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Phytochemicals, Antioxidant and Anti-Microbial Screening of some Palestinian Grape Genotypes (*Vitis vinifera* L.)

By

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Declaration

I certify that the results reported in this thesis are conducted of my research, except where otherwise acknowledged, and this thesis has not been submitted for the higher degree to any other university or institute.

Signed.....

Dana Khdour

Dedication

This thesis is dedicated:

I am grateful to my father and mother for everything they have done for me, whose words of encouragement and push for the tenacity to finish this thesis. To my brother and sisters who have never left my side and are very special. To my dearest husband: for his endless support and encouragement.

Dana Khdour

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List of Abbreviations

$ABTS^{\bullet+}$	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
ADHD	Attention deficit-hyperactivity disorder
ADP	Adenosine diphosphate
ADR	Antimicrobial Drug Resistance
AMD	Age-related macular degeneration
APR	April
ATP	Adenosine 5′-triphosphate
C°	The degree Celsius
Ca	Calcium
CFS	Chronic fatigue syndrome
Cm	Centimeter
COVID-19	Coronavirus Disease 2019
CP	Chronic Polyneuropathy
Cr	Chromium
Cu	Copper
CVD	Cardiovascular disease
DNA	Deoxyribonucleic Acid
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl hydrate
DW	Distilled water
E. coli	*Escherichia coli*
EMB	Eosin-methylene blue
FAO	Food and Agriculture Organization
FCV	Feline calicivirus
Fe	Iron
Feb	February
FRAB	Ferric reducing antioxidant power
G	Gram
GA	Gallic acid
GC-MS	Gas chromatography-mass spectrometry
HAV	Hepatitis A virus
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HPLC	High Performance Liquid Chromatography
ICP-OES	Inductively coupled plasma - optical emission spectrometry
In	Inch

K	Potassium
K. pneumonia	*Klebsiella pneumonia*
LDL	Low-density lipoprotein
Mg	Magnesium
MIC	Minimal inhibitory concentration
Mm	Millimeter
Mn	Manganese
MNV	Murine nor virus
MS	Mass spectrometry
MSA	Mannitol Salt Agar
N	Nitrogen
Ni	Nickel
NRCS	Natural Resources Conservation Service
OIV	International Organization of Vine and Wine
P	Phosphorus
P. aeruginosa	*Pseudomonas aeruginosa*
P. mirabilis	*Proteus mirabilis*
Pb	Lead
PCBS	Palestinian Central Bureau of Statistics
RD	Refsum's Disease
RNA	Ribonucleic Acid
ROS	Reduce Oxidative Stress
Rt	Retention time
RZCP	Rhizomelic Chondrodysplasiapunctata
S. aureus	*Staphylococcus aureus*
SO_2	Sulfur dioxide
SSR's	Simple sequence repeats
TMS	Tetramethylsilane
TNF	Tumor necrosis factor
TPC	Total phenolic content
UV	Ultraviolet
VASP	Vasodilator-stimulated phosphoprotein
WHO	World Health Organization
ZDHA	Zellweger's disease hyperpipecolic academia

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Abstract

Grape (*Vitis vinifera* L., Vitaceae) is a vital plant for the Palestinian economy and folkloric culture. In addition to its valuable part in our diet, grapes can also be used as a traditional medicine to treat many illnesses including reducing blood pressure, improving blood flow, reducing oxidative damage, improving collagen levels and bone strength, inhibiting infectious growth, and reducing cancer risk. In this study, minerals, antioxidants capacities, total phenols, phytochemicals characters and antibacterial properties, were determined in grape leaves, seeds, and fruit-skins of two selected genotypes namely, Red-Halawani and Black-Betuni, which both are commonly grown in Palestine. Mature fruits and well-developed leaves were collected from Halhul city. Seeds and fruit-skin were separated from fruits. All *V. vinifera* parts were air-dried until no change in mass was recorded. The minerals were measured using (ICP-OES); the antioxidant activities were assessed by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging assays; total phenolic content (TPC) was determined using the Folin-Ciocalteau method; the volatile compounds were determined using GC-MS equipped with electron impact mode and the antimicrobial activities were examined by well and disk diffusion methods against G+ and G- bacteria.

Among the two examined genotypes, our results revealed significantly higher ash, fat, and protein contents in grape leaves over seeds and fruit-skins. However, grape seeds exhibited significantly higher fiber content compared to leaves and fruit-skins.

Similarly, Nitrogen (N), Phosphorus (P), Potassium (K), calcium (Ca), Magnesium (Mg), Iron (Fe), and Manganese (Mn) were also higher in the leaves of the two grape genotypes followed by grape seeds and grape fruit-skins, respectively.

Leaves in both genotypes exhibited higher antioxidant capacity compared with seeds and fruit-skins. Leaves from Red-Halawani and Black-Betuni genotypes showed 86.84% and 79.62%, respectively, scavenging as assessed by $DPPH^{\bullet}$ and 90.92% and 87.59% respectively, scavenging as assessed by $ABTS^{\bullet+}$. Both seeds and fruit-skins scavenging capacities were less than 80% using both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging assays. Moreover, seeds in both Red-Halawani and Black-Betuni genotypes showed the highest value of total phenolic content (TPC) 82.68% and 61.26%, respectively. While, both leaves and fruit-skins were less than 60%.

Analysis of 14 phytochemical components revealed that grape leaves, seeds, and fruit-skins are good sources of cardiac glycosides, phenolic groups, saponins, steroids and terpenoids. Furthermore, tannins were found in leaves and seeds, however glycosides are present in leaves and fruit-skins. Leaves in both genotypes expressed a spectrum of volatile compounds identified as hydroquinone (*Rt* = 3.52 min), cyclohexanone (*Rt* = 3.56 min), ionone (*Rt* = 5.55 min), caryophyllene (*Rt* = 5.60 min), menthol (*Rt* = 5895 min), and phytol (*Rt* = 8.76 min) in methanolic extract. Under our experimental condition, no volatile compounds were detected in the seed and fruit-skins of grapes genotypes.

Seeds methanolic extracts in both genotypes showed significant antimicrobial activity against the gram-positive bacteria (*S. aureus*) and the gram-negative bacteria (*P. mirabilis* and *P. aeruginosa*) bacteria. The zone of inhibition (expressed as % of the positive control) using both well and disk diffusion methods against *S. aureus* in the methanolic extracts of Red-Halawani 98% and 78%, respectively. In Black-Betouni genotypes the zone of inhibition recorded was 97 % and 88%, respectively. In addition, the zone of inhibition recorded against the gram-negative bacteria *P. mirabilis* and *P. aeruginosa* was less evident compared to gram-positive bacteria.

This study was the first to screen and evaluate phytochemical compounds in selected Palestinian grape genotypes for their antibacterial and antioxidants activity. These results suggest the seed of both grape genotypes have antioxidants activities with a pronounced antimicrobial activity comparable to positive controls. Our results revealed a considerable variation in the ash, fiber, fat, proteins, minerals, phytochemicals, antioxidant activity and antibacterial activity for the different parts of the two grape genotypes studied.

Keywords: Grape, minerals, proximate analysis, antioxidant, phytochemical, GC-MS and biological activity.

Chapter 1: Introduction

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Grape (*Vitis vinifera* L.) is one of the most economically important and widespread fruits worldwide. It is mainly used to make wine, and juice, pressed and processed mostly into sauces and raisins, and can also be eaten fresh (Khorramifar *et al*., 2022). Along with some 70 other species of the genus *Vitis*", this species is a fascinating subject for evolutionary studies (Grassi and Arroyo-Garcia, 2020).

Statistics from the Food and Agriculture Organization (FAO) showed that the global grape production in 2021 was estimated at 24.54 million metric tons, in the major grape production comes from China, Italy, and the United States with 16.9%, 10.8% and 8.7% respectively (FAOSTAT, 2021). Remarkably, about 71% of the world's grapes production is heading to make wine, 27% as fresh fruit and 2% as dried fruit (Khan, 2020). Unusually, more than 13,000 genotypes of grapes in the world, representing a relatively huge diversity of grapes is existing (OIV, 2013).

In Palestine, the grape is one of the most valuable fruit crops after olive, covering an area of about 29117 dunums with approximately 4.3% of the total horticultural area (PCBS, 2021). Due to geographical and climate concerns, its cultivation and production are still limited to the southern region of the West-Bank (Basheer-Salimia and Hamdan, 2014), comprising about 85% of the total grape production. Previous studies based on grape morphological and fruit-skin color characteristics revealed that about 30 grape genotypes were existing in Palestine. Amongst the two most common genotypes harvested in Palestine include the Red-Halawani and Black-Betuni. However recent studies depending on genetic SSR's were narrowing this number into 16 different genotypes (Basheer-Salimia *et al*., 2014).

Climate is considered the determinate factor for the suitability of certain grape cultivars for a particular region as well as the limit factor for grape production and quality (Santos *et al*., 2020). Generally, the grape is affected by temperature in which fruits ripening requires a temperature between 25 and 40˚C. As temperature increases, sugar accumulation increases however, certain metabolites such as anthocyanins are negatively affected as well as the acidity of grapes (particularly their malic acid content) decreases (Field *et. al*., 2009; Kok, 2017). Regarding the soil requirements, grapes could grow well in a wide range of well-drained soil types with high soil pH range in which 6.5-8.0 are considered ideal (Warmling *et al*., 2018).

Remarkably, regular consumption of grapes and grape products was found to reduce the incidence of some chronic illnesses such as cancer, cardiovascular disease, ischemic stroke, neurodegenerative disorders, and aging; accordingly decreasing the complication and risk factors of these diseases in the context of the Mediterranean dietary tradition (Iriti *et al*., 2009; Nasser *et al.*, 2020; Sabra A. *et al*., 2021). In fact, grapes contain an interesting number of secondary metabolites that have many biological activities and consequently have enormous potential to support different pharmaceutical properties such as antioxidants, anti-inflammatory and antiplatelet aggregations, antibacterial, and antifungal effects and more (Hasanaliyeva, 2020; Zhang, 2021). Moreover, grape extracts were found to have various ash (2.47%), lipids (1.75%), protein (21%), carbohydrates (60.72%), fibers (2.20%), vitamins (B_2 and C), and diverse minerals including N, P, K, Mg, Mn, Ca, Fe, Cu, Cr, Pb and Ni (Ullah *et al.*, 2008; Antoniewicz, 2022; Baroi *et al.*, 2022).

The main goal of the present study is to describe and evaluate the nutritional constitutes, malaxate analysis (protein, fiber, fat, etc), antioxidants, phytochemical and antimicrobial effects of two grape genotypes (Halawani and Betuni), that are commonly grown in Palestine.

Chapter 2: Literature Review

Chapter 2: Literature Review

2.1. General aspects

Grape classification (NRCS, 1996).

Kingdom: Plantae

Division: Tracheophytes

Class: Angiosperms

Order: *Vitales*

Family: *Vitaceae*

Genus: *Vitis*

Species: *V. vinifera*

Scientific Name: *Vitis vinifera* L.

2.1.2. Origin and distribution

In face of the widespread world in the cultivation of grapes, the origin and cultivation started in Asia, North America, and Europe, and then by human migration and moved between countries, grape cultivation spread across many continents. In addition, grape cultivation has greatly expanded in both Mediterranean and Subtropical areas due to the convenient climate (Terral *et al.*, 2010).

2.1.3. Production areas and levels

According to indicators and statistics, grapes are grown all over the world. The top ten countries in terms of productivity are China, France, the United States, South Africa, Italy, Chile, Iran, Turkey, Spain, and Argentina (FAO, 2020). The Chinese production in 2017 was at the top among ten counties with about 13,083,000 tons, while the South African was tenth with about 2,032,582 tons (FAO, 2012). In Palestine, there are approximately 50,065 tons, planted on 7,784.3 hectares (PCBS, 2018). About 71% of

the world's grapes production is heading to make wine, 27% as fresh fruit, and 2% as dried fruit (Khan, 2020).

2.1.4. Description of the plant

Grapes are all woody, climbing vines planted and cultivated on a trellis, fence, or other structure for support. Grapevines attach themselves to tall-growing plants using tendrils. Shoots grow nearly a meter a year because most of the energy is devoted to length growth rather than girth. When they are exposed to another object, tendrils coil automatically opposite leaves. The leaves are Palm-sized with multi-lobes. Buds are compound in grapes, meaning that they have multiple growing points or meristems. Flowers are hermaphrodites in clusters that are small, 3-4 mm (0.12-0.16 in), and white in color. Then it is fertilized and then produces bunches of grapes. Grape's fruit is true berries, small, round to oblong, and consisting of up to four seeds. Berries are often glucose, having a fine layer of wax on the surface. The fruit-skins of the grape are thin and is the source of the anthocyanin compounds that give rise to red, blue, purple and black (dark purple) colors (Toda FMD, 2018).

2.1.5. Genotypes and cultivars

Some Palestinian genotypes and cultivars were discussed in detail in (**Table 2.1**)

Table 2.1: Descriptors of some common Palestinian grape genotypes and cultivars.

Descriptor	Cultivars	
	Halawani	**Betuni**
Beginning of ripening.	10-20 September	1-15 September
Harvested period.	More than 60 days	More than 40 days
Fruit External color.	Red	Purple black
Skin cracks	No cracked	No cracked
Fruit shape	Round	Round
Fruit weight	5-15 g	4-8 g
Fruit firmness	Firm	Firm
Fruit Neck length	-	-
Fruit Stalk length	0.5-0.8 cm	0.5-0.8 cm
Ostiole type	Open	Open
Fruit-skins peeling	Hard	Hard
Internal color	Yellowish	Reddish
Bud break	May	May
Leaf color	Dark green	Dark green
Lobes number	Five	Five
Leaf venation	Apparent	Apparent
Apex shape	Triangle-obtuse	Triangle-obtuse
Leaf roughness	Smooth	Smooth
Leaf area	About 20 - 25 cm^2	About 16 - 22 cm^2
Petiole length	5-25 cm	5-25 cm
Beginning of leaf	March	March

2.2. Environmental requirements

2.2.1. Climacteric requirements

In general, grapes are perennial plants; the growth and development of grapes are dependent on many factors, primarily weather, soil, cultural practices, and water

requirements. To some extent, the climatic requirement of grapes depends on the cultivar (Pisciotta *et al.*, 2022). Moreover, the optimal conditions for grape production are temperate and rainy winters, temperature range of 12–22 ℃ during the growing season. To initiate the growing/vegetative cycle, as well as to store carbohydrate reserves in perennial organs for subsequent years, grapes respond optimally to daily temperatures between 20 and 35 °C; however, bud dormancy with a base temperature of 10 °C must be broken (Droulia, *et al.*, 2022).

2.2.2. Soil requirements

There are many types of soil that grapes thrive on, well-drained, deep, fertile loams are excellent, and thrive in other types, including clay, slate, gravel, shale, and sand (Wang R. *et al*, 2015). Due to grapes' deep-rooting habits, the soil depth needs to be at least 30 inches (David *et al.*, 2006). The ideal pH should be between 6.5 to 7.5 (Havlin J. *et al.*, 2022).

2.3. Cultivation practices

2.3.1. Plant propagation

Generally, stem cutting is the most common method of vegetative propagation for grapes. The application of Auxin enhances the formation of callus and tissue and the differentiation of vascular tissue histologically. Moreover, fruit crops are propagated more quickly by cutting and grafting than by seed propagation, however, are one option for propagation (Singh, *et al*., 2022).

2.3.2. Planting

Mostly, it is possible to transplant grapes from the nursery at any time of the year. However, proven that planting grapes from late winter (Feb) until spring (April) is the best (Demir, 2014). Planting distances in grapevine are very important but not limited to, productivity, utilization of unit area, economic return, and soil quality in terms of ventilation, moisture, and available elements (Hunter, 1998). Medium-spaced (2 x 2 m, 2 x 1 m) consistently performed optimally (Hunter, 1998).

2.3.3. Irrigation and fertilization

About the fertilizers, a mulch of straw manure applied as a mulch during the growing season is a good practice. Furthermore, grape needs Nitrogen, Phosphorus, and Potassium fertilizers for leaves and fruit growth (Havlin J. *et al.*, 2022). If necessary, irrigate grapes to help them survive dry periods and mature their fruit. During mid to late summer, gradually decrease water amounts if vines grow so vigorously that they develop dense canopies, increased disease, and reduced fruit production (Cabral *et al.*, 2022).

2.3.4. Pests and diseases control

In general, pests and diseases reduce grape productivity. A number of these diseases and pests are sensitive to grapes and they are not resistant to them. Phylloxera, grape erineum mite, Wasp's hornets, and Rodents are among grape pests that cause serious damage (Barah, 2015). Moreover, the common diseases of grape are Crown gall, Botrytis fruit rot, and Powdery mildew, therefore the use of insecticides, fungicides, herbicides, acaricides, and nematicides is approved for grape to minimize damage caused by insects, fungal infections, and other pests (Sanghavi, 2018).

2.3.5. Harvesting

There are several indications of the ripening of grapes, the most famous of which are color, sweetness, brown stem, and many others. As well as a difference in maturity between the varieties, some of which ripen early during the month of May, and some continue to ripen until September. The cluster of grapes mostly should be harvested when they are ripe and mature (Pezzi, 2015).

2.4. Post-harvesting handling

2.4.1. Field heat removal

The field boxes should be placed under shade in the interval between the haves and transported to the packinghouse, because direct exposure of grapes to the sun will increase the internal temperature and speed up chemical processes in fruits such as rancidity in fruits, deterioration of chlorophyll pigments and flavor changes. Lipid oxidation and non-enzymatic browning are also possible chemical changes. Fruits' color

and flavor were altered as a result, leading to a significant defect in sensory quality. As a result, temperature management requires rapid removal of heat, which could be achieved by hydro-cooling, packaging in iced containers, evaporating cooling, room cooling, and vacuum cooling. To ensure that the value chain of fruits is maintained from the farm gate to the consumer, a cold chain system is essential (Ayam *et al.,* 2021).

