Deanship of Graduate Studies

Al- Quds University

Anticancer, Antioxidant, Antimicrobial, and Enzymes Inhibitory Effects of *Terfezia Arenaria* from Palestine

Ahmed Mutasem Tawfiq Al-Khader

M.Sc. thesis

Jerusalem-Palestine

2023-1444

Anticancer, Antioxidant, Antimicrobial, and Enzymes Inhibitory Effects of *Terfezia Arenaria* from Palestine

Prepared by:

Ahmed Mutasem Tawfiq Al-Khader

B.Sc. Pharmacy at An Najah National University / Nablus. Palestine

Supervisor: Dr. Sawsan Salameh

Co- supervisor: Mohannad Qurie

A Thesis Submitted in Partial Fulfillment of requirements for the degree of Master pharmaceutical sciences Program.

Al-Quds University – Palestine

1444\2023

Al- Quds University

Deanship of Graduate Studies

Pharmaceutical sciences program

Thesis approval

Anticancer, Antioxidant, Antimicrobial, and Enzymes Inhibitory Effects of *Terfezia Arenaria* from Palestine

Prepared by: Ahmed Mutasem Tawfiq Al-Khader

Registration no: 22010044

Supervised by: Dr. Sawsan Salameh.

Co-supervisor: Dr. Mohannad Qurie.

Master thesis submitted and accepted, Date: 29/04/2023

The names and signatures of the examining committee members are as follows:

1- Head of Committee: Dr. Sawsan Salameh Signature:

2- Co-supervisor: Dr. Mohannad Qurie Signature:

3- Internal Examiner: Dr. Mahmoud AL khatib Signature:

4- External Examiner: Dr. Nidal Jaradat Signature:

Jerusalem-Palestine

2023\1444

Dedication:

I thank God who helped me with this great achievement and helped me climb a step on the ladder of success until I reach my goal.

I dedicate this work to my father, who has been encouraging me to continue my education and providing me with everything necessary for success and improvement.

To my beloved mother who accompanies me with her prayers wherever I am and supports me with all her love and tenderness, my sisters, brother, and friends.

To the souls of my uncles, Abu Tawfiq and Abd Al Sattar, who passed away two years ago before living this happy moment with us.

Declaration:

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

Signed: 

Ahmed M Al-Khader.

Date: 29/04/2023

Acknowledgments:

I would like to thank all those at Al-Quds University who helped me to get my M.Sc. degree.

I would like to express my deep respect and gratitude to my honorable supervisors Dr. Sawsan Salameh, Dr. Mohannad Qurie and great thanks to Mr. Ibrahim ayyad.

Contents

Abstract:

Terfezia arenaria belongs to the Terfeziaceae truffle family and is one of the most important desert truffles in Palestine. It is an edible truffle with many medicinal folks uses in the past and present, so the study was designed to consolidate knowledge about this truffle and to validate the medicinal uses of the natural products.

In the beginning, we used a variety of extraction methods including Soxhlet extraction, sonication extraction, and exhaustive extraction, as well as various solvents with varying polarity properties including methanol, ethanol, water, acetone, hexane, dichloromethane and ethyl acetate. We relied on sonication extraction with methanol as a solvent because it yielded the highest extract, whereas other solvents yielded negligible amounts of extract.

Qualitative tests were performed to investigate the ingredients of the methanolic extract, which revealed the presence of flavonoids, phenols, glycosides, carbohydrates, and steroids. The flavonoids and phenolic compounds were then determined quantitatively, using Folin-Ciocalteu's method for phenolic contents and aluminum chloride method for flavonoids. This work also investigated the expected effect of these compounds in addition to the discovery of new effects of the *T. arenaria*. We tested the enzyme inhibition effect on amylase enzyme, which is naturally present in the human intestine and plays an important role in carbohydrates digestion; inhibiting the enzyme may be useful to reduce the absorbed amount of glucose and thus improve the treatment of diabetic patients, so we measure the absorbance using UV spectrophotometer and higher absorbance means higher inhibition, and we use acarbose which is an antidiabetic drug as a positive control. The other enzyme we tested for inhibition effect is lipase enzyme, which is found normally in humans and other mammals and digests lipids, so inhibiting it may also aid in the treatment of obesity, orlistat used as a reference in this test and using UV spectrophotometer same as amylase inhibition.

Another important test we carried was the extraction of antioxidant because oxidative stress is a factor in the progression of many diseases, and a positive antioxidation effect

may be beneficial in reducing Alzheimer, dementia, cancer, and many other diseases. In our study we used reducing power assay method to determine the antioxidant activity.

The antibacterial activity was tested on different bacterial species, gram-positive and gram-negative bacteria, to validate one of the most well-known uses. Cytotoxicity activity was also investigated in order to test the ability to treat cancer cells in vitro, and two different cell lines used, which could be the first step in the discovery of a new lead compound for cancer therapy.

In our results we found our desert truffle to have various biochemical compound and good phenolic and flavonoids contents, so this will have positive impact on our results. And also, the results of enzymes inhibition activity were good enough to build on, as it showed good inhibition comparatively with the positive controls. For the anticancer activity there was obvious cell growth inhibition on two different cell lines used, while there was no antibacterial activity.

List of tables:

List of figures:

List of abbreviations:

Symbols	Abbreviation
ABS	Absorbance
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CC	Column chromatography
Conc	Concentration
DM	Diabetes mellitus
DMSO	Di methyl sulfoxide
DCM	Dichloromethane
Eth Ac	Ethyl acetate
FRAP	Ferric reducing antioxidant power assay
GAE	Gallic acid equivalence
Hr	Hour
HIV	Human immune deficiency virus
HPLC	High-performance liquid chromatography
DAD	Diode array detection

IC 50	Inhibition concentration of 50 %
I %	Inhibition %
Min	Minute
M	Molar
MAb	Mono clonal anti-body
PNPB	Para nitrophenyl butyrate
ROS	Reactive oxygen species
RF	Retention factor
RPM	Round per minute
TPC	Total phenolic content
TFC	Total flavonoid content
T. Arenaria	*Terfezia arenaria*
UV-Vis	Ultra violet- visible
WHO	World health organization

Chapter 1

Introduction

1.Introduction

In both allopathic and conventional systems of medicine around the world, medicinal plants, which are employed in a number of ways are defined as plants used to preserve health and treat specific problems. It is a fact that 20–25% of medicines are made from plants, and even those which have only ever used allopathic medication are probably partly dependent on them (1).

80% of the world's population, according to the World Health Organization (WHO), relies exclusively or mostly on traditional remedies for medical care (2). According to some estimates, over than two billion People may rely extensively on medicinal herbs (3).

Therapeutically active plant products have attracted a lot of attention throughout history due to their significance in the discovery of novel molecules as particular drugs. From the very beginning of the human existence, people have employed plants to treat a variety of diseases and create cosmetics (4).

About 80% of communities in underdeveloped countries still rely on phytotherapeutics as their primary form of healthcare because they are less likely to cause adverse effects, less expensive, more compatible with human bodies, and more socially acceptable (5). Furthermore, even in the majority of industrialized nations, their use has dramatically expanded during the past twenty years. For instance, in the United States, sales of herbal remedies and dietary supplements in health food stores reached $8 billion in 2017, while it was $4 billion in 1996 (6).

Despite its long history in medicine, phytotherapy frequently lacks the required scientific confidence confirmation. Nevertheless, it is recognized as a complementary technique for prophylactic treatment and disease prevention. The judicious use of phytopharmaceuticals should be supported by appropriate laboratory research and valid clinical studies. Old wives' tale that has only been proven in a few instances is that though herbal extracts typically have a wide range of effects, they have fewer negative

ones than pure single-active ingredients. Numerous academic papers on the topic state that phytotherapeutics is particularly well suited for the long-term management of chronic illnesses, in geriatric and convalescent patients, for follow-up treatment, in the prevention of degenerative, infectious, and metabolic diseases. Unfortunately, people usually assume these claims to be true. The existing state of the art could be improved with proper estimation, evaluation, and implementation of cutting-edge research like proteomics, genomes, and metabolomics (7).

Natural products, whether as pure chemicals or as standardized extracts, offer an endless number of options for new therapeutic discoveries due to the unequaled abundance of chemical variety(8). According to the World Health Organization (WHO), more than 80% of the world's population receives their primary healthcare through traditional medicine. Asia's long and significant history of human interactions with the environment is reflected in the usage of herbal therapy there. There are many different compounds found in traditional medicinal natural products that can be utilized to treat both acute and chronic diseases (9).

It is well known that using pure active chemicals compared with a natural products extract (Phyto complex) can have very different effects. Under these circumstances, looking for active components in a specific natural products extract may be pointless other than for pharmacological purposes. Because natural products extracts are so complex, creating evidence-based phytotherapy is a challenging endeavor that requires a lot of analytical work and manufacturing expertise to create well-defined, standardized Phytopreparations. Pharmacologists are faced with additional difficulties in understanding how complex combinations affect biochemical processes in health and illness due to the advent of phytomedicine (10).

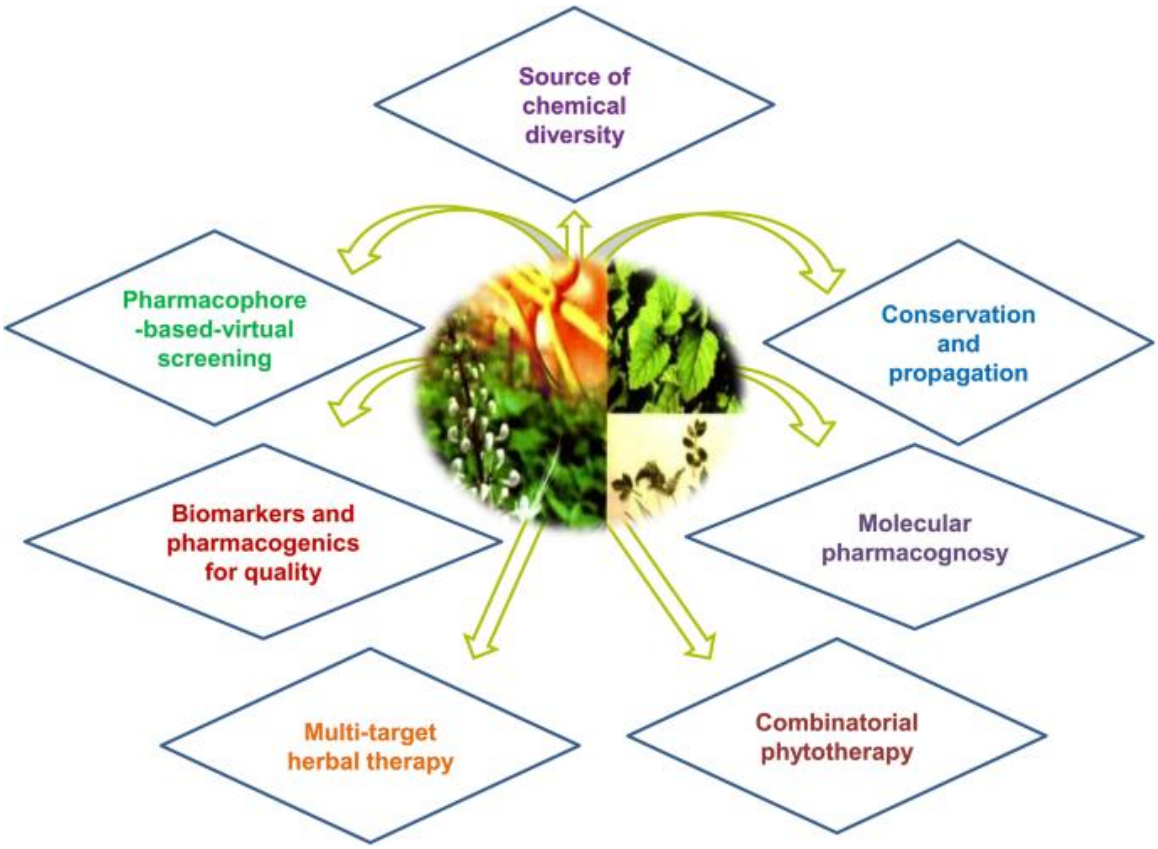


Figure 1: Phytotherapy and its impact on drug development copyright photo: (11).

