Faculty of Graduate Studies

Chemistry Department/ Birzeit University

Phytochemical Analyses and Bioactivity Studies of *Arum Palaestinum* Spadix

تحليل المواد النباتية الثانوية والنشاط البيولوجي لزهرة اللوف

Submitted by:

Baydaa' J. Habash

Supervisor:

Dr. Ghassan Albarghouti

September, 2022

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Baydaa' J. Habash

(1175184)

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Dr. Ghassan Albarghouti

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This Thesis was prepared under the supervision of Dr. Ghassan Albarghouti and has been approved by all members of the examination committee.

Examination committee

Dr. Ghassan Albarghuoti

(Chairman of the committee) Signature: .....

Dr. Hani Shtaya (Member) Signature: .....

Dr. Waseem Abo-Oun (Member) Signature: .....

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The findings, interpretations and conclusions expressed in this study, do not necessary express the views of Birzeit University, the views of the individual members of M. Sc. Committee or the views of their respective employers.

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Contents

List of Abbreviations

GENERAL CONCEPTS

CAM: Complementary and Alternative Medicine

WHO: World Health Organization

SAR: Structure Activity Relationship

A. P: Arum Palaestinum

INSTROMENTATION AND TECHNIQUES

TLC: Thin Layer Chromatography

UV: Ultra-Violet Spectroscopy

HPLC: High Performance Liquid chromatography

LC-MS: Liquid chromatography Mass Spectrometry

GC-MS: Gas chromatography Mass Spectrometry

^{1}HNMR: Hydrogen Nuclear Magnetic Resonance

ELISA: Enzyme Linked Immunosorbent Assay

CHEMICAL REAGENTS

DPPH: 1, 1-diphenyl-2-picrylhydrazyl

DMSO: Dimethyl Sulfoxide

BIOLOGICAL CONCEPTS

P53: Tumor Suppressor Gene

COX-2: Cycloxygenase-2

ROS: Reactive Oxygen Species

L6: Tumor Associated Antigen

P-gp: *P*-Glycoprotein1

MRP1: Multidrug Resistance Protein1

BACTERIA AND FUNGI

MHA: Mueller Hinton Agar Medium

MRSA: Methicillin-resistant staphylococcus Aureus

P. Auroginosa: Pseudomonas Auroginosa

E. Faecalis: Entercoccus Faecalis

L. Monocytogenes: Listeria Monocytogenes

S. Aureus: staphylococcus Aureus

E. Coli: Escherichia Coli

C. Albicans: Candida Albicans

CANCER CELL LINES

DMEM: Dulbecco's Modified Eagle Medium

HEK293T: Human Embryonic Kidney Cancer Cells

HeLa: Henrietta Lackes' Immortal Cancer Cells

HCT-116: Human Colorectal Carcinoma

MFC-7: Breast Cancer Cells

Hep2: HeLa Marker Chromosoms

HL-60: Human Leukemia cancer Cells

HL-60/DOX: Doxorubicin Resistant Human Leukemia cancer Cells

MDA-MB-231: Epithelial Human Breast Cancer Cells

HepG2: Human Liver Cancer Cells

HepG2/ADM: Human Multidrug Resistant Hepatocellular Carcinoma

K562: Chronic Meyelogenous Leukemia

K562/DOX: Doxorubicin Resistant Chronic Meyelogenous Leukemia

MES-SA/DX5 : Human Multidrug-Resistant Cancer Cells

EACC: Ehrlich Ascites Carcinoma Cells

1301: Human T-cell Leukemia

A549: Human Non Small Lung Adenocarcinoma

C2Cl2: Muscle Precursor cell line

3T3-L1: Embryonic Albino Swiss Mouse Fibroblast cells

HNSCC: Head And Neck Squamous Cell Carcinoma

SGC7901/ VCR: Human Multidrug Vincristine Resistant Gastric Cell line

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Abstract

Arum Palaestinum is a wild edible, parietal, unisexual, perennial plant that belongs to the *Araceae* family and grows in different regions in the Middle East and among many other herbal plants, it is used as a remedy for the treatment of many maladies, above all, it is best known for being used as colon cancer therapy. Historically, its areal parts are commonly used for cooking and studying purposes, and were believed to have rich bioactive compounds such as alkaloids, polyphenols, flavonoids, terpinoids and many other contents that have been under investigation since so long and revealed antioxidant, antimicrobial, anti-inflammatory and anticancer activity. Its leaves-extracts showed toxicity towards lymphoblastic carcinoma as well as HCT-116 colon cancer, MFC7 breast cancer, Hep2 and HeLa cancer cell lines. But, the lack of phytochemicals analysis about the dark purple spadix, as well as its rhizomes and stems, parts of the plant has led to this research in which separation, components identification, characterization in addition to bioactivity, antioxidant activity, cytotoxicity and anticancer studies will be done via HPLC, GCMC, LCMS, ^{1}HNMR and ELISA techniques. Two compounds with their suggested possible structures were separated in addition to twelve compounds that were analyzed by GCMS. Neither antibacterial nor antifungal activity of the *Arum Palaestinum* spadices extract were reported on *E. Coli, S. Aureus, P. Aeruginosa* bacteria and *C. Albicans* fungi. On the other hand, significant anti-inflammatory effects was reported at concentration 25 mg/mL. *Arum Palaestinum* aqueous XAD and butanol extracts were recorded to have the best anti-proliferative activity against HeLa and HEK932T cancer cell lines among all its extracts that were used in this research work.

ملخص

اللوف الفلسطيني، هو نبات بري، جداري، ثنائي الجنس، معمر ينتمي إلى عائلة الأراكايا وينمو في مناطق مختلفة في الشرق الأوسط، ويستخدم كعلاج للعديد من الأمراض من اهمها السرطان وقد اشتهر على مدى عقود باستخدامه كعلاج لمرض سرطان القولون. كان يُستخدم بشكل شائع في الطهي لغرض الدراسة، ويُعتقد أنه يحتوي على مركبات نشطة بيولوجيًا غنية مثل القلويات ومركبات البوليفينول والفلافونويد والتربينويد والعديد من المحتويات الأخرى التي خضعت للدراسة منذ فترة طويلة وكشفت عن وجود مضادات الأكسدة ومضادات الميكروبات ومضادات الالتهابات ومضادات للسرطنة. أظهرت مستخلصات أوراقه فعالية سمية تجاه العديد من انواع السرطان كسرطان الخلايا الليمفاوية, وسرطان القولون, وسرطان الثدي والعديد من الخلايا السرطانية. ان نقص الدراسات على زهرة اللوف ومكوناتها كانت الدافع لإجراء هذا البحث الذي سيتم فيه فصل وتحديد المكونات والتوصيف بالإضافة إلى دراسة النشاط الحيوي والنشاط المضاد للأكسدة بالإضافة إلى دراسة السمية الخلوية السرطانية عبر تقنيات الفصل الكروماتوغرافي, وتقنيات قياس الطيف الكتلي عبر الكروماتوغرافيا المسالة والغازية بالإضافة الى تقنية الرنين النووي المغناطيسي. تم فصل مركبين واقتراح البنية الهيكلية الممكنة لهما بالإضافة الى اثني عشر مركب آخر تم تحليله كروماتوغرافيا. لم يتم تسجيل أي نشاط مضاد للجراثيم أو مضاد للفطريات للمستخلص الكحولي لزهرة النبتة على كل من البكتيريا الإشريكية القولونية, والبكتيريا المعوية البرازية, والبكتيريا الزائفة الزنجارية وفطر المُبيضَّة البيضاء. لكن من ناحية اخرى, تم تسجيل تأثير واضح مضادة للالتهابات عند التركيز 25 مجم / مل. لقد تم تسجيل افضل فعالية مضادة للتكاثر السرطاني في هذا البحث على خلايا السرطان HeLa وHEK932T لكل من مستخلص نبتة اللوف المائي ومستخلص البيوتانول الكحولي من بين جميع المستخلصات الاخرى.