2.4.2. Sorting and grading

Sorting and grading are the most important steps in packaging and marketing grapes. During sorting, damaged fruits, such as those that have rotted or been afflicted with diseases, are separated from healthy products. A grading system categorizes products according to their color, size, maturity stage, or degree of ripening. In order to maintain postharvest shelf life and quality of grapes, both processes are essential. As a result of sorting, infectious microorganisms cannot spread from bad fruits to healthy ones during storage. Handlers can categorize grapes based on a common parameter, allowing for easy handling (Liguori *et al.*, 2021).

2.4.3. Packaging and branding

Despite their low respiration rate, grapes undergo several physicochemical, biochemical, and microbiological changes during storage that reduce their shelf-life. Gray mold, weight loss, and stem browning are the main factors that reduce table grape marketability. The most popular treatment is sulfur dioxide, but it is not recommended for minimally processed table grapes that will be consumed within a few days of packaging. there are several efforts are being made worldwide to find alternative technologies to replace sulfur dioxide in ready-to-eat table grapes, as well as, passive and active modified atmosphere, avoiding the use of chemicals (Liguori *et al*., 2021).

2.4.4. Storage

Storage is the most important post-harvest practice of grapes to reduce losses. Cold storage is mainly built to control the rate of transpiration, respiration, and microorganism growth and to preserve the grape in the most usable form for the consumer. The cold storage slows down the biological activities of the fresh commodities while avoiding chilling injury and showing the microorganism growth. Furthermore, this reduces transpiration losses to avoid various undesirable processes

including sprouting, elongation, rotting, greening, and toughening of certain (Mahajan *et al.*, 2009).

2.4.5. Chemical and physical treatments

In view of overall findings regarding the control of postharvest pathological disorders, fumigation with SO_2 could be proven as a powerful method for table grape storage at present. Therefore, SO_2 application usually causes injury to table grapes and for harm full to human health (Unal, 2022).

2.4.6. Marketing

Internationally, wine is the most expensive grape product, and it even earns more income from selling fresh grapes. However, the forms of products have gone beyond. There are juices, jams, raisins, and many products from grapes, all of which reap profits (Laswai *et al.*, 2018). It is worth noting that grapes are of great importance and festivals take place around the world (Katarína, 2021), especially in Palestine. Moreover, the grape festival provided farmers with a chance to sell and marketed fresh grapes, and grape products.

2.5. Food, medicinal and therapeutic

2.5.1. Grape as food

Grape is one of the most important fruit crops in the Mediterranean diet, can be eaten fresh as the grape leaf is used as a food and table grapes, used for making wine, jam, grape juice, jelly, grape seed extract, vinegar, and grape seed oil, or dried as raisins, currants, and sultanas. Furthermore, grapes are rich in vitamins and minerals. In religion, Christians have traditionally used wine during worship services as a means of remembering the blood of Jesus Christ, which was shed for the remission of sins (Shi *et al.*, 2003).

2.5.2. Grape as a cosmetic ingredient

The high content of phenols, flavonoids, and alkaloids in the grape extract which acts as antioxidants, anti-aging, anti-carcinogenic, anti-microbial, and anti-fungal has enhanced the use of the extract in cosmetic manufacturing. Cosmetics are very rich in

the presence of grape extracts, especially grape seed oil as it is used as a preservative for cosmetics. Furthermore, it is natural and safer than chemicals used parallel (Burnett, 2011).

2.5.3. Medicinal uses

2.5.3.1. Traditional uses as medicine

Grape is used for preventing diseases of the heart and blood vessels, varicose veins, hemorrhoids, hardening of the arteries (atherosclerosis), high blood pressure, swelling after injury or surgery, heart attack, and stroke. Some people also use grapes as a mild laxative for constipation. Moreover, the grape seeds are used for diabetes complications such as nerve and eye problems, improving wound healing, preventing tooth decay, and preventing cancer, an eye disease called age-related macular degeneration (AMD), poor night vision, liver disorders, and hay fever. In addition, dried grapes, raisins, or sultanas (white raisins) are used for cough. The grape leaf is used for attention deficit hyperactivity disorder (ADHD), chronic fatigue syndrome (CFS), diarrhea, heavy menstrual bleeding, uterine bleeding, and canker sores (Nasser *et al.*, 2020).

2.5.3.2. Nutritional compounds

2.5.3.2.1. Macro-nutrients (carbohydrates, proteins, and fat)

2.5.3.2.1.1. Carbohydrates

In terms of health and fitness, carbohydrates constitute a major part of food and support the building of body strength, since they are considered an excellent energy provider after being broken down biologically. In addition to serving as a structural material (cellulose), a component of ATP, and a recognition site on cell surfaces, carbohydrates are also one of the main three components of DNA and RNA (Khowala *et al.*, 2008). Further, dietary fibers are a grape constituent that humans can digest (break down) because they are complex carbohydrates. Due to its ability to increase motility in the small intestine, decrease transient times, and hold water to release constipation, dietary fibers prevent stomach and intestinal problems, such as constipation. It also helps control cholesterol levels and blood sugar levels, therefore reducing the risk of cardiovascular diseases and diabetes. (Lunn and Buttriss, 2007; Kumar *et al*., 2017).

2.5.3.2.1.2. Proteins

A protein is made up of amino acids, which are organic nitrogenous compounds that make up most of the body's weight (about 20%). Among their functions, grape contains a small amount of proteins, which play an important role in bodybuilding, tissue repair, and tissue maintenance, adjusting the acid-base balance of tissues when acting as buffers. They also play a crucial role in the synthesis of antibodies, plasma proteins, enzymes, hormones, etc. (Kumar *et al.*, 2017).

2.5.3.2.1.3. Lipids

Fats are a subset of a class of nutrients called lipids, which also include phospholipids and sterols. Usually when people refer to fats and oils, they speak of triglycerides, the most common form of fat in the diet. Triglycerides come in many sizes and several varieties, but they all share a common structure: a backbone of glycerol to which three fatty acids are attached. While all glycerol molecules are alike, the fatty acids may vary in two ways: length and degree of saturation. Grapes contain little amount of fat, including Triglycerides which are mainly responsible for storing energy and insulating the body and organs (Lyndsey *et al.*, 2016). They also transport vitamins that are fat-soluble and contribute to taste and feeling full after eating a meal. Lipids in grapes have shown positive results in reducing the risk of CVD (Lupoli *et al.*, 2020)

2.5.3.2.2. Micronutrients (vitamins and minerals)

In general, vitamins are organic, while minerals are inorganic substances. To stay healthy and grow, our bodies require higher amounts of minerals, such as calcium. In addition to boosting the immune system, vitamins and minerals support normal growth and development, as well as support the functions of cells and organs (Antoniewicz *et al.*, 2022). Many vitamins and minerals are found in grapes, such as vitamins K, E, and C, as well as calcium, copper, iron, magnesium, manganese, phosphorus, potassium, silicon, and sulfur (Daccak *et al.*, 2022).

2.5.3.3. Phytochemical compounds

2.5.3.3.1. Volatile compounds

The volatile compounds were classified into different categories, namely, terpenes, esters, alcohols, aldehydes, ketones, alkanes, alkynes, acids, and benzodiazepines. The proportion of volatile components in each part of the grape differed among tissue types. High levels of hexanal were only detected in some grape genotypes. The grapes also have higher concentrations of esters (3-hydroxymandelic acid, ethyl ester, di-TMS, acetate, and butyl ester), terpenes (geraniol, linalool, and D-limonene), alcohols (octanol, silanediol, dimethyl-, 2,6-octadien-1-ol, 3,7-dimethyl-, (Z)-, trans-2-hexenol, and 2-hexene-1-alcohol), aldehydes (phenylacetaldehyde, benzaldehyde, and 2-hexenal), and benzodiazepines (cyclopropane, propyl-, and octamethyl-). Moreover, aldehydes (2,2-dimethyl-3-hydroxypropionaldehyde, phenylacetaldehyde, and hexanal), benzodiazepines (cyclopropane, propyl, and octamethyl), and trans-β-ionone (Zheng, 2021). Among the most volatile compounds in grapes and wines are terpenes (monoterpenes, sesquiterpenes), fatty acids derivatives, sulfides, and methoxypyrazine, while acetyl-CoA is the precursor to terpene synthesis. As one of the main classes of secondary metabolites, isoprenoids include quinones, sterols, polyterpene alcohols, chlorophyll, carotenoids, and phytohormones. The smell of grapes is enhanced by isoprenoids (Kaya *et al.*, 2022).

2.5.3.3.2. Non-volatile compounds

A limited number of previous studies reviewed the non-volatile compounds, specifically the study of non-volatile substances in wine and very rare studies about non-volatile compounds in grapes (Rodríguez-Bencomo J. *et al.*, 2011).

2.5.3.4. Biological activities

2.5.3.4.1. Antioxidant activity of grapes

Antioxidants are stable natural chemical compounds capable of neutralizing reactive oxygen species (ROS) and subsequently reducing their harmful effects on humans. In general, the antioxidant efficiency of herbal extracts depends largely on the content of phenolic compounds and the reaction activity of the phenol because of the chain-

carrying peroxyl radicals and on the stability of the phenoxyl radicals formed in the reaction. It is identified that typical phenolics possess antioxidant activity. The pharmacological value of grapes lies in the presence of some organic compounds such as alkaloids, saponins, flavonoids, tannins, anthraquinones, steroids, and terpenoids. These compounds are synthesized by the secondary metabolism of living organisms. Secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure functions. Amongst the most important functions of some of the secondary compounds is their ability to scavenge ROS due to the presence of structurally diverse phenolic compounds (Castro-Lópeza *et al.*, 2019).

In grapes, extracts of different parts have exhibited varied antioxidant activities as determined by DPPH, ABTS, and FRAB free radicals scavenging capacities (Sochorova et al., 2020; Zhou *et al.*, 2022), depending mainly on grape genotypes and cultivars (Castro-Lópeza, et al., 2019). Although a grape is known to be a source of phenolic compounds. Polyphenols have recently received a lot of attention due to their diverse biological activities, from pharmacological and therapeutic perspectives, and the antioxidant properties of polyphenols, such as free radicals scavenging and inhibiting lipid peroxidation, are the most important (Baroi *et al.*, 2022).

2.5.3.4.2. Anti-inflammatory of grape

The grape extracts have the capacity to inhibit major pathways responsible for the activation of oxidative systems and the expression and release of pro-inflammatory cytokines and chemokines (Harbeouiabc *et al*., 2019). Furthermore, the regulation of immune cells by polyphenols is illustrated with special reference to the activation of T-regulatory cells (Baroi *et al.*, 2022).

2.5.3.4.3. Anti-microbial activity of grape

2.5.3.4.3.1. Anti-bacterial activity of grape

Increased numbers of bacterial strains showing resistance to antibiotics have received much interest in recent years. According to the World Health Organization (WHO), antibiotic resistance is a serious threat affecting every country with the potential to affect anyone, at any age, in the world, and Palestine. Antimicrobial Drug Resistance (ADR) limits access to infectious diseases and poses a threat in cases of infectious disease cases and during routine surgery, cancer treatments, and organ transplants,

therefore a large number of sicknesses and deaths are caused by fighting bacteria worldwide and this needs global commanding actions to prevent ADR. At present, there is an insistent need to explore new antimicrobial medications with various mechanisms of action. That is at most endorses to the up growth of infections affected by bacterial strains that are greatly strong to antibiotics and the presence of chronic diseases that could not be cured effectively in all cases with the existing modern medications. Even though the healing of patients cured with grapes appears to be a slow progression, they are becoming more common due to their specific side effects associated with current medications. It is difficult to restrict the influence of antibiotic resistance in terms of death rate and budget (Ghendov-Mosanu A. *et al.*, 2022).

2.5.3.4.3.2. Anti-fungal activity of grape

Commonly, plants extract possesses strong antimicrobial and potentially antifungal properties. For example, grape seed extract found to have antifungal properties that make it a good defense against candidiasis. (Insanu *et al*., 2021). This was demonstrated by experiments using 16 g/mL of pterostilbene added to poly (lactic-coglycolic acid) nanoparticles to decrease the growth of C. albicans biofilms by 63% and mature biofilms by 50% (Simonetti *et al.*, 2019).

2.5.3.4.3.3. Anti-viral activity of grape

The grape extract has antiviral activities against hepatitis A virus (HAV) and human nor virus surrogates (feline calicivirus (FCV-F9) and murine nor virus (MNV-1)) (Insanu *et al*., 2021). Furthermore, resveratrol also suppressed the replication of MERS-CoV RNA in high concentrations. Concomitant administration of resveratrol with MERS-CoV or after that had been shown to stop MERS-CoV infection (Lin *et al*., 2017).

2.5.3.4.4. Anti-cancer activity of grape

Grape is one of some medicinal plants that have a role as an anti-tumor due to its ability to inhibit the proliferation of various cancer cell lines. A study was performed to explain the anti-cancer effect of f. carica latex in a different concentration, the result revealed that the 5mg/mL concentration gives the maximum inhibition effect on stomach cancer cell line growth. The main cytotoxic agent in grape latex which applies the inhibition

effect on the proliferation of various cancer cell lines, is 6-*O*-Acyl-Beta-D-glucosyl-beta sitsterols (Insanu *et al.,* 2021).

2.5.3.4.5. Anti-hypertension activity of grape

Grapes are used for treating cholesterol and hypertension. However, it has a high interaction with statins and must be used cautiously because may lead to reduce ability or reduce statins drug toxicity. Therefore, it should be used in specific amounts.

Grape prevents the absorption of cholesterol inside the digestive tract and can aid LDL decreasing as well, grape leaves extract performance as induced inhibition of lipid accumulation during adipogenesis, particularly *via* improvement of triglyceride-rich lipoprotein catabolism, inhibition of adipocyte differentiation lipogenesis. Simvastatin, pravastatin, and lovastatin are inhibitors of HMG-CoA reductase, the rate-limiting step in cholesterol synthesis reduced lipid peroxidation leads to decrease absorption of statin. Moreover, many studies correlate with the hypertension-lowering effect in grapes, based on the use of the active ingredient in grapes, resveratrol (Baroi *et al.*, 2022).

2.5.3.4.6. Prevent bone loss activity of grape

The Proanthocyanidin-rich red grape seed extract was able to stop bone resorption (Insanu M. *et al*., 2021). In mice exposed to lipopolysaccharide, the proanthocyanidin decreased inflammatory osteolysis while preventing osteoclast differentiation, apoptosis, and increasing proliferation (Kwak *et al.,* 2020)

2.5.3.4.7. Anti-Alzheimer activity of grape

The fruit-skins, seeds, and fruit of grapes all had anti-Alzheimer's properties (Insanu *et al.,* 2021). As anti-Alzheimer's treatments, grape powder and ethanolic extract improved recall and recovered memory loss in Alzheimer's rats, then decreased mRNA expression of amyloid precursor protein and cleared tau tangles (Ma *et al*., 2018).

2.5.3.4.8. Anti-acne activity of grape

Propionibacterium acnes is responsible for the condition known as acne vulgaris (Insanu M. *et al.,* 2021). According to Nelson's research, grape leaf extracts had MIC_{50} and MIC_{90} values of 64 g/mL against *P. acnes*, indicating that they had anti-acne activity (Nelson *et al.,* 2016).

2.5.3.4.9. Anti-asthma activity of grape

The alcoholic extract of *V. vinifera* dried fruits, which contains gallic acid, can be used to treat asthma. The alcoholic extract of *V. vinifera*, which has a concentration of 31 mg/kg and 42.5 mg/kg, inhibits histamine release and reduces cytokine production (IL-4, IL-5, TNF, IL-1) as part of its anti-asthma action. Then comes another mechanism, which enhances lung function by reducing cellular infiltration, increasing lumen size, and decreasing the number of leukocytes and white blood cells (Insanu *et al.*, 2021).

2.5.3.4.10. Antiplatelet activity of grape

Poliflavan-3-ol, a component of grape fruit-skins extract, could operate as an antiplatelet by preventing the aggregation of human platelets (Insanu M. *et al.*, 2021). According to research on antiplatelets by Bijak et al. (2019), where grape seeds extract was examined using the vasodilator-stimulated phosphoprotein (VASP) assay, it might lessen adenosine diphosphate (ADP)-induced aggregation in white blood (Bijak *et al.*, 2019).

2.6. Problem statement and motivation of the study

Few studies have been conducted on Palestinian herbs, but their composition, effectiveness, and safety remain unknown. In addition, due to the undesirable side effects of orthodox synthetic medications such as toxicity and carcinogenicity, and the emerging microbial resistance to available antimicrobial agents, the interest in finding naturally occurring antioxidants and antimicrobial compounds suitable for food and/or medicine has greatly increased.

As a result of its long medicinal reputation among Palestinians, Palestinian grape genotypes (*Vitis Vinifera* L.) were chosen for this study. Research on indigenous grapes

in Palestine was motivated by a lack studies of phytochemical composition of their volatiles, semivolatiles, and minerals, as well as a lack of pharmacological studies.

2.7. Aim of the study

This study aims to screen grape genotypes (Red-Halawani and Black-Betuni) commonly grown in Palestine, secondary metabolites, and minerals by using SD-GC-MS and ICP-OES and to examine some of their claimed pharmacological activities. The test will include antioxidant and anti-bacterial biological activities.

2.8. Objectives of the study

1. To extract the major crude volatile compounds present in *Vitis vinifera* L. genotypes (Red-Halawani and Black-Betuni) by methanol (80%) and to analyze the extract by GC-MS technology at the electron impact (EI) mode.

2. To compare the type and composition of the volatile compound of the *Vitis vinifera* L. genotypes leaves, seeds, and fruit-skins.

3. To compare the biological activities, total ash, fibers, fats, Proteins, and minerals content of the *Vitis vinifera* L. genotypes leaves, seeds, and fruit-skins.

4. To assess the relation between *Vitis vinifera* L. genotypes leaves, seeds and fruit-skins concerning the contents and biological activities.

