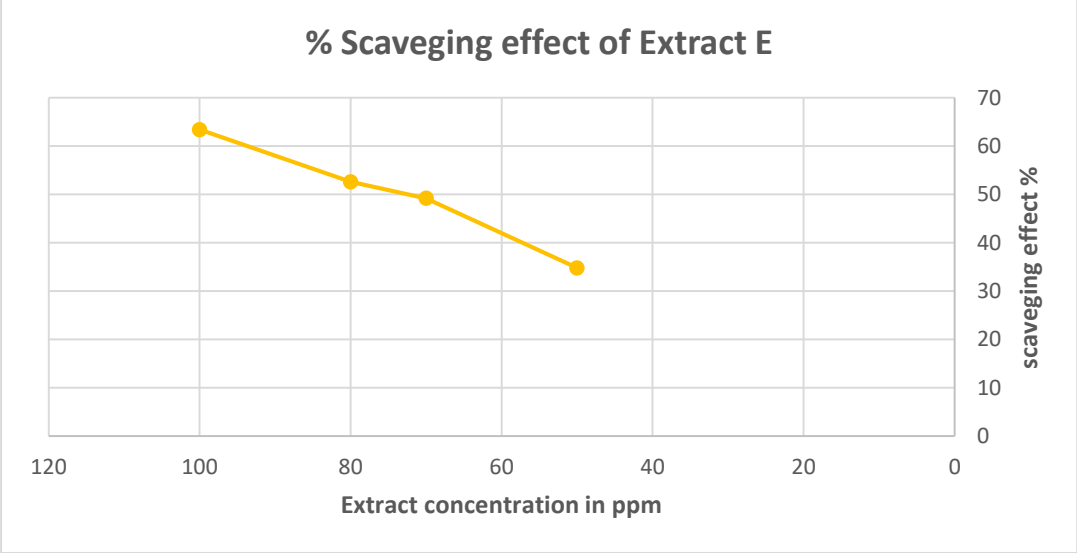


Figure 17: DPPH scavenging activity of Extract E (Ethanol extract) in different concentration.

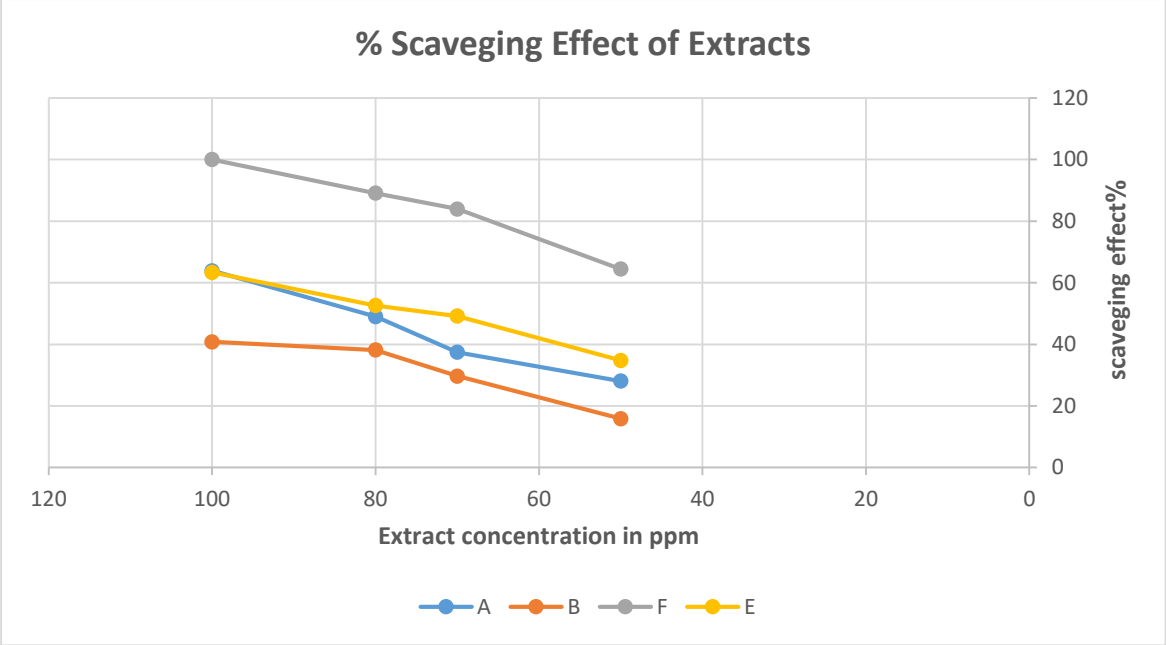


Figure 18: The comparison of concentration dependent effects of extracts: Sonication method extract (A), Reflux method extract (B), Ethanol extract (E) and Acetone extract (F) on DPPH scavenging activity.

4.5.2. Reducing power FRAP assay

The polyphenol in each type of PPE has a mechanism of reducing ferric ions (Fe+3) to ferrous ions (Fe+2). The yellow color of the ferric ions solution is converted to blue and pale green according to the concentration of extracts. The highest concentration gives a blue color, which indicates the reducing steps of ferric ions by antioxidants. In figures 19,20,21,22 & 23 dose-dependent of FRAP of each extract and positive control BHT from low dose to high dose, with increase dose of each extract the reduction process increase. Figure 24 depicts a comparison between the four types of extracts (A, B, E, and F) by antioxidant potency. Extract A shows a higher antioxidant capacity at a concentration of 1000 ppm than extracts F, B, and A sequentially. All extracts except extract E indicate higher antioxidant potency compared to positive control BHT.