Chapter. 1 Background

1.1 Traditional Medical Products

The screening of flora for biological and pharmacological active compounds started in the late nineties [1]. That is because the wild edible plants have always been of interest in medications and treatment of maladies. In the last two decades, herbal-based drugs, averaged about 25-28% of drugs, and about 50% of all modern medicines are directly or indirectly derived from plants [2]. By the year 1990, 70% of all drugs became herbal based in one way or another, i.e. drugs from synthetic or semisynthetic origins [3]. There are about 260,000 higher plants, over 200,000 primary and secondary metabolites may be present in the plant kingdom. In fact, there are around 120,000 plant species that can be utilized to produce biologically active products, which are used for the treatment of different maladies [4]. Nearly up to 60% of drugs used for cancer treatment worldwide have been isolated from natural products, as an example, *Taxol, Vinblastine* and *Vincristine* [5, 6]. There are more than 3000 plants with anti-cancer properties and contain high levels of antioxidants phytochemicals of low or relatively non-toxic nature that can stimulate the immune system. Thus, locate tumors and destroy their cells, inhibit angiogenesis and causes a widespread expression of the tumor cells suppressor gene (P53) [7]. To mention, alkaloids such as *pyrrolidines, quinolines, indoles, colchicines* and poly phenols as *flavonols, flavones, isoflavones* and *chalcones* are major examples of phytochemicals that are very well known for their bioactive role in the medical field [8].

Epidemiologic studies indicates that diets that contain high amounts of fruits and vegetables are associated with a lower risk of different cancer types. For instance, several reports demonstrated that a high consumption of soybean plant products in Asian countries reduces the incidence of colon cancer, and a high consumption of vegetables decreases the risk of colon cancer mortality [9-11].

World Health Organization (WHO) estimates 65-80% of world population use traditional herbal based medicine [2, 12]. Since the nineteenth century, the bioactivity of

these compounds has been utilized for the production of therapeutic and psychoactive drugs [13]. According to WHO, a third of the world's population has no regular access to essential modern medicine, and about 80% of people who are living in rural areas in developing countries use traditional medicine for their primary health care needs[14]. According to its latest reports, even advanced countries have adapted traditional system of herbal treatment including; as an example, Australia (48%), China (40%), France (49%), Canada (70%) and Germany (77%), likewise, the less developing countries i.e. India (70%), Chile (more than 70%) and Colombia (40%) [2].

Historically, the Middle East was a strategic area and was therefore dominated by different eastern and western civilizations, e.g. (Greeks, Romans, Turks, Mongolians, etc). These visiting nations also influenced local cultures in terms of traditional and folk plant medicines[5]. The Eastern region of the Mediterranean has been known over the years for its rich herbal medicine inventory. Some distinguished Arab scholars, chemists, pharmacologists, herbalists, and physicians were popular for their achievements in utilizing the herbal plants in the medicine area via extraction of active ingredients, purification and preparation of remedies. Also, combining chemistry, medicine, pharmacology, agriculture and plant science to develop new aspects in the treatment field. They studied cancer and several diseases and applied various medicines and surgical means for their treatment [15, 16].

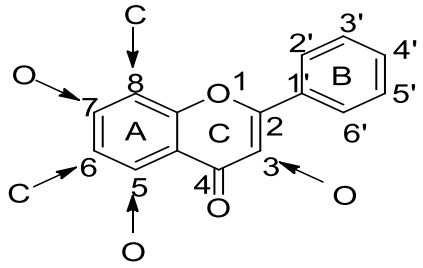
Herbal medicine is still being used in Arab and Islamic societies, especially the Mediterranean region. Its diet was and still known for its strong protective effect against cardiovascular disease and cancer for its variety of ingredients, from fruits, vegetables, herbs, seeds and cereals to fibers and legumes. The thing that makes it rich in antioxidants, antibiotics and immunostimulators as carotenoids, vitamins, tocopherols, phenolic acids and so on, which help enhancing all of the body physiological functions. Throughout Muslim history, the Greco-Arab and Islamic herbal medicine was the first choice of treatment for many maladies involving epilepsy, infertility, depression and cancer [16].

Due to its geographical location, different zoogeographic and phytogeographic zones as well as climate and natural biodiversity [17], Palestine is the country with the second greatest number of anticancer herbal species (54 family, 113 species) after Ghana (55 family, 117 species). Followed by India, Nigeria, Brazil, Kenya, Pakistan, and South Africa [18], as reported by M. Aumeeruddy, and partners (2021). The West Bank is home to approximately 700 wild edible and medicinal plant species and their secondary metabolites [19]. About 345 species of wild plants in the West Bank, and 120 in Gaza Strip were reported to be in traditional use. The most popular ones are *Arum Palaestinum* (Louf), *Urtica pilulifera* (Nettle), *Allium sativum* (Garlic), *Olea europaea* (Olives), *Nigella ciliaris* (black cumin) *and Allium cepa* (Onions) [20].

Polyphenols, the most abundant secondary metabolites of plants, with more than 8000 phenolic known structures, differing from simple molecules such as phenolic acids to highly polymerized substances like tannins. These compounds play important role in the development of the plant. In addition to protection of plants from predators and ultra violet-*B* radiations as well. In fact, their bioactivity rise from their powerful antioxidant activity [21].

It is estimated that about 2% of all the carbon that are photosynthesized by plants, up to about 1×10^9 tons per year, is converted into flavonoids or related compounds [22]. Flavonoids phytochemicals, a class of polyphenols, are well investigated low molecular weight water soluble secondary metabolites that are based on 2-phenyl-chromone nucleus (Figure. 1). This nucleus consists of a heterocyclic pyrane ring C fused with the ring A and linked to the benzene ring B, and it is usually present in glycosidic form and found in various plants and herbs. The flavonoids name is originated from the Latin word "*Flavus*" which means yellow. There are more than 9000 flavonoids which are responsible for taste, coloration, detoxification and enzymes, vitamins and the whole plant protection from both radiations and pathogens. They are also involved in regulation of growth hormones, morphogenesis and sex determination in the plant and also they take a part in photosynthesis and energy transfer as catalysts, and as regulators of iron channels involved in phosphorylation [22, 23]. Besides, they act in symbiotic nitrogen

fixation, physiological regulation, self-healing and auxin transport inhibition. They may also transfer or accept light energy to or from other molecules, i.e. they act as sensitizers or quenchers [24, 25]. For instance, researches have postulated that flavonoids exposure to UV radiation induces higher levels of flavonoids in plants [25].



2- phynyl-chromone

Fig. 1 Main structure of phenyl chromone, Common O- and C-glycosylation positions are indicated with an arrow [22, 26].

They are of common C6–C3–C6 chemical skeleton divided into various classes based on the degree of oxidation of C3, annularity of the C ring, unsaturation and substitution pattern of ring C, and connection position of ring B. Common substitution reactions as hydroxylation, methoxylation, and glycosylation occur to produce thousands of flavonoids derivatives that all have in common a fifteen-carbon chain skeleton with two phenyl rings connected by a three-carbon bridge. Flavonoids reveal a high diversity of glycosylation sites. Mainly via hydroxyl glycosylation or C-C glycosylation by direct linkage between the sugar and the basic flavonoid nucleolus (Figure. 1). Flavonoids O-glycosides which have sugar substituents bound to a hydroxyl group of the aglycones favoring the 7-hydroxyl group in flavones, flavanones and isoflavones. The 3- and 7-hydroxyls are predominant in flavonols and flavanols while the 3- and 5-hydroxyls are favoured in anthocyanidins, and the latter hydroxylation is predominate in the whole plants. Whereas, in flavonoids C-glycosides the sugar substituents are attached to the aglycone through a carbon-carbon bond [4, 22, 26].