Chapter 3: Materials and Methods

Chapter 3: Material and Methods:

3.1. Sample Collection

Grape leaves and fruits were collected from two grape genotypes namely red-Halawani and black-Betuni from Halhul city – Hebron governorate. Leaf and fruit samples were collected from the middle region of the shoots from late September to early October 2020. The two targeted grape genotypes were visually identified and confirmed at the laboratories of the college of agriculture, Hebron University.

Figure 3.1: Collection of grape leaves and fruits.

3.2. Sample Preparation

Grape leaves and fruits were directly shifted to Hebron University laboratories. The collected leaves were cleaned to remove dust particles and then placed under shade drying at room temperature for about two to three weeks. Meanwhile, seeds and fruit-skins were washed from dust then separated from each other, and then placed under shade drying at room temperature for about five to six weeks. Thereafter, the samples (dried plant samples) were crushed to a fine texture powder by a grinding machine, then transferred into jars and stored at room temperature away from light until processed.

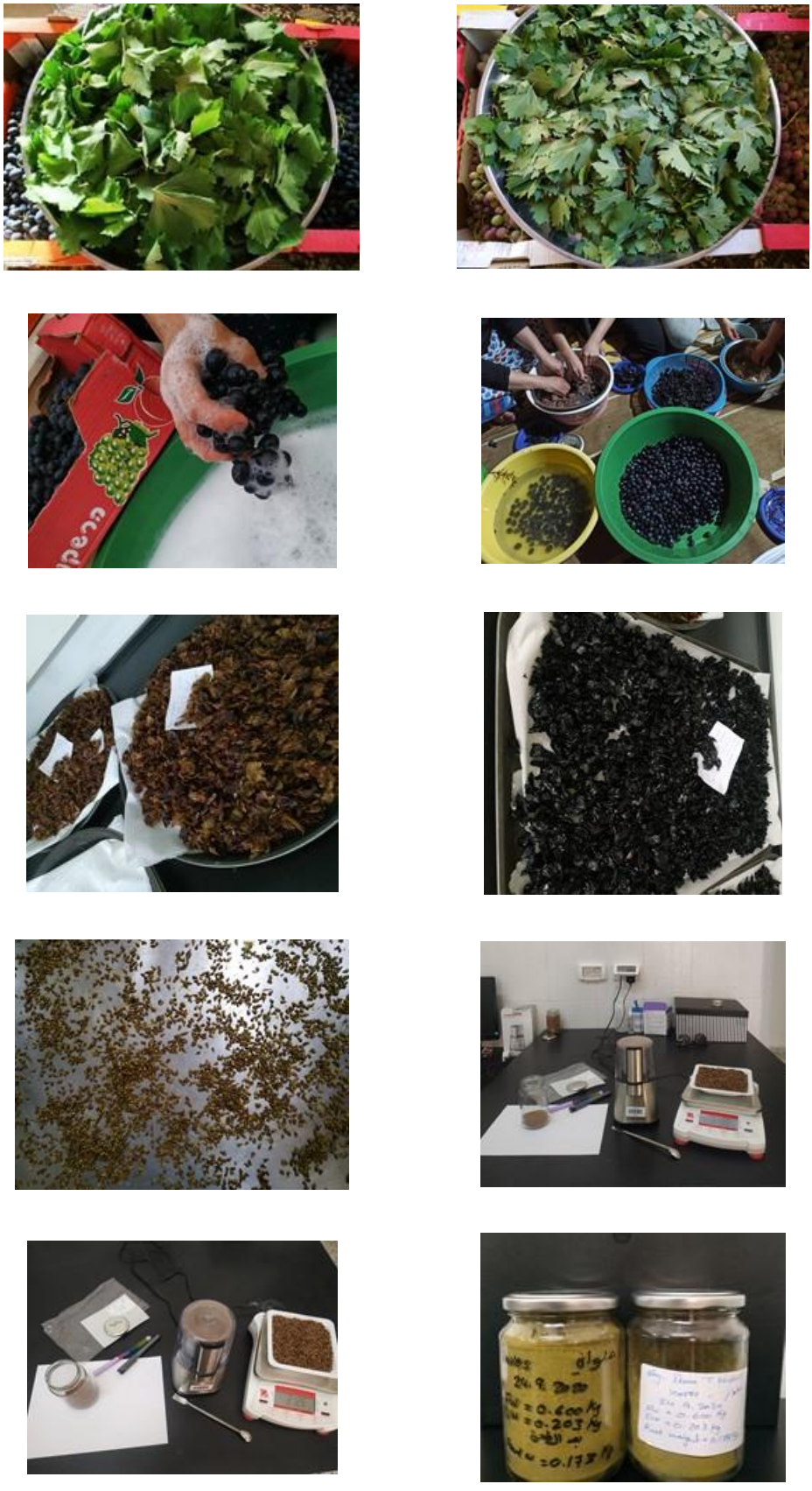


Figure 3.2: Preparation, crushing and storage of grape leaves and fruits.

3.3. Ash

The empty crucible was weighed, and subsequently 1 g of the sample was placed in an empty crucible and placed in a muffle furnace at 550 °C to burn the samples (Mukherjee, 2019). Ash percentage was calculated according to the following formula:

%Ash = ((weight of crucible with ash- weight of crucible)/weight of sample) *100

Figure 3.3: Grape samples before and after burning on a muffle furnace at 550 °C.

3.4. Minerals

3.4.1. Estimation of Ca, Mg, Fe, and Mn

Estimation of Calcium, Magnesium, Iron and Manganese amount in Grape samples, 6 ml of 2N HCl was added to the previous ash samples to extract, following filtrated; the sample was transferred in a volumetric flask, then added distilled water up to 100 ml. The absorbance of the standard for each mineral was then measured before the samples were taken, and the samples were recorded using atomic absorption, which is used to determining K, Ca, Mg, Fe, and Mn. The measurements were carried out with flame atomization, and a deuterium lamp was used as background correction. The measurements were repeated three times for each sample.

Figure 3.4: Filtration of the grape samples after extraction from ash.

3.4.2. Estimation of P

Estimation of P phosphor in Grape samples,10 mL of previous extract ash was added to 10 ml of the reagent (ammonium vanadomolybdate), diluted with distilled water up to 100 ml, and eventually have been read the absorbance using spectrophotometer at 410 nm.

Figure 3.5: The reagent and grape samples to estimate the amount of Phosphorus.

3.4.3. Estimation of N

The Kjeldahl method was used to determine the nitrogen content in Grape samples. Nitrogen was converted into ammonium sulfate by digestion with sulfuric acid, and it is alkalized and then the ammonia obtained by distillation is determined. Kjeldahl analysis works in three steps processes: digestion, distillation, and titration (Michalowski T. *et al*., 2013).

3.4.3.1. Digestion

The Grape samples taken is 0.5 g of each sample, which firstly treated with (K_2SO_4-$CuSO_4.5H_2O$-Se; 100g-10g-1g). Also, about 12 mL of sulfuric acid (H_2SO_4) was added. The solution was boiled at an extremely high temperature for 2 hours on Foodalyt SBS 2000 system. The acid solution digested the sample and produced an ammonium sulfate solution.

3.4.3.2. Distillation

This process is a combination of boiling and condensation. An excess of the base was added to the formed solution to convert the ammonium sulfate solution to NH_3 gas.

3.4.3.3. Titration

To finally quantify the nitrogen present in the Grape samples, the obtained product from the previous process is titrated with 1N H_2SO_4 by using burette in order to give the final required results [the color change from violet to green by using methylene red and blue as the indicator] (Radha, 2021).

$$\mathbf{N_{(g/100g)} = titration\ volume_{(mL)} \times 0.35017_{(mg/mL)} / sample\ mass_{(mg)} \times 100_{(g/100g)}}$$

Figure 3.6: Digestion of the grape samples by using Foodalyt SBS 2000 system.

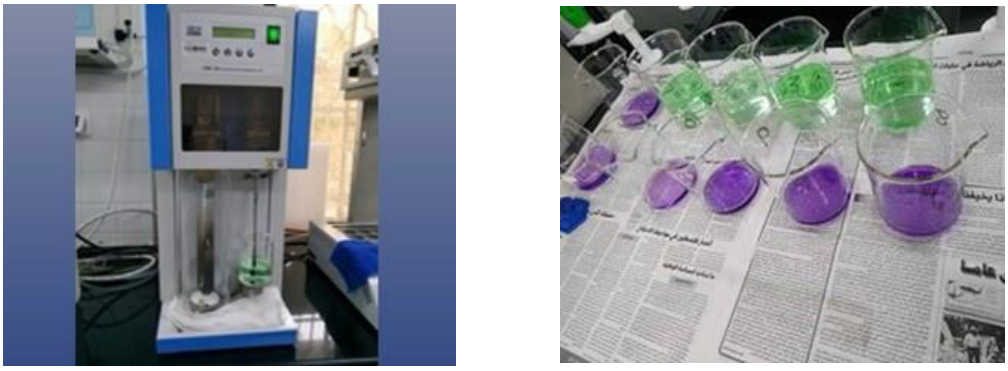


Figure 3.7: Distillation by using Kjeldahl and titration of the grape samples.

3.5. Proximate analyses

3.5.1. Estimating the Fibers

Estimating the fiber content in different grape samples was accomplished through a fiber analyzer, ankom 200, in which filter paper was used to weigh 1g of each sample. Here, we used (1.25%) H_2SO_4 and heated it to 100°C for 30 min; next we used (1.25%) KOH and heat it to 100°C for 30 min, and then dried the samples at 100°C for 3 hours. Then we took the weight of the samples again (Hirn and Bauer, 2006).

Figure 3.8: Grape samples in filter paper before it was in the fiber analyzer and after drying.

3.5.2. Estimating the Fats

The empty beaker for the Soxtherm and the thimble were weighed. The two weights were recorded, then about 2g of the sample have been added, and after that, 10 ml of diethyl ether have been added. The sample was placed on a Soxtherm device for 80 minutes, and then in the oven to dry, and the weight after drying was determined (Min and Ellefson, 2010; Dasari and Goud, 2013).

Figure 3.9: Fat extraction of grape samples by Soxtherm device using diethyl ether.

3.5.3. Estimating the Proteins

The Kjeldahl technique is used to measure the entire nitrogen content material of a Grape extract of leaves, seeds, and fruit-skins, which is used to estimate the crude protein content, with the aid of using the conversion factor. Most proteins incorporate 16% of nitrogen; therefore, the conversion factor is 6.25 (Michalowski *et al.*, 2013).

3.6. Antioxidants

Extraction

One gram of each sample was weighed, and then 10 ml of (80%) methanol was added to a 25 ml beaker. After overnight (24hr.) in a shaking incubator, the samples were filtered (Dowek, 2020).

Figure 3.10: Extraction of grape samples to estimate antioxidants.

3.6.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH•) assay

The extracts were tested for their ability to scavenge free radicals using the free radical 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH•), which is a molecule containing a stable free radical (Sharma & Bhat, 2009). DPPH• scavenging capacity of extracted solutions was assayed based on the methods described in (Barros, et al., 2007) with minor modification, DPPH• stock solution was prepared by dissolving 2.3 mg of DPPH• (Sigma Aldrich-STBD4146V) with 5.57 mL of [methanol (80% v/v]. A 200 µL of DPPH• stock solution was mixed with 2 mL 80% methanol and 20 µL of diluted plant extract (1:5, sample) or 20 µL of methanol (80%, control) in plastic cuvettes. All the cuvettes were mixed by a vortex and incubated in a dark at room temperature for 1h. The absorbances of plant extracts (A sample) and the methanol (A control) were

measured at 517 nm using a UV or Visible spectrophotometer (Dowek, 2020). The radical scavenging activity was calculated as a percentage of DPPH$^{\bullet}$ discoloration using the following equation:

$$\textbf{DPPH}^{\bullet}\ \textbf{Scavenging (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

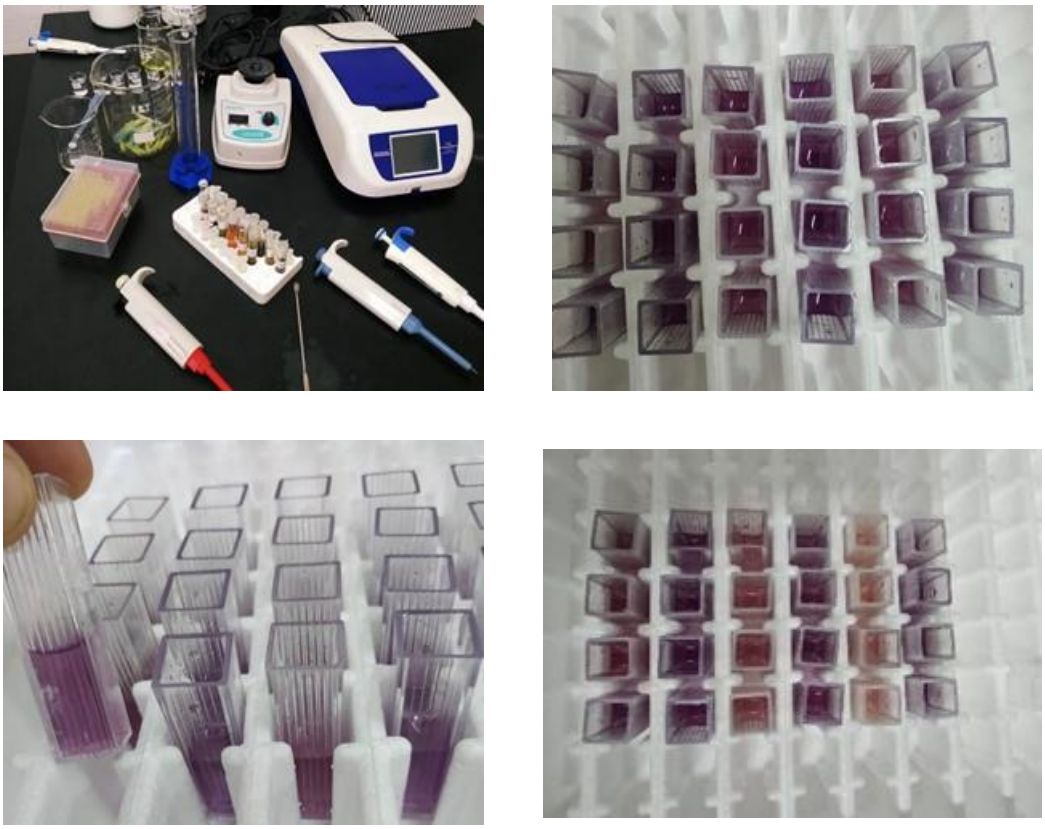


Figure 3.11: Estimation of the antioxidant of grape samples by DPPH procedure.

3.6.2. ABTS$^{\bullet+}$ Assay

The ABTS$^{\bullet+}$ stock solution was prepared following the protocol described by (Qawasmeh *et al.*, 2012), in which 18 mg of ABTS$^{\bullet+}$ (Sigma Aldrich, Palestine) dissolved in 5 mL distilled water to get a final concentration of 7 mmol. An aliquot (88 µL) of potassium persulfate solution (2.45 mmol) was added to ABTS$^{\bullet+}$ solution. The mixture was incubated in the dark overnight before use. The working solution of ABTS$^{\bullet+}$ was prepared by diluting a stock solution of ABTS$^{\bullet+}$ with methanol (80% v/v) to final absorbance of 0.7000 ± 0.02 at 734 nm. A 30 µL of diluted plant extracts (1:5) solutions were mixed with 3 mL ABTS$^{\bullet+}$ working solution in micro cuvettes. For control, 30 µL methanol (80%) was mixed. All cuvettes were mixed by a vortex (Lab net international Inc. U.S.A) and incubated in a dark for 30 min at room temperature.

The absorbances of plant extracts (A sample) and the methanol (A control) were measured at 734 nm using UV or Visible spectrophotometer (Cole-Parmer Ltd, UK) (Dowek, 2020). The percentage scavenging of $ABTS^{\bullet+}$ was calculated according to the equation:

$$\mathbf{ABTS^{\bullet+}\ Scavenging\ (\%) = [(A_{control} - A_{sample}) / A_{control}] \times 100\%}$$

Figure 3.12: Estimation of the antioxidant of grape samples by ABTS procedure.

3.6.3. Determination of total phenols using Folin–Ciocalteu

Preparing Gallic acid as a stock solution

Gallic acid (GA) was prepared by dissolving 250 mg of GA in 5 ml of (80%) methanol (because the GA dose did not dissolve in water), then diluted with distilled water up to 50 ml. The different concentrations of GA were prepared by adding a sex-different volume of GA (50, 100, 200, 300, 500 and1000 µl) in a cuvette, then adding (80%) methanol up to 10ml to use as a reference for total phenols determination in the *V. vinifera* samples. The extracts of *V. vinifera* samples prepared above were used to determine the total phenols as described in Dowek, 2020.

Sodium carbonate (Na_2CO_3) solution preparation

Na_2CO_3 was prepared by dissolving 5g of Na_2CO_3 in 20 ml D.W. in a beaker and then heating it to a boil. The solution was cooled and filtered, and finally, adding D.W. up to 25 ml.

To determine the total phenols in *V. vinifera* samples, the spectrophotometer was used (λ max= 765 nm, D.W. the blank). In each cuvette, 20 µl of the extract, 1.58ml of D.W., and 150 µl of Folin–Ciocalteu reagent were added, after mixing the solution well about 300 µl of Na_2CO_3 solution was added to the solution in the cuvette. The resulting

solution was kept for 1 hr. in the dark before taking the reading. The absorbance of the resulting solution was measured at λ max of 760 nm. The blank used was water and the assay were done in triplicate

For the GA stock solution, 20 µl of GA was added (from each concentration in 10 cuvettes) instead of the extract. Data were expressed as milligrams of gallic acid per gram of dried plant samples (mg GAE/g). (Dowek, 2020).