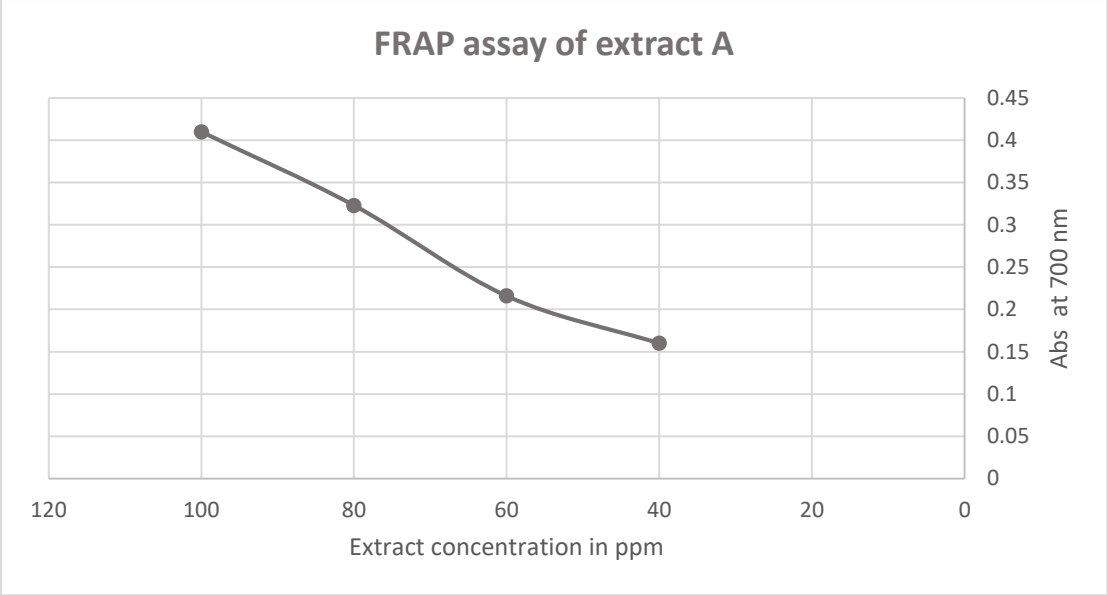


Figure 19: FRAP assay of Extract A (Sonication method extract) in different concentration.

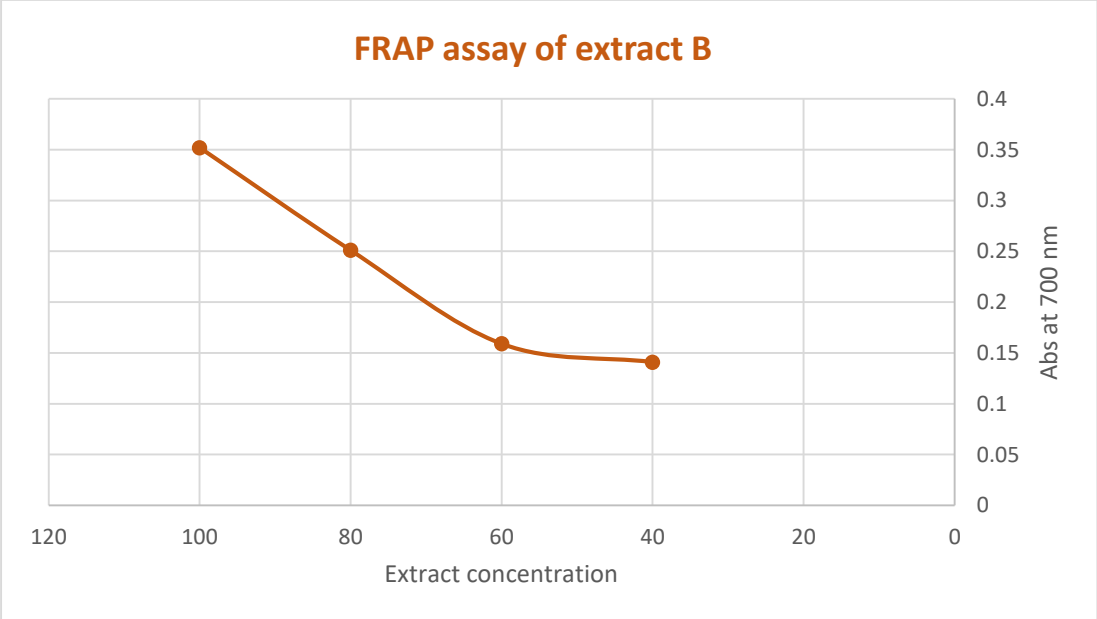


Figure 20: FRAP assay of Extract B (Reflux method extract) in different concentration.

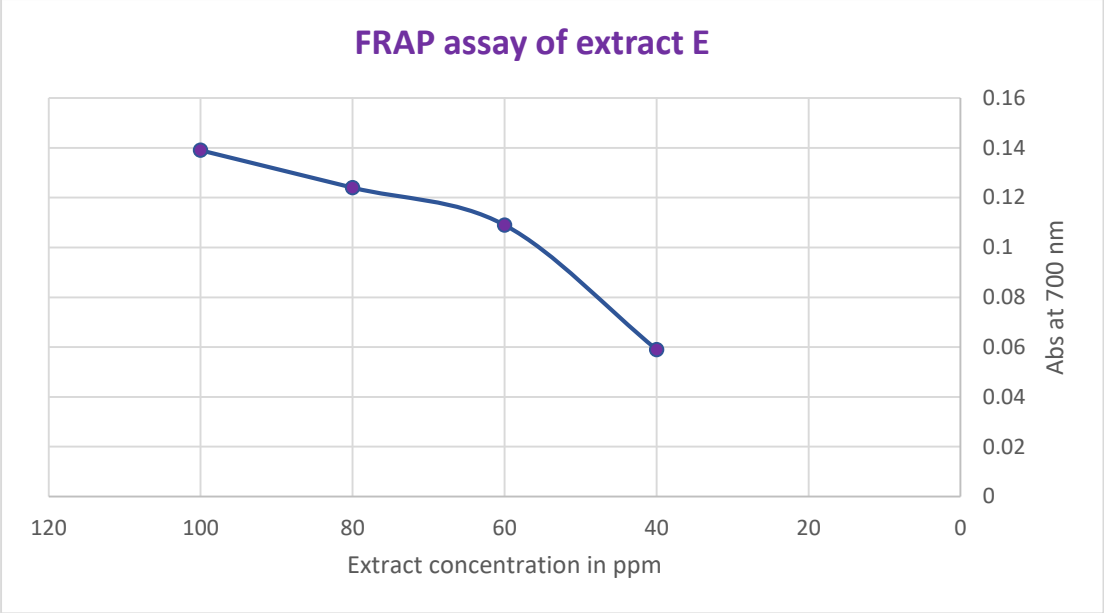


Figure 21: FRAP assay of Extract E (Ethanol extract) in different concentration.