Sometimes, both types of bonds may be observed simultaneously on one aglycone as O,C-glycosid. The most common sugars that can be found in flavonoid glycosides are

hexoses like glucose, mannose and galactose, or their 6-deoxyderivatives as rhamnose, chinovose and fucose, or pentoses as arabinose and xylose. Sometimes, disaccharides like rutinose and neohesperidose are also found. The flavonoid aglycones differ in the number of hydroxyl and methyl groups substituted to the aromatic core of the C6–C3–C6 structure [4, 22, 26, 27].

Continuous exploration of flavonoids emphasizes that they are promising candidates in the medical field and natural drugs discovery and innovation. They are well known to have anti-inflammatory, antiviral, anti-allergic, anti-ageing and strong antioxidant and free radical scavenging effects. As consequent of their lower redox potentials, they are able to reduce highly oxidizing free radicals by forming less reactive flavonoid radicals and so prevent for example lipid peroxidation [23, 28]. Also, they play role against cardiovascular, neurodegenerative diseases, coronary heart diseases atherosclerosis, cancer and Alzheimer's disease [24, 29- 31] .

They gained their biological and pharmacological properties from the existence of hydroxyl groups on rings A and C, the double bonds especially the C2-C3 bond and oxo carbonyl group (C=O), as well as hydroxyl groups substitution pattern on the ring B. These structural characteristics leads to their ability of reactive oxygen species (ROS) scavenging (Figure. 2a), and also oxidase and cycloxygenase-2 (COX2) inhibition [32]. In addition to α-tocopheryl radical reduction, and metal chelation (Figure. 2b) and as well as relaxing the oxidative stress triggered by nitric oxides. Flavonoids also increase the activity of several detoxifying and antioxidant enzymes. In nature, large proportion of flavonoids occur in the form of water soluble glycosides that are usually isolated intact as their aglycones (Figure. 3) [23, 33- 35].

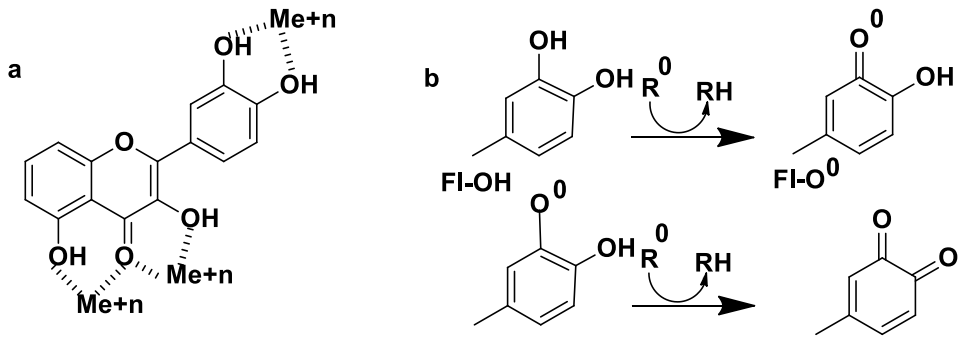


Fig. 2 Flavonoids **a**- binding sites for trace metals and **b**- their Scavenging of ROS [23].

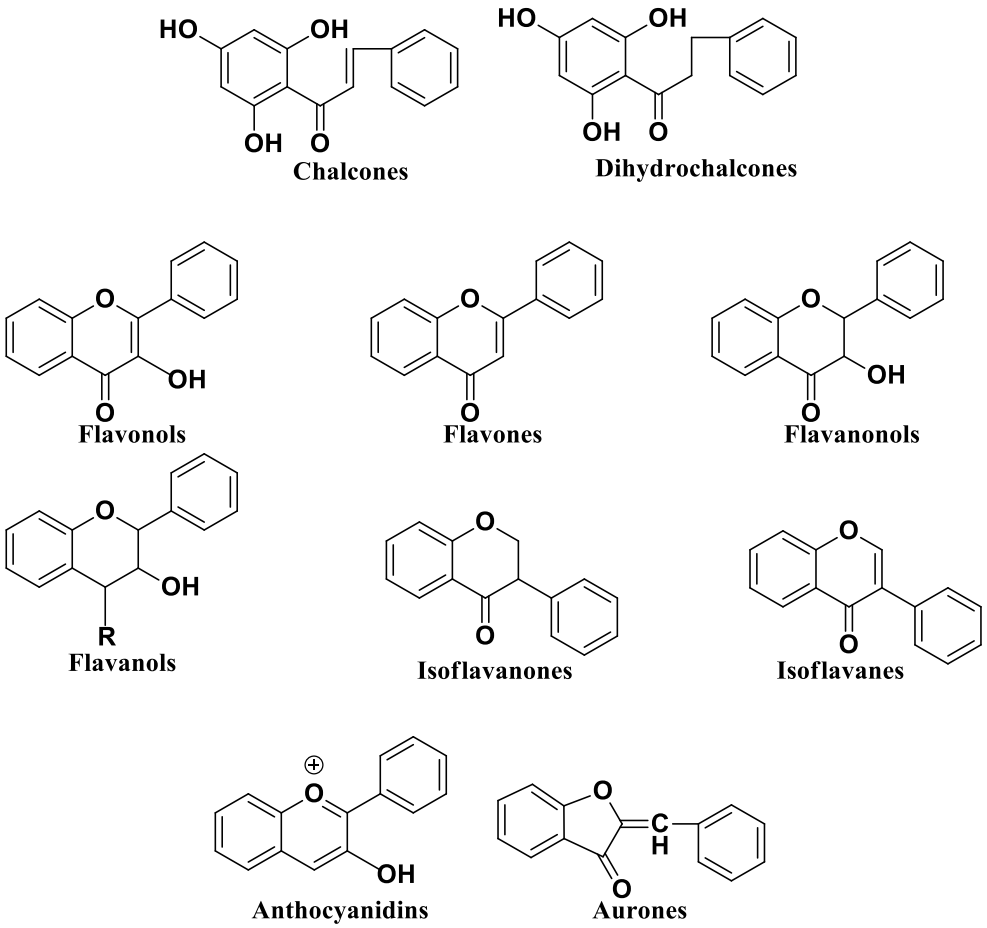


Fig. 3 Basic structures of the flavonoids aglycones [26].

Flavones, which comprise for the pale yellow pigmented compounds found in apple skin, citrus fruits, celery flowers and leaves and absorb only UV, they can give rise to many derivatives of this basic skeleton. Isoflavonoids, a distinct sub class of phytoestrogens, they mainly present in the *Legominosae* and *Fabaceae* plant family, and they are usually colorless compounds. Flavonols are mostly abundant in food such as onions, apples, tea, and chalcones, which are ring C-opening isomers of dihydroflavones, both contribute to the yellow color of plants [34, 35].

Anthocyanins and anthocyanidins are a group of chromene pigments that are important for characteristic color of plants, found particularly in flowers and fruits, and are responsible for the red, pink, blue and purple colors that attract pollinators. They are known to be natural pH indicators [26, 33-36] and they are the most important chromogenic substances in the metabolism of flavonoids [37]. Catechins, they are distributed widely in the plant kingdom, for instance, they are present in tea and red wine, they are reduction products of dihydroflavonols especially flavan-3-ols [26, 34-36]. Chalcones and dihydrochalcones also belong to flavonoids for their common synthetic pathways despite having an open structure. finally, aurones are rarely occuring five-membered ring C benzofuran derivatives, they exist in two isomeric forms, *E* and *Z* configurations [24].