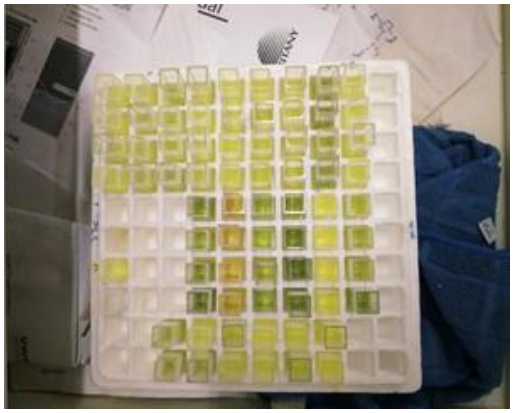


Figure 3.13: The varying color of grape extracts from corresponding to total phenols content.

3.7. Phytochemical screening

Extract preparation (Mujeeb *et al*., 2014)

About 3g of each sample of *V. vinifera* was added to a beaker with 60 ml of (80%) methanol and kept for 24 h at 25 ºC in a shaking incubator. The resulting extracts were filtered through a filter paper and used to screen the phytochemicals content in each sample as the following:

Figure 3.14: Samples of grape extraction for phytochemical screening.

3.7.1. Test for Alkaloids

In a test tube, 1 mL of 1% HCl was added to 2 mL extract then a few drops of Meyer's reagent were added to the mixture. The presence of white precipitate indicates positive for alkaloids.

3.7.2. Anthraquniones

In a test tube, 1 mL of 10% NH_3 solution was added to 2 mLextract, which was mixed with benzene, the presence of red, pink, or violet color indicates positive for Anthraquniones.

3.7.3. Anthocyanin

In a test tube, 1 mL of 1N NaOH was added to 1ml extract and heated for 5 min. The formation of bluish-green color indicates a positive for anthocyanin.

3.7.4. Cardiac glycoside

In a test tube, 2 mL of glacial acetic acid, and 1 mL of conc. H_2SO_4 and a few drops of $FeCl_3$ were added to the 2 mL extract. The formation of a brown ring indicates a positive for cardiac glycosides.

3.7.5. Coumarins

In a test tube, 1 mL of NaOH was added to 1 mL extract and kept in a boiling water bath for few minutes; the appearance of yellow color indicates positive Coumarins.

3.7.6 Flavonoids

In a test tube, a few drops of 1% NH3 solution was mixed with 2 mL extract. The presence of yellow color indicates a positive for Flavonoids.

3.7.7. Glycosides

In a test tube, 2 mL of 50% H_2SO_4 was added to the 2 mL of extract. After 5 min of heating a mixture in a water bath, a few drops of Fehling's solution were added and boiled. The presence of red prick precipitate indicates positive for Glycosides.

3.7.8. Phenolic groups

In a test tube, 2 mL of distilled water and a few drops of 10% $FeCl_3$ were added to 1 mL extract. The formation of blue or black color indicates positive for phenolic groups.

3.7.9. Phlobatannins

In a test tube, 1 mL of 10% NaOH was added to 2 mL extract. The formation of yellow color indicates a positive for Phlobatannins.

3.7.10. Quinones

In a test tube, 1 mL of conc. H_2SO_4 was added to 1 mL of extract. The presence of red color indicates a positive for quinines.

3.7.11. Saponins

In a test tube, 5 mL of distilled water was shaken with 2 mL of extract; the formation of foam indicates positive Saponins.

3.7.12. Steroids

In a test tube, 2 mL of $CHCl_3$, and 1 mL of H_2SO_4 were added to 1 mL extract, and the appearance of a reddish-brown ring indicates positive for steroids.

3.7.13. Tannins

In a test tube, 1 mL of distilled water and 1-2 drops of $FeCl_3$, were added to 2 mL extract, the presence of green or blowback color indicates positive for tannins.

3.7.14. Terpenoids

In a test tube, 2 mL of $CHCl_3$, and 3 mL conc. H_2SO_4 were mixed with 2 mL extract. The formation of a reddish-brown layer indicates a positive for Terpenoids.

3.8. GC-MS

GC–MS analysis volatile compounds in grape leaves, seeds, and fruit-skins (1 g) were extracted in absolute 80% methanol (10 mL) overnight and analyzed using a GC–MS fitted with a BD-5 ms capillary column (30 m, 0.25 μm film thickness, 0.25 μm bore

diameter) based on the method described by Qawasmeh and others (Qawasmeh A. 2011) with minor modifications as described below. The injection volume was 1 µl. The oven temperature was maintained at 80 °C for 2 min. and was programmed to rise to 280 °C at the rate of 30 °C/min. The temperatures of the injector and the detector were maintained at 250 °C and 260 °C, respectively. Helium was used as the carrier gas; the total-gas flow and velocity were maintained at 134.3 mL/min and 43.1 cm/s, respectively. MS scan speed was 1000 amu/s and the molecular masses (M/Z) of the compounds between 50 and 500 M/Z were acquired. The analysis for each sample was repeated 2 times. Compounds were tentatively identified using the NIST05 mass spectral library, and when applicable, their mass spectra were compared with those published in the literature review (Mujeeb *et al.*, 2014).

Figure 3.15: GC-MS automatically injector.

3.9. Antibacterial

3.9.1. Study Design, Location, and Ethical Considerations

The proposed quantitative experimental study has been conducted in accordance with ethical guidelines approved by the Ethical and Protocol Review Committee of Hebron University.

3.9.2. Extract preparation

To conduct the extraction of plant material, twenty-five grams (25 g) of previously crushed and dried samples of grape leaves, seeds, and fruit-skins were weighed out using an analytical weighing scale. It was mixed with 100 milliliters of 80% v/v methanol and macerated overnight at 25°C in a shaking incubator. After 24 hours, the 80% methanol was decanted and filtered through a Whatman No. 1 filter paper. Extracts were then concentrated in a beaker on a hotplate in a laminar flow cabinet with relatively lower heat at 30°C and agitated by magnetic stirring for 24 hours before being kept in a refrigerator at 4°C.

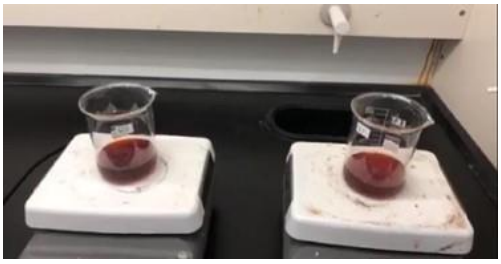


Figure 3.16: The extraction of grape samples for antimicrobial activity.

3.9.3. Media preparing

The media that will be used in the next step for bacterial culturing and identification was prepared as follows before pouring into Petri dishes:

- EMB: 35,96 g/ 1000 mL, for 250 mL, approximately 8,99g needed.
- Mannitol salt agar base (MSA): 111,2/1000ml, for 250ml, about 27,755g needed.
- MacConky agar: 51,53g/1000ml, for 250ml, about 12,883g needed.
- Nutrient agar: 28g/1000l, for 250ml, about 7g is needed.

Prepared small amounts of each type of media for identification of isolated bacteria and prepared three bottles of Muller Hinton (M.H.) media to be used for culturing the bacteria and testing the sensitivity of the plant extract with positive and negative controls. Approximately 28.5 g of M.H. powder was weighed in an autoclavable bottle, and then 750 mL of distilled water was added. The bottles were then heated on the hot plate until they became clear (using a magnetic stirrer), wait 20 minutes, close the bottle and cover it with aluminum foil, and autoclave the media (EMB, MSA, MacConky,

N.A; B.A; and M.H.) for an hour and a half to become sterile. After an hour and a half of sterilization in the autoclave, the media was poured into the Petri dishes as follows, where 25 mL of M.H was poured into each Petri dishes and the quantity filled was about 70 Petri dishes. In addition, differential media was poured into Petri dishes until the circle was covered. Finally, all the dishes were kept in the refrigerator at 4°C until needed.

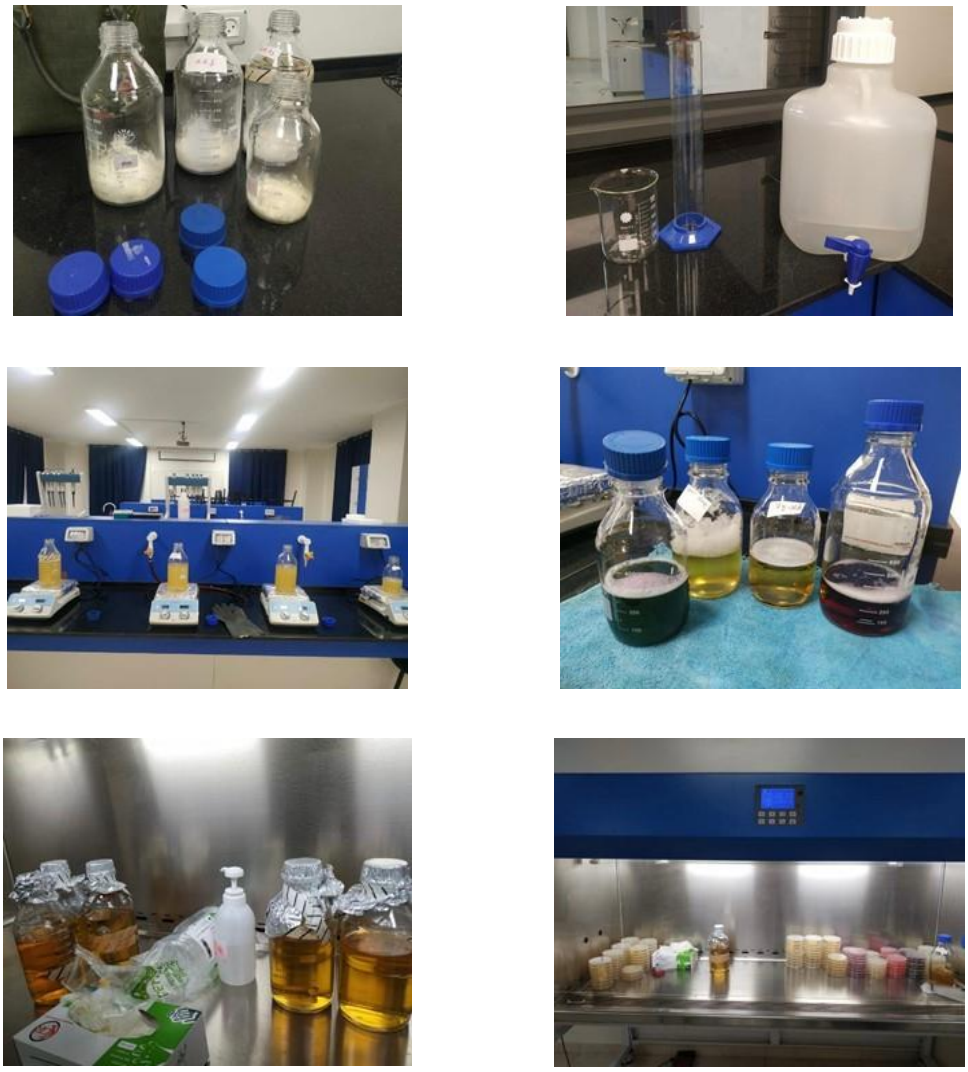


Figure 3.17: Media preparing for antimicrobial activity.

3.9.4. Identification of Bacteria Isolates

At the Microbiology Department of the University of Hebron, bacterial isolates were obtained from the Microbiology laboratory. Bacterial isolates (*E. coli, Proteus, Pseudomonas, Staphylococcus aureus, and Klebsiella*) were authenticated by the secondary culture of the archived isolates. After thawing the isolates in 80% methanol,

they were cultured on differential media plates (Owusu E., 2021). Differential media of EMB, mannitol salt agar base (MSA), MacConky, agar and Nutrient agar aided in distinguishing between Gram-negative bacteria and Gram-positive bacteria. For Proteus: By using cotton swab, one touch of bacteria was put at the center of the petri dish. Whereas following the spread of other types of bacteria in the petri dish (primary, secondary, and tertiary). Then stayed in the incubator at 37°C for 24 hours.

Isolates were characterized phenotypically using colonial morphology and Gram staining. A detailed description of the isolates' characterization follows:

a. *Escherichia coli (E. coli):* These isolates were characterized based on their appearance using two differential media EMB and MacConky; a greenish metallic sheen forms colonies of *E. coli* on Eosin Methylene Blue agar (EMB), whereas a pink colony of *E. coli* fermenting lactose forms on MacConky agar.

b. *Proteus*: The strains were characterized by their appearance as swarming growth forms on Nutrient agar plates.

c. *Pseudomonas aeruginosa*: produce pigment Pyoverdine, which causes them to appear green on nutrient agar.

d. *Staphylococcus aureus*: the appearance of golden-yellow colonies on Mannitol Salt Agar (MSA).

e. *Klebsiella*: These isolates were characterized based on their appearance on using two differential media MacConky and Eosin methylene blue agar (EMB); in both MacConky agar and EMB, Klebsiella pneumonia colonies were pink in color due to the lactose fermentation.

3.9.5. Antimicrobial Activities Evaluation of Plant Extracts by Agar Well Diffusion Method.

Distilled water was added into 4 test tubes about 1 ml in each one, then by cotton swap one touch of bacteria colony -that was isolated previously and identification was taken and added to the distilled water and shaken. The density/turbidity of the inoculum and

the absorbance should be about 0.07 Abs by using a spectrophotometer with lambda max 450 nm, adjusted to 0.5 McFarland turbidity standard, resulting in a suspension of 1.5 × 108 CFU colony forming units.

Mueller Hinton agar plates were seeded with the test organisms and the plates were left for five minutes to dry. After drying, five wells were made in the agar using sterile tips of a micropipette measuring 9 mm in diameter, 3 for the extracts of each genotype, one for the negative control, and other for the positive control. A hundred microliters (100 µL) of the methanolic extract of samples were dispensed into the labeled wells, Meropenem disk was used as a Positive control of E. coli, Proteus, Pseudomonas aeruginosa and Klebsiella, while Vancomycin disk used for Staphylococcus aureus. In addition, 80% methanol was used as a negative control because it was used to prepare the extract. The plates were then incubated at 37 ◦C for 24 h (h) and the zones of inhibition were measured in millimeters. Analysis was done in triplicates (Enid Owusu, 2021).

Figure 3.18: Antimicrobial activity evaluation of grape extracts by agar well diffusion method.

3.9.6. Antimicrobial Activity Evaluation of Plant Extracts by Disk Method.

Distilled water was added into 4 test tubes about 1 ml in each one, then by cotton swap one touch of bacteria colony -that was isolated previously and identification- was taken and added to the distilled water and shaken. The density/turbidity of the inoculum and the absorbance should be about 0.07 Abs by using a spectrophotometer with lambda max 450 nm, adjusted to 0.5 McFarland turbidity standard, resulting in a suspension of 1.5 × 108 CFU colony forming units. Then Whatman filter papers, were cut as disks (about 6 mm in diameter) and put in the extract of grape leaves, seeds, or fruit-skins until the next step.

Mueller Hinton agar plates were seeded with the test organisms and the plates were left for five minutes to dry. Then, Whatman filter paper discs, containing the extract of grape leaves, seeds or fruit-skins at a desired concentration, were placed on the agar surface after labeled the petri dish, also positive disk control (Meropenem disk was used as a Positive control of E. *coli, Proteus, Pseudomonas aeruginosa* and *Klebsiella*, while Vancomycin disk used for *Staphylococcus aureus*) and negative control disk (80% methanol) were placed. The plates were then incubated at 37 ˚C for 24 hours and the zones of inhibition were measured in millimeters. Analysis was done in triplicates (Balouiri, 2016).

Figure 3.19: Antimicrobial activity evaluation of grape extracts by disk diffusion method.

Chapter 4: Results

Chapter 4: Results

4.1. Ash

As shown in **Figure (4.1)**, the ash content of the Red-Halawani genotype exhibited significantly higher content than the Black-Betuni genotype for the three targeted grape parts including leaves, seeds, and fruit-skins. Furthermore, maximum values of ash content were revealed in fruit-skins of Red-Halawani genotype, followed respectively by grape leaves and grape seeds. In contrast, higher ash content was exhibited in the leaves of the Black-Betuni genotype compared to fruit-skins and seeds. In both genotypes, the lowest ash contents were presented in the seeds.