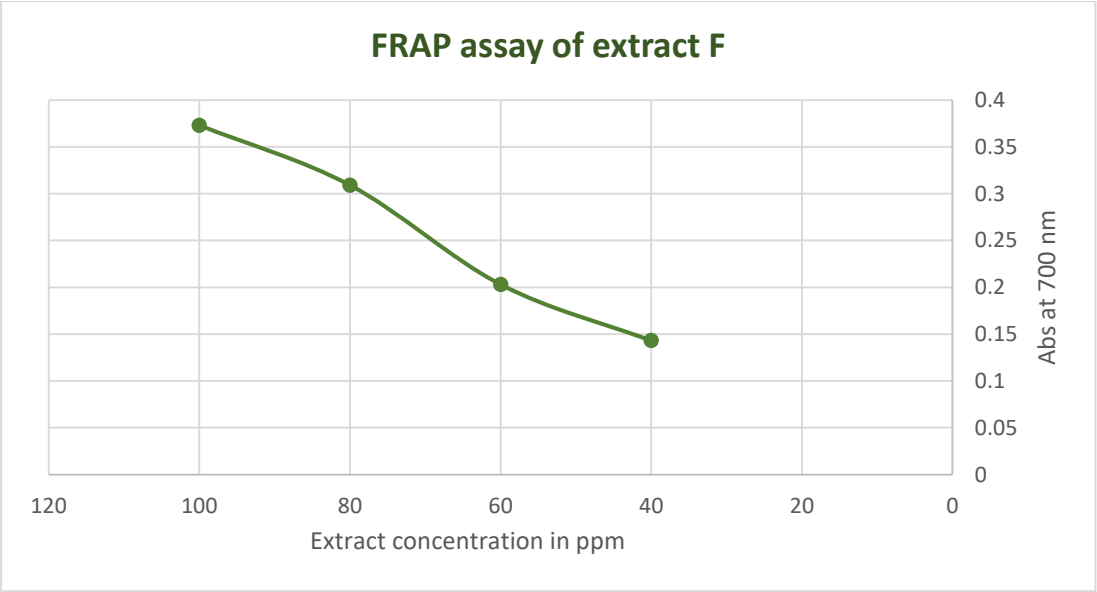


Figure 22: FRAP assay of Extract F (Acetone extract) in different concentration.

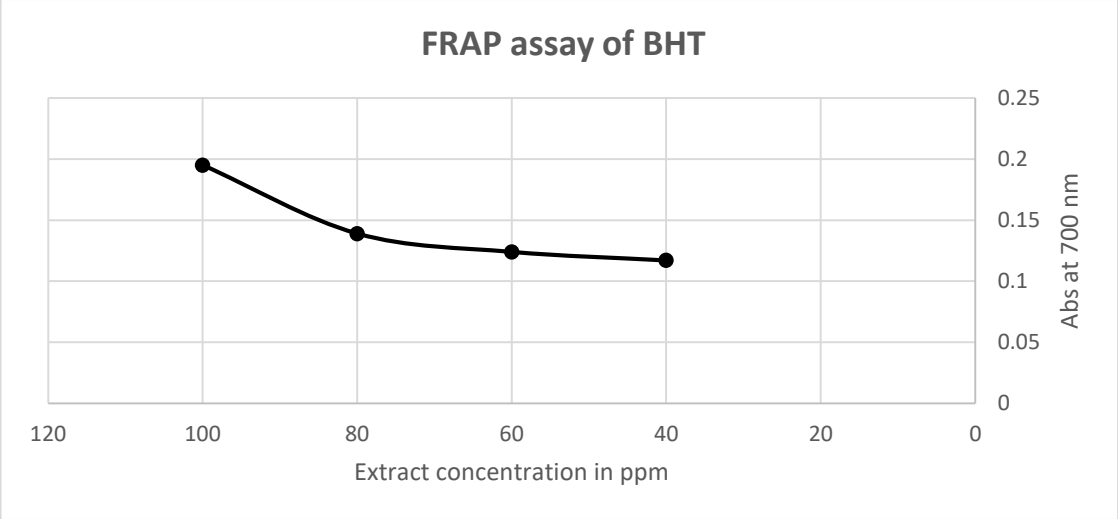


Figure 23: FRAP assay of BHT positive control in different concentration.

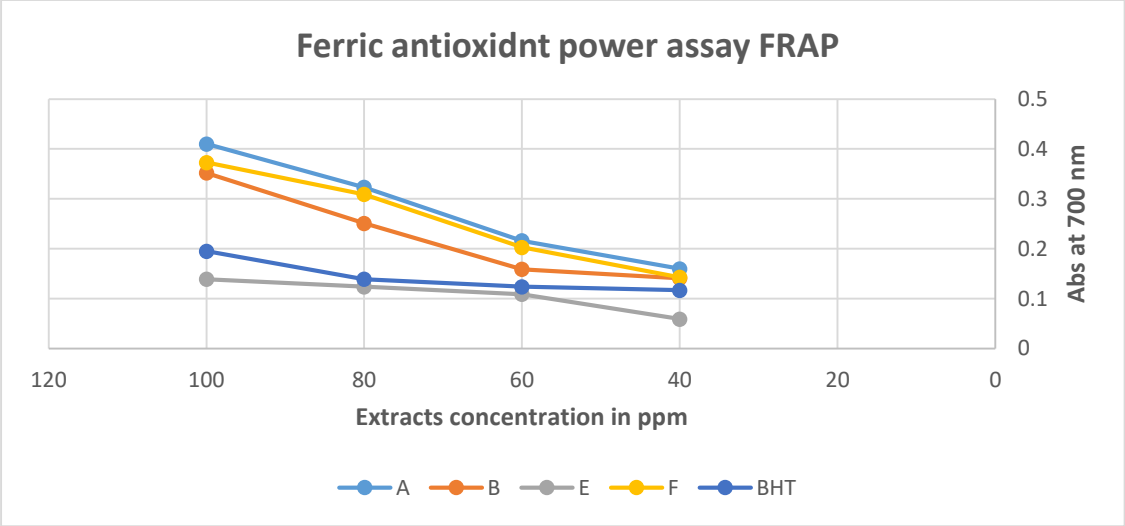


Figure 24: The comparison of concentration dependent effects of extracts: Sonication method extract (A), Reflux method extract (B), Ethanol extract (E), Acetone extract (F) and BHT as positive control on Ferric oxidation activity.

4.5. Antibacterial assay

Chemical compounds such as phenols, anthocyanin, and tannins in pomegranate peel extract have a tendency to kill and inhibit the growth of several types of gram positive and gram-negative bacteria as a natural and cheap alternative to synthetic compounds. Each type of PPE shows a different capacity in the inhibition of growth of E. coli, pseudomonas, MRSA, and S. Inhibition zone of extracts vary according to phytochemicals component in each extracts that prevent bacteria growth as mention in table 6 and figure 29.

Table 6: Inhibition zone in cm of each extracts: Sonication method extract (A), Reflux method extract (B), Ethanol extract (E), Acetone extract (F) against gram positive bacterial (MRSA and S. aureus) and gram negative bacterial (E. coli and Pseudomonas).