About 12% of the natural products currently available on the Western market lacked significant published scientific investigations on their qualities; about 1 out of 200 of them were poisonous or allergic, and, therefore, their usage should be limited or forbidden (12).

It is upsetting to see that there is no scientific evidence to justify the widespread usage of various phytopreparations, nor is there any pertinent pharmacokinetic or pharmacodynamic data (12).

1.1. Oxidative stress

Free radicals can be externally formed in the human body by splitting water molecules to produce hydroxyl radicals.(13) Indeed, a wide range of diseases such as asthma, atherosclerosis, diabetes, cancer, Alzheimer, senile dementia, Parkinson and obesity have been connected to the development of unregulated free radicals in the human body, which can lead to cell injury and tissue denaturation (14) .

1.2. Obesity

Being overweight and obese are significant risk factors for a variety of illnesses, primarily cardiovascular diseases which are the world's leading cause of death. Obesity is also a significant risk factor for insulin resistance and diabetes (15). As a result, numerous studies are currently concentrating on creating novel tactics to reduce obesity and enhance general health (16).

1.3. Diabetes mellitus (DM)

Due to problems caused in the micro- and macrovascular systems, diabetes mellitus is one of the most serious metabolic conditions and a leading cause of mortality and morbidity worldwide, a study in 2021 prove that a strong correlation between diabetes and neuropathy, nephropathy and retinopathy (17).

1.4. Antimicrobial resistance

In recent years, the availability of efficient treatments and preventive strategies for a variety of infectious diseases brought on by bacteria, fungi, parasites, and viruses has come under serious danger from antimicrobial resistance. All civilizations, as well as the public and private health sectors, must take action to address this major threat to global health systems, and without adequate and potent antimicrobial medication, the efficacy of

cancer treatment and all surgical procedures will be in jeopardy (18). Global estimates show that from 2016 more than half a billion people developed multi-drug resistance to tuberculosis alone and that the fight against malaria and HIV is also becoming more challenging due to antimicrobial resistance (19).

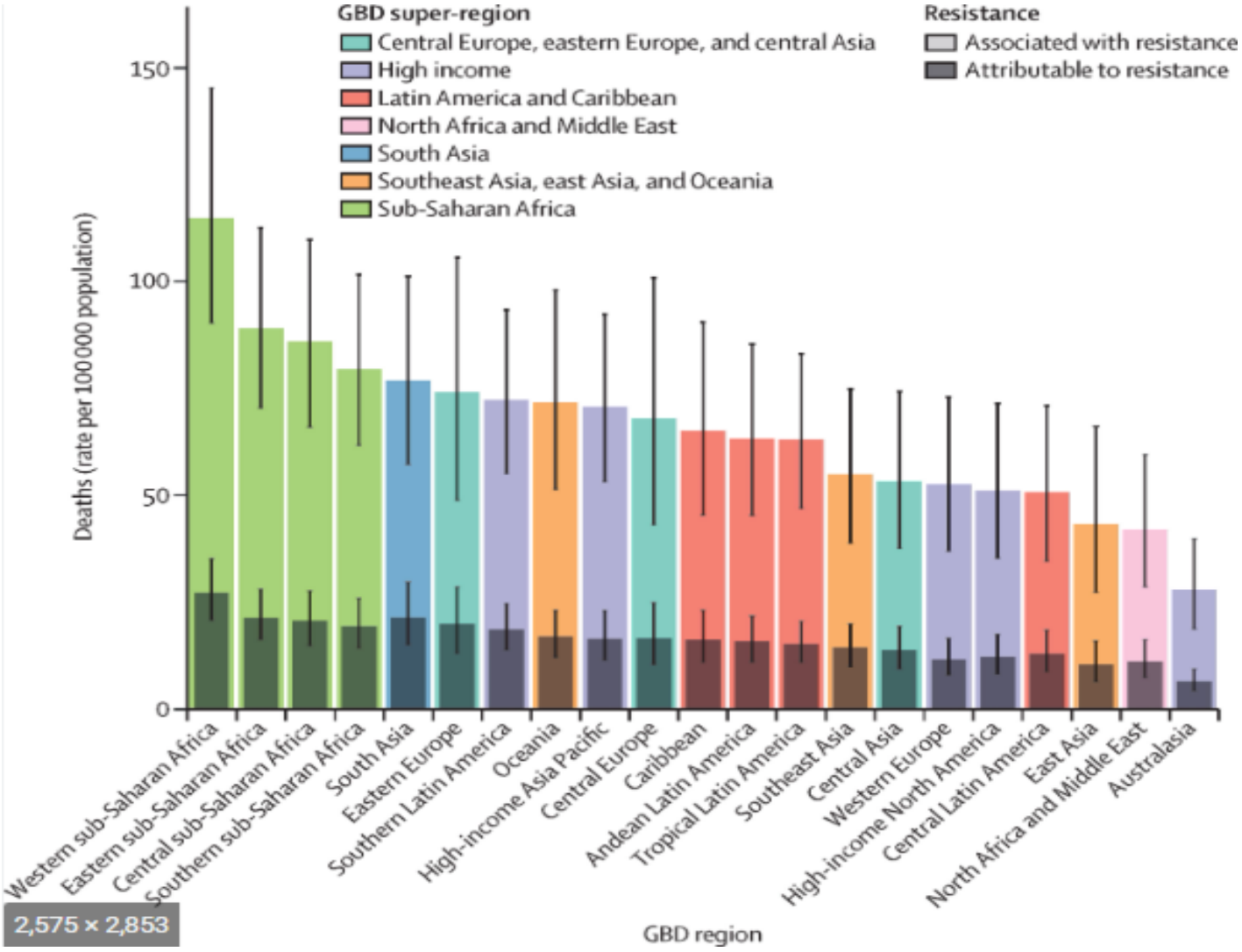


Figure 2: All-age rate of deaths attributable to and associated with bacterial antimicrobial resistance by GBD region, 2019. Photo Copyright (20).

1.5. Cancer

Cancer is a serious hazard to human life and a problem for public health in the twenty-first century. The focus of global resources must be on initiatives for finding preventive measures for this disease and immediate medical care(20). In fact, most cancer chemotherapy medications come from natural products, mainly, vinblastine from the Vinca and taxol from the bark of the Pacific yew (21).

1.6. Desert truffle Extraction

Different extraction methods are used in truffle extraction around the world; some are chosen to be suitable for the natural prodruct itself, while others are chosen to obtain specific extracts with specific properties such as lipophilicity, solubility, or oily extracts (22).

The sonication extraction method is used to extract compounds with the solvent property used in the extraction using ultrasonic power to extract deeply rather than shake (23).

Another extraction method is Soxhlet extraction, which is a thorough extraction technique widely used on thermally stable natural product. The extraction solvent is continuously cycled through the natural product by boiling and condensation, with the natural product being collected in the hot solvent (24).

CO_2 extraction, also known as supercritical carbon dioxide extraction, which yields a pure, clean, and safe result. This is a well-liked technique for extracting different components from natural product. The supercritical state of carbon dioxide is reached at 1071 pressure and 31.1 °C. A molecule has both liquid and gas properties when it is in a supercritical state, and at the end the result of the extraction is hydrophobic (25).

1.7. Desert truffle

Several desert truffles have been discovered in the Palestinian desert Al-Naqab, and their ability to produce bioactive substances such anticancer, antimicrobial and enzyme inhibitory properties is under investigation.

Based on morphological analysis, and the identification analysis in pharmacognosy department in Al Quds university the collected strains were recognized as *T. Arenaria*, a member of the Terfeziaceae truffle family. There are about thirty-four species of Terfezia general species, which are found in different deserts, particularly in Mediterranean regions.

Figure 3: Desert Truffle "*Terfezia arenaria*" fresh truffle.

The fruit bodies of Desert Truffle "*Terfezia arenaria*" are large, spherical to turbinate in shape, thick-walled, and solid. Marbled veins interspersed with sterile tissue form the asci, which are cylindrical to spherical, indehiscent (they do not split open when mature), and occasionally stain blue in iodine. Ascospores are hyaline to pale brown, spherical, and uninucleate, with a similar appearance to a traditional truffle, weighing between 30 and 300 grams and measuring about 5 cm in diameter (26).

As an effective antibacterial agent for the treatment of numerous bacterial illnesses of the eye, gastrointestinal tract and urinary systems, Terfezia species truffle are widely employed in Arabian and Islamic medicine. In some regions, they are also tasty (27).

Some research had previously been conducted on *T. Arenaria*, but what distinguishes our study is that no research had previously been conducted on Palestinian desert truffle, or tested the anti-enzymatic activity of the extracted; moreover, poor characterization had been applied on the truffle content.

1.7.1. **Nutritional value**

These truffles are generally consumed for their flavor, but their high protein content, which includes important amino acids and antioxidants, as well as minerals, fiber, omega-3, and 6 acids, make them nutritionally fascinating. Because it is intended to replace a significant amount of the proteins derived from meat with proteins of fungal origin, desert truffles are currently a very fascinating product (28).

1.7.2. **Cultivation**

Although historically these mushrooms were taken from the wild, they have recently been tamed and are now grown in farms. The term "thermi culture" refers to this kind of horticulture. Desert truffles are usually symbionts with truffle of the genus Helianthemum, but can also occur in symbiosis with other genera, such as Avena or Pinus. Although there are several species of these truffles, the most commonly used for cultivation is the species *T. claveryi*, in symbiosis with Jarilla (Helianthemum almeriense). Mycorrhization is challenging and difficult to produce in a large-scale, but once it is successfully implanted, it regenerates continuously because as the Marilla seeds fall on the ground, they become impregnated with the mycelium present in the soil allowing the mycorrhizae to persist (29).

1.8. Significance of the study

We are the first to test the truffle anti-diabetic and anti-obesity effects, as well as its antibacterial and cytotoxicity effects. Thus, the success of this study may lead pharmaceutical companies to focus on the improvement of this type of medication, particularly here in Palestine because it is available in our desert, and may lead to increase confidence in the general community in the folk uses of this truffle.

The objectives of the study are as follows:

- To extract the truffle using the best techniques and solvents available.
- To assess the antioxidant activity of the truffle extract.
- To assess the anti-enzymatic activity of the extract of the truffle.
- To study the cytotoxicity and antibacterial activity of the truffle extract.

Chapter two:

Literature review

2. Literature review

Terfezia arenaria has previously been studied for its antioxidant, antibacterial, anticancer, and antifungal properties.

In 2012, Algerian researchers Samir Neggaz and Zohra Fortas tested the antibacterial and anti-fungal activity of the Algerian desert truffle (30). They used the Soxhlet extraction method with ethyl acetate yielded 4%, and then this extract entered the column chromatography (CC) using petroleum ether and increasing concentration of ethyl acetate as a mobile phase. The results were six fractions, and each analyzed by TLC by 6:1 petroleum ether to ethyl acetate (30).

In the antibacterial activity test, Staphylococcus aureus, Enterococcus aeruginosa faecalis, Pseudomonas, Escherichia coli, and Candida albicans were used.

It was discovered that the antimicrobial activity of different fractions separated from crude ethyl acetate extract of *Terfezia pinoyi*, especially with fractions no 2 and 6, support the usage of truffle extract traditionally treating some infection (30).

In 2013, Hasan Hüseyin Doan and colleagues (31) conducted a research on the antibacterial effect, antioxidant activity, and phenolic content of the desert truffle in Turkey. They tried different extraction solvents: methanol, acetone, and chloroform. The DPPH scavenging activity method and reducing power assay was used in the anti-oxidant activity test, in addition to BHA and BHT were used as a reference for antioxidants. The results show that economically important and edible mushrooms may have significant antioxidant activity by giving IC50 lower than BHA and higher than BHT in the DPPH assay (31).