Nitrogenous compounds such as alkaloids, are also a low molecular weight secondary metabolites that are found in plants. They form about 20% - 25% of plant based secondary metabolites [38, 39]. And they also can be found in bacteria, fungi and some animals like frogs and lizards and marine organisms. They were believed to function as a defense mechanism against herbivores and pathogens. In their structure, they have a nitrogen atom in their heterocyclic ring that cause the alkalinity of these compounds. There are more than 40000 known alkaloids [39] that are usually colorless, odorless of bitter taste crystalline solids with some exceptions. Moreover, they exist in the plant as alkaline that mostly combines with acid to form organic salts such as citrate, oxalate, tartrate, succinate salts and a few present as inorganic salts as berberine. However a very few alkaloids present in their free form such as amide alkaloids. Most of them are poisonous and they are recognized for their anti-inflammatory, anticancer, analgesics, anesthetic,

antimalarial, antiarrhythmic, antihypertensive, neuropharmacologic, antimicrobial, hypnotic, psychotropic, antimitotic and antifungal activities. They gained their activity due to their electron-withdrawing substituents on the ring, i.e. the functional nitrogen that is originated from amino acid precursors, tyrosine, lysine, tryptophan, phenyl alanine, arginine, glycine, histidine and ornithine [13]. Alkaloids include important drugs with characteristic physiological effects, like morphine, codeine and cocaine, they are often insoluble amine salts, referred to as the hydrochlorides [36].

The term alkaloid is derived from the Arabic word "*Al-kali*" that is related to the plant from which soda was first isolated. It was coined by the German chemist Carl. F. W. Meissner in 1819 [38]. The true heterocyclic alkaloids (Figure. 4) are classified into indole alkaloids, pyrrole alkaloids, isoquinoline alkaloids, piperidine, tropane, pyridines, pyrrolizidine, pyrrolidine, purine, imidazole, indolizidine, aporphine, xanthine, steroids, and quinolizidine alkaloids. And they all have their spectacular biological, pharmacological and medicinal properties [34, 35, 38, 40-42].

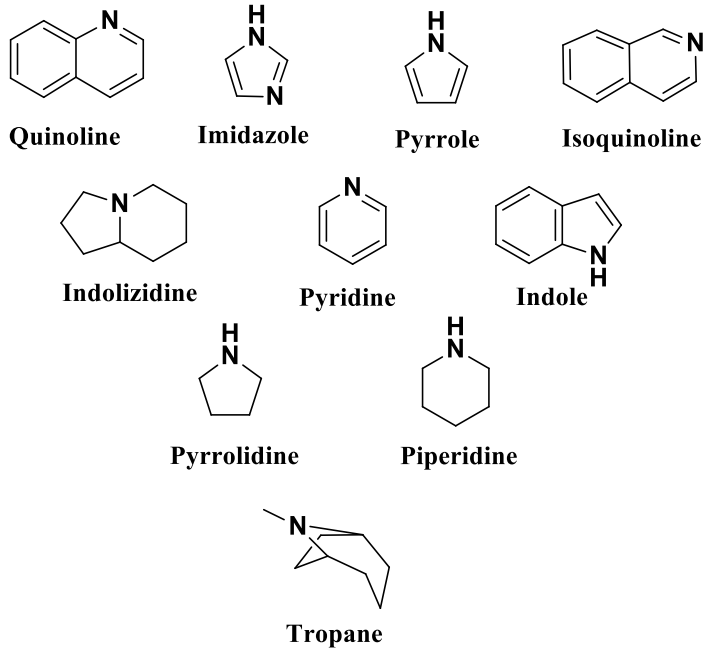


Fig. 4 Skeletal structure of true alkaloids [39].

The non-heterocyclic protoalkaloids, a kind of alkaloids that are recognized by the presence of the nitrogen atom outside the ring, for example, *Ephedrine* and *Colchicine*. And the pseudoalkaloids, which are heterocyclic compounds containing nitrogen but are derived from acetate, pyruvic acid, adenine/guanine, or geraniole instead of amino acids. The diterpenoid alkaloids (composed of 18, 19, and 20 carbons) are an example of this group [39].

1.2 Araceae Family

The Arum family, often known as aroids, belongs to monocotyledonous flowering plants [43]. It is one of the most morphologically diverse plant families that generally inhabits the tropical regions. And recognized by having calcium oxalate crystals that are possessing a spadix of small, bisexual or unisexual flowers, confronted by a spathe, and they lack ethereal oil cells. It consists of over 3700 species that are distributed in 118 genera and branched into eight subfamilies: *Gymnostachydoideae, Orotioideae, Lemnoideae, Pothoideae, Monsteroideae, Lasioideae, Aroideae, Zamioculcadoideae* [44, 45].

Archeological evidence indicated that they have been under human use since ancient times [46]. For reproduction purposes, *Araceae* depends on flies and beetles such as *Drosophila phalerata*, *D. immigrans*, *D. hydei*, *melanogaster* and *D. simulans* as the main pollinators [47,48]. The family Araceae contain various classes of secondary metabolites including flavones, flavonols, C-glycosyl flavone, polyphenols, proanthocyanidins, phenolic acids as well as terpenoids and alkaloids. Arum subspecies are well known for their odor that basically comes from volatile amines like skatole and indole in addition to other volatile compounds such as eugenol and bicyclogermacrene [46]. In most subspecies the scent is foul, reminiscent of fermenting rotten fruits, closely matches volatile profiles of red wine and balsamic vinegar [49]. As in *Arum Palaestinum* that its appendix emits mainly ethyl acetate odor and smells like an alfactory mimic of fermentation in order to attract drosophilids. It also contains acetoin, acetoin acetate, butanediol mono- and diacetates. These substances elicit physiological responses in the olfactory system of drosophilids [50] which drives an innate sensory preference in the fly. That, in turn, mediates the innate attraction of the fly to this flower, therefore, the communication between *Arum Palaestinum* boiss and its drosophilid pollinators occurs [51].

1.3 Arum Palaestinum

Kingdom: plantae

Subkingdom: Viridaeplantae

Phylum: Tracheophyta

Subphylum: Euphyllophytina

Class: Spermatophyta

Subclass: Arecidae

Order: Alismatales

Family: Araceae

Genus: Arum

Species: Arum Palaestinum [61]

Arum Palaestinum Boiss (Figure. 5) is one of about 26 species flowering plants of the *Arum* genus that belongs to the *Araceae* family. It is a parietal, perennial plant [45] that is distributed in Europe, Western Asia, Northern Africa and has the highest diversity in the Mediterranean region where it grows in spring months at mountains, rocky places, forests, red soils, alluvial soils, near water canals, and shrubby plains [62- 65, 71]. It is widely used as a folk traditional medicinal plant and a complementary drug in Arabic and Greek traditions. And it may have other common names such as *Solomon's Lily, Kardi, Black Calla Lily, Priest's Hood ,Nooa'h Loof* and *Uḍn ilFil* (which means Elephant Ear and it's driven from the leaf's shape) [66]. It is most popularly in the Levant and may have the names *Al-Luf* [67] and *Mekhalet El-Ghoule* (the paintbrush of the witch) [68]. And, it has been utilized for treatment several diseases such as diabetes, obesity, cough, constipation, stomach acidity, atherosclerosis, heart burn, hemorrhoids, internal bacterial infection, prostate disorder, hypertension, poisoning, urinary retention, kidney infections, ameba, circulatory system problems, worms in the gastrointestinal tracts [5, 8, 61, 65, 67, 68, 71]. In fact, it is one of the most important traditional medicinal herbs in the middle eastern countries and among the three most commonly used medicinal herbs that's used as a remedy for treatment of several types of cancer especially breast, liver, colon and kidney in Palestine [17, 70].

Fig. 5 Black Calla / Arum Palaestinum Leaves (on the left) and Spadix surrounded by spathe (on the right). The image on the right was adopted by Dr. Albarghoti permission.

Its leaves are commonly prepared by decoction (leaves are boiled in water and fried with onions, tomatoes and olive oil). Sometimes, it can be taken as tea, used as powder added to food, or capsulated (leaves are dried and filled in a capsule, 3-4 capsules daily before breakfast) [18].

In the Palestinian region, about 60.9% of cancer patients use medical herbs for curing purposes [72]. *A. Palaestinum* is one of the most common plants that's believed to cure colon cancer besides its efficacy in suppressing tumors in prostate glands [7]. It's distributed widely in many regions in Palestine and has its superior medical features in Salfeet region, Jaradat, and co-researchers (2015) [73]. Nearly 40% of patients who ingested the plant revealed a probable cure [74].