Gram positive bacterial				
Bactria name	Inhibition zone (cm) of Extract A	Inhibition zone (cm) of Extract B	Inhibition zone (cm) of Extract E	Inhibition zone (cm) of Extract F
MRSA	0.63 ±0.073	0.63±0.083	0.73 ±0.060	0.67 ±0.117
S. aureus	0.65 ±0.076	0.68 ±0.109	1.5 ±0.029	1.7 ±0.132
Gram negative bacterial				
E. coli	0.63 ±0.033	0.67 ±0.067	0.73 ±0.067	0.77±0.033
Pseudomonas	0.72 ±0.033	0.88 ±0.033	1.17 ±0.033	1.63 ±0.033

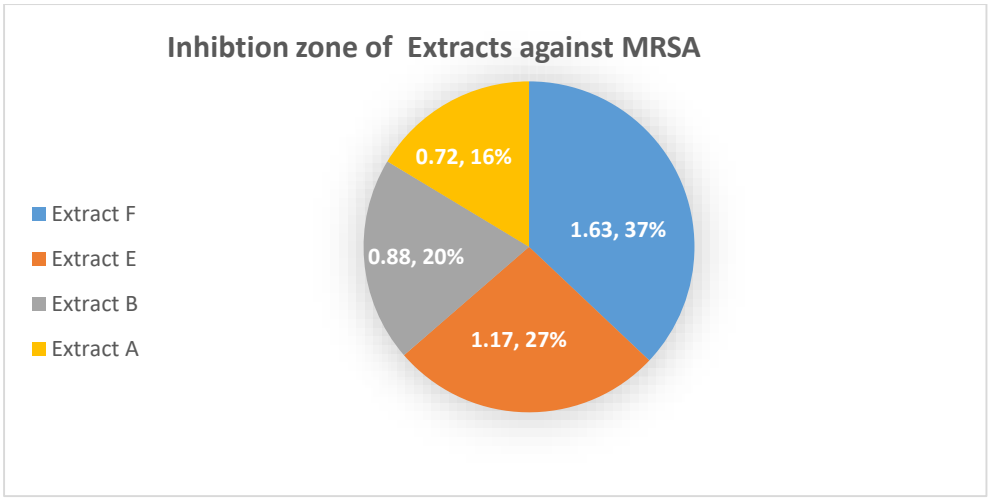


Figure 25: Inhibition zone of extracts (A, B, E and F) against MRSA.

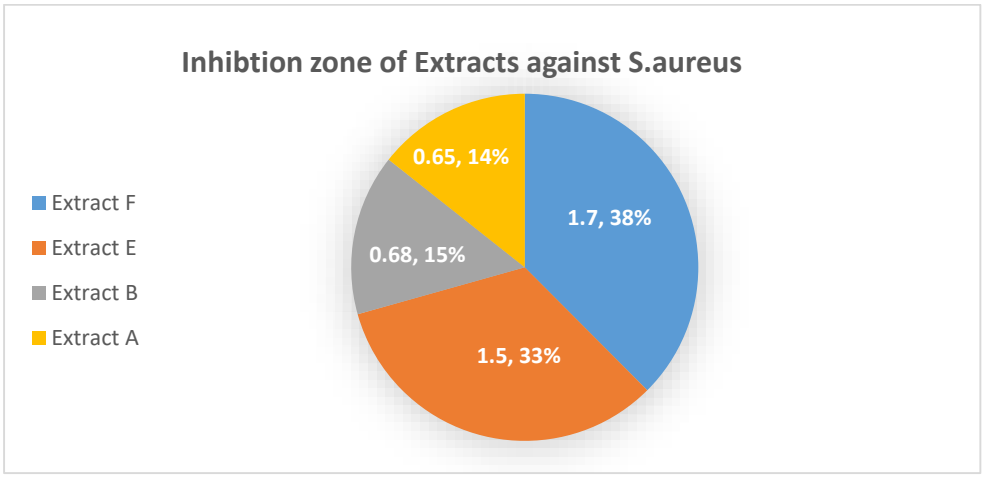


Figure 26: Inhibition zone of extracts (A, B, E and F) against S.aureus.

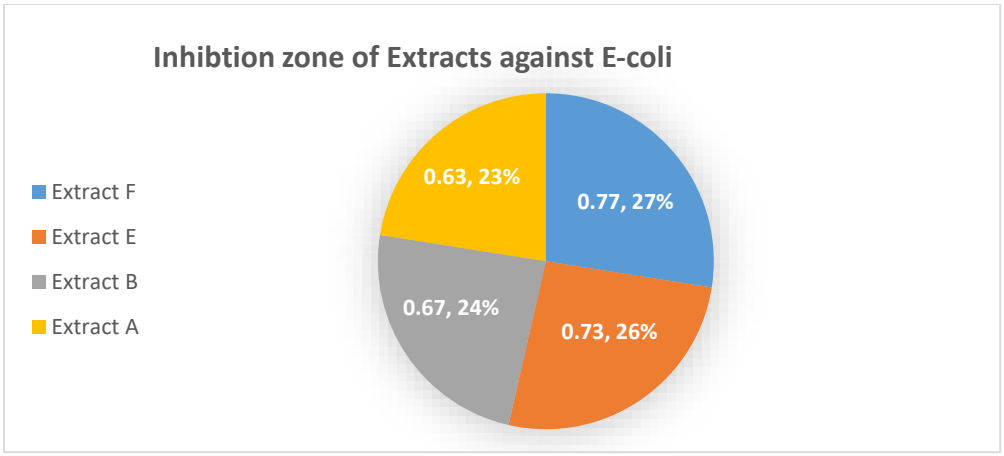


Figure 27: Inhibition zone of extracts (A, B, E and F) against E-coli.