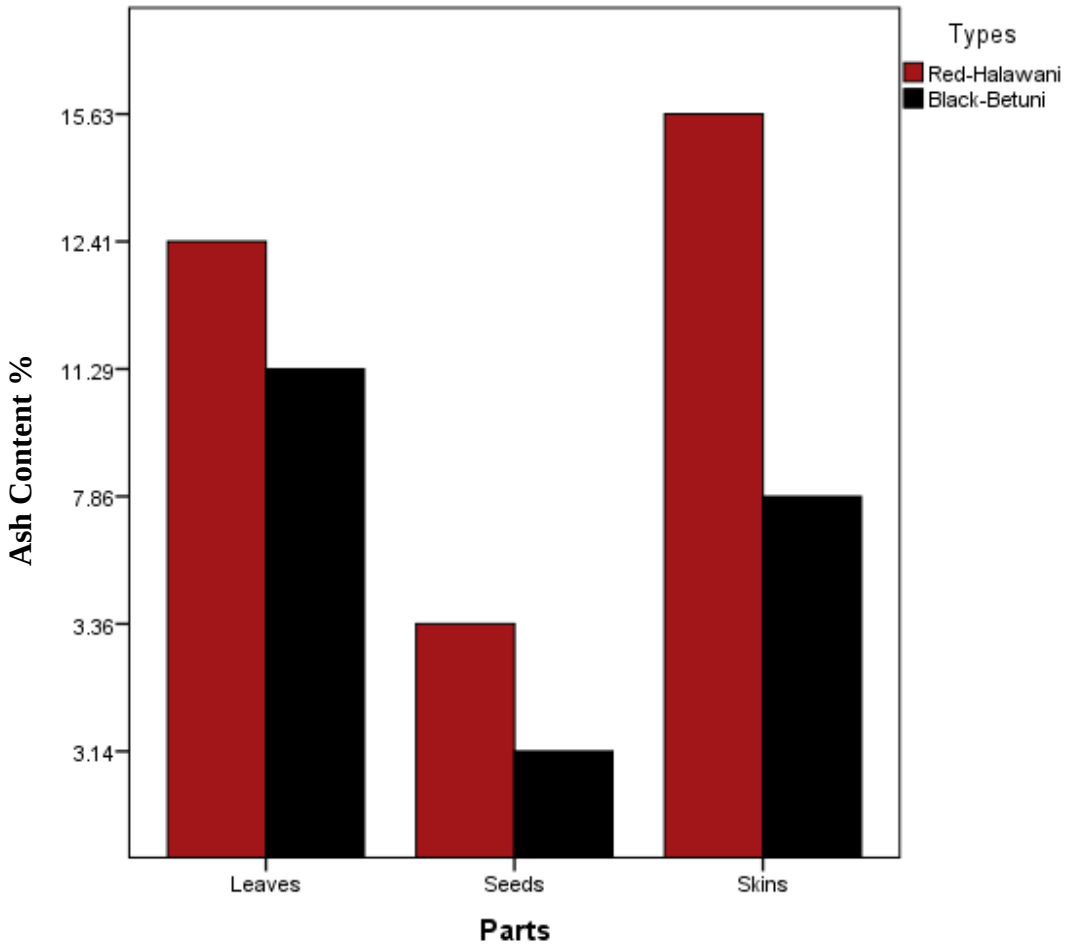


Figure 4.1: Ash content (%) of different parts of Red-Halawani and Black-Betuni grape genotypes

4.2. Minerals

In general, a significantly higher calcium (Ca) percentage was revealed in the leaves of both genotypes over the other evaluated minerals. Among the two examined grape genotypes compromising Red-Halawani and Black-Betuni; leaves presented significantly higher calcium (Ca), nitrogen (N), magnesium (Mg) and iron (Fe) over grape seeds and fruit-skins. In addition, potassium (K) was higher in the fruit-skins of the two grape genotypes followed by grape leaves, and grape seeds. Additionally, no significant differences were observed for phosphor (P) and manganese (Mn) among the different evaluated parts of the two grape genotypes **(Table 4.1).**

Table (4.1): Chemical composition of Red-Halawani and Black-Betuni grape genotypes.

Grape genotypes	Parts	Conducted Minerals (%)						
		N	P	K	Ca	Mg	Fe	Mn
Red-Halawani	Leaves	1.83 ±0.005	0. 002 ±0.001	0.23 ±0.002	2.52+0.003	0.31 ±0.002	0.023 ±0.002	0.001 ±0.001
	Seeds	1.58 ±0.001	0. 002 ±0.001	0.15 ±0.001	0.23 ±0.002	0.16 ±0.001	0.005 ±0.001	0.002 ±0.001
	Fruit-skins	1.35 ±0.005	0. 001 ±0.001	0.42 ±0.002	0.15 ±0.001	0.08 ±0.001	0.006 ±0.001	0.003 ±0.001
Black-Betuni	Leaves	2.07 ±0.003	0. 002 ±0.001	0.18 ±0.001	2.43 ±0.003	0.34 ±0.002	0.026 ±0.002	0.006 ±0.001
	Seeds	1.47 ±0.001	0. 002 ±0.001	0.13 ±0.001	0.42 ±0.002	0.17 ±0.001	0.008 ±0.001	0.001 ±0.001
	Fruit-skins	0.77 ±0.005	0. 001 ±0.001	0.30 ±0.002	0.04 ±0.001	0.04 ±0.001	0.008 ±0.001	0.001 ±0.001

Values expressed as means (%) ± standard deviations. Significant difference at 0.05 level, n=3.

4.3. Proximate Analysis

4.3.1. Fiber

As shown in **Figure (4.2)**, significant maximum values of fibers content were exhibited in the seeds of the Red-Halawani genotype, were as the lowest was registered in the fruit- fruit-skins of the Black-Betuni genotype. Leaves presented a general intermediate fiber content. Moreover, for both genotypes the highest fiber content was shown in the seeds part over leaves and fruit-skins.

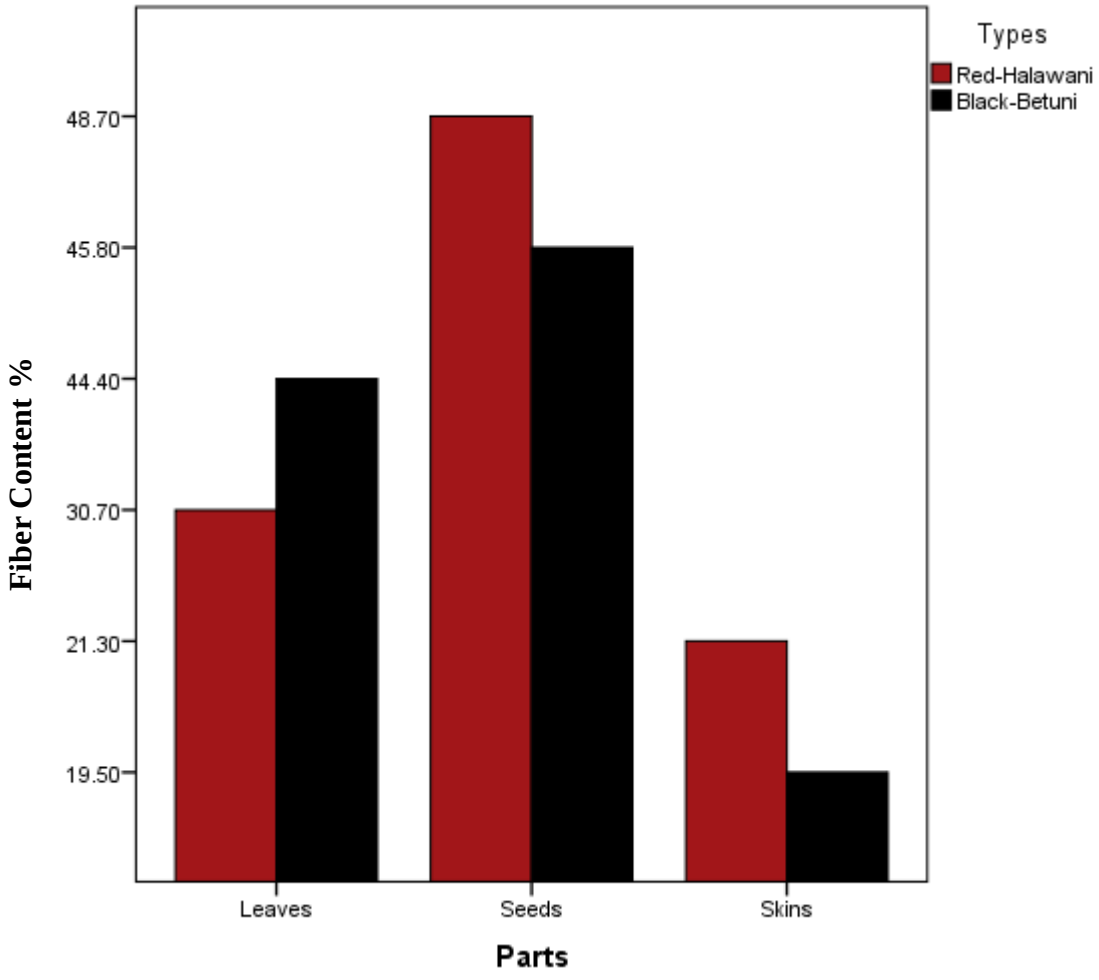


Figure 4.2: Fiber content (%) of different parts of Red-Halawani and Black-Betuni grape genotypes

4.3.2. Fat

As shown in **Figure (4.3)**, the fat content of the Red-Halawani genotype exhibited significantly higher content than the Black-Betuni genotype for the three targeted grape parts including leaves, seeds, and fruit-skins. Furthermore, maximum values of fat content were revealed in leaves of the Red-Halawani genotype, followed respectively by grape seeds and fruit-skins. In contrast, higher fat content was exhibited in the seeds of the Black-Betuni genotype compared to leaves and fruit-skins. In both genotypes, the lowest fat contents were presented in the fruit-skins.

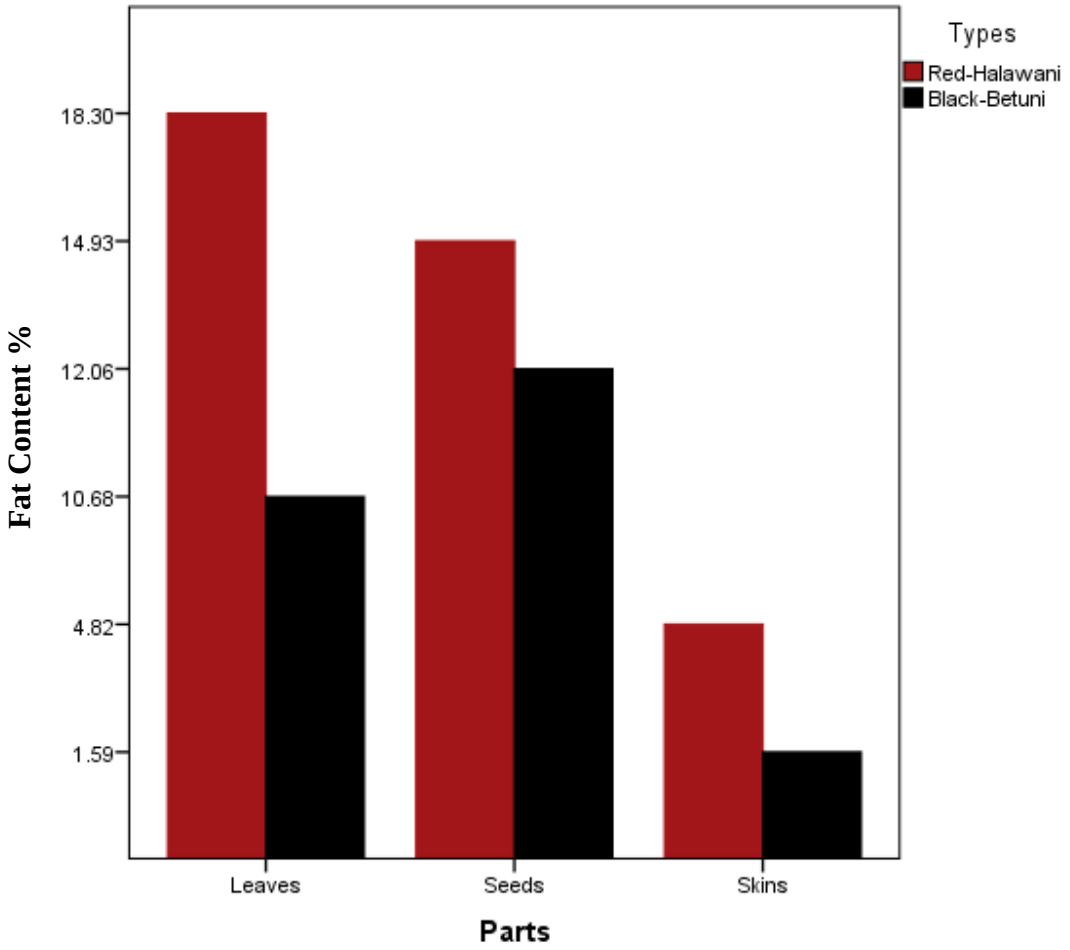


Figure 4.3: Fat content (%) of different parts of Red-Halawani and Black-Betuni grape genotypes.

4.3.3. Protein content (%)

As shown in **Figure (4.4)**, in general significantly higher protein content was revealed in the leaves of both genotypes compared to seeds and fruit-skins. Furthermore, significantly higher protein content was registered in the leaves of the Black-Betuni than in the Red-Halawani genotype, whereas the lowest content was exhibited in the fruit-skins of the Black-Betuni genotype. Here, seeds showed intermediate protein content compared to leaves and fruit-skins.

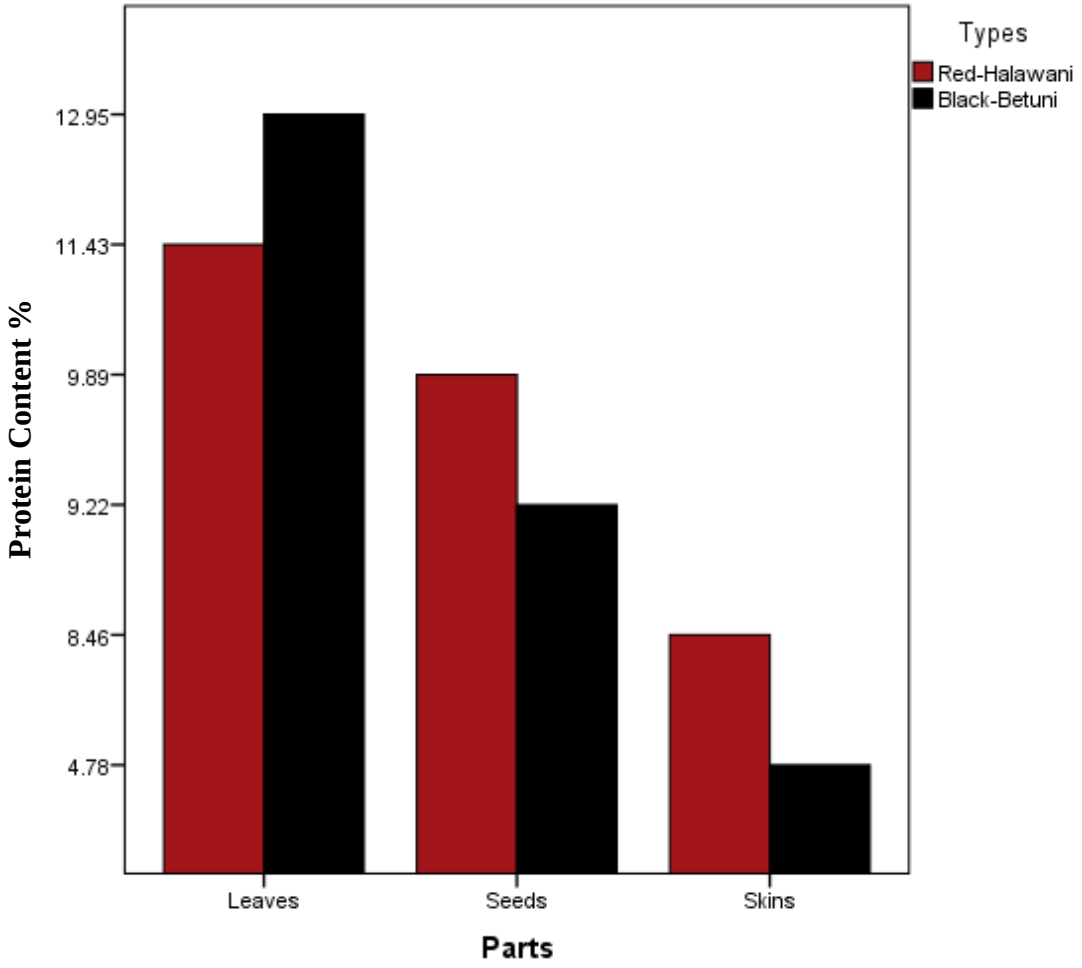


Figure 4.4: Protein content (%) of different parts of Red-Halawani and Black-Betuni grape genotypes.

4.4. Antioxidant activities

4.4.1. DPPH• scavenging capacity

Diluted methanol extracts (1:5) for selected grape parts of both genotypes including Red-Halawani and Black-Betuni showed pronounced antioxidant activity as assessed by DPPH• free radical scavenging assay **(Figure 4.5).** The percentage of scavenging of diluted methanolic extracts of leaves, seeds and fruit-skins was 86.8%, 85.9%, 15.6%, and 79.6%, 80.6%, and 56.4% for Red-Halawani and Black-Betouni cultivars, respectively.

A high percentage of antioxidants was found in grape leaves of both genotypes according to DPPH• scavenging, and the highest percentage was found in the leaves of the Red-Halawani grape genotype, whereas the lowest percentages were found in fruit skins of both genotypes, and the lowest percentage was found in fruit-skins of the Red-Halawani genotype.

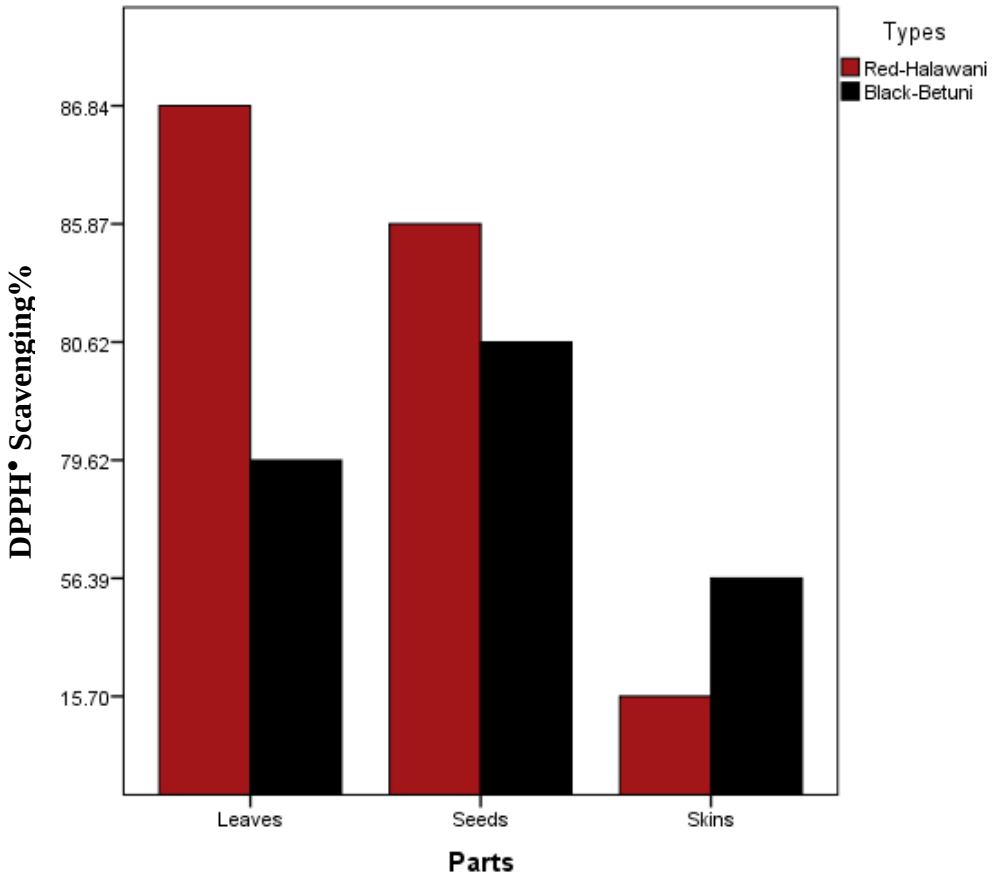


Figure 4.5: Antioxidant capacity (%) of the methanolic extracts of different parts of Red-Halawani and Black-Betuni grape genotypes using DPPH• free radical scavenging assay.