In Jordan, four arum species called Luf, and sometimes called Spotted Arum, Green Arum, Water Arum and Palestine Arum, all are distributed in Ajlun, Jarash, Irbid, Al-Balqa' and Wadi-Shua'ib, in addition to Amman and Jordan valley. These species are *Arum dioscorides*, *Arum elongates*, *Arum hygrophilum* (that is best known for its antibacterial activity against the most resistant types of bacteria, its aqueous and polar organic extracts were reported to have a growth inhibition of *P. Auroginosa*, *E. Faecalis*,

L. Monocytogenes, *S. Aureus*, and *Methicillin-resistant staphylococcus aureus* (MRSA)). And the fourth one is *A. Palaestinum*[65, 75] which has other local names such as Slaglag or Lisan al-ejail [76].

The flowering part of the plant is an erect slender dark-purple unisexual spadix with the flowers of both sexes are in distinct zones separated by zone of sterile flower. It is surrounded by a pale-green with 16-50 cm purplish-spots spathe holding the vibrant red seeds from the inside arranged as the females at the base and the males above. This cryptic ethyl acetate-odourly part grows within the arrow shaped leaves that are generally 20-60 cm tall with tuberous roots [47, 66, 77, 78, 79]. Its dark spathe gained its color from the high accumulation of anthocyanins and procyanidins components that are distributed in the mesophyll cells in the upper and lower epidermis [37].

1.4 Reactive Oxygen Species ROS

Free radicals, or as called, reactive oxygen species (ROS), defined as chemical species possessing an unpaired active electron in their valence shell, they are produced by the body from aerobic metabolic processes. And they are responsible for biological damage of some of the body components as proteins, enzymes, cellular membranes and DNA leading to oxidative stress. Which is one of the main causes of several human chronic diseases like heart diseases, stroke, arteriosclerosis, diabetes, cardiovascular diseases, neural disorders, Alzheimer's disease, Parkinson's disease and aging, as well as cancer [3, 80]. Hydroxyl (HO•), superoxide radicals (O_2•$^-$) and nitric oxide (NO•), peroxyl (ROO•) and alkoxyl (RO•) are significant examples of free radicals [23].

1.5 Cancer

Cancer, commonly, an abnormal and uncontrolled cellular growth due to accelerated cell proliferation and low apoptosis rate. That will cause enlarging mass that is called tumor, or in other words, neoplasm. Which, in turn, aggressively invades other tissues

through direct cell migration or through the bloodstream and lymphatic system, then, to other organs consuming their oxygen and nutrients and metastasize in their space. So, the cell out-divides, out-grows, and forces the body to form new blood vessels to feed itself in a process called angiogenesis. Cancer is among the main causes of mortality around the world, and the number of new cases is expected to ascent by about 70% over the next two decades [5]. In fact, in the developing countries, cancer is one of the three main causes of death, in the Arab World, breast, prostate, liver, bladder and lung carcinoma are the most common [72].

Chemical carcinogenesis process occurs in three stages. It begins with the initiation stage in which occurs the mutations in the genes that control cell growth and division. Secondly comes the promotion stage that involves mass formation, cell proliferation, chronic inflammation, loss of growth control and imbalance between metastasis (it accounts for 90% of cancer-associated mortality) [81] and apoptosis process (programmed cell death). Persistent gene mutations and impairment of immune function will sequentially contribute to cancer final progression stage [82].

Conventional therapies such as surgery, radiotherapy, and chemotherapy are the fundamental therapies currently practiced for the management of cancer. But, they lack of selectivity and cytotoxicity and work on destruction of both malignant and healthy cells. In addition to their harmful and costly side effects [83]. It's important to point out that one of the main challenges in cancer treatment besides selectivity, is the renewable development of multidrug resistance to chemotherapy agents. So, the need to alternative drugs with low toxicity and minimal side effects is increasing [39].

Chapter. 2 Literature Review

2.1 Arum Palaestinum Anti-cancerous Activity

Several uprising, in vivo and in vitro, studies are concerning the bioactivity of the phytochemicals derivatives (Tables. 1 and 2) to overcome the need for conventional drugs. Due to their hydroxyl groups and functional carbonyl group, various phenolic acids such as caffeic acid, gallic acid, tannic acid and gingerol had been studied and showed chemopreventive effects against different cancerous cell lines [84]. The studied cytotoxicity of a drug containing the diterpene alkaloid (*taxol)* represents both inhibition of cell proliferation and cell death, it also blocked cells in the G2/M phase of the cell cycle and induced apoptosis [84]. And they have also been demonstrating that some Tannin-related compounds were reported to induce cytotoxicity and inter-nucleosomal DNA cleavage in HL-60 cells [84]. And its noteworthy to point out that the poly phenol caffeic acid has significant effects on the reduction of hepatocarcinoma cells (HCC) by preventing the production of ROS (reactive oxygen spices) [85].

Other bioactive plant metabolites like Terpenoids, also referred as terpenes and isoprenoids, was examined and showed antibacterial, antifungal, antiviral and antiprotozal activities [67, 68]. Saponins, are also distinguished for their antibacterial properties owing to their ability to complex with sterols in the bacterial membranes forming pores and serious damage to them [86]. They were reported to have antifungal, antitumor, cytotoxic and anti-platelet aggregation activities, as well as their effect in lowering blood cholesterol and triacylglycerol [27].

Table. 1 Some flavonoids derivatives and their bioactivity [34, 35].

Some bioactive flavonoids derivatives			
Flavonoids	**Derivative**	**Source**	**Bioactivity**
Flavones	*Resveratrol*	Red wine	Lowers heart attacks
Flavanones	*Hesperidin*	Citrus fruit	Common treatment against influenza virus
			Have anti-inflammatory properties
Isoflavones	*Genistein*	Soy plants	Lowers blood pressure and cholesterol levels
			Reduce the risk of cardiovascular diseases
			Inhibit the growth of prostate and breast tumors
Flavonols	*Quercetin*	Plant tissues	Antioxidant
			Prevent oxidation of low density lipoprotein

Table. 2 Some alkaloids based drugs [34, 35, 38, 40-42].

Some bioactive drugs alkaloids			
Alkaloids	**Drug**	**Source**	**Bioactivity**
Indoles	*Vincristine*	Catharanthus roseus	Anti-cancerous
Isoquinolines	*Morphine*	Papaver somniferum	Narcotic drug
Quinolines	*Quinine*	Cinchona officinalis	Antimalarial drugs
Tropanes	*Cocaine*	Erythroxylum coca	Stimulant
Piperidines	*Coniine*	Conium maculatum	Neurotoxin

The interest in *A. Palaestinum* plant is increasing for its bioactive phytochemicals that were discovered and that are still being under discovery. They were isolated, characterized and subjected to bioactivity assays by several researchers. Based on a comprehensive study that was achieved by Abu-Reidah and his group (2015), it contains a total of 180 phytochemical metabolites, basically, flavonoids, phenolic acids, alkaloids, terpinoids, iridoids, saponins, tannins, amino acids and many other compounds that were separated and studied by analytical and spectral techniques [67].

Its in-vitro and in-vivo anti-tumor cytotoxicity has been studied since decades and still being studied nowadays. A study that had been done by Aboul-Enein, and his group (2012) on traditional plants in Egypt concluded that ethanolic extract exhibited a strong inhibition of ehrlich ascites carcinoma cells (EACC) [87]. Besides, a study was done by A. I. Husien work team (2014) on 6 different traditional plants including *A. Palaestinum*, stated that the ethanolic extract of arum leaves showed intermediate free radical scavenging capacity and antioxidant activity, and moderated cytotoxic inhibition of MFC-7 cancer cells. The same study indicated that *A. Palaestinum* has the highest phenolic content among the six plants studied, however, it has the lowest flavonoid content among them all according to this study [80]. Also, another study demonstrated by A. I. Husien, and partners (2014) reported that its ethanolic extract exhibited inhibitory effects against dermatophytes fungi, *Trichophyton rubrum* and *Microsporum canis* [1].