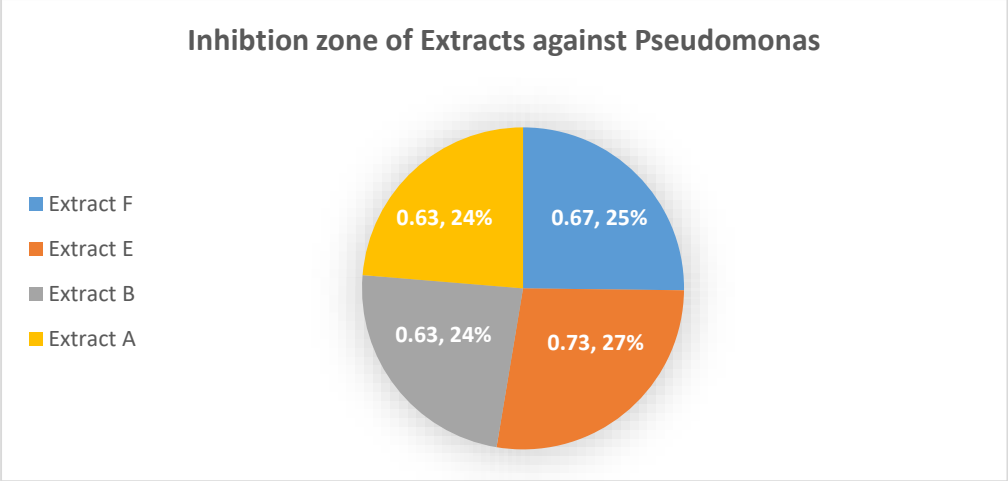
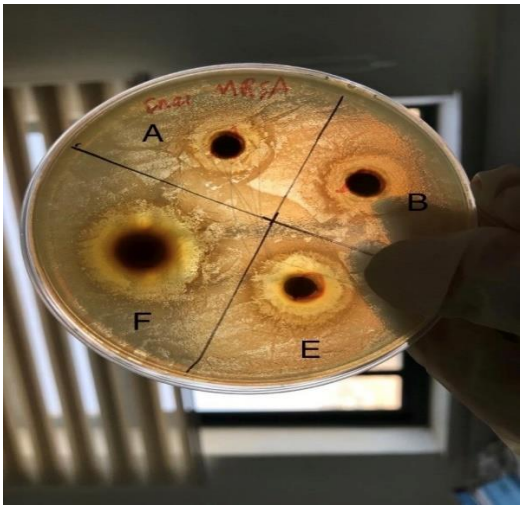


Figure 28: Inhibition zone of extracts (A, B, E and F) against Pseudomonas.

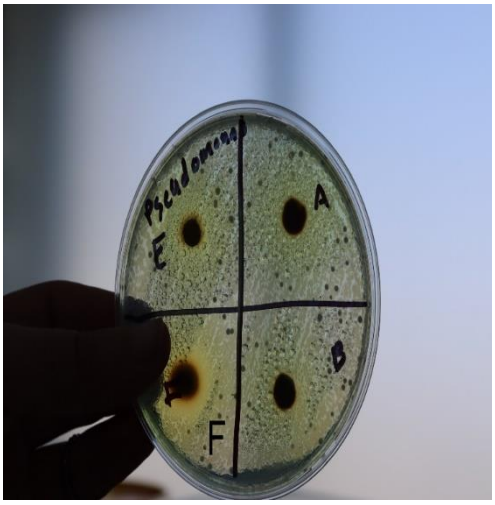
By measurement of the inhibition zone at a specific concentration (0.02g/ml) for extracts against gram bacteria, 37% of MRSA was suppressed by extract F, 27% by extract E, 20% and 16% by extract B and A, respectively (Figure 25). Extract F shows a higher ability to inhibit the growth of S.aureus by 38% of inhibition, followed by extract E with 33% of inhibition, then Extract B & A with 16% and 15% inhibition, respectively, (Figure 26). For E.coli bacteria, the extracts have close proportions of 27%, 26%, 24% & 23% for Extracts F, E, B, and A, respectively (Figure 27). 27% of pseudomonas growth was suppressed by extract E, and 25% by extract F, the same inhibition percent for extract A & B (figure 28).

As a result, Extract F is more effective against bacteria than other extracts, with the exception of pseudomonas, where it is less effective than Extract E by less than 2%.The specific processes of cell damage have not really been identified because of the complexity of the process of phenols in the inhibition of the growth of bacteria. The most likely mechanism of phenolic extracts' antibacterial action is that it's been suggested that it's because of the disturbance of the membrane of a cell. [52]

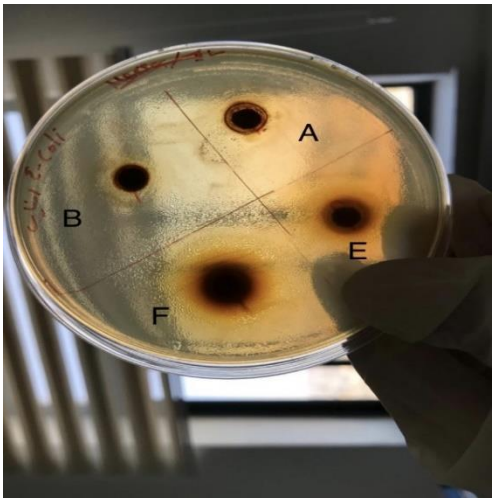


Antibacterial effect of Extracts against MRSA

Antibacterial effect of Extracts against S-aureus



Antibacterial effect of Extracts against Pseudomonas



Antibacterial effect of Extracts against E.coli

Figure 29: Inhibition zone of extracts (A, B, E& F) against gram positive bacteria :(MRSA and S –aureus) and gram negative bacteria: (E-coli and Pseudomonas).

The PPE in four routes of extraction gave efficient results, such as antioxidant effects, total antibacterial effects, total phenols, total flavonoids, and anti-glycation effects. The

correlation involving total phenol, DPPH, FRAP, antibacterial activity, and anti-glycation ability of various extracts depends on the variation of phytochemical properties, which is based on the method of extraction and solvents used in extraction. The result of each extract was convergent, especially in hydrozable tannin extracts A and B, which were the same biochemical compounds with different ratios. It is the same for the whole extract of PP extracted with ethanol and acetone. Extract A performed the best in the anti-glycation assay because it contained the most tannins, phenols, and flavonoids compared to the other extracts. However, in the antibacterial assay, Extract F performed the best. Extracts E and F have higher potency against four types of bacteria; the explanation returns to the presence of more chemical compounds, as opposed to Extracts A and B, which only contain hydrozable tannins.

Based on the glucose-BSA test, agricultural plants with high tannin content, antioxidative, and radical scavenging capacity were reported to have in vitro anti-glycation efficacy [53], antioxidants in PPE reduce the formation of reactive oxygen species and carbonyl groups during the glycation process as well as increase GLO I activity to limit MG development, which can both diminish Amadori product synthesis and therefore AGE development.[54] According to studies, flavonoids are the polyphenols with the greatest potential for inhibiting glycoxidation. Also, the inhibitory effects of polyphenols on glycation are assumed to be mostly owing to their antioxidant rather than metal-chelating capabilities. [55]

Chapter five

Conclusion and future work

Conclusion

The present study showed the potency of biochemical in pomegranate peel extracts in the inhibition of floursense glycation end products and antibacterial effects due to the high content of antioxidant compounds such as phenol and flavonoids. This finding suggests that pomegranate peel extract might be used as a food supplement as it is safe, cheap, and effective in inhibiting glycation formation and, therefore, diabetic complications.