4.4.2. ABTS$^{\bullet+}$ scavenging capacity

Diluted methanol extracts (1:5) for selected grape parts of both genotypes compromising Red-Halawani and Black-Betuni showed pronounced antioxidant activity as assessed by ABTS$^{\bullet+}$ free radical scavenging assay **(Figure 4.6).** The percentage of scavenging of diluted methanolic extracts of leaves, seeds and fruit-skins of Red-Halawani and Black-Betouni cultivars was 87.6%, 90.8%, 53.4%, and 90.9%, 93.9%, 89.8% for Red-Halawani and Black-Betuni genotypes, respectively.

A high percentage of antioxidants was found in grape seeds of both genotypes according to ABTS$^{\bullet+}$ scavenging, and the result shows very trending in percentage among the parts in both genotypes while the highest percentage in seed followed by leaves then fruit-skins.

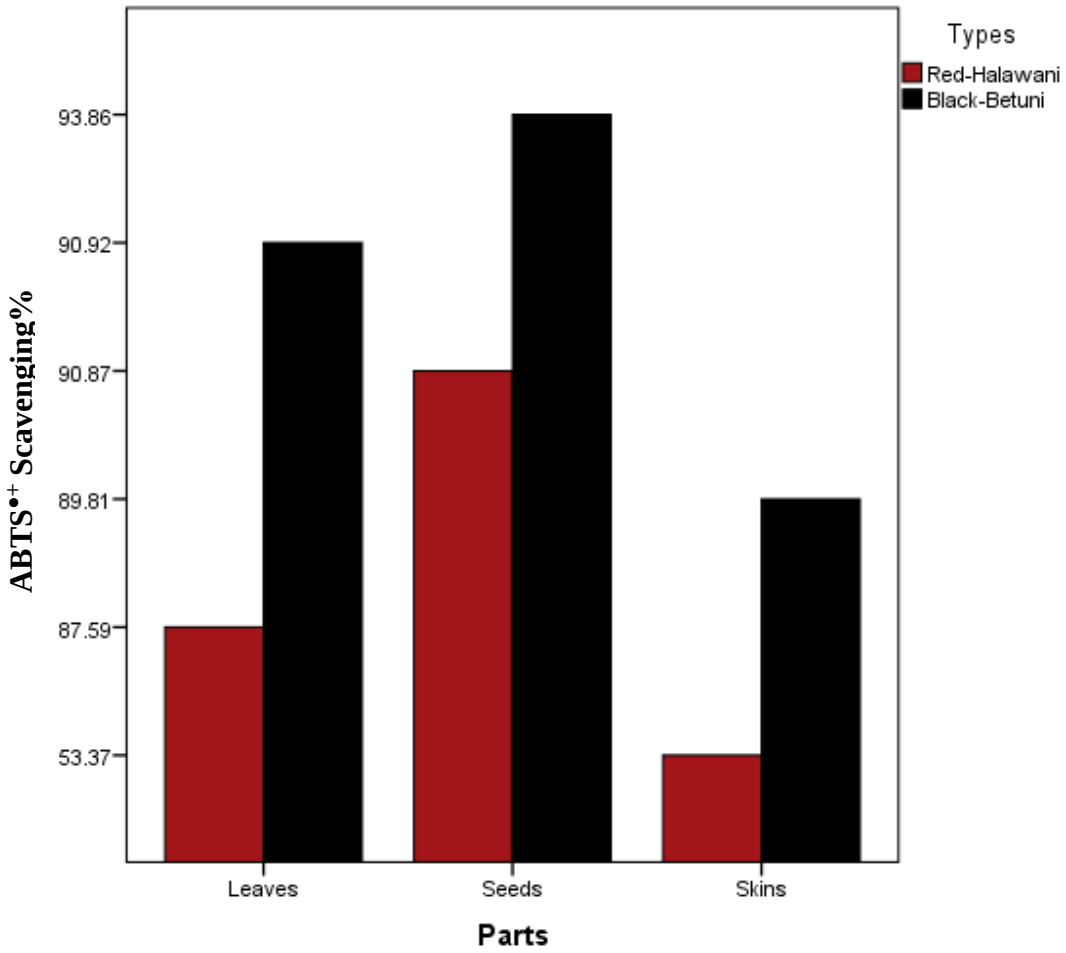


Figure 4.6: Antioxidant capacity (%) of the methanolic extracts of different parts of Red-Halawani and Black-Betuni grape genotypes using ABTS$^{\bullet+}$ free radical scavenging assay.

4.4.3. Quantitative estimation of total phenols

Gallic acid was used to estimate total phenolic content, which was expressed as mg/L of Gallic acid equivalent (GAE) **(Table 4.2; Figure 4.7)**. Quantitative analysis of total phenols of both genotypes including Red-Halawani and Black-Betuni revealed significant amounts of total phenols in all parts.

Table 4.2: Absorbance values of Gallic acid standard (mg/L) n=2

GAE (mg/L)	1	2	Mean ±SE (mg/GAE)
100	0.033	0.050	0.04 ±0.00
200	0.249	0.635	0.39±0.12
300	0.689	0.800	0.69±0.06
500	1.328	1.013	1.16±0.09
1000	2.208	1.883	2.19±0.17

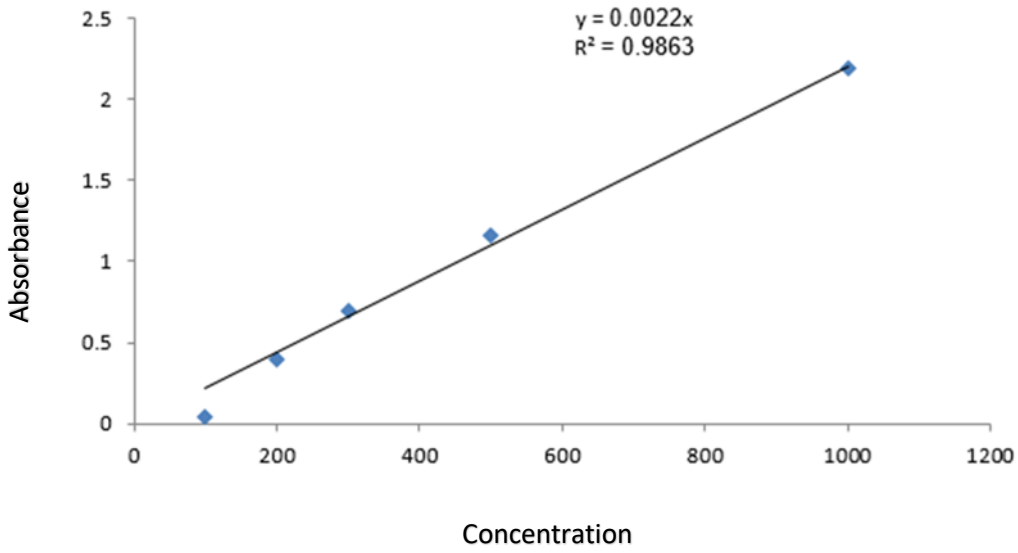


Figure 4.7: Calibration curve of Gallic acid. Each point represents the mean of duplicates.

Furthermore, Red-Halawani and Black-Betuni genotypes parts diluted (1:5) in methanol showed pronounced phenols as assessed by Folin–Ciocalteu reagent. The percentage of scavenging of diluted Methanolic extracts of leaves, seeds and fruit-skins of Red-Halawani and Black-Betouni cultivars was 25.13%, 82.68%, 49.27%, and 30.50%, 61.326%, 56.67% for Red-Halawani and Black-Betuni grape genotypes, respectively.

According to **(Figure 4.8)**, seeds in both Red-Halawani and Black-Betuni genotypes showed the highest value of total phenolic content (TPC) 82.68% and 61.26%, respectively. While, both leaves and fruit-skins in two genotypes were less than 60%.

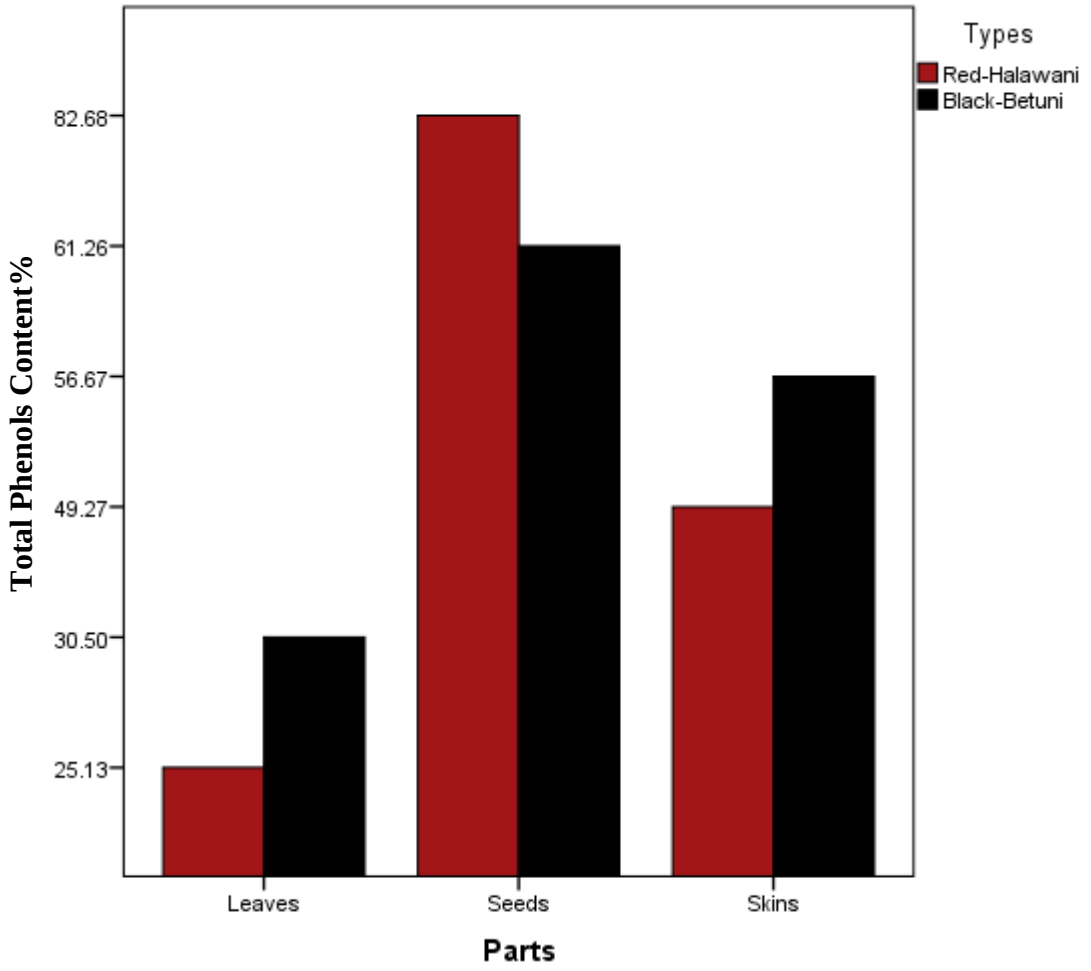


Figure 4.8: Total phenols (%) of the methanolic extracts of different parts of Red-Halawani and Black-Betuni grape genotypes using Folin–Ciocalteu reagent.

4.5. Qualitative phytochemical screening

The preliminary phytochemical analysis of the three parts in Red-Halawani and Black-Betuni genotypes is summarized in **(Table 4.3)**, which showed the presence of cardiac glycosides, phenolic group, saponins, steroids, and terpenoids in all parts.

The qualitative analysis showed that Alkaloids, Anthraquinones, Anthocyanins, Coumarins, Flavonoids, Phlobatannins, and Quinones were not present in the three parts of both grape genotypes (Red-Halawani and Black-Betuni).

Both grape genotypes contain some phytochemicals that are present in some parts, but absent in others. For example, Glycosides were present in both leaves and fruit-skins, but absent from seeds in both grape genotypes. Tannins were also present in leaves and seeds, but absent from fruit-skins in both grape genotypes.

Table 4.3: Phytochemical screening for the methanolic extracts of Grape samples, n=3

Grape genotype	Red-Halawani			Black-Betuni		
Part/Phyto	Leaves	Seed	Skin	Leaves	Seed	Skin
Alkaloids	-ve	-ve	-ve	-ve	-ve	-ve
Anthraqinones	-ve	-ve	-ve	-ve	-ve	-ve
Anthocyanin	-ve	-ve	-ve	-ve	-ve	-ve
Cardiac glycosides	+ve	+ve	+ve	+ve	+ve	+ve
Coumarins	-ve	-ve	-ve	-ve	-ve	-ve
Flavonoides	-ve	-ve	-ve	-ve	-ve	-ve
Glycosides	+ve	-ve	+ve	+ve	-ve	+ve
Phenolic group	+ve	+ve	+ve	+ve	+ve	+ve
Phlobatannis	-ve	-ve	-ve	-ve	-ve	-ve
Quinones	-ve	-ve	-ve	-ve	-ve	-ve
Saponins	+ve	+ve	+ve	+ve	+ve	+ve
Steroids	+ve	+ve	+ve	+ve	+ve	+ve
Tannins	+ve	+ve	-ve	+ve	+ve	-ve
Terpenoids	+ve	+ve	+ve	+ve	+ve	+ve

4.6. GC-MS analysis

The GC-MS analysis of a methanolic extract of different parts of Red-Halawani and Black-Betuni grape genotypes are illustrated in **Figures (4.9, 4.10, 4.11, 4.12, 4.13, 4.14).** The GC-MS analysis revealed the presence of some interesting bioactive compounds in leaves of both grape genotypes including Hydroquinone, Cyclohexanone, Ionone, Caryophyllene, Menthol, and Phytol. These identified compounds with their retention time and molecular weight are shown in **Table (4.4)**.

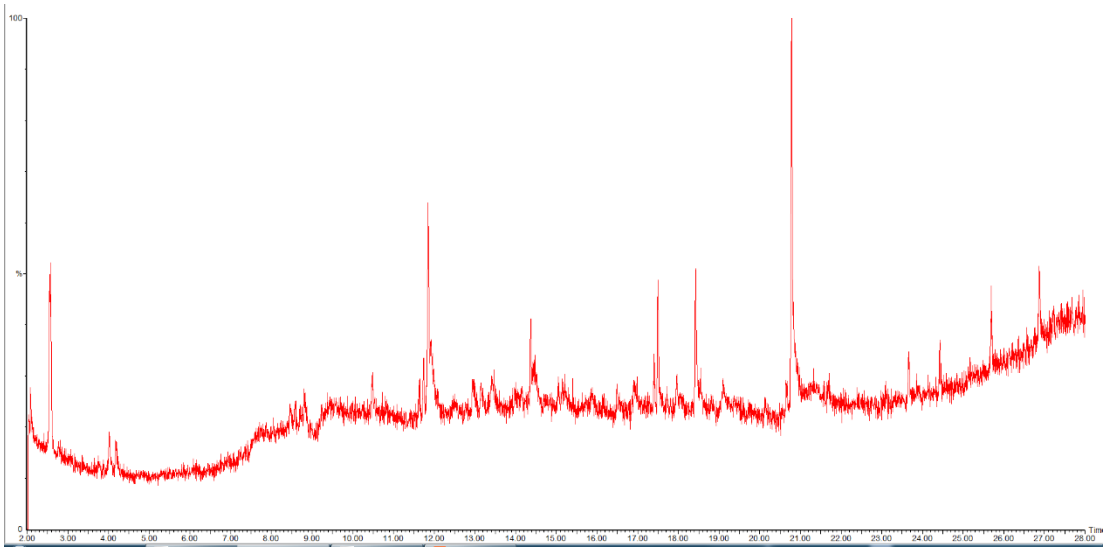


Figure 4.9: Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of Red-Halawani leaves.

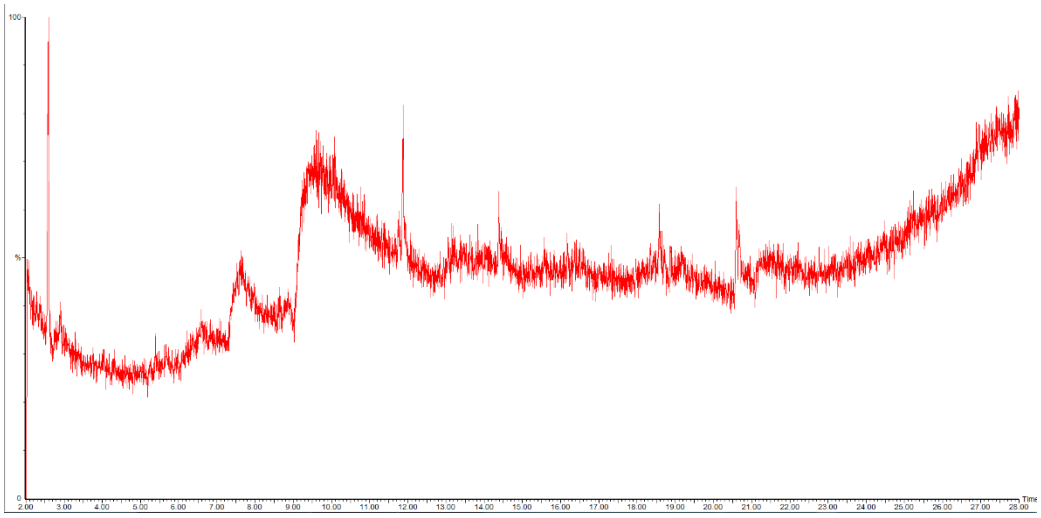


Figure 4.10: Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of Red-Halawani seeds.