In this aspect, studies revealed the oven dried Arum leaves have the largest phenolic content, which is best known as the major antioxidant source. As an example, Rosmarinic acid that has antimicrobial anti-carcinogenic, anti-inflammatory, anti-Alzheimer and antidepressant properties is a main component in this group [67, 68]. Moreover, Shogaol has been reported by Zhu and coworkers to exhibit significant antioxidant and anti-inflammatory activities [32]. On the other hand, it is noteworthy to point that the total phenolic content in *A. Palaestinum* bioss leave-extract (collected from Jordan Valley and Palestine among other plants) was of lower antioxidant activity comparing to the other plants extracts that was studied. However, the extract did not reveal any considerable anti-inflammatory activity [88].

Otherwise, a study carried out by Jaradat, and coworkers (2016) demonstrated that the antioxidant activity of *A. Palaestinum* is the highest amongst the arum species, this is correlated with its high phenolic contents [89]. Furthermore, an investigation carried out by M. Diab-Assaf research group (2012) declared that the highest phenolic and flavonoid contents were observed in the methanolic followed by chloroform and ethyl acetate extracts respectively, and, they induced dose dependent reduction in cell proliferation values, after 48 hours incubation [90].

Consequently, its bioactivity against different cancer cells was recognized by many researcher. As an example, an In-vitro inhibition for both chronic myelogenous leukemia (k562) and colon (HCT-116) cancer cell lines by its ethyl acetate extract was also reported by Hatmel research team (2017) followed by methanolic and chloroform extracts [61]. Water, chloroform, ethanol and ethyl acetate extracts of *A. Palaestinum* boiss leaves were investigated by many researchers during the last decade. El-Desouky, and his group (2007) reported that ethyl acetate was of the best proliferation suppression for breast cancer cell lines (MCF-7) and lymphoblastic leukemia cells (1301). In addition to strong DPPH (1, 1-diphenyl-2-picrylhydrazyl) radicals scavenging [63]. Furthermore, the alkylated piperazirum compound (3α,5α-diisobutyryl-6α-isopropyl-piperazine-2-one) that had been isolated from n-butanol extract showed in vitro cytotoxicity against cultured tumor cell lines as stated by El-Desouky, and his group (2007) [62]. Also, piperazine derivatives, 3-hydroxypiperazine-2,5-dion and piperazine-2,5-dion were isolated from leaves' water extract by El-Desouky, and co-workers (2014) exhibited mild in-vitro cytotoxic activity against human multidrug-resistant (MES-SA/DX5) cell culture [91]. It is worth noting to point out that 3,6,8-trimethoxy, 5,7,3',4'-tetrahydroxy flavone was discovered for the first time by El-Desouky and co-workers (2007) [63].

The major flavonoid in the *A. Palaestinum* plant is Isoorientin (luteolin 6-C glucoside) that was reported by Afifi and his group (1999), to have mylotic activity on uterine smooth muscle of rats and guinea pigs. Together with vitexin (apigenin 8-C glucoside), they both were found to exert antimicrobial activity towards *Staphylococcus Aureus*, *Bacillus Subtilis* and *Pseudomonas aeruginosa*. Besides, luteolin 7-galactoside

was reported to have anti-inflamatory activity, and Chrysoeriol and isovitexin compounds, which were reported to be isolated for the first time by Farid, and his group (2015), also isolated from the plant. And the previously mentioned four compounds were investigated and reported to have anti-proliferative activity against epidermal carcinoma of larynx (Hep2), cervix (HeLa), liver (HepG2) and breast (MFC7) carcinoma cell lines [64, 92, 93]. In the same study, the diethyl ether and dichloromethane fractions of the crude arum extract exhibited a strong antiproliferative activity against the previous cell lines [64].

The polyhydroxy alkaloid compound, (s)-3,4,5-trihydrooxy-1H-pyrrol-2(5H)-one which was isolated from the ethyl acetate fraction of *A. Palaestinum* was proven to suppress the proliferation of both breast cancer cells and the lymphoblastic leukemia cells. In addition to its strong scavenging for the DPPH radicals [63]. Also, the n-butanol isolated Piperazirum compound showed cytotoxicity against cultured tumor cells. Furthermore, piperazine and its substituted derivatives are essential pharmacophores that can be found in many drugs, such as the *Merck HIV protease inhibitor* and *Crixivan* [62].

Additionally, other compounds extracted from *A. Palaestinum* were reported to have several biological importance. Linolenic acid and Linoleic acid revealed anti-carcinogenic, antihistaminic and antioxidant effects. Phytol that have a cytotoxic activity and Phytol acetate showed antimicrobial, antidiuretic and anti-inflammatory properties [64, 94]. As well as several other compounds which were isolated such as Tannins, that help in blood clotting, reduce blood pressure, decrease lipid levels, posse antimicrobial activity and increase the immune response. Moreover, Betalains, which have anticancer, antioxidant, antilipidemic and antimicrobial activity as well as Carotenoids, which help in reducing the risk of lung cancer, prostate cancer, head, neck and breast cancer. And Glucosinolates, which have therapeutic effects in cancer and dementia problems. In addition to vicenin, coffiec acid, xanthuric acid, hexadecanoic acid, isovanillic acid hexoside, isoferulic acid, 6-shogaol, vitamin B4 and cumarin and kaempferol derivatives [64, 67, 78].

2.2 Other Therapeutic Properties of *Arum Palaestinum*

Recent regional studies in Palestine and Jordan have investigated the anti-diabetic, anti-inflammatory and antimicrobial properties of *A. P* among other wild medicinal herbs. *A. P* seemed to have the modest effect against *Propionibacterium Acnes* with minimum inhibition concentration, and a unique potent inhibitory effects on interleukin-1 alpha release [95] in addition to its significant effects in controlling diabesity [96]. On the other hand, it has been reported by Hawash and his group (2020) that it's lypophilic extract has strong pancreatic α-amylase inhibitory effect [97, 98]. Moreover, *A. P* was discovered to have analgesic and anti-nociceptive effects, its methanolic extract acts on the mechanism of activation of opioid receptors, lipoxygenase and cyclooxygenase as stated by Qnais and his research team (2017) [99].

Lately, Mustafa, and his research group (2021) have investigated the COVID-19 possible inhibitory effects of *A. P* leaves methanolic extract using docking methods after analyzing its phytochemical constituents by GC-MS. Nineteen compounds including α-D-Glucopyranose, α-Tocopherol, Androstan-3-one, 1,3-Propanediol, 3-Hydroxydodecanoic acid, 3-Hydroxysebacic acid, 3-Hydroxytetradecanedioic acid, Cyanuric acid, Undecanedioic acid, Palmitic acid, Malic acid, L-Ascorbic acid, Isobutyric acid, D-Glucuronic acid, N-Butylglycine, Ethylamine, Maltose, Phenobarbital and β-Sitosterol. Among which the latter has revealed a high affinity towards the two coronaviral proteins 3CLpro and Nsp15 active sites receptors. And thus, a promising potential inhibitory efficacy against the virus after more potent investigations [100].

On the other side, this plant, *A. P* was reported to contain phthalate derivatives such as dibutyl phthalate, dioctyl phthalate and diisobutyl phthalate, were separated for the first time by Husein, and his research group (2014), which are hazardous, mutagenic and carcinogenic. They are distributed in different concentrations with the highest amounts in leaves and inflorescences, and the lowest in the stem and the inflorescence base [101, 102]. As well as the needle-shaped calcium oxalate salts, that are held in bundles (raphides), and decomposed to the toxic oxalic acid, they are distributed in many parts of the plant

and cause irritation to tissues when being in contact, specially, the linings of oral cavity and the gastrointestinal tract and violent choking. Sometimes, it may cause suffocation if esophagus is blocked by swelling of the tissues around the base of the tongue and throat. Hence, it can cause a mild poisoning symptoms, that's why it needs a special treatment before being eaten to avoid high lethal dose [68, 73].