Future work

1. Developed microemulsion as carrier of tannins to enhance the solubility and bioavailability.

2. Determine the minimum inhibitory concentration (MIC) of each extract against the previously mentioned bacteria in the research.
3. Separation by Column chromatography of hydrozable tannins and indication of the potency of each compound separately as antioxidants, antiglycation, and antibacterial agents

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Appendices

Appendix (a): Anti-glycation results for samples & positive control:

Negative Control Samples	
Fluorescence Response	Average Fluorescence Response
5.469	5.394
5.213	
5.500	

Sample A					
concentration (ppm)	Fluorescence Response	% of inhibition	Average % of inhibition	Standard Deviation	Standard error
150	1.889	64.9796	64.2628	0.6219	±0.3591
	1.945	63.9414			
	1.949	63.8673			
120	2.126	60.5858	58.8802	2.8743	±1.6595
	2.397	55.5617			
	2.131	60.4931			
90	2.8252	47.6233	44.7213	3.2248	±1.8619
	2.951	45.2911			
	3.169	41.2495			
30	3.932	27.1042	23.0009	4.1033	±2.3690
	4.029	25.3059			
	4.499	16.5925			

Sample B					
concentration (ppm)	Fluorescence Response	% of inhibition	Average % of inhibition	Standard Deviation	Standard error
150	2.038	62.2173	63.9167	2.0183	±1.1653
	1.975	63.3852			
	1.826	66.1476			
120	2.327	56.8595	57.5763	2.4170	±1.3955
	2.143	60.2707			
	2.395	55.5988			
90	3.315	38.5428	40.5759	1.9524	±1.1272
	3.196	40.7490			
	3.105	42.4360			
30	4.046	24.9907	20.8750	4.1918	±2.4201
	4.498	16.6110			
	4.260	21.0234			
Sample F					
concentration (ppm)	Fluorescence Response	% of inhibition	Average % of inhibition	Standard Deviation	Standard error
150	3.180	41.0456	41.6018	0.8843	±0.5105
	3.175	41.1383			
	3.095	42.6214			
120	3.330	38.2647	39.0063	0.8496	±0.4905
	3.240	39.9333			
	3.300	38.8209			

concentration (ppm)	Fluorescence Response	% of inhibition	Average % of inhibition	Standard Deviation	Standard error
90	3.412	36.7445	35.1131	1.8570	±1.0721
	3.609	33.0923			
	3.479	35.5024			
30	4.598	14.7571	13.3791	5.4982	±3.1744
	4.999	7.3230			
	4.420	18.0571			
Sample E					
concentration (ppm)	Fluorescence Response	% of inhibition	Average % of inhibition	Standard Deviation	Standard error
150	3.714	31.1457	31.1334	0.0386	±0.0223
	3.713	31.1643			
	3.717	31.0901			
120	3.985	26.1216	25.7014	0.6492	±0.3748
	4.048	24.9537			
	3.99	26.0289			
90	4.349	19.3734	20.9368	1.5666	±0.9045
	4.265	20.9307			
	4.180	22.5065			
30	4.829	10.4746	10.0915	0.3801	±0.2194
	4.870	9.7145			
	4.850	10.0853			

Quercetin					
concentration (ppm)	Fluorescence Response	% of inhibition	Average % of inhibition	Standard Deviation	Standard error
150	1.677	68.9099	69.2065	0.4211	±0.2431
	1.671	69.0211			
	1.635	69.6885			
120	1.803	66.5740	66.1970	1.0618	±0.6130
	1.779	67.0189			
	1.888	64.9981			
90	2.296	57.4342	59.1212	1.6343	±0.9436
	2.199	59.2325			
	2.120	60.6971			
30	3.451	36.0215	35.7867	1.9941	±1.1513
	3.577	33.6856			
	3.363	37.6529			

Appendix (b): Total phenolic Content results for all tested samples

The standard curve of the Gallic Acid gives the following liner equation: Y=0.0097X-0.1555					
Extract A 0.0001g/ml	Absorbance	mg GAE \ g dry extract	Average mg GAE \ g dry extract	Standard deviation	Standard error
Sample1	0.276	445	453.67	8.51	±4.91
Sample 2	0.244	454			
Sample 3	0.293	462			

Extract B 0.1 mg/ml	Absorbance	mg GAE \ g dry extract	Average mg GAE \ g dry extract	Standard deviation	Standard error
Sample 1	0.242	410	409.00	3.61	±2.08
Sample 2	0.244	412			
Sample 3	0.238	405			
Extract E 0.0002 mg/ml	Absorbance	mg GAE \ g dry extract	Average mg GAE \ g dry extract	Standard deviation	Standard error
Sample 1	0.260	214	208.33	6.03	±3.48
Sample 2	0.249	209			
Sample 3	0.237	202			
Extract F 0.00015mg/ml	Absorbance	mg GAE \ g dry extract	Average mg GAE \ g dry extract	Standard deviation	Standard error
Sample 1	0.324	329	335.33	14.57	±8.41
Sample 2	0.317	325			
Sample 3	0.357	352			

Appendix (c): Total Flavonoids content results for all tested samples

The standard curve of the Quarectin gives the following liner equation: y = 0.0097x + 0.0168					
Samples A 0.002mg/ml	Absorbance	g QEQ\ g dry extract	Average mg QEQ \ g dry extract	Standard deviation	Standard error
Sample1	0.561	28.05	28.14	0.081	±0.047
Sample 2	0.564	28.21			
Sample 3	0.563	28.15			
Samples B 0.002 mg/ml	Absorbance	mg GAE \ g dry extract	Average mg GAE \ g dry extract	Standard deviation	Standard error
Sample 1	0.454	22.54	22.49	0.956	±0.552
Sample 2	0.434	21.51			
Sample 3	0.471	23.41			
Sample F 0.002mg/ml	Absorbance	mg GAE \ g dry extract	Average mg GAE \ g dry extract	Standard deviation	Standard error
Sample 1	1.159	58.88	59.05	1.552	±0.896
Sample 2	1.194	60.68			
Sample 3	1.134	57.59			

Sample E 0.002mg/ml	Absorbance	mg GAE \ g dry extract	Average mg GAE \ g dry extract	Standard deviation	Standard error
Sample 1	0.391	19.29	19.37	0.076	±0.044
Sample2	0.394	19.44			
Sample3	0.393	19.39			

Appendix (d): DPPH result for all tested samples

Control	Absorbance	Average absorbance	Standard deviation	Standard error
Reading 1	1.273	1.299	0.066	±0.038
Reading 2	1.374			
Reading 3	1.250			