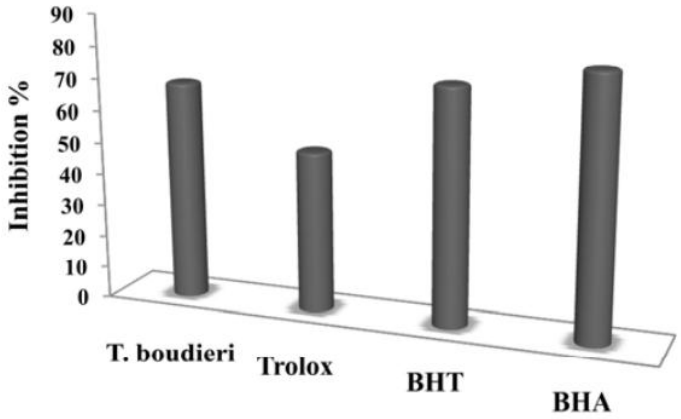


Figure 4: Statistical comparison between *T. boudieri* and antioxidant references (Trolox, BHA, and BHT) the inhibition % graph (31).

The reducing power of *T. boudieri* extract showed parallelism with its increased concentration. The highest reducing power was observed with BHA, and it was followed by BHT and *T. boudieri* **figure (6).** This highest reducing power is described in the study carried out by *T. Boudieri.*

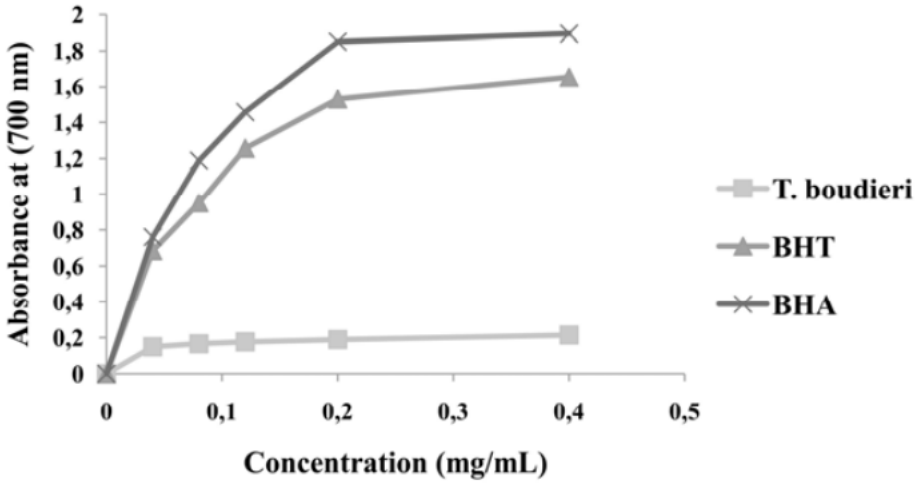


Figure 5: Absorbance values of *T. boudieri* and synthetic antioxidant compounds.(31)

In terms of antibacterial activity, different extracts, particularly acetone and chloroform, were found to have a significant effect on the tested bacterial species with MIC less than 100 mcg/ml.

In order to identify certain specified phenolic acids and flavonoids in the extract, they were separated using HPLC and putting in comparison with real standards. This helps us to know the total phenolic and phenolic compounds of *T. boudieri.*

Antioxidant, antimicrobial, antiallergy, and anticancer effects of catechin were reported in some previous studies, and in the study, it was found at 20 mg/g which is quite a high ratio. Moreover, in this present study, antimicrobial activities appeared in high impact that can be related to high catechin levels.

In 2019, Harir M. and colleagues (32) tested the antimicrobial activity of *T. arenaria* extracts collected from the Saharan Desert against bacteria and filamentous fungi. Two extraction methods were used in the study (normal extraction with methanol and Soxhlet with dichloromethane) yielded 15.5% and 0.48%, respectively. The study used five bacterial strains, three were gram-negative (Escherichia coli, Pseudomonas aeruginosa, Salmonella Typhimurium) and two were gram-positive (Staphylococcus aureus, Enterococcus faecalis). Regarding the *T. arenaria* extracts obtained by the Soxhlet method with dichloromethane, they presented strong antibacterial activities against all the tested bacteria (S. aureus, E. faecalis, E. coli, and P. aeruginosa) for all the extract concentrations tested, while on the other hand, the extracts by the maceration method have no activity at low concentrations against all bacterial strains, and this activity gets a little higher at higher concentrations. The same thing happened for antifungal activity. It is generally noted that the antifungal potential and especially against the Candida albicans was stronger with Soxhlet extraction with DCM than previously reported studies; the maceration method extraction with methanol was used.

The quantitative analysis tests for the total phenolic and flavonoids contents were conducted, the dichloromethane extracts by Soxhlet contains 55.02mg GAE/mg extract of polyphenols and 5.68 mg QE/mg extract of flavonoids content. While the methanolic extract by maceration method contains 48.99 mg GAE/mg extract of polyphenols and 9.79 mgQE/mg extract of flavonoids content (32).

In 2020**,** Al Obaydi MF and colleagues (33) tested the anticancer and immunomodulatory activity of desert truffle (*Terfezia boudieri*). They used different solvents in the extraction and found that the aqueous and aqueous-methanol were the highest-yield extracts.

MCF-7, T47D, MDA-MB231, HCT-116, Hela, and Vero cell lines were used to test the cytotoxicity activity of *T. boudieri* by different (33).

The results showed that hexane and ethyl acetate extracts have the highest activity against the MCF-7 cell line with minimum IC50, for T47D and MDA-MB231 cell lines, and showed good activity in growth inhibition with a noted increase in hexane activity. n HCT-116, Hela, and VERO extracts showed dose-dependent activity with lower toxicity for Vero cells (33).

The aqueous and aqueous-methanol extracts have the highest phenolic contents, determined according to the Folin-Ciocalteu procedure. The TPC increased in a concentration-dependent manner.

The study tested the activity on immune systems, as immune cells, cytokines, and immune responses effects were taken into account.

Testing the activity of aqueous-methanol extract on VEGF showed good activity on the T47D cell line compared to the anticancer drug doxorubicin which inhibits the angiogenesis process. The same extract was tested on caspase-3 to Assis the effect on apoptosis compared to doxorubicin.

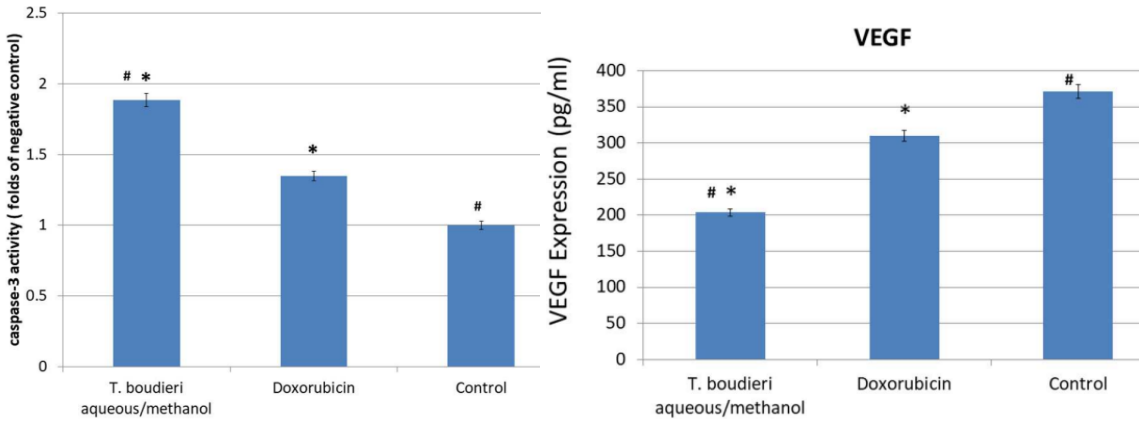


Figure 6: The in vitro biological activity of *T. boudieri* on immune systems (33).

(a) The effect of *T. boudieri* aqueous/methanol extract at a concentration of (25 mg/ml) and doxorubicin (250 nM) on caspase 3 activity expressed by number of folds of negative control.

(b) The effect of *T. boudieri* aqueous/methanol extract at a concentration of (12.5 mg/ml) and doxorubicin (250 nM) on VEGF (vascular endothelial growth factor) expression (pg/ml) in T47D cells.

The study concluded that the truffle *T. bouderi* is rich in biologically active phytochemicals. It contains substances that have anticancer and immune-modulating properties. Its anticancer properties are mediated by apoptosis induction and angiogenesis suppression. The immune system's innate and acquired defenses are activated, which has an immunomodulatory effect (30).

Chapter three:

Methodology

3. Methodology

3.1. Instrumentation

- Sonicator (ULTRASONIC CLEANER DC150H).
- HPLC (Nexera X2 LC30AD), column oven (Nexera X2 cto-30A), autosampler (Nexera x2 sil-30AC), communication bus mobile (CBM-20A), Degassing unit (DGJ-20A), DAD (Prominence SPD-M20A).
- UV-visible spectrophotometermeter-1601-shimadzu
- Rotary evaporator (stuart)
- Soxhlet extractor shimadzo.
- Vortex agitator (classic advanced vortex mixer, VELP SCINTIFICA).
- Centrifugator (thermo scientific, medifuge).

3.2. Chemicals and materials

Water purified in lab, methanol was purchased from Lobachemie, hexane purchased from Frutarum, dichloromethane purchased from sigma-aldrich, acetone purchased from Frutarum, 3-5-dinitro salicylic acid purchased from sigma-aldrich, corn starch purchased from Palestinian market, Trolox purchased from sigma-aldrich, DMSO purchased from sigma-aldrich, amylase purchased from sigma-aldrich, lipase purchased from sigma-aldrich, Ninhydrin solution, Millon's reagent, *Fehling's solutions, Benedict's reagent, Molisch's solution* all were obtained from Gadot(Israil), magnesium ribbon, H_2SO_4 were obtained from SDFCL, *Iodine*, $FeCl_3$ obtained from (Riedeldehan, Germany), HCl were obtained from SDFCL, potassium acetate, AlCl3, NaOH, acetic acid, chloroform, NaHCO3, gallic acid, Rutin obtained from MP-Biomedical (USA), folin-ciocalteo reagent purchased from sigma-aldrich, S,Aureus 23235, P,Aeruginosa 9027, S,Pneumonia 13883, E,coli 25922.

3.3. truffle collection and preparation

The truffle was collected from Al Naqab Palestinian deserts by volunteer students from Al Quds University. The truffles were washed several times with water and cleaned well from the sand, and then cut into small pieces, dried by oven at 45 C, and finally milled to be a dry powder.

3.4. Experimental part

3.4.1. Extraction

3.4.1.1. Sonication extraction

Sonicator was used to extract the entire truffle with various solvents.(34) Sonication fractionation was performed using different solvents with varying degrees of polarity, including methanol, water-methanol, water, ethyl acetate, ethanol, ether, acetone, hexane, and dichloromethane. A 5 g of dried milled truffle was placed in a glass bottle and extracted with 100 ml of each solvent separately. Each truffle and solvent bottle were placed on a Sonicator at room temperature for 24 hours. Each extract was filtered using centrifugation, and then dried using a rotary evaporator.

3.4.1.2. Soxhlet extraction

The phytochemical extraction was performed using organic solvent extraction. The extraction was done by taking 3 gm of dried truffle powder and placed into a glass thimble, and then extracted with 250 ml of methanol. The extraction processes carried on till the solvent in the siphon tube of the Soxhlet apparatus becames colorless. After that,

the extract was evaporated by the rotary evaporator till the extract became dry. Finally, the truffle crude extract was kept in a dry cool place for their future use.

3.4.1.3. Exhaustive extraction

It is the simplest extraction method used in the study; water and various organic solvents with different polarity were used in the extraction process. The truffle was soaked in the solvent for 24 hours each time, with or without shaking.