A study done on female rats has indicated that different doses of *A. P* leaves ethanol extract can exert toxic effects on liver cells. The thing that may induce hepatocyte necrosis and liver fibrosis due to aroin, saponins, cyanogenic glucosides, and calcium oxalate compounds which, when penetrate mucosal cells, they facilitate the entry of toxins that interfere with central functions in an animal leading to liver disturbances [103, 104].

2.3 Synergistic Effects (Combinatorial Efficacy)

Tumors develop a chemotherapy resistance to a particular drug, the fact that weaken its efficiency against them [5]. Thus, contentious work has been done on different natural cancer-agents that are derived from plants in order to obtain synergistic combinations that have the capability to rise the therapeutic efficacy against cancer. For example, *Quercetin*, the flavonoid compound, restores *Daunorubicin* chemo-sensitivity in resistant HL-60/DOX and K562/DOX cell lines by suppression of P-gp expression. In the same manner, *Curcumin* inhances *Vincristine* chemotherapy activity in SGC7901/ VCR cell lines and it is still being under studies of combinatorial drugs [105].

Recently, studies started testing combinations of *A. P* with other pharmaceutical compounds to test their synergistic effects, since, unlike other natural plant-derived anticancer therapies, *A. P* has no herb-drug contradictions with synthetic drugs [46]. For instance, GZ17 is a fortified formulation of the targeted plant with higher levels of isovanillin, linolenic acid and β-sitosterol, this fortified version caused a reduction in prostate cancer spheroids in mice, Cole, and partners (2015) [74]. Lately, three nutraceutical

compounds have been examined for their antitumor impact on head and neck squamous carcinoma cells (HNSCC). They are GZ17-S (*A. P.* extract, *Peganum harmala* and *Curcuma longa* combination), GZ17-05.00 (16 synthetic compounds of GZ17-S) and GZ17-6.02 (3 synthetic components of GZ17-S, curcumin 10%, harmine 13% and isovanillin 77%). And the latter was the most effective combination among all, it reduced HNSCC progression in-vitro but failed in-vivo, and enhanced cisplatin cytotoxicity against the aforementioned cancer cell line[106]. Moreover, this combinatorial drug has had effects on prompting DNA damage in the GI tumor cells, hepatocellular carcinoma, pancreatic adenocarcinoma, colorectal adenocarcinoma and cholangiocarcinoma as reported by Booth and co-partners (2020) [107].

Latest investigations have declared that GZ17-6.02 may target both the tumors and their associated fibroblasts (stroma). Furthermore, the drug may damage super-enhancer networks in the tumor cells and thus affecting genes that play important roles in pancreatic cancer [108]. Over and above, Booth, and his research team (2020), have demonstrated that the interaction between a combination of GZ17-6.02 and Doxorubicin increased autophagosome formation and autophagic flux and also caused elevated death receptor signaling resulting in mitochondrial dysfunction and tumor cell death [109]. Nowadays, ongoing studies are being carried out on GZ17-6.02 in a combination with cancer drugs such as *Trametinib* and *Dabrafenib* to inspect their ability to kill mutated cutaneous melanoma cancer cells [110].

In this work, the flowering spadices and its surrounding parts, rhizomes and stems, are going to be our main goal to study, separate, analyze, characterize and define the bioactivity of their phytochemical components.

Chapter. 3 Methodology

The phytochemical separation and analysis of the herbal plant *A. P* as well as the different bioactive assays that were done in this work, were based on the accumulative studies that had been achieved during the past years. In this work, the spadices, stems and rhizomes parts of the plant were separated, analyzed and characterized.

3.1 Plant Collection

Arum Palaestinum (*A. P*) flowers were collected from Ramallah mountains/ west bank, air dried in a dark place and stored at Birzeit university labs, The dark purple spadices of the plant were separated from their surrounding spathes, cleaned, grinded and weighed.

3.2 Sample Preparation

The *A. P.* spadices sample powder (41.4880 g) was soaked twice, each was in a mixture of 360 mL methanol (MeOH), 40ml distilled water (D. W) and 2 mL trifluoroacetic acid (TFA), then, left at room temperature for 72 hours with changing the solvent every 24 hours. The resultant solutions were suction-filtrated, evaporated by rotatory evaporator at temperature 35^{o}C, weighed and dried with Nitrogen. In order to complete the bioactivity investigations, another 41.7713 g arum spadices powder sample alongside with 35.3189 g of arum spathes powder samples were prepared using the same aforementioned procedures [64, 92, 101].

3.3 Crude Sample Separation

The dried obtained *A. P.* extract (10.2385 g out of the total 23.3274 g extract) was partitioned with 120 mL distilled water and 80ml n. hexane for 3 times, and the partitioning process was continued with diethyl ether (DEE), chloroform,

dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (n. BuOH) respectively following the same procedures mentioned above.

7.2096 g of the *A. P.* spadices alongside with 3.9416 g of *A. P.* spathes crude extracts were taken to bioactivity studies. The aqueous sample was prepared to polar separation and the other organic samples were analyzed by thin layer chromatography TLC, followed by reversed phase HPLC. GC, LCMS and ^{1}HNMR were used for wider identification of the most pure samples' components if possible.

3.4 Polar Separation

After the crude separation, the aqueous phase that was subjected at first to XAD column chromatography using the solvent mixture (10% D.W, 90% MeOH, 2ml TFA) as an eluent and 2.0751 g concentrated was obtained. Then, 0.9849 g of the resultant XAD extract was followed by Sephadex LH-20 column chromatography [35, 111] starting with 20% MeOH: 80% DW: 0.50ml TFA eluent with increasing the concentration of MeOH gradually up to 80%. 51 fractions were collected (Table. A1, appendix. 1), analyzed by UV and *Shimadzu* HPLC (Conditions are as follows: for crude extracts, ODS-Hypersil column, 20 × 0.5 cm, length × i.d., 5 µm; Supelco, Bellefonte, PA, USA, 10 μL injection volume, 0.5 mL/min flow rate, see Table. A2, appendix. 1. For fractions, ZORBAX RRHT column, 0.075 × 105 mm, 1.8 μm with 5% ACN: 5% D. W: 90% MeOH. At 40 C, 10 μL injection volume, 0.5 mL/ min flow rate and at different wave lengths (280, 360, 400 and 520 nm) in order to be identified by LCMS and ^{1}HNMR. 0.7671 g of the XAD extract was exported to bioactivity assays.

3.5 Non Polar Crude Preparation

73.9478 g sample of the rhizomes part of the plant was soaked twice, each in 320ml DCM [112], then, basified with ammonium hydroxide (NH_4OH) until the solution alkalinized at pH 8-9, then left for 4 hours, filtrated and concentrated to yield 1.6275 g of

crude alkaloids. Another 35.8376 g of stems powder were macerated twice in total 300 mL DCM as the same method above to yield 0.3821 g.

48.9208 g of rhizomes and stems powder mixture was soaked twice for four hours, each in 300 mL ethyl acetate and alkalinized with NH_4OH (until PH 9), then, concentrated and dried to produce 0.7352 g that was prepared to be silica chromatographed. Another 35.8376 g sample of stems powder were macerated twice in total 300 mL DCM as the same method above to yield 0.3821 g.

3.6 Non Polar Separation

0.7384 g of rhizomes alkaloid crude sample was subjected to silica chromatography (about 37 g silica), with 5%: 5%: 90% ethyl acetate: diethyl ether: hexane solvent mixture as an eluent with increasing the polarity in the final separation stage, TLC preliminary tests were done to end with two main fractions that were analyzed by HPLC and GCMS [38].