Absorbance of control (400µL of ethanol 96% and 1.5 mL of DPPH) = 1.299

Sample A

concentration (ppm)	scavenging capacity (Absorbance)	Scavenging effect %	Average Scavenging effect %	Standard Deviation	Standard error
50	0.919	29.253	28.099	1.633	±1.155
	0.949	26.944			

concentration (ppm)	scavenging capacity (Absorbance)	Scavenging effect %	Average Scavenging effect %	Standard Deviation	Standard error
70	0.886	31.794	37.452	8.002	±5.658
	0.739	43.110			
80	0.674	48.114	49.038	1.307	±0.924
	0.650	49.962			
100	0.483	62.818	63.895	0.816	±0.577
	0.468	63.972			
Sample B					
concentration (ppm)	scavenging capacity (Absorbance)	Scavenging effect %	Average Scavenging effect %	Standard Deviation	Standard error
50	1.094	15.781	15.858	0.109	±0.077
	1.092	15.935			
70	0.929	28.483	29.677	1.688	±1.194
	0.898	30.870			
80	0.792	39.030	38.183	1.198	±0.847
	0.814	37.336			
100	0.777	40.185	40.801	0.870	±0.615
	0.761	41.416			

Sample E					
concentration (ppm)	scavenging capacity (Absorbance)	Scavenging effect %	Average Scavenging effect %	Standard Deviation	Standard error
50	0.835	35.720	34.796	1.307	±0.924
	0.859	33.872			
70	0.662	49.038	49.192	0.218	±0.154
	0.658	49.346			
80	.6220	52.117	52.618	0.708	±0.501
	0.609	53.118			
100	0.483	62.818	63.395	0.816	±0.577
	0.468	63.972			
Sample F					
concentration (ppm)	scavenging capacity (Absorbance)	Scavenging effect %	Average Scavenging effect %	Standard Deviation	Standard error
50	0.403	68.976	64.473	6.369	±4.504
	0.520	59.969			
70	0.172	86.759	83.949	3.974	±2.810
	0.245	81.139			

concentration (ppm)	scavenging capacity (Absorbance)	Scavenging effect %	Average Scavenging effect %	Standard Deviation	Standard error
80	0.125	90.377	89.107	1.796	±1.270
	0.158	87.837			
100	0	100.00	100.00	0.000	±0.000
	0	100.00			

Appendix (e) Reducing power (FRAP) for all tested samples results:

Sample A	Absorbance	Average absorbance	Standard deviation	Standard error
100 ppm	0.419	0.410	0.017	±0.010
	0.421			
	0.390			
80 ppm	0.325	0.323	0.004	±0.002
	0.319			
	0.326			
60 ppm	0.212	0.216	0.005	±0.003
	0.215			
	0.221			
40 ppm	0.154	0.160	0.006	±0.003
	0.159			
	0.166			
Sample B	Absorbance	Average absorbance	Standard deviation	Standard error
100 ppm	0.355	0.352	0.009	±0.005

	0.358			
	0.342			
80 ppm	0.251	0.251	0.003	±0.002
	0.254			
	0.248			
60 ppm	0.158	0.159	0.002	±0.001
	0.159			
	0.161			
40 ppm	0.136	0.141	0.005	±0.003
	0.143			
	0.145			
Sample E	Absorbance	Average absorbance	Standard deviation	Standard error
100 ppm	0.142	0.139	0.0030	± 0.0017
	0.136			
	0.139			
80 ppm	0.123	0.124	0.0007	± 0.0004
	0.124			
	0.124			
60 ppm	0.111	0.109	0.0020	± 0.0012
	0.107			
	0.109			
40 ppm	0.062	0.059	0.0026	±0.0015
	0.058			
	0.057			

Sample F	Absorbance	Average absorbance	Standard deviation	Standard error
100 ppm	0.370	0.373	0.004	±0.002
	0.377			
	0.374			
80 ppm	0.287	0.309	0.022	±0.013
	0.331			
	0.309			
60 ppm	0.235	0.203	0.030	±0.017
	0.175			
	0.199			
40 ppm	0.155	0.143	0.012	±0.007
	0.131			
	0.143			
BHT	Absorbance	Average absorbance	Standard deviation	Standard error
100 ppm	0.194	0.195	0.0010	±0.0006
	0.196			
	0.195			
80 ppm	0.147	0.139	0.0080	±0.0046
	0.139			
	0.131			
60 ppm	0.120	0.124	0.0036	±0.0021
	0.127			
	0.125			
40 ppm	0.117	0.117	0.0010	±0.0006
	0.118			
	0.116			

Appendix (f): Gram positive bacterial (MRSA)

Samples A 0.02mg/ml	Width of inhibition zone (cm)	Length of inhibition zone (cm)	Average of inhibition zone (cm)	Average of inhibition zone for reading (cm)	Standard deviation	standard error
Sample 1	0.6	0.6	0.6	0.72	0.126	±0.073
Sample 2	0.7	0.7	0.7			
Sample3	0.9	0.8	0.85			
Samples B 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	1	1.1	1.05	0.88	0.144	±0.083
Sample 2	0.8	0.8	0.8			
Sample3	0.8	0.8	0.8			
Samples E 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading (cm)	Standard deviation	standard error
Sample 1	1.2	1.2	1.2	1.17	0.104	±0.060
Sample 2	1	1.1	1.05			
Sample3	1.2	1.3	1.25			

Samples F 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading (cm)	Standard deviation	standard error
Sample 1	1.4	1.5	1.45	1.63	0.202	±0.117
Sample 2	1.8	1.9	1.85			
Sample3	1.6	1.6	1.6			

Appendix (g): Gram positive bacterial (S. aureus)

Samples A 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.6	0.6	0.6	0.65	0.132	±0.076
Sample 2	0.5	0.6	0.55			
Sample3	0.8	0.8	0.8			
Samples B 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading (cm)	Standard deviation	standard error
Sample 1	0.6	0.6	0.6	0.68	0.189	±0.109
Sample 2	0.6	0.5	0.55			
Sample3	0.9	0.9	0.9			

Samples E 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading (cm)	Standard deviation	standard error
Sample 1	1.5	1.6	1.55	1.5	0.050	±0.029
Sample 2	1.5	1.5	1.5			
Sample3	1.5	1.4	1.45			
Samples F 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone (cm)	Average of inhibition zone (cm)	Average of inhibition zone for reading (cm)	Standard deviation	standard error
Sample 1	1.4	1.6	1.5	1.7	0.229	±0.132
Sample 2	1.6	1.7	1.65			
Sample3	1.9	2	1.95			