3.4.2. **Phytochemical analysis**

The purpose of the phytochemical screening tests was to find both primary and secondary metabolic groups such as proteins, carbohydratess, starch, flavonoids, saponins, glycosides, alkaloids, tannins and phenols.(4)

3.4.2.1. Qualitative test:

Proteins Test

- Millon’s test: 2 ml of Millon's reagent was mixed with 2 ml of truffle crude extract, and then the test tube was placed in a water bath for about 2 minutes. A white precipitate was observed, which turned out to be red upon gentle heating, indicating the presence of protein in the truffle.
- Ninhydrin test: 2 ml of the 2% ninhydrin solution was added to extract solution. The test tube was placed in a warm water bath for about 5 minutes. The presence of amino acids was indicated by the deep blue/violet color.

Carbohydrates Tests

- Fehling's solutions test: a Fehling solutions A and B were boiled and added to crude truffle extract in equal parts. The presence of reducing sugars was indicated by a red precipitate.
- Benedict's reagent test: A crude extract was boiled with 2 ml of Benedict's reagent, resulting in a reddish-brown color indicating the presence of carbohydrates.
- Molisch's solution test: 2 ml Molisch's solution was shaken with crude truffle extract, and then 2 ml concentrated H_2SO_2 was carefully poured along the side of the test tube. A violet ring appeared at the test tube's interphase, indicating the presence of carbohydrates.
- Iodine test: A crude truffle extract was mixed with 2 mL of iodine solution. The presence of carbohydrates is indicated by the presence of purple or dark blue colors.

phenols and tannins test

- Two milliliters of 2% solution of $FeCl_3$ were mixed with the crude extract. Black or blue-green color indicating the presence of tannins and phenols.

flavonoids tests

- Shinoda test: pieces of magnesium ribbon and HCl concentrated were mixed with crude truffle extract, and after a few minutes pink colored scarlets appeared, indicating the presence of flavonoids.
- Alkaline reagent test: 2 ml of 2% NaOH solution was mixed with truffle crude extract, resulting in an intense yellow color that turned out to be colorless when 2 drops of diluted acid were added to the solution, indicating the presence of flavonoids.

Saponins Test

- In a test tube, five milliliters of distilled water were added and vigorously shaken with the crude truffle extract. The presence of saponins was indicated by the formation of foam.

Glycosides tests

- Liebermann's test: A total of 2 mL of acetic acid and 2 mL of chloroform were mixed with the entire truffle crude extract. After cooling and adding concentrated H_2SO_4, the green color indicated the entity of the aglycone steroidal part of glycosides.
- Salkowski's test: A concentrated H_2SO_4 solution (approximately 2 mL) was added to the entire truffle crude extract. The presence of a reddish-brown color indicated the presence of the steroidal aglycone component of the glycoside.

Steroid tests

• The entire truffle crude extract was mixed with two milliliters of chloroform and concentrated H_2SO_4. The presence of steroids was indicated by the presence of red color in the lower chloroform layer.

• Another experiment was carried out by mixing 2 ml of each acetic acid with H_2SO_4 concentrated and crude extract with 2 ml of chloroform. The entity of steroids was represented by the green color.

Terpenoids test

• Two milliliters of chloroform were mixed with the truffle extract and evaporated on the water path before being boiled with two milliliters of concentrated H_2SO_4. Terpenoids were represented by a grey color produced.

3.4.2.2. Quantitative tests:

Determination of total phenol content in truffle extract

The total phenolic content (TPC) of truffle methanolic and ethanolic extracts was determined using a modified spectrophotometric method [25]. In the analysis, 1 mg/ml

aqueous solutions of methanolic extract were prepared. Mixing 0.5 mL of truffle extract solution, 2.5 mL of 10% Folin-reagent Ciocalteu's dissolved in water, and 2.5 mL of 7.5% $NaHCO_3$ aqueous solution yielded the reaction mixture. The samples were then incubated for 45 minutes in a thermostat set to 45 degrees Celsius. The absorbance was measured with a spectrophotometer at 765 nm. For each analysis, the samples were prepared in triplicate and the mean absorbance value was determined. The calibration line was constructed using the same procedure for the standard solution of gallic acid at various concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mcg/ml). The concentration of gallic acid equivalent is expressed as (mg of GA/g of extract) based on the measured absorbance.(35)

Determination of flavonoid concentrations in the truffle extract

Aluminum Chloride Colorimetric Method:

The aluminum chloride colorimetric method was adapted from the Woisky and Salatino procedure.(36) The calibration curve was created using quercetin. The standard solutions (1 mL) were separately mixed with 3 mL of methanol, 200 mcg of 10% aluminum chloride, 100 mcg of 1M potassium acetate, and 5 mL of distilled water. The absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer after 30 minutes at room temperature (Kyoto, Japan). In the blank, the same amount of 10% aluminum chloride was replaced by the same amount of distilled water. Similarly, the methanolic extract, like the flavonoid standard solution, was reacted with aluminum chloride to determine flavonoid content as previously described(37).

3.4.3. **Anti-oxidant activity reducing power assay**

The reducing power was calculated using the Oyaizu method (1986).(38) *T. arenaria* methanolic extracts (2.5 ml) in various concentrations were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. For 20 minutes, the mixture was incubated at 50 degrees Celsius. The mixture was centrifuged at 650 rpm for 10 minutes after 2.5 ml of 10% trichloroacetic acid (w/v) was added. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1 ml of 0.1%

ferric chloride, and then the absorbance at 700 nm was measured: higher absorbance indicates greater reducing power. The tests were done in triplicate, and the results are given as mean values with standard deviations. The extract concentration providing 0.5 of absorbance (EC50) was calculated from the absorbance versus extract concentration graph at 700 nm. As controls, Trolox solution which is a vitamin E derivative used as positive controls.(39)

3.4.4. **Cytotoxicity procedure**

The cytotoxicity test was performed on two cancer cell lines: breast cancer MCF-7 and colon cancer HT-29. Cell culture and cytotoxicity tests had taken place on the Al-Raihan campus of Arab American University AAUP.

MCF-7 and HT-29 cells were cultured in 5 ml of RPMI 1640 medium containing 20% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 g/ml). Cells were cultured every 3 days at 37° in a humidified atmosphere with 5% CO2 in the air. In 96-well plates or Petri dishes, exponentially growing cells were seeded at a density of 2104 cells/ml. Following an overnight incubation to allow for attachment, the cells were exposed for 24 or 48 hours to 100 µL diluted in 10 ml of distilled water.(40)

3.4.5. **Porcine pancreatic lipase enzyme inhibitory method**

A stock truffle dilution of one mg/ml was prepared by dissolving 1 g of each truffle fraction in 100 ml of Dimethyl sulfoxide (DMSO). The resulting solution was diluted several times to achieve various concentrations (50, 100, 200, 300, and 400 mg/ml). A lipase enzyme stock solution (1 mg/ml) was also prepared directly before use by dissolving 25 mg of lipase enzyme powder in 25 ml of 10% DMSO. 20.9 mg of p-nitrophenyl butyrate (PNPB) was dissolved in 2 ml of acetonitrile to make the stock solution. In 0.2 ml, each truffle fraction was prepared. Serial dilutions were mixed with 0.1 ml of lipase enzyme stock solution and Tris-HCl solution to achieve 1 ml of volume. Orlistat, a potent anti-lipase drug, was used as a positive control and went through the

same procedures as the truffle fraction. A spectrophotometer (UV-vis) was used to measure absorbance at 405 nm.

The lipase enzyme inhibitory potential was calculated using the following equation:

I (%) = [ABS blank – ABS test] / [ABS blank]) *100 %

Where, I (%), is the percentage inhibition of lipase enzyme.(41)

3.4.6. **α-Amylase inhibitory method**

By combining 25 mg of each truffle fraction with 10% DMSO, a truffle working solution (1 mg/ml) was created. The buffer was then used to dilute this solution to obtain different dilutions (10, 50, 70, 100, and 500 mg/ml).

Subsequently, an α-amylases enzyme stock solution (2 units/ml) was prepared by dissolving 12.5 mg of α-amylase enzyme in a minimum amount of 10 % DMSO– and the buffer solution was added up to 100 ml. Corn starch solution was prepared by dissolving 1 g of starch in 100 ml distilled water. A 200 µl from each truffle fraction stock solution was mixed with 200 µl of α-amylase stock solution and incubated for 10 min at 30 °C in a water bath. After that, 200 µl of corn starch solution was added and incubated at 30 °C for 3 minutes. Furthermore, 3,5-dinitro salicylic acid was added and boiled in a water bath at 85–90 °C for 10 minutes. After cooling, 5 ml of distilled water was added and blank solutions were prepared throughout the replacement of the *T. arenaria* fractions with 200 µl of buffer solution. Acarbose, a commercial anti-diabetic drug, was also used as a positive control. A UV–vis spectrophotometer was used to measure the optical activity of the prepared solutions at 540 nm. The following equation was used to calculate the -amylase inhibitory potential:

I (%) = [ABS blank – ABS test] / [ABS blank]) *100 %

Where I (%), is the α-amylase inhibitory percentage.(42)

3.4.7. **Antibacterial test**

The antimicrobial activity of the methanolic extract was determined by using the broth microdilution method of methanolic extract of *T. Arenaria*.(43) The screened strains, however, included Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa; each bacterial strain tested was streaked onto nutrient agar medium to obtain isolated colonies. After incubation at 37 °C overnight, we selected three well-isolated colonies and transferred the growth to a tube of sterile nutrient broth (10 mL), and then incubated at 37 °C for 24 h. After that, the bacterial suspension was agitated on a vortex mixer immediately, and then compared to the 0.5 McFarland standard (108 CFU/mL) to adjust the turbidity of the inoculum suspension. 15 mL of the molten Mueller Hinton Agar was poured evenly into Petri plate (9 cm diameter). Using a sterile pipette, 1 mL of the 24 h test bacterial broth culture (1 × 106 CFU/mL) was spread over the surface of the dried agar plates using a sterile glass spreader, and then allowed to absorb in the agar for 10 min; the plates dried, inverted, at 37 °C for approximately 30 min until the bacterial overlay had dried on the surface. Sterile filter discs (6 mm diameter) were impregnated with 20 µL of each fraction of the crude extract of *T. arenaria*; three filter discs were used, methanol as a negative control and antibiotic "penicillin and azithromycin" as positive control, and all were impregnated in the agar by the filter paper.(44)

3.4.8. **Characterization:**

- **Thin layer chromatography (TLC)**

Variant solvents used in the TLC attempt to get separated layers under UV light. We used in the study too many mixtures of solvents with different polarities such as DCM: METH 10:1, Hex: Meth 15:1, Ethyl AC, Ethyl AC: Meth 10:1, Ethyl AC: M Eth 5:1, Ethyl AC: METH 1:1.

- **Ultra-performance liquid chromatography (UPLC)**

An Ultra-Performance Liquid Chromatograph (UPLC, shimadzu) coupled to a photodiode array detector (PDA, shimadzu) was used. Chromatographic separation was done using an UPLC column (UPLC® C18, 100 mm × 3 mm, 1.7 µm, shimadzu). A gradient mobile phase was used: mobile phase (A) 70% water: 30% methanol for 10 min, (B) 80% water: 20% methanol for 10 min, and (C) 70% water: 30% methanol. The column temperature was maintained at 25℃, flow rate at 1 ml/min, wavelength range between 210 and 400 nm, and 10 µl sample was injected.

Chapter four:

Results and discussion

4. Results and discussion

4.1. Extraction:

The extraction process varied depending on the solvents and extraction techniques used.

Table 1: the percentage yields of different solvents used in the extraction of *T. arenaria*.

Solvent	water	methanol	hexane	acetone	ethanol	DCM	Eth Ac	Water-methanol	Ether
Yield %	30	35	6	1	16	1	4	36	8

According to the results in the table, which compare the extraction yields of various solvents using the sonication extraction method, methanol, water, and water-methanol have the best extraction yields with 35, 35, and 36, respectively, while hexane, ethanol, and ether have less efficient extraction yields with 6, 16, and 8 respectively. Additionally, acetone and DCM were completely insolubilizing the truffle's entire materials and did not exhibit any extract following evaporation.

According to prior research on our truffle and other members of the same family of truffles, the methanol extract was the most compatible extract to utilize in our test, and also produced the maximum yield.