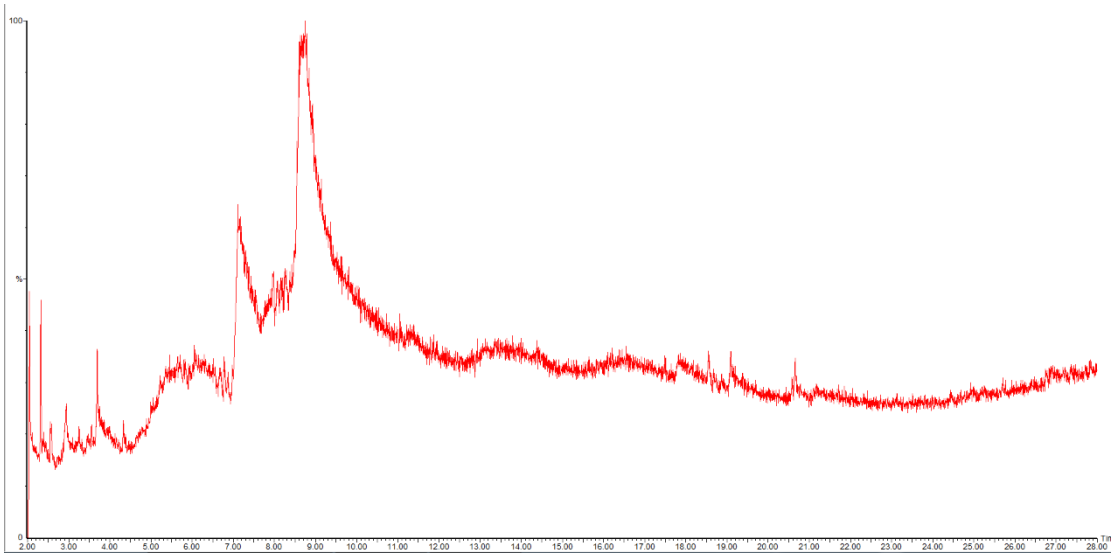


Figure 4.11: Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of Red-Halawani fruit-skins.

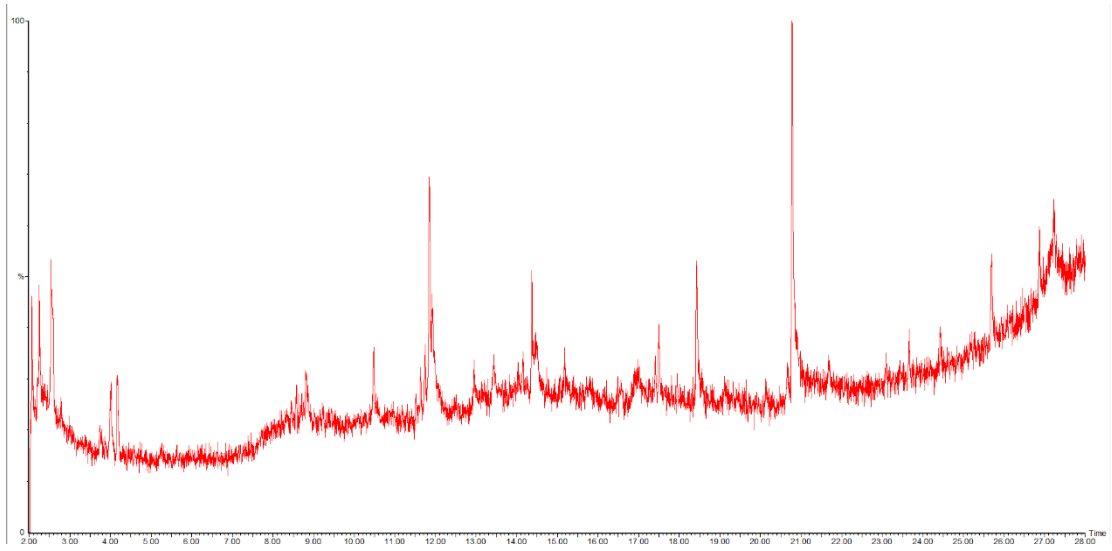


Figure 4.12: Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of Black-Betuni leaves.

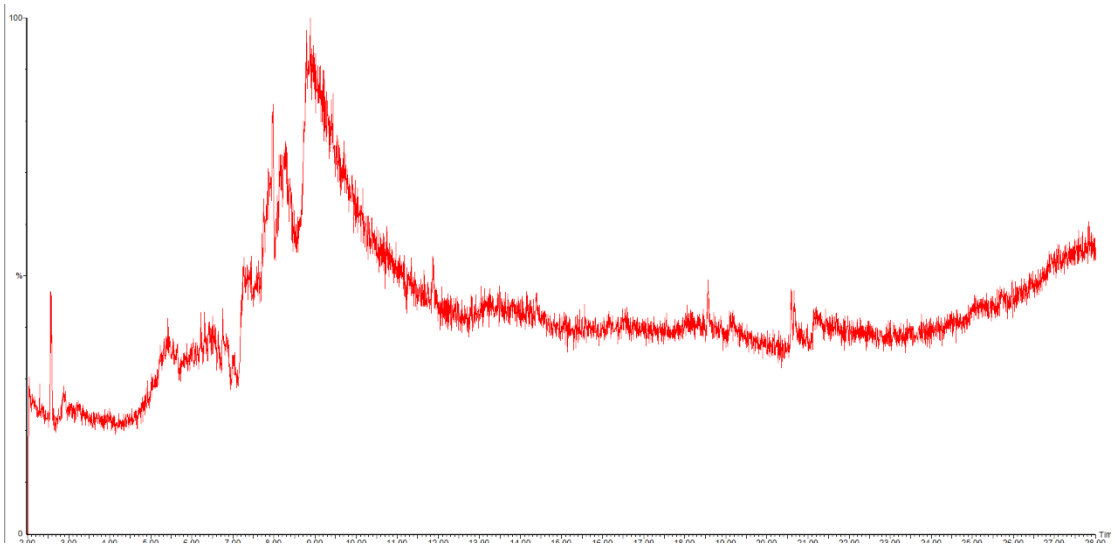


Figure 4.13: Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of Black-Betuni seeds.

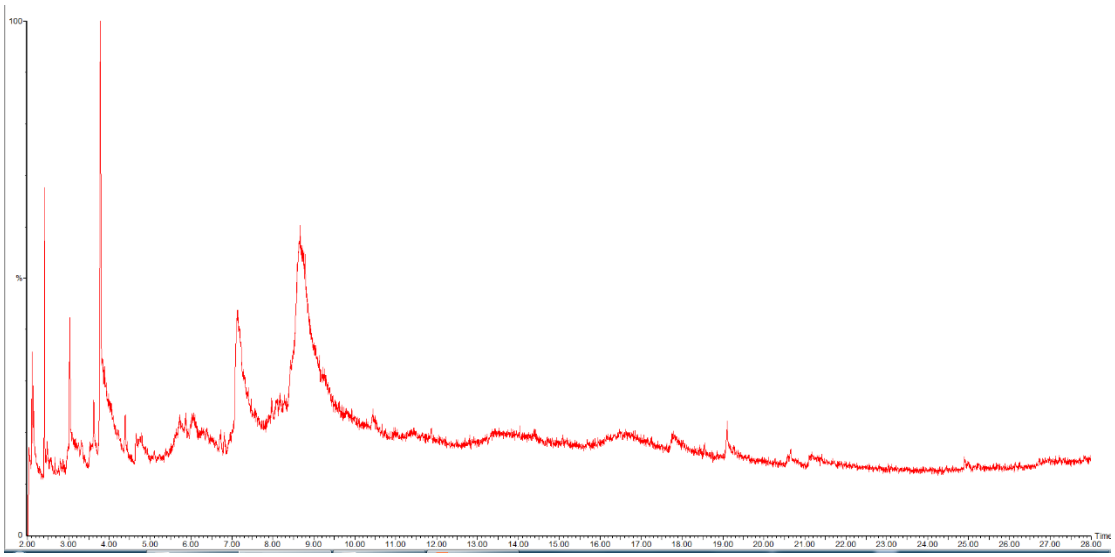
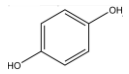
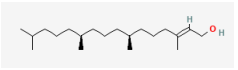


Figure 4.14: Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of Black-Betuni fruit-skins.

Table 4.4: Major compounds detected in two grape genotypes extracts with their retention time (Rt) and molecular weight (MW), and molecular mass (M/Z).

No	*Rt*	M/Z	Compound Identification	Formula	MW	Structures
1	3.519	40,91,77	Hydroquinone	$C_6H_6O_2$	110	
2	3.564	110,109,96	Cyclohexanone	$C_6H_{10}O$	152	
3	5.555	142,91,60	Ionone	$C_{13}H_{20}O$	192	
4	5.600	192,163,91	Caryophyllene	$C_{15}H_{24}$	204	
5	5895	207,71,96	Menthol	$C_{10}H_{20}O$	156	
6	8.756	207,95,57	Phytol	$C_{20}H_{40}O$	296	

4.7. Antibacterial activities

Methanolic extracts of the seeds of Red-Halawani and Black-Betuni genotypes showed significant antimicrobial activities against the gram-positive *S. aureus*, and the gram-negative *P. mirabilis* and *P. aeruginosa*, bacteria. The zone of inhibition recorded (expressed as % of the positive control (Vancomycin for gram-positive bacteria and Meropenem for gram-negative bacteria)) using well and disk diffusion methods against *S. aureus* for the methanolic extracts of Red-Halawani was 98% and 78%, respectively; while in Black-Betouni genotype were 97% and 88%, respectively. In addition, the zone of inhibition recorded against the gram-negative bacteria. *P. mirabilis* using well and disk diffusion methods for the methanolic extracts of Red-Halawani were 48% and 39%, respectively; while Black-Betouni genotype were 48% and 45% respectively. Moreover, *P. aeruginosa* using well and disk diffusion methods for the methanolic extracts of Red-Halawani were 43% and 28% respectively, and Black-Betouni were 46% and 50% respectively **(Table 4.5)**.

Table 4.5: Antimicrobial activity % against the gram-positive *S. aureus*, and the gram-negative *P. mirabilis* and *P. aeruginosa* bacteria of methanolic extracts of seed in both grape genotypes.

Grape	***S. aureus***		***P. mirabilis***		***P. aeruginosa***	
Red-Halawani	Well	Disk	Well	Disk	Well	Disk
100%	93.30%	96.60%	43.40%	42.70%	31.30%	30.40%
80%	98.30%	80.95%	51.30%	36.20%	63.20%	10%
60%	100%	93.30%	52.20%	37.30%	47.80%	34.40%
40%	100%	39.30%	44.80%	39.60%	31.30%	36.70%
AVG	**98%**	**78%**	**48%**	**39%**	**43%**	**28%**
Black-Betuni	Well	Disk	Well	Disk	Well	Disk
100%	100%	91.60%	45.20%	44.80%	52.10%	36.70%
80%	92.10%	96.70%	49.40%	44.80%	56.00%	72.50%
60%	96.30%	84.80%	55.10%	45.50%	42.20%	50.00%
40%	100%	78.30%	42.20%	45.50%	32.20%	40.00%
AVG	**97%**	**88%**	**48%**	**45%**	**46%**	**50%**

Chapter 5: Discussion

Chapter 5: Discussion

Grapes and their different parts are presenting a high level of safety and nutritional value. Generally, the fruits of this tree are among the healthiest and most widely consumed, as it has been used for the treatment of various ailments (diabetes, hypertension, high cholesterol, activating blood circulation, reducing fatigue and exhaustion, cancer, etc.).

In Palestine, numerous cultivars are existed mightily due to the diverse set of environmental conditions (Basheer-Salimia, 2015). In this study, the chemical composition, quality parameters, and pharmacological properties of two selected grape genotypes (cultivars) and their parts were investigated. The obtained ash values in the leaves (11.29-12.41%) and seeds (3.14-3.36%) of both grape genotypes were within the range of many other similar studies on different grape genotypes (Deng *et al*., 2011; Abbas and Khauoon, 2021); however, the ash content was very high in fruit-skins reaching a maximum of 15.63% in Red-Halawani grape genotype **(Figure 4.1)**. Indeed, ash content contributes to the identification of its organic, inorganic, and impurity content, helps in determining whether a material is carbon-free, and boundaries which minerals are soluble and insoluble in plant samples (Ilodibia C. *et al*., 2016).

Concerning minerals, usually they are present in small amounts in comparison with the composition of most of any plant materials; however, they play a significant role in improving health and preventing disease (Singare, 2010). Our study presented significantly higher values of Ca, N, Mg, and Fe in the leaves of both grape genotypes over grape seeds and fruit-skins **(Table 4.1)**, compared to the lower values of P and Mn might be attributed to the differences in plant genotypes, climatic conditions, soil abundance, or soil fertilization (Daccak, 2022; Rana, 2022). Since both cultivars are from the same field with similar environmental conditions, therefore we might assume that such differences are related to the plant's genetic makeup.

Indeed, these minerals are crucial for many biological activities and medicinal uses, for example calcium is vital for strong teeth and bones, improving structural rigidity, and helping blood clots (Indrayan *et al*., 2005); nitrogen is essential to building blocks of life, it is also needed to make nucleic acids, which form DNA and RNA (Kitadai *et al.*, 2018); magnesium is vital to energy metabolism, enzymatic activity, blood pressure,

and cardiac activity (Volpe, 2013); and iron is a critical mineral for the production of hemoglobin, oxygen transport, and immune function (Kruczek, 2005).

Our results revealed higher content of potassium in the fruit-skins over grape leaves and seeds which played an important role in producing fruit-skin of higher quality and condition. According to previous studies, potassium plays a significant role in reducing the firmness and acidity of fruit collected from covered trees, but increasing caliber and weight when compared to fruit collected from uncovered trees. (Mirza M. *et al.*, 2018).Some of its key functions include acting as a precursor for vital cells and tissues, helping in controlling human physiology and pathology, and aiding in the functioning of organs such as the heart and muscles (Karla G., *et al.*, 2021). Additionally, it is vital to manage blood pressure and keep smooth muscle tissue contracting normally in the digestive system and extremities (Pohl, *et al.*, 2013).

Regarding fiber content, our samples of the two grape genotypes and their parts presented higher fiber contents with 46-49% (in seeds), 30-44% (in leaves), and 20-21% (in fruit-skins) **(Figure 4.2**); in which these ranges are in general exceeding many of other similar studies performed in grapes with 36% in grape seeds and 20% in grape leaves (Rabia, 2021), and 10% in fruit-skins (Sunil *et al.*, 2022), respectively. Such differences might be related to different environmental conditions and analytical methods. Furthermore, the higher amount of fibers in grape seeds than in grape leaves and fruit-skins may be related to the time of harvest, since grape harvest generally lasts from August to November (Spinei, *et al.*, 2021). Even though fiber is not considered a nutritional element, it has many health benefits including but not limiting to preventing constipation, regulating blood glucose levels, and protecting against cancer (Colombo, *et al*., 2019).

The revealed differences in the fat content among both genotypes and their parts might be related to the plant genetic material and maturity stage of each genotype. Our findings for the fat content range (11-19% in grape leaves, 12-15% in grape seeds, and 2-5% in fruit-skins), are exceeding the finding of Karovičová (2015) who stated 9.7% in grape leaves, 8.6% in grape seeds, and within the range for fruit-skins by 4.9%. In general, grape components have low lipid levels in the form of monounsaturated fatty acids which are recognized for their health benefits, especially to the cardiovascular system (Rockenbach *et al*., 2011) **(Figure 4.3)**.

Concerning protein content, for both grape genotypes, grape leaves exhibited significantly higher percentages followed by grape seeds and fruit-skins respectively (**Figure 4.4)**. The diverse ranges among the three grape parts are related to the N levels (Figure 4.1), which presented a similar regime (higher in leaves, intermediate in seeds, and low in fruit-skins). Indeed, these differences might be attributed to the plants genetic make-up, the nature of the climate, the soil, and the added fertilizers to the soil (Karovičová J. *et al*., 2015). In fact, plant proteins differ in their chemical, physical, functional, and structural properties, so bioproducts made from them have a variety of properties, such as being crucial for the proper growth and development of the young organism, preventing weight gain, lowering the risk of heart disease, and lowering the incidence of type 2 diabetes, among others (Sim *et al.,* 2021).