Appendix (h): Gram negative bacterial (E. coli)

Samples A 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.7	0.7	0.7	0.63	0.058	±0.033
Sample 2	0.6	0.6	0.6			
Sample3	0.6	0.6	0.6			

Samples B 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.6	0.6	0.6	0.67	0.116	±0.067
Sample 2	0.8	0.8	0.8			
Sample3	0.6	0.6	0.6			
Samples E 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.8	0.8	0.8	0.73	0.116	±0.067
Sample 2	0.8	0.8	0.8			
Sample3	0.6	0.6	0.6			
Samples F 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.8	0.8	0.8	0.77	0.058	±0.033
Sample 2	0.8	0.8	0.8			
Sample3	0.7	0.7	0.7			

Appendix (i): Gram negative bacterial (Pseudomonas)

Samples A 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	Standard error
Sample 1	0.6	0.6	0.6	0.63	0.058	±0.033
Sample 2	0.7	0.7	0.7			
Sample3	0.6	0.6	0.6			
Samples B 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.6	0.6	0.6	0.63	0.058	±0.033
Sample 2	0.7	0.7	0.7			
Sample3	0.6	0.6	0.6			
Samples E 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.8	0.8	0.8	0.73	0.058	±0.033
Sample 2	0.7	0.7	0.7			
Sample3	0.7	0.7	0.7			

Samples F 0.02mg/ml	Width of inhibition zone(cm)	Length of inhibition zone(cm)	Average of inhibition zone(cm)	Average of inhibition zone for reading(cm)	Standard deviation	standard error
Sample 1	0.7	0.7	0.7	0.67	0.058	±0.033
Sample 2	0.6	0.6	0.6			
Sample3	0.7	0.7	0.7			

الصفات الإيجابية والتأثيرات البيولوجية للمستخلصات النباتية الفلسطينية من بونيكا كرانتيم (الرمان)

اعداد : ايناس شقير أحمد شقير

المشرف الرئيسي: الأستاذ الدكتور ابراهيم كيالي

المشرف الثانوي: الأستاذ الدكتور فؤاد الريماوي

الملخص

الرمان هي شجرة قديمة تنمو في فلسطين والوطن العربي التي تمتلك العديد من الفوائد في الغذاء والتجميل والصحو والادوية في معالجة الامراض مثل السكري. يولد السكر غير الأنزيمي العديد من المركبات التي تعرف بالمنتجات النهائية للسكر المتقدمة (AGEs) التي تتراكم في الجسم وتسبب تطور الأمراض المزمنة لدى الإنسان ، مثل مرض السكري من النوع 2 وتصلب الشرايين والزهايمير. لذلك ، فإن تطوير مثبط طبيعي للـ AGEs يحتاج إلى تحقيق بحثي مكثف. تُظهر مستخلصات قشر الرمان (PPE) الغنية بمضادات الأكسدة مثل التانين والفينولات قدرة كبيرة كمثبطات طبيعية في علاج مرضى السكر. في هذه الدراسة ، تم استخلاص العفص (التانين) القابل للتحلل من قشر الرمان PP بطريقة الامواج فوق صوتية (الاهتزاز) (المستخلصA) وطريقة الغليان (المستخلص B) بنفس نوع المذيبات. للمقارنة مع التانين القابلة للتحلل، تم استخدام مذيب الإيثانول (مستخلص E) ومذيب الأسيتون (مستخلص F) لاستخراج التانينات والمواد الكيميائية الأخرى كمزيج. تم تقييم الإنتاج المضاد للسكلر للمنتجات النهائية بأربعة أنواع من المستخلصات باستخدام اختبار مصل الزلال (الألبومين) الجلوكوز البقري (BSA) في المختبر. تم تقييم التأثيرات المضادة للأكسدة بطريقتين: باستخدام طريقة الجدور الحرة (DPPH) وطريقة اختزال ايونات الحديد(+Fe3) (FRAP). تم استخدام طريقة فولين سيوكالتيو لتحديد المحتوى الفينولي الكلي ، و فحص الفلافونويد باستخدام طريقة المقاييس اللونية. تم تقييم التأثيرات المضادة للبكتيريا بواسطة طريقة الانتشار القرصي لفحص منطقة التثبيط لكل مستخلص مقابل البكتيريا موجبة الجرام (المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA)و المكورات

العنقودية الذهبية (S.aureus)) والبكتيريا سالبة الجرام(الإشريكية القولونية (E. Coli) و الزائفة الزنجارية (pseudomonas)).

تظهر النتائج االنهائية أن تركيزات 30-150 جزء في المليون من المستخلصات تشير إلى تأثير تثبيط لتكوين AGE. يُظهر المستخلص A أعلى نطاقات تثبيط عند 150 جزء في المليون بنسبة 64.62٪ ، يليه المستخلص B و F و E بنسب مثبطة بنسبة 63.92٪ و 41.60٪ و 31.130٪ على التوالي. ترجع هذه الفاعلية في قمع المنتجات النهائية AGE إلى وجود مواد كيميائية نشطة بيولوجيًا في أربعة مستخلصات ، والتي تم إثباتها من خلال فحوصات مجموع الفينولات و الفلافونويد، 453.675 ميليغرام مكافئة للجاليك أسيد لكل غرام من المستخلص الجاف ، 28.14 ميليغرام مكافئة للكوارستين لكل غرام من المستخلص الجاف A ، 409.00 ميليغرام مكافئة للجاليك أسيد لكل غرام من المستخلص الجاف، 22.49 ميليغرام مكافئة للكوارستين لكل غرام من المستخلص الجاف B، 335.33ميليغرام مكافئة للجاليك أسيد لكل غرام من المستخلص الجاف 59.05 ميليغرام مكافئة للكوارستين لكل غرام من المستخلص الجافF ، 208.33 ميليغرام مكافئة للجاليك أسيد لكل غرام من المستخلص الجاف ، 19.37 ميليغرام مكافئة للكوارستين لكل غرام من المستخلص الجاف E. مع طريقة الجدور الحرة (DPPH) ، يُظهر المستخلص F تثبيطًا بنسبة 100 ٪ عند 100 جزء في المليون ، بينما في اختبار FRAP ، يُظهر المستخلص A فاعلية أعلى من المستخلصات الأخرى. يوضح الفحص المضاد للبكتيريا قدرة المستخلصات على تثبيط نمو أربعة أنواع من البكتيريا. يمكن أن تكون مستخلصات قشر الرمان بديلاً آمنًا ورخيصًا لعلاج أمراض السكري.