Comparing several extracts and their yields was crucial to save time and effort, as well as to save the truffle powder, which is not readily available in sufficient quantities.

In comparison to methanol alone, water and water-methanol produce very good yields, but they were not chosen for the study because they are more difficult to evaporate, require a more involved process like freeze drying and this is not easily available in our lab, take longer time to complete, and are not sufficiently supported by prior studies as methanol.

4.2. Phytochemical analysis:

4.2.1. Qualitative test:

In an initial research on phytochemicals as the Table below shows, that while terpenoids and saponins were absent on the one hand, proteins, carbohydrates, glycosides, steroids, tannins, flavonoids, and phenols were present on the other hand, as determined by routine analytical testing.

Table 2: qualitative analysis of compound contents of the methanolic extract of *T. Arenaria*.

phenols	flavonoids	terpenoids	glycosides	carbs	protein	tannins	saponins	steroids
+	+	-	+	+	+	+	-	+

While the presence of phenols and flavonoids supports the usage of the truffle as an anti-inflammatory for various infections in folk uses, which will be proven in our study, the presence of carbohydrate and protein supports that the truffle is edible and rich in nutrients, and that is why it is considered a food in many cultures around the world.

To understand how a phytochemical effect might occur, phytochemical analysis and compound group detection in the extract are crucial.

Flavonoids:

Flavonoids are a diverse group of polyphenolic compounds with the structural element benzo-pyrone that are present in many natural product. According to reports, a number of pharmacological actions are caused by secondary metabolites of a phenolic character, particularly flavonoids. The phenylpropanoid pathway generates these. Flavonoids are hydroxylated phenolic chemicals that are known to be produced by natural product; their activities rely on their structural makeup. It is well known that microbial infection causes the production of flavonoids. The chemical composition of flavonoids is influenced by

their structural class, degree of hydroxylation, different replacements and conjugations, and degree of polymerization.(45)

There are numerous pharmacological actions that flavonoids can make, however the following are the most important:

Activity of Antioxidants: Although flavonoids have a wide range of biochemical properties, most of them are well-known for their capacity to act as antioxidants. Some of the processes through which antioxidants work include chelating trace elements that form ROS, blocking the enzymes that produce ROS, and scavenging ROS. Controlling or safeguarding antioxidant defenses are examples of additional processes.(46)

It is not surprising that flavonoids, which natural product produce in response to microbial infection, demonstrated its effective antimicrobials in vitro against a variety of bacteria. Numerous natural product species with high flavonoid concentrations have been found to possess antibacterial properties.(47)

Numerous flavonoids including catechin, flavonoid-rich naringenin, rutin, and venoruton, have been linked to hepatoprotective effects(48).

A typical biological response to tissue damage, microbial pathogen infection, and chemical irritation is inflammation. Anti-Inflammatory Activity: Hesperidin, apigenin, luteolin, and quercetin are only a few flavonoids that are thought to have analgesic and anti-inflammatory activities. Flavonoids may have a direct effect on protein kinases, particularly tyrosine and serine-threonine protein kinases, which are crucial for the generation of inflammatory processes.(49)

Dietary factors are important in the management of cancer with its anticancer effect. According to reports, the flavonoids found in fruits and vegetables can prevent cancer.(50)

Natural compounds are an important source for the discovery and development of novel antiviral medications due to their accessibility and predicted lack of adverse effects. Since the 1940s, it has been known that flavonoids which is found in nature have antiviral effects, and there are several papers published on the antiviral properties of various flavonoids.(51)

4.2.1 Quantitative test:

4.2.1.1. Total phenolic content

The quantitative determination of the total phenolic content was carried out using the Folin–Ciocalteu reagent. The TPC was calculated from the calibration curve (Y = 0.0097x-0.0064, R^2 = 0.995) of standard gallic acid and expressed as mg GAE/g dry extract weight. The TPC of the studied truffle sample was 50 mg GAE/g dry extract weight, which is considered high amount of these compound.

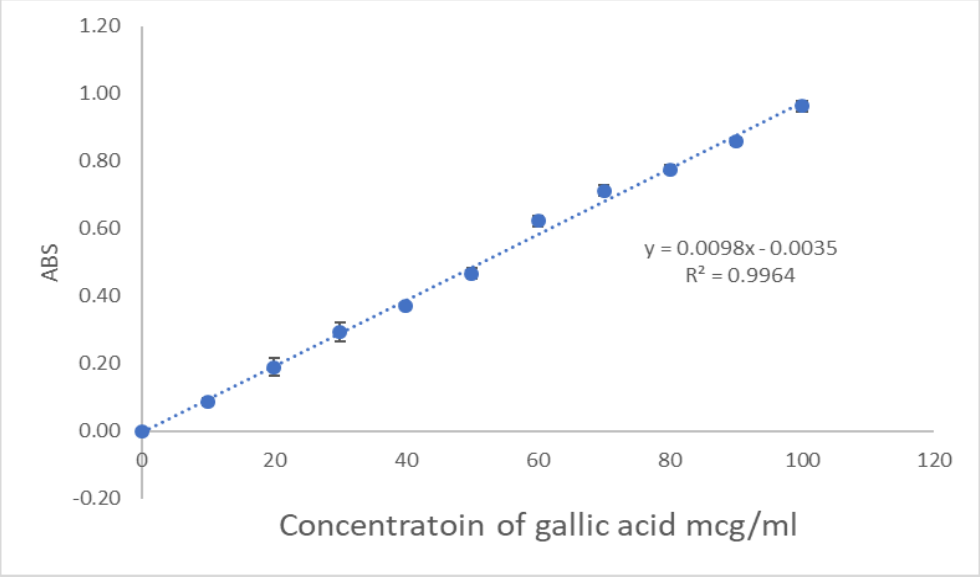


Figure 7: gallic acid calibration curve.

4.2.1.2. Total flavonoid content

The analysis of the flavonoid concentration in truffle extract is crucial because, as mentioned before, flavonoids play a key role in antioxidant, antibacterial, anti-inflammatory, and other functions. Additionally, a number of factors have been theorized to be essential for the production of the flavonoid-AlCl3 complexes including reaction

duration, reagent concentration (AlCl3 and flavonoid content/truffle material), and polyphenol chemical structure (48).

Using AlCl3 technique, the total flavonoid content was quantitatively determined. The TPC was determined using the calibration curve of standard quercetin (Y = 0.024x+0.0018, R2 = 0.9998) and expressed as mg GAE/g dry extract weight.

The result of the total flavonoid contents of the crude extract of *T. arenaria* appear in the figure below. The total flavonoid content in the crude extract was 32 mg quercetin/g weight.

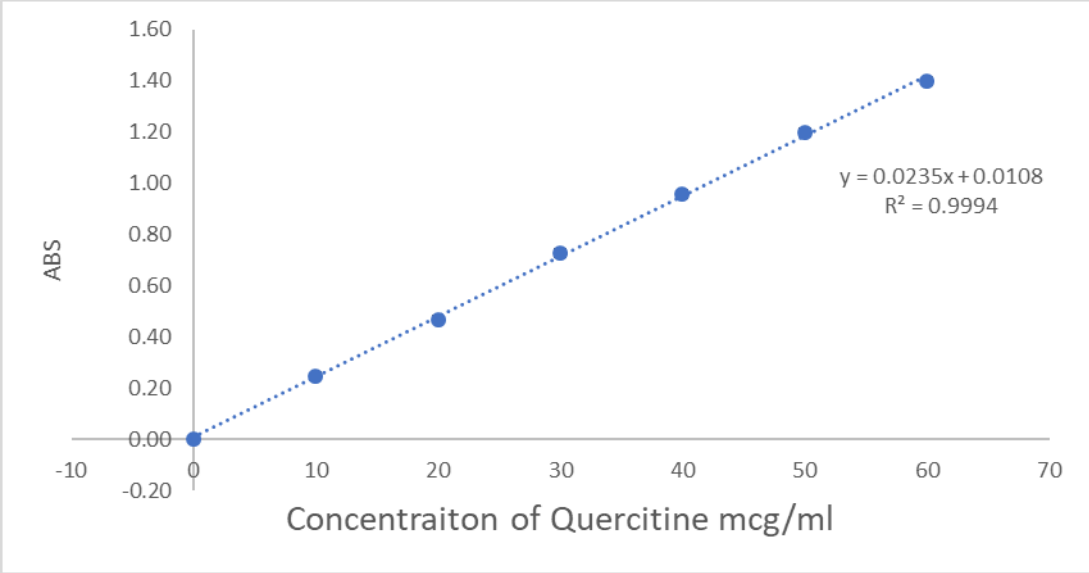


Figure 8: quercetin calibration curve.

4.3. Antioxidant activity: Ferric-reducing antioxidant power assay (FRAP)

In the form of phenolic compounds, there are nitrogen compounds, vitamins E and C, terpenoids, endogenous molecules, and natural product that contain significant levels of antioxidants. Other phenolic compounds are phenolic acids, flavonoids, coumarins, tannins, and lignans. (49). These antioxidants prevent the onset of chronic diseases like diabetes, cancer, and cardiovascular problems by protecting cells from oxidative damage. They are believed to be the main cause of this activity due to their redox properties,

which are essential in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. (50) The ferric-reducing ability (FRAP) assay is often used to evaluate the antioxidant content of dietary polyphenols. (51) It is found that there is a linear correlation between phenolic concentration and antioxidant activity. The antioxidant activity of *T. arenaria* methanolic extract was assessed using the FRPA free radical scavenging assay, which is often used to gauge the antioxidant potential of natural products extracts. As a positive control, Trolox, a synthetic antioxidant, was used. The outcomes showed that the ability of *T. arenaria* methanolic extract to scavenge free radicals was dose-dependent.

The methanolic extract of *T. arenaria* has a clearly higher anti-oxidant activity than the Trolox solution as shown in the graph below. This action is expected given that the methanolic extract contains phenolic and flavonoid compounds, which support earlier studies that examined the antioxidant activity of *T. Arenaria.*

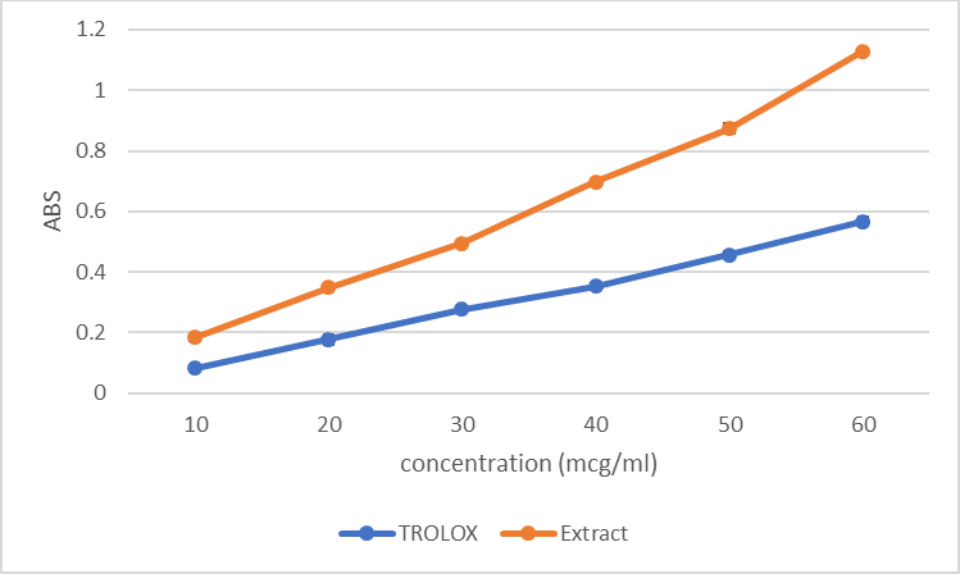


Figure 9: Trolox vs methanolic extract for antioxidant activity using FRAP assay.