Free radicals are extremely unstable chemical entities that primarily have one or more unpaired electrons and are in charge of harming other molecules by stealing their electrons in order to become stable. The human body constantly produces free radicals, which are necessary for energy production, detoxification, chemical signaling, immunological function, and other processes. Free radicals, however, can also be dangerous even if the body needs them. It is possible for ionizing radiation, UV light, chemical interactions, and metabolic activities to cause the creation of reactive oxygen species (ROS) (Zhou *et al*., 2016). The oxidation of biomolecules, including lipids, amino acids, proteins, and DNA, is thought to be the main source of oxidative stress, which in turn causes cell damage and a number of diseases. Oxidative stress is caused by an imbalance between antioxidants and reactive oxygen species (cancer, Parkinson's disease, and others) (Li *et al*., 2015). The damage caused by free radicals may be repaired with the assistance of a number of enzymes, including glutathione reductase, catalase, superoxide dismutase, and others. Because antioxidants (polyphenols, vitamin A, vitamin C, and others) are able to neutralize or scavenge reactive oxygen species (ROS) through hydrogen donation, they are essential in the treatment of a number of human diseases, including cancer, cardiovascular disease, and inflammatory diseases. In addition, antioxidants play a key role in reducing oxidative stress in cells (Baiano *et al.*, 2016). Organic compounds like tannins, alkaloids, carbohydrates, steroids, and terpenoids, among others, that have definite physiological effects on the human body come primarily from medicinal plants, which are mostly used as spices and food plants. Due to their capacity to act as reducing agents and hydrogen donors, the phenolics

group, which is primarily found in leaves, flowering tissues, and woody parts like stems and barks, provides a significant capacity to scavenge free radicals. As a result, they may have a significant impact on the prevention of certain diseases (Li *et al*., 2014).

As a stable free radical method, the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays were used to determine the antioxidant activities of grape samples. These assays are an easy, quick, and sensitive way to examine the antioxidant activities of a specific compound or plant extract, according to Baliyan *et al.*, 2022. Interestingly, as phenolic components like flavonoids, phenolic acids, and phenolic diterpenes increased, so did $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity simultaneously. Red-Halawani and Black-Betuni leaves showed the highest antioxidant activity of the two grape genotypes and their parts, with a high percentage of scavenging activity above 80% for $DPPH^{\bullet}$ and more than 90% for $ABTS^{\bullet+}$. Our $DPPH^{\bullet}$ and ABTS assays results are consistent with those of Shen Y. and others (2019), who discovered that the $DPPH^{\bullet}$ scavenging activity of *Vitis vinifera* leaves extract ranged from 52% to 87%. Additionally, the $ABTS^{\bullet+}$ assays were comparable to those of our study in 94% of cases. However, the fruit-skins showed the lowest antioxidant capacity, with a percentage of scavenging activity ranging between 15.70 - 56.39% and 53.37 - 89.81% for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ respectively. This is consistent with Shen Y. and others (2019) study obtained that the grape leaves and seeds exhibited the highest antioxidant activity compared with grape pulps because they contained the highest concentrations of phytochemicals (**Figure 4.5** and **Figure 4.6**).

The total phenolic contents of grape samples methanolic extracts were determined using the FolinCiocalteu method. The highest TPC was found in Black-Betuni and Red-Halawani seeds respectively **(Figure 4.8**). The values are less than those obtained by Castro-López L. and others (2019) who possessed that the grape seeds TPC ranged from 76.3 to 139.8 mg GAE/g. However, fruit-skins revealed the lowest total phenolic contents. Which supported by Castro-López and others (2019) who showed that the TPC of different cultivars of *Vitis vinifera* fruit-skins ranged higher than our range. During the present work, the leaves of the two grape genotypes possessed the strongest antioxidants activities followed by seeds, and the fruit-skins had the weakest activity, these results agree with Shen Y. and others (2020) who obtained that the seeds of six different grape genotypes have the strongest antioxidant activities comparing with fruit-skins. These studies findings suggest that grape seeds and leaves could be an important

natural source of antioxidants that could be used to prevent or slow the progression of diseases caused by oxidative stress.

Phytochemicals are naturally occurring compounds that are primarily found in fruits, vegetables, legumes, beans, nuts, and whole grains. Phytochemicals include numerous compounds like phytosterols, saponins, flavonoids, terpenes, and others that are responsible for the health benefits of these plant-based foods and beverages (Brindha, 2016). Plant food's color, flavor, and smell are all caused by phytochemicals, a diverse group of chemical compounds that are also considered multifunctional food components because of their important biological properties and antioxidant activity. *V. vinifera* contains stilbenoid compounds, phenolic compounds, aromatic acids (hydroxycinnamic and hydroxybenzoic acid), flavonoids, proanthocyanidin, and a few other classes of secondary metabolites in all of its parts, according to phytochemical research. The numerous pharmacological activities that were discovered showed that each component and compound contained in it had advantages for humans. The grape component and the extract type determine the pharmacological activities. As a result, *V. vinifera* can benefit humans through traditional use (Insanu M. *et al*., 2021). Phytochemical analysis of grape samples included a screening of saponins, steroids, tannins, terpenoids, phenolic groups, and other secondary metabolites, was revealed that grape leaves, seeds, and fruit-skins are a good source of cardiac glycosides, phenolic group, saponins, steroids, and terpenoids. On the other hand, glycosides were present in both leaves and fruit-skins, but absent from seeds in both grape genotypes and tannins were also present in leaves and seeds, but absent from fruit-skin in both grape genotypes (**Table 4.3**).

Using the Electron Impact (EI) mode of GC-MS, the volatile components of grape samples' methanolic extract were compared to the NIST library, and their Kovats Index (KI) was calculated. Grape samples were analyzed using GC-MS, and at least six volatile compounds were found in **Figures (4.9, 4.10, 4.11, 4.12, 4.13, 4.14)**. The bioactive compounds identified with their retention time and molecular weight are shown in **Table 4.4** The major volatile compounds detected in the methanolic extract of grape samples were Hydroquinone (*Rt* = 3.519 min), Cyclohexanone (*Rt* = 3.564 min), Ionone (*Rt* = 5.555 min), Caryophyllene (*Rt* = 5.600 min), Menthol (*Rt* = 5895 min), and Phytol (*Rt* = 8.756 min). The major compounds are found in a high quantity

in the leaves, while less amount of these appeared in the seeds and fruit-skins of both grape genotypes. The majority of studies were conducted on grape juice, not on leaves, seeds, or fruit-skins and the majority of analyses were performed using other instruments like HPLC, so there are no studies to support the findings. The primary compound that can be found in grape juice and grape leaves is called phytol; it is a non-cyclic liquor of diterpene habitually accessible in specific sweet-smelling plants' medicinal oils. Phytol and its derivatives have been shown to have cytotoxic, anti-inflammatory, anti-diabetic, anti-hyperalgesic, antibiotic chemotherapy, antimicrobial, antitumor, antifungal, anti-mutagenic, anti-teratogenic, anticonvulsant, anti-schistosomal, lipid restriction, antispasmodic, anti-scratching behavioural effects, anxiolytic, hair growth facilitator, antide It is necessary for the human body to reap the benefits of phytol. As a vital biomarker for a number of diseases, including chronic polyneuropathy (CP), Refsum's disease (RD), Zellweger's disease hyperpipecolic academia (ZDHA), and Rhizomelic chondrodysplasiapunctata (RZCP), phytol may also be considered a new drug aspirant (Taj T., 2021).

One of the main causes of morbidity and mortality worldwide is infectious diseases, which account for roughly half of all deaths in tropical nations. Multidrug-resistant bacteria have developed as a result of the overuse of antibiotics. These bacteria are a major cause of treatment failure and are regarded as a major issue that limits drug effectiveness worldwide. Therefore, to resolve these issues, it is urgently necessary to investigate a novel approach to infectious disease treatment and prevention. In the past ten years, chemical compounds that were isolated from medicinal plants have been used as a model for many clinically proven drugs. These drugs are now being reevaluated as antimicrobial agents because there are fewer new antibacterial drugs, there is more resistance to antimicrobial drugs, and there is a need to treat new emerging pathogens (Mahady, G., 2005). Multiple mechanisms, including disruption of the cytoplasmic membrane, inhibition of the synthesis of nucleic acids, energy metabolism, cell wall synthesis, cell membrane synthesis, and others, allow medicinal plants to exert their antibacterial properties (Al-Snai, A., 2019). Additionally, the discovery of natural antimicrobials may provide useful solutions to the global issue of antibacterial resistance. Subsequently, distinguishing new wellsprings of regular cancer prevention agents and antimicrobials is significant.

The present study investigated the antioxidant as well as antibacterial activities of grape genotypes, which are traditional Palestinian fruits. However, Methanolic extract of Red-Halawani and Black-Betuni grape genotypes were investigated for their antimicrobial activities against five bacterial strains, one gram-positive (*Staphylococcus aureus*), and four gram-negative (*Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli,* and *Proteus*) using agar well and disk diffusion methods for determining the inhibitory zone diameters. Interestingly, our results revealed that the methanolic extract of both Red-Halawani and Black-Betuni grape genotypes seeds displayed antibacterial activities against gram-positive *S. aureus* with a reasonable zone of inhibition, compared with the positive control (vancomycin). Moreover, displayed antibacterial activity against the gram-negative bacteria. *P. mirabilis* and *P. aeruginosa* with a reasonable zone of inhibition, compared with the positive control (Meropenem) **(Table 4.5)**. Grape seed extracts exhibited no activity against *E. coli* or *K. pneumoniae* for either genotype. These findings align with Ranjitha C.Y, and others (2014) who showed that the *Vitis vinifera* seeds extracts was able to inhibit the growth of *S. aureus* (Ranjitha C., 2014). Further quantitative analysis of volatile compounds is needed to explain the responsible constituent of antibacterial activities against *S. aureus, P. mirabilis* and *P. aeruginosa* in grape seeds genotypes. In addition, studies also are needed to estimate the minimum inhibitory concentration (MIC) and the safety of the grape methanolic extracts.

Conclusions

Based on the results obtained in this study, it was revealed that the methanolic extract of the selected grape genotype: Red-Halawani and Black-Betuni harvested from Halhul in Palestine have the following valuable effects:

1. Both grape genotypes and their parts are rich in nutrients (mainly Ca, N, Mg, Fe, and K), fibers, and protein content.

2. Both grape genotypes have high antioxidant activities due to the high content of phenolic compounds like cardiac glycosides, glycosides, phenolic group, saponins, steroids, tannins, and terpenoids, these compounds exhibit anti-aging activity, anticancer, antioxidant, antibacterial, and anti-inflammatory activities.

3. Phytochemical's screening of both grape genotypes shows the presence and detection of various plant secondary metabolites present in the methanolic extract of leaves of these selected plants like cardiac glycosides, glycosides, phenolic groups, saponins, steroids, tannins, and terpenoids compounds which detected by different phytochemical screening tests and GC-MS analysis.

4. The antimicrobial studies of grape seeds showed remarkable antimicrobial activities against some gram-negative and gram-positive bacterial strains, suggesting that these Palestinian folkloric medicinal plants possess broad-spectrum antibacterial activities.

5. Our Palestinian grape has an interesting zone inhibition against Gram +ve bacteria. These results are to be investigated to enhance the pharmacological industry.

Recommendations

The followings are some suggestions that may be taken into consideration for future investigation:

- The current research used the compounds found in the plant while it is dried. It is recommended to examine the components in the different stages of the plant to note the differences with the application of the same protocols and compare the results.

- As grapes are rich in minerals, it is recommended to do more comprehensive tests for more minerals. Furthermore, it recommended checking the grape vitamins contents.

- Work on more tests on these plants, for example, testing the anti-cancer and antifungal activities.

- As for the antioxidant test, it is recommended to do several tests, such as IC_{50}, Trolox equivalent antioxidant capacity (TEAC) assay, the ferric reducing ability of plasma (FRAP) assay, and the copper reduction (CUPRAC) assay. Moreover, compare them with the results we obtained with $ABTS^{\bullet}$ & $DPPH^{\bullet}$ antioxidant activities assays. Additionally, measure the total oxidant scavenging capacity (TOSC).

- It is recommended to raise the temperature, increase the separation period of vehicles inside the GC-MS, and monitor the exit of new compounds if they appear.

- It is recommended to use the headspace to separate the volatile compounds and compare them with the results we got in this study.

- As grapes proved to have valuable antioxidant activities, it is recommended that these plants could be used as a therapeutic agent to treat various infectious diseases including COVID-19 infection, the effect of grape on the COVID-19 virus, and its signs and symptoms are yet to be understood.

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Abstract in Arabic

الملخص

فحص المواد الكيميائية النباتية ومضادات الأكسدة والمضادات الميكروبية لبعض أصناف العنب الفلسطيني

العنب نبات حيوي للاقتصاد الفلسطيني والثقافة الفولكلورية. بالإضافة إلى كونه جزءًا مهمًا من نظامنا الغذائي، يمكن أيضًا استخدام العنب كدواء تقليدي لعلاج العديد من الأمراض، بما في ذلك خفض ضغط الدم، وتحسين تدفق الدم، وتقليل الأكسدة، وتحسين مستويات الكولاجين وقوة العظام، وتثبيط النمو المعدي، و تقليل مخاطر الإصابة بالسرطان. في هذه الدراسة، تم تحديد الخصائص الكيميائية النباتية، والمعادن، والقدرة المضادة للأكسدة، والخصائص المضادة للبكتيريا في أوراق وبذور وأغشية ثمار نمطين وراثيين (الحلواني والبيتوني) التي تزرع بشكل شائع في فلسطين. تم جمع عينات العنب من الثمار الناضجة والأوراق من (حلحول -الخليل) في فلسطين وبعد فصل البذور والجلد عن الفاكهة. تم تجفيف جميع الأجزاء بالهواء حتى لا يتم تسجيل أي تغيير في الكتلة. تم قياس المعادن باستخدام (ICP-OES)؛ تم تقييم الأنشطة المضادة للأكسدة بواسطة مقايسات الكسح $DPPH^{\bullet}$ و $ABTS^{\bullet+}$ تم تحديد إجمالي محتوى الفينول باستخدام طريقة Folin-Ciocalteu؛ ثم تم تحديد المركبات المتطايرة باستخدام GC-MS المجهزة بوضع تأثير الإلكترون وتم فحص الأنشطة المضادة للميكروبات بطريقتين ضد بكتيريا موجبة غرام وبكتيريا سالبة غرام.

من بين الطرز الوراثية التي تم فحصها، كشفت نتائجنا عن محتوى أعلى بكثير من الرماد والدهون والبروتين في أوراق العنب مقارنة بالبذور وأغشية الثمار. ومع ذلك، أظهرت بذور العنب محتوى ألياف أعلى بكثير مقارنة بالأوراق والأغشية. وبالمثل، كان النيتروجين (N) والفوسفور (P) والبوتاسيوم (K) والكالسيوم (Ca) والمغنيسيوم (Mg) والحديد (Fe) والمنغنيز (Mn) أيضًا أعلى في أوراق نمطي العنب الجيني يليه بذور العنب وأغشية ثمار العنب، على التوالي.

أظهرت الأوراق في كلا الطرازين قدرة مضادة للأكسدة أعلى مقارنة بالبذور وأغشية الثمار. أظهرت الأوراق من التراكيب الجينية الحلواني 86.84% والبيتوني79 62.% حسب تقييم $DPPH^{\bullet}$. كما واظهرت النتائج حسب تقييم $ABTS^{\bullet+}$ للصنفين الحلواني والبيتوني 90.92٪ و87.59٪ على التوالي. بينما كانت النتائج في باقي الأجزاء للصنفين أقل من 80%. علاوة على ذلك، أظهرت البذور في كلا الطرز الوراثية الحلواني والبيتوني أعلى قيمة لمحتوى الفينول الكلي 82.68% و61.26٪ على التوالي. بينما كانت نسبة الأوراق وأغشية الثمار أقل من 60٪. وأظهر تحليل 14 مكونًا كيميائيًا نباتيًا أن أوراق العنب والبذور والجلود مصدر جيد غليكوسيد والفينولات والصابونين، الستيرويدات والتربينات. علاوة على ذلك، تم العثور على العفص؟؟؟؟؟؟ في الأوراق والبذور، والجليكوسيدات موجودة في الأوراق وأغشية الثمار. عبّرت الأوراق في كلا الطرازين عن طيف من المركبات المتطايرة التي تم تحديدها على أنها هيدروكينون (Rt = 3.52 دقيقة)، سيكلوهكسانون (Rt = 3.56 دقيقة)، أيونون (Rt = 5.55 دقيقة)، كاريوفيلين (Rt = 5.60 دقيقة)، منثول (Rt = 5895 دقيقة)، والفيتول (Rt = 8.76 دقيقة)

في المستخلص الميثانولي. في ظل ظروفنا التجريبية، لم يتم اكتشاف أي مركبات متطايرة في الأنماط الجينية لبذور وأغشية الثمار العنب.

أظهرت المستخلصات الميثانولية للبذور في كلا الطرازين نشاطاً معنوياً مضاداً للميكروبات ضد البكتيريا موجبة الجرام (*S. aureus*) والبكتيريا سالبة الجرام (*P. mirabilis* و *P. aeruginosa*). منطقة التثبيط (معبرًا عنها كنسبة مئوية من المضادات الحيوية المستخدمة لغرض الاختبار الإيجابي) باستخدام طرق الانتشار والقرص ضد *S. aureus* في المستخلصات الميثانولية للصنف الحلواني 98% و78٪ على التوالي. في المستخلصات الميثانولية للصنف البيتوني كانت منطقة التثبيط المسجلة 97٪ و88٪ على التوالي. بالإضافة إلى ذلك، كانت منطقة التثبيط المسجلة ضد البكتيريا سالبة الجرام *P. mirabilis* و *P. aeruginosa* أقل وضوحًا مقارنة بالبكتيريا موجبة الجرام.

كانت هذه الدراسة هي الأولى من نوعها لفحص وتقييم المركبات النباتية الكيميائية في طرز وراثية مختارة من العنب الفلسطيني من حيث نشاطها المضاد للبكتيريا ومضادات الأكسدة. تشير هذه النتائج إلى أن بذور كلا النوعين الجيني للعنب لها نشاط مضاد للأكسدة مع نشاط مضاد للميكروبات واضح مقارنة بالضوابط الإيجابية. كشفت نتائجنا عن تباين كبير في الرماد والألياف والدهون والبروتينات والمعادن والمواد الكيميائية النباتية والنشاط المضاد للأكسدة والنشاط المضاد للبكتيريا للأجزاء المختلفة من صنفي العنب المدروسين.

الكلمات المفتاحية: العنب، المعادن، مضادات الأكسدة، النشاط الكيميائي النباتي، النشاط الحيوي.