4.4. Anti-microbial activity

The antibacterial activity of the methanolic extract of *T. arenaria* were done in two different conditions. In figure 17, the antibacterial activity was tested in AL QUDS university labs, and the results showed no activity and the absence of inhibition zone around the filter paper of *T. arenaria* methanolic extract, while it was positive for the positive control for all the used strains of bacteria, and negative activity for the negative control "methanol" mostly due to the rapid evaporation and the continuous grouth of bacteria.

The test was also carried out in AAUP labs using the same working method and the results showed weak activity against the used bacterial strains as shown in Figure 18.

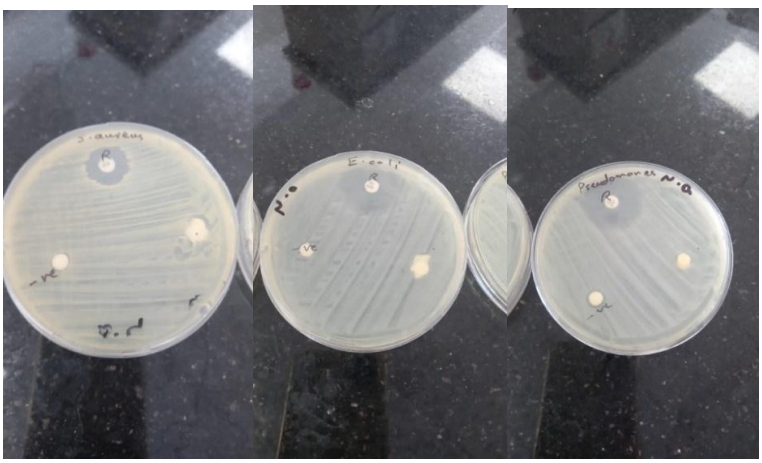


Figure 10: antimicrobial activity of methanolic extract, s. aureus, E. coli, and P. aeruginosa.

Figure 11: antimicrobial activity of the methanolic extract against P. aeruginosa and streptococcus pneumonia.

4.5. Cytotoxicity activity

In order to test the effect of *T. arenaria* on cancer cells, we treated MCF7 breast and HT29 colon cancer cells with 100 ul of crude extract. 24 and 48hrs later we counted the cells. As shown in figure 17 and 18, the treatment lowered cell count of MCF7 to ~46% and ~35% after 24hrs and 48hrs, respectively. Treating HT29 cells also, lowered cell count to ~65% and ~35% after 24hrs and 48hrs, respectively. We found that when we counted cells in the presence of trypan blue, we didn't notice cell death. In conclusion, our results show that *T. arenaria* crude extract treatment inhibited the growth of two cancer cell lines.

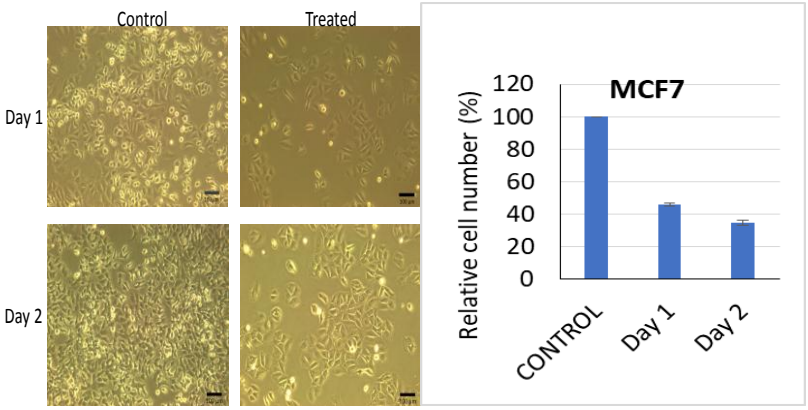


Figure 12 (a): to the left MCF-7 cell line before and after treatment with truffle extract, to the right statistical analysis of the MCF-7 cell line in day 1 and day 2 compared with the untreated cells.

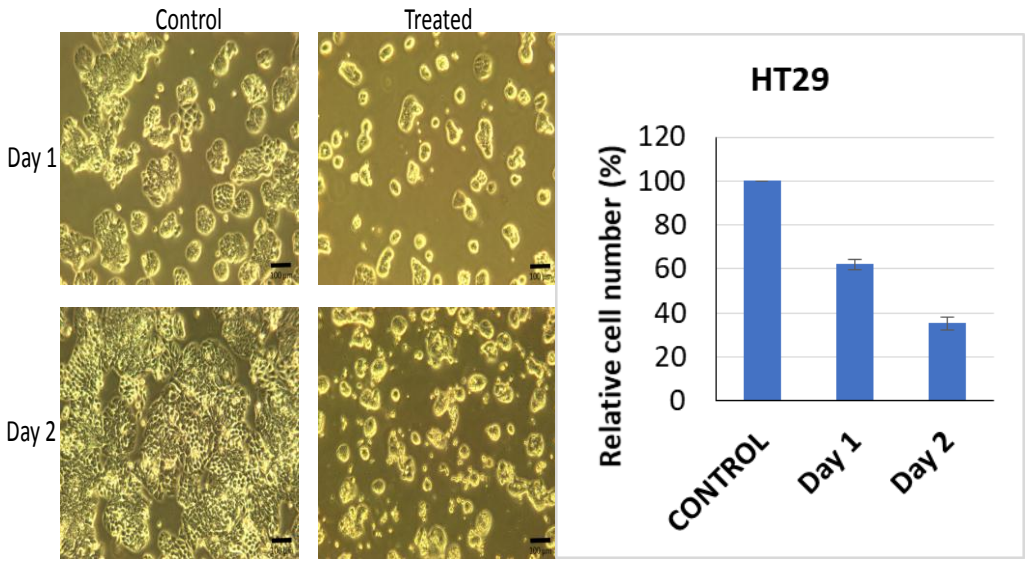


Figure 12 (b): to the left HT29 cell line before and after treatment with truffle extract, and to the right statistical analysis of the MCF-7 cell line in day 1 and day 2 compared with the untreated cells.

4.6. Anti-lipase:

The hydrolysis of p-nitrophenyl butyrate to p-nitrophenol was used to measure the impact of the methanolic extract of *T. arenaria* on the pig pancreatic lipase enzyme. Both the positive control drug (Orlistat) and the *T. arenaria* truffle extract showed a dose-dependent suppression of pig pancreatic enzymes. *T. arenaria* truffle extract and several solutions of orlistat were made in ascending doses. The drug's and truffle extract's IC50 values were determined, and the degree of lipase inhibition was displayed as shown in Fig (21). When compared to the positive control (Orlistat), which has antilipase activity with an IC50 value of 12.3 0.35 g/ml, the results showed that the methanol TA extract has anti-lipase potential, with an IC50 value of 150 mcg/ml.

The triple investigation evaluated both the medication and the truffle extract at various quantities.

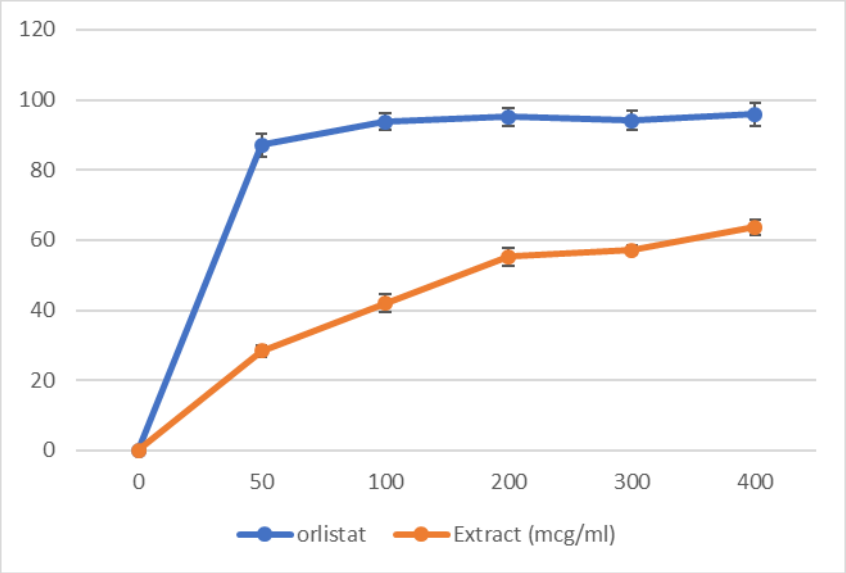


Figure 13: the inhibition activity of amylase enzyme of methanolic extract of *T. arenaria* compared to lipase inhibitor drug orlistat using different concentrations.

4.7. Anti-amylase:

To ascertain the T. arenaria medicinal Palestinian truffle extract in vitro anti-diabetic efficacy, its -amylase inhibitory action was examined. The T. arenaria truffle's ability to inhibit -amylase was compared to that of an anti-diabetic drug (Acarbose).

Fig. 1 displays the effectiveness of the commercial anti-diabetic drug Acarbose and *T. arenaria* extract in inhibiting amylase (22) The findings demonstrated that the methanol extract of the T. Arenaria truffle has strong -amylase inhibitory activity, with IC50 values of (100mcg/ml). Acarbose, the positive control used, has an IC50 of 10 mcg/ml.

When compared to the FDA-approved anti-diabetic medicine acarbose, the truffle's methanolic extract exhibits good activity and very powerful inhibition of the -amylase enzyme activity.

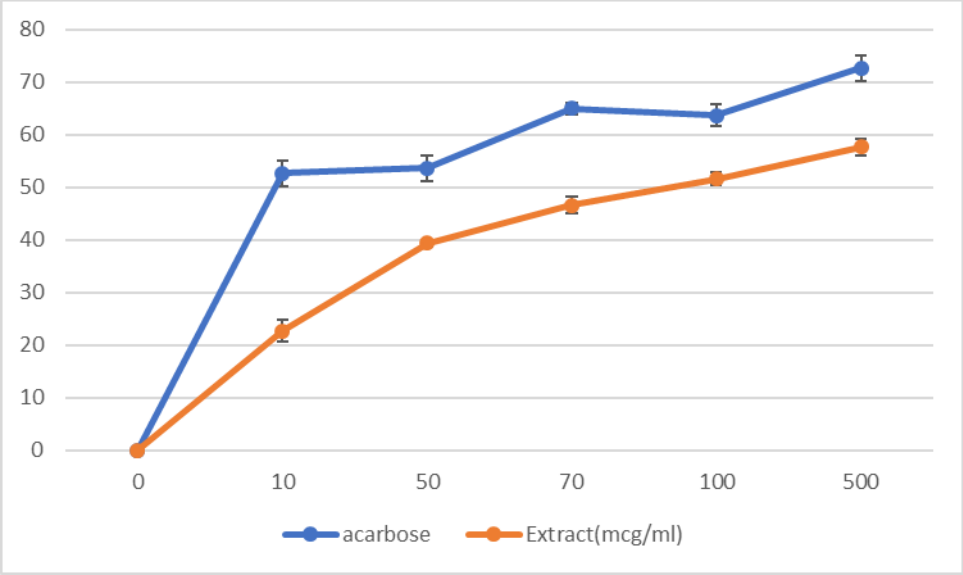


Figure 14: the inhibition activity of amylase enzyme of methanolic extract of *T. Arenaria* compared to amylase inhibitor drug acarbose using different concentrations.

Table 3: Comparison between *T. arenaria* in our study and other studies

Author	year	Truffle's origin	extraction method	solvent	tests	results
Negagaz	2012	Algeria	soxhlet	ethyl acetate	Antibacterial test	Positive antibacterial activity against s.aureous, E. aeruginosa, and e. coli
					antifungal test	positive antifungal activity against candida albicans
huseyin Dogan	2013	Turkey	soxhlet	methanol	antioxidant	positive antioxidant activity
			normal		Antibacterial test	negative antibacterial activity against gram +ve and -ve bacteria
			normal		phytochemical analysis	presnse of catechin, cinnamic acid, ferulic acid , p-coumaric acid
			normal	acetone	Antibacterial test	positive antibacterial activity against gram +ve and -ve bacteria
			normal	chloroform	antibacterial	positive antibacterial activity against gram +ve and -ve bacteria
Harir M	2019	Saharan desert	normal	methanol	antibacterial	negative antibacterial activity against gram +ve and -ve bacteria
					phytochemical analysis	presence of phenols and flavonoids
			soxhlet	DCM	antibacterial	positive antibacterial activity against gram +ve and -ve bacteria
					phytochemical analysis	presence of phenols and flavonoids
Al Obaydi MF	2020	Jordan	normal	methanol	anticancer	The results showed that hexane and ethyl acetate extracts have the highest activity against the MCF-7 cell line with minimum IC50, for T47D and MDA-MB231 cell lines, and showed good activity in growth inhibition with a noted increase in hexane activity.
				aqueous	anticancer	
				hexane	anticancer	
				ethyl acetate	anticancer	
khader A.	2023	palestine	sonication	methanol	phytochemical	presence of Flavanoids, phenols, proteins, tannines, glycosides and steroides
					antioxidant	showed strong antioxidant activity
					Lipase inhibitory	positive inhibitory effect for lipase enzyme
					Amylase inhibitory	positive inhibitory effect for amylase enzyme
					Antibacterial	negative antibacterial acticity against P. aregonosa, E.coli, S. pneumonia, S. aurous
					anticancer	positive anticancer activity against MCF7 and HT29 Cell lines

4.8. Identification and characterization

Although it is true that truffle's extracts frequently contain mixes of different kinds of bioactive compounds or phytochemicals with distinct polarity ranges, separating these mixtures remains a significant difficulty to the identification and characterization of bioactive substances. It is standard procedure to use a variety of separation techniques, including TLC, column chromatography, flash chromatography, Sephadex chromatography, and HPLC, in the isolation of these compounds in order to get pure substances. The structure and biological activity are then determined using pure molecules. The bioactive chemicals can also be obtained and identified more easily using non-chromatographic methods such as Fourier-transform infrared spectroscopy (FTIR), phytochemical screening assays, and immunoassays, which employ monoclonal antibodies (MAbs) (52).

4.8.1. Thin layer chromatography

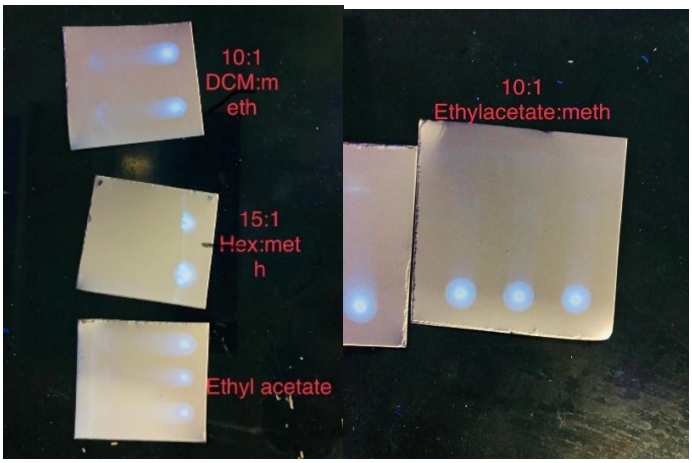
TLC is a simple, efficient, and inexpensive procedure that gives the researcher a prompt response on the number of elements in a mixture or the status of the reaction. TLC is also used to identify an unknown chemical when the Rf of one compound is compared to the Rf of another well-known substance. The plate can also be examined under UV light, and additional tests can be performed using phytochemical screening reagents, which alter color based on the phytochemicals present in a truffle extract. It is also employed to confirm the legitimacy and purity of isolated compounds (53).

As previously explained, the separation process is used in our TLC test to produce a pure product, after which we may evaluate its activity for various uses based on what is required or anticipated to have.

Hexan15:1 methanol did not show any separation under UV light, while DCM 10:1 methanol showed a mild separation in the spot. We tried using ethyl acetate alone for the

separation obtained from previous studies, but this did not show a good separation to consider for the future ongoing work on *T. Arenaria*. Instead, using various mixtures of ethyl acetate and methanol revealed that ethyl acetate 1: 1 methanol.

(A)



(B)

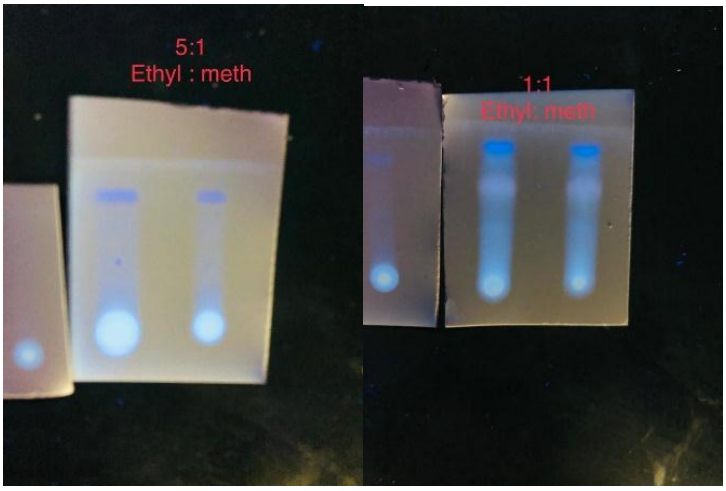


Figure 15: TLC papers for the truffle extract dissolved in methanol using different solvent mixtures as a mobile phase.

4.8.2. **Ultra-Performance Liquid Chromatography (HPLC):**

UPLC-PDA showed at 300nm wl the presence of two main peaks, the first with RT of 3.9 min with peak area of 69% and the second at 7.7 min with peak area 22%.

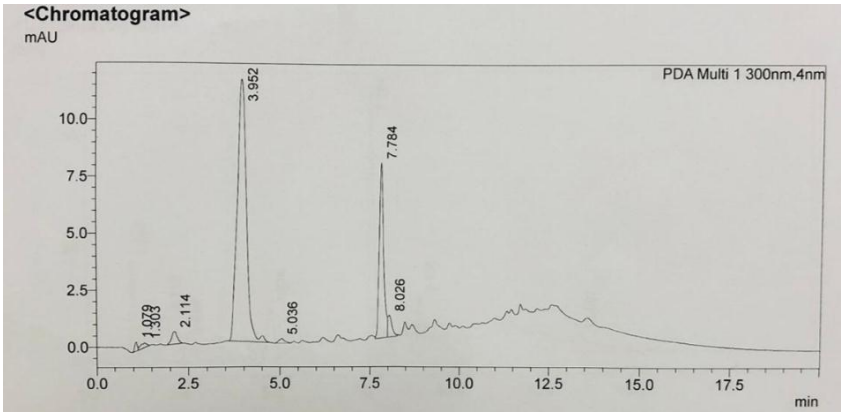


Figure 16: UPLC-PDA chromatogram of extract.

Table 4: peak's retention times, area's and area's % of UPLC chromatogram.

PDA Ch1 300nm

Peak#	Ret. Time	Area	Area%
1	1.079	2155	0.754
2	1.303	2583	0.904
3	2.114	5976	2.090
4	3.952	199505	69.788
5	5.036	2206	0.772
6	7.784	65738	22.995
7	8.026	7712	2.698
Total		285874	100.000

Chapter five

Conclusion and recommendations

5. Conclusion and recommendations:

5.1. Conclusion:

At the end of the study, the desert truffle "*Terfezia arenaria*" has been investigated, and, particularly, the quantitative and qualitative tests of content give us the biological activity estimation which we estimated to have. The results of our test for amylase and lipase enzymes inhibition action are good enough, and it is possible to build on what we have found in the future for further investigations. The antioxidant activity is positive and confirms the results of previous studies for the desert truffles family. Cytotoxicity shows a good activity in treating cancer cells in the in vitro activity test, while the antibacterial test shows no or weak activity. In the characterization process, the TLC and UPLC showed good separation and the possibility to test each part alone.

5.2. Recommendations:

The study of the benefits of truffle to cure is an endless path; this needs limitless medical research. I recommend other researchers to carry out tests similar to ours but with different extractions. It is important for any researcher in such a field of study to isolate and identify compounds and biological activities desired, but this process, as we have found, needs huge funds and a lot of time.

I hope researchers will work on our field in the future because there is a need for more clarification of the differences between *T. Arenaria* and other similar truffles by means of clarifying the qualitative and quantitative contents of each.

6. References:

1. Rates SMK. Plants as source of drugs. Toxicon. 2001 May 1;39(5):603–13.

2. Fokunang C, Ndikum V, Tabi O, Jiofack R, Ngameni B, Guedje N, et al. Traditional Medicine: Past, Present and Future Research and Development Prospects and Integration in the National Health System of Cameroon. Afr J Tradit Complement Altern Med. 2011 Apr 2;8(3):284–95.

3. No L John,Srivastava, Jitendra,Vietmeyer. Medicinal plants : rescuing a global heritage [Internet]. World Bank. [cited 2023 May 6]. Available from: https://documents.worldbank.org/pt/publication/documents-reports/documentdetail/538541468242090002/Medicinal-plants-rescuing-a-global-heritage

4. Jaradat N, Qadi M, Abualhasan MN, Al-lahham S, Al-Rimawi F, Hattab S, et al. Carbohydrates and lipids metabolic enzymes inhibitory, antioxidant, antimicrobial and cytotoxic potentials of Anchusa ovata Lehm. from Palestine. Eur J Integr Med. 2020 Feb;34:101066.

5. Structural Simplification of Natural Products | Chemical Reviews [Internet]. [cited 2023 May 7]. Available from: https://pubs.acs.org/doi/abs/10.1021/acs.chemrev.8b00504

6. US Sales of Herbal Supplements Increase by 8.6% in 2019 - American Botanical Council [Internet]. [cited 2023 May 7]. Available from: https://www.herbalgram.org/resources/herbalgram/issues/127/table-of-contents/herbalgram-127-herb-market-report-american-botanical-council/

7. Application of the "-Omic-" technologies in phytomedicine - PubMed [Internet]. [cited 2023 May 7]. Available from: https://pubmed.ncbi.nlm.nih.gov/17188482/

8. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: how to develop a stronger in vitro "proof-of-concept." J Ethnopharmacol. 2006 Jul 19;106(3):290–302.

9. Dahiru D, Onubiyi JA, Umaru HA. PHYTOCHEMICAL SCREENING AND ANTIULCEROGENIC EFFECT OF MORINGA OLEIFERA AQUEOUS LEAF EXTRACT. Afr J Tradit Complement Altern Med. 2006 Mar 27;3(3):70–5.

10. Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. Plants. 2017 Sep 22;6(4):42.

11. Singh DB, Pathak RK, Rai D. From Traditional Herbal Medicine to Rational Drug Discovery: Strategies, Challenges, and Future Perspectives. Rev Bras Farmacogn

[Internet]. 2022 Apr 1 [cited 2023 May 6];32(2):147–59. Available from: https://doi.org/10.1007/s43450-022-00235-z

12. Cravotto G, Boffa L, Genzini L, Garella D. Phytotherapeutics: an evaluation of the potential of 1000 plants. J Clin Pharm Ther. 2010 Feb;35(1):11–48.

13. Tas S, Tas B, Bassalat N, Jaradat N. In-vivo, hypoglycemic, hypolipidemic and oxidative stress inhibitory activities of Myrtus communis L. fruits hydroalcoholic extract in normoglycemic and streptozotocin-induced diabetic rats. Biomed Res. 2018 Aug 3;29(13):2727–34.

14. [PDF] Traditional Palestinian medicinal plant Cercis siliquastrum (Judas tree) inhibits the DNA cell cycle of breast cancer – Antimicrobial and antioxidant characteristics | Semantic Scholar [Internet]. [cited 2023 May 7]. Available from: https://www.semanticscholar.org/paper/Traditional-Palestinian-medicinal-plant-Cercis-the-Amer-Jaradat/b465e58cdd50daaf9edbf3ccfc11cae3f622dbf9

15. Mirończuk-Chodakowska I, Witkowska AM, Zujko ME. Endogenous non-enzymatic antioxidants in the human body. Adv Med Sci. 2018 Mar;63(1):68–78.

16. An-Najah Staff [Internet]. [cited 2023 May 7]. Available from: https://staff.najah.edu/en/publications/8093/

17. Saini DC, Kochar A, Poonia R. Clinical correlation of diabetic retinopathy with nephropathy and neuropathy. Indian J Ophthalmol. 2021 Nov;69(11):3364–8.

18. Antimicrobial resistance [Internet]. [cited 2023 May 7]. Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

19. Tuberculosis (TB) [Internet]. [cited 2023 May 7]. Available from: https://www.who.int/news-room/fact-sheets/detail/tuberculosis

20. Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Aguilar GR, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet [Internet]. 2022 Feb 12 [cited 2023 May 6];399(10325):629–55. Available from: https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(21)02724-0/fulltext

21. Demain AL, Vaishnav P. Natural products for cancer chemotherapy. Microb Biotechnol. 2011 Nov;4(6):687–99.

22. Abubakar AR, Haque M. Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. J Pharm Bioallied Sci. 2020;12(1):1–10.

23. Ultrasound Assisted Extraction - an overview | ScienceDirect Topics [Internet]. [cited 2023 May 7]. Available from:

https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/ultrasound-assisted-extraction

24. Soxhlet Extraction - an overview | ScienceDirect Topics [Internet]. [cited 2023 May 7]. Available from: https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/soxhlet-extraction

25. Supercritical Carbon Dioxide (CO2) Extraction Method from Cole-Parmer [Internet]. [cited 2023 May 7]. Available from: https://www.coleparmer.com/tech-article/supercritical-co2-extraction-method

26. Fagga or Desert Truffles | N&N bakers [Internet]. [cited 2023 May 7]. Available from: https://nicknnikhbakers.wordpress.com/2010/02/18/fagga-or-desert-truffles/

27. Innovatione. Desert truffle [Internet]. Innovatione. 2021 [cited 2023 May 7]. Available from: https://innovatione.eu/en/2021/10/29/desert-truffle/

28. Lee H, Nam K, Zahra Z, Farooqi MQU. Potentials of truffles in nutritional and medicinal applications: a review. Fungal Biol Biotechnol. 2020 Jun 17;7:9.

29. Morte A, Kagan-Zur V, Navarro-Ródenas A, Sitrit Y. Cultivation of Desert Truffles—A Crop Suitable for Arid and Semi-Arid Zones. Agronomy. 2021 Aug;11(8):1462.

30. Neggaz S, Fortas Z. Tests of antibiotic properties of Algerian Desert Truffles against bacteria and fungi. T--T J Life Sci. 2013 Mar 1;Vol.7, n°3:259–66.

31. Doğan HH, Aydın S. Determination of antimicrobial effect, antioxidant activity and phenolic contents of desert truffle in Turkey. Afr J Tradit Complement Altern Med AJTCAM. 2013;10(4):52–8.

32. Harir M, Bendif H, Yahiaoui M, Bellahcene M, Zohra F, Rodríguez-Couto S. Evaluation of antimicrobial activity of Terfezia arenaria extracts collected from Saharan desert against bacteria and filamentous fungi. 3 Biotech. 2019 Jul;9(7):281.

33. Al Obaydi MF, Hamed WM, Al Kury LT, Talib WH. Terfezia boudieri: A Desert Truffle With Anticancer and Immunomodulatory Activities. Front Nutr [Internet]. 2020 [cited 2023 May 7];7. Available from: https://www.frontiersin.org/articles/10.3389/fnut.2020.00038

34. Annegowda HV, Bhat R, Min-Tze L, Karim AA, Mansor SM. Influence of sonication treatments and extraction solvents on the phenolics and antioxidants in star fruits. J Food Sci Technol. 2012 Aug;49(4):510–4.

35. Kumar S, Sandhir R, Ojha S. Evaluation of antioxidant activity and total phenol in different varieties of Lantana camara leaves. BMC Res Notes. 2014 Aug 22;7:560.

36. Magalhaes L, Almeida MI, Barreiros L, Reis S, Segundo M. Automatic Aluminum Chloride Method for Routine Estimation of Total Flavonoids in Red Wines and Teas. Food Anal Methods - FOOD ANAL METH. 2012 Jun 1;5.

37. Elsanhoty RM, Soliman MSM, Khidr YA, Hassan GOO, Hassan ARA, Aladhadh M, et al. Pharmacological Activities and Characterization of Phenolic and Flavonoid Compounds in Solenostemma argel Extract. Mol Basel Switz. 2022 Nov 22;27(23):8118.

38. Gülçin İ, Huyut Z, Elmastaş M, Aboul-Enein HY. Radical scavenging and antioxidant activity of tannic acid. Arab J Chem. 2010 Jan 1;3(1):43–53.

39. Trolox - an overview | ScienceDirect Topics [Internet]. [cited 2023 May 7]. Available from: https://www.sciencedirect.com/topics/chemistry/trolox

40. Dailey OD, Wang X, Chen F, Huang G. Anticancer activity of branched-chain derivatives of oleic acid. Anticancer Res. 2011 Oct;31(10):3165–9.

41. Jaradat N, Zaid AN, Hussein F, Zaqzouq M, Aljammal H, Ayesh O. Anti-Lipase Potential of the Organic and Aqueous Extracts of Ten Traditional Edible and Medicinal Plants in Palestine; a Comparison Study with Orlistat. Medicines. 2017 Dec;4(4):89.

42. Ali H, Houghton PJ, Soumyanath A. α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to Phyllanthus amarus. J Ethnopharmacol. 2006 Oct 11;107(3):449–55.

43. Wikler M. Performance standards for antimicrobial susceptibility testing: seventeenth informational supplement: clinical and Laboratory Standards Institute, 2007. J Biol Res-Thessal. 2015;22(4):7–12.

44. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 2016 Apr 1;6(2):71–9.

45. Kumar S, Pandey AK. Chemistry and Biological Activities of Flavonoids: An Overview. Sci World J. 2013 Dec 29;2013:162750.

46. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010;4(8):118–26.

47. Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents. 2005 Nov;26(5):343–56.

48. Flavonoid content assay: Influence of the reagent concentration and reaction time on the spectrophotometric behavior of the aluminium chloride - Flavonoid complex | Request PDF [Internet]. [cited 2023 May 7]. Available from: https://www.researchgate.net/publication/11895604_Flavonoid_content_assay_Influen

ce_of_the_reagent_concentration_and_reaction_time_on_the_spectrophotometric_behavior_of_the_aluminium_chloride_-_Flavonoid_complex

49. Chagas M do SS, Behrens MD, Moragas-Tellis CJ, Penedo GXM, Silva AR, Gonçalves-de-Albuquerque CF. Flavonols and Flavones as Potential anti-Inflammatory, Antioxidant, and Antibacterial Compounds. Oxid Med Cell Longev. 2022 Sep 6;2022:9966750.

50. Rodríguez-García C, Sánchez-Quesada C, Gaforio JJ. Dietary Flavonoids as Cancer Chemopreventive Agents: An Updated Review of Human Studies. Antioxidants. 2019 May 18;8(5):137.

51. Godinho PIC, Soengas RG, Silva VLM. Therapeutic Potential of Glycosyl Flavonoids as Anti-Coronaviral Agents. Pharmaceuticals. 2021 Jun 7;14(6):546.

7. Appendix:

Table 5: calibration curve of the lipase inhibitory activity, conc vs abs.

ORLISTAT			SDV	EXTRACT			SDV
0	0	0	0	0	0	0	0
85.5	91	85	3.329164	30	28	27	1.527525
96.4	93	92	2.306513	45	41	40	2.645751
97.9	94.5	93	2.510644	58	55	53	2.516611
91.5	97	94	2.753785	58.4	56	57	1.205543
92.1	97.6	98	3.296968	66	62	63	2.081666

Table 6: calibration curve of the amylase inhibitory activity, conc vs abs.

acarbose			SDV	extract			SDV
0	0	0	0	0	0	0	0
50	53	55	2.516611	25	22	21	2.081666
51	54	56	2.516611	40.1	39	39	0.635085
66	64	65	1	46.9	48	45	1.517674
63	66	62	2.081666	51	53	51	1.154701
75	73	70	2.516611	56	58	59	1.527525

Table 7: calibration curve of quercetin for TFC.

flavanoids					
quercitine mg/ml	A	B	c	Av	SDV
0	0.00	0.00	0.01	0.00	0.0069
10	0.25	0.24	0.26	0.25	0.0135
20	0.47	0.46	0.48	0.47	0.0135
30	0.73	0.72	0.74	0.73	0.0135
40	0.95	0.95	0.98	0.96	0.0151
50	1.19	1.19	1.22	1.20	0.0151
60	1.44	1.43	1.46	1.40	0.0135

Table 8: calibration curve for gallic acid for TPC.

phenols					
gallic acid mcg/ml	A	B	C	average	SDV
0	0.00	0	0	0.00	0.0000
10	0.10	0.08	0.08	0.09	0.0115
20	0.22	0.17	0.18	0.19	0.0265
30	0.31	0.31	0.26	0.29	0.0289
40	0.38	0.36	0.37	0.37	0.0100
50	0.45	0.47	0.48	0.47	0.0153
60	0.61	0.62	0.64	0.62	0.0153
70	0.73	0.7	0.71	0.71	0.0153
80	0.77	0.79	0.77	0.78	0.0115
90	0.87	0.86	0.85	0.86	0.0100
100	0.98	0.95	0.96	0.96	0.0153

Table 9: antioxidant curve chart, Trolox solution as a control sample and extract.

TROLOX			sdv
0.09	0.08	0.08	0.005774
0.19	0.18	0.16	0.015275
0.28	0.27	0.28	0.005774
0.35	0.36	0.35	0.005774
0.45	0.47	0.45	0.011547
0.57	0.55	0.58	0.015275

Extract			sdv
0.1	0.09	0.11	0.01
0.183	0.17	0.16	0.011533
0.219	0.212	0.22	0.004359
0.34	0.34	0.35	0.005774
0.4	0.42	0.431	0.015716
0.555	0.57	0.56	0.007638

8. Abstract in Arabic:

الخصائص المضادة للسرطان والجذور الحرة والميكروبات والتثبيطية للإنزيمات لنبات الكمأ الصحراوي في فلسطين.

إعداد الطالب: أحمد معتصم توفيق الخضر

المشرف الرئيسي: الدكتورة سوسن سلامة

المشرف الثانوي: الدكتور مهند قريع

الملخص:

تعد النباتات مصدراً مهماً للعلاج، حيث أنه منذ الزمن القديم تم استخدامها بشكل مباشر لعلاج الكثير من الأمراض للإنسان، وفيما بعد أصبحت مصدراً لاستخراج واستخلاص الأدوية والعلاجات منها. وأصبحت الدراسات تتوالى وتتلاحق لاكتشاف المزيد من هذه النباتات وامكانية استخدامها لتلبية مطالب الإنسان المتزايدة للدواء وإمكانية تطويره.

في هذه الدراسة نستعرض نبات الكمأ الصحراوي الفلسطيني الذي ينبت في صحراء النقب لدراسته بشكل تفصيلي ودراسة المركبات التي يحتويها، وامكانية استخدام هذه المركبات لعلاج بعض الأمراض التي قد تصيب الإنسان كأمراض السمنة والسكري والإختلالات العصبية وكذلك دراسة تأثيره على النمو البكتيري والخلايا السرطانية وبعض الإنزيمات الهاضمة في جسم الإنسان.

وكانت نتائج الدراسة إيجابية، حيث وجد أن تأثير النبات كان ملحوظاً على كل ما سبق ذكره، حيث كان له تأثير تثبيطي واضح على الإنزيمات المستخدمة في التجربة، وكذلك على النمو للخلايا السرطانية، وبشكل جزئي على البكتيريا المستخدمة في التجربة. وحيث أن هناك إمكانية في الحصول على دواء نقي من هذا النبات، قمنا بعمل بعض الفحوصات لفصل هذه المكونات للنبات، لإعادة فحصها بشكل معمق للحصول على نتائج جديدة مرجوة.