A mixture of rhizomes and stems crude sample, 0.7352 g, was subjected to silica chromatography with the eluting mixture 87.5% petroleum ether (PE) and 12.5% ethyl acetate, then, 80% PE : 20% EA, and gradually continued with increased polarity, one main fraction was obtained.

The entire rhisomes crude mixture as well as another 3 fraction from rhisomes and stems parts were analyzed by GCMS (*PerkinElmer Clarus* 600), and some alkaloids and flavonoids fractions samples, that seemed to have the possibility to form crystals, were diluted in a suitable solvent that were allowed to be evaporated slowly in order to help forming the suitable crystals for X-ray crystallography analysis [35].

3.7 Bioactivity Studies

3.7.1 Anticancer Assay

The HEK-293T cancer cells were grown in a suitable *Dulbecco's Modified Eagle Medium* (DMEM), which contains fatal bovine serum to support cells growth, penicillin and streptomycin antibiotics to enhance cells protection, then, they were incubated in CO_2 incubator at 37^oC for cytotoxicity anticancer assays. After grown and duplicated, the cells were counted and introduced into 961-plates, each well contained 100 μL of the DEME cell culture and 10 μL of different concentrations of the four extracts, *A. P.* spathes methanol extract, *A. P.* spadix methanol extract, *A. P.* spadix aqueous extract and *A. P.* spadix n-butanol extract. The dilution solvent DMSO was used as control. After 48 hours, *cell-titer 96 aqueous one solution* reagent was added to the plate for two hours, then, the plate was introduced to ELISA reader in order to analyze the absorbance at 490 nm. The same methodology was applied on HeLa cells. DMSO and Methanol were used as control.

3.7.2 Antibacterial and antifungal assay

Mueller Hinton Agar Medium (MHA) in D. W was autoclaved and sterilized as manufactured, and then added to petri-dish at room temperature for 24 hours then refrigerated. The *E. Coli, S. Aureus and P. Aeruginosa* bacteria in addition to *C. Albicans* fungi were all separately sub-cultured and colonized in sterile D. W, then, they were inculcated in petri dish plates before being treated with eight different concentrations of both *A. P.* spathes and *A. P.* spadices extracts (concentration range started from 100 mg/mL to 0.78 mg/mL obtained via serial dilution method), each well treated with 100 μL of the extract). Then, they were incubated for 48 hours before observing the inhibition zone.

Chapter. 4 Results and Discussion

4.1 Expected Flavonoids Structures From Spadices

The preliminary HPLC separation and analysis of the *A. P.* spadices crude extract showed that it had at least 4 to 5 major compounds that have the highest possibility to be separated. The crude separation of *A. P.* spadices MeOH crude extract (Figure. 6) revealed that the n. Hexane, DEE, chloroform, DCM and EtOAc are of small amounts and not enough to proceed and complete the separation stages, they were lost in the TLC, UV and HPLC analysis. Also, their TLCs and HPLC chromatograms revealed that they are of less phytochemical compounds comparing to the aqueous and alcoholic n. BuOH extracts. And therefore, their separation couldn't be accomplished. Unlike the aqueous extract that have gone through continuous separation steps in order to separate flavonoids.

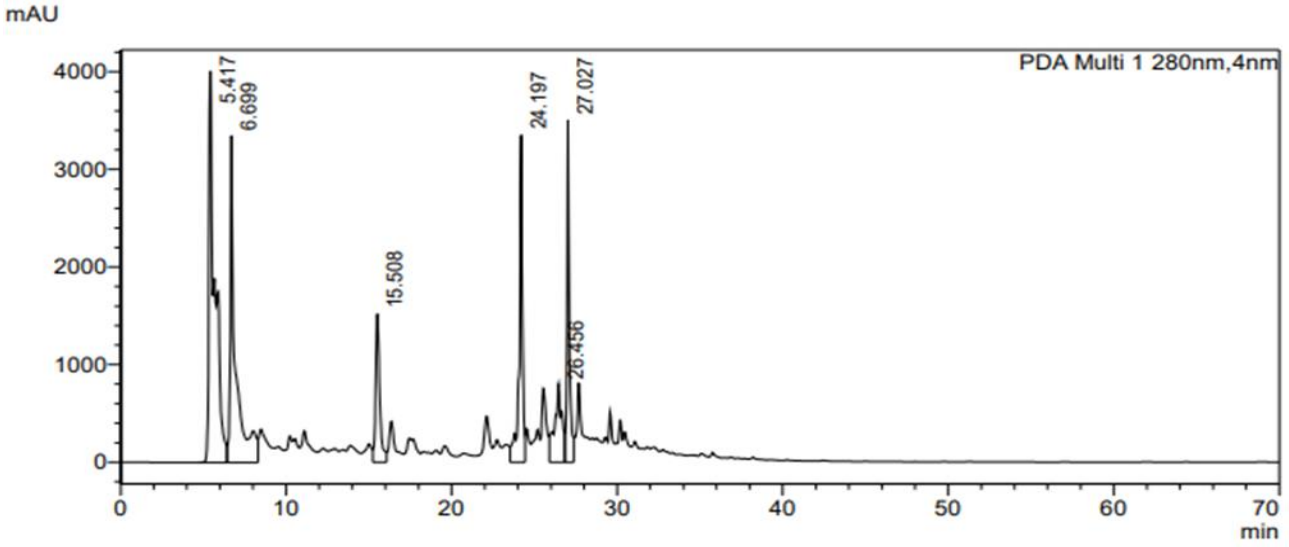


Fig. 6 The HPLC chromatogram of crude *A. P.* spadices extract.

In general, flavonoids usually separated by paper chromatography, column chromatography and HPLC, purified and identified by UV, LCMS and NMR. Generally, they are absorb UV wavelengths at 254 nm regarding to their aromatic ring (π, π*

excitation), 280 nm due to their carbonyl group (n, π* excitation) and 330 nm for the presence of a carbonyl group conjugated with the aromatic ring, and certain polyphenolic chalcones may absorb light in the visible region [25, 113].

The preliminary step before separation was HPLC run for the XAD extract (Figure. 7) with a mixture of seventeen phenolic standard (Table. 3) in the same conditions (see Table. A3, appendix. 1) to check for any known compound in the extract. The extract seemed to have four major compounds, as can be noticed from the four major peaks, but, none of these peaks has similar attitude in the HPLC column which can be concluded from their retention time values. So that, none of the 17 known phenolic standards was in common with any compound in the extract, which can be an evidence to conclude that the major compounds in the *A. P.* spadices are not one of the compounds in the Table. 3.

The polar separation and analysis of the XAD aqueous extract (Figure. 7) produced 51 fractions that were examined by *Shimadzu* HPLC indicated that fractions number 8 (23 mg) and 33 (20.3 mg) are the most suitable to be analyzed by *BRUKER CAB-AV4 500 MHz* ^{1}HNMR (solvent, deuterated methanol (MeOD)). As can be seen from Figures. 8 and 9 respectively, these two fractions are seemed to be two possible compounds. It is worth to mention that the other two compounds were exist in the fractions of less purity that wasn't analyzed by ^{1}HNMR, the HPLC chromatograms of them are in the appendix. 2 (Figures A1 and A2).

Table. 3 The Phenolic standards and their Retention time values (this table was obtained in corporation with Dr. Fouad Al-Rimawi research group).

Standard #	Standard name	Retention time
1	Gallic acid	8.2
2	3,4-Dihydroxybenzoic acid	13.8
3	3,4-Dihydroxyphenylacetic acid	16.5
4	Chlorogenic acid	21.6
5	4-hydroxyphenylacetic acid	24.5
6	Vanallic acid	25.4
7	Caffeic acid	26.9
8	Syringic acid	27.7
9	Isovanallic acid	28.5
10	p-Coumaric acid	37.8
11	Ferrulic acid	42.6
12	Sinapic acid	43.1
13	Rutin	45.9
14	Verbascoside	49.9
15	Quercetin	67
16	Trans-cinnamic acid	68.6
17	Kaempferol	72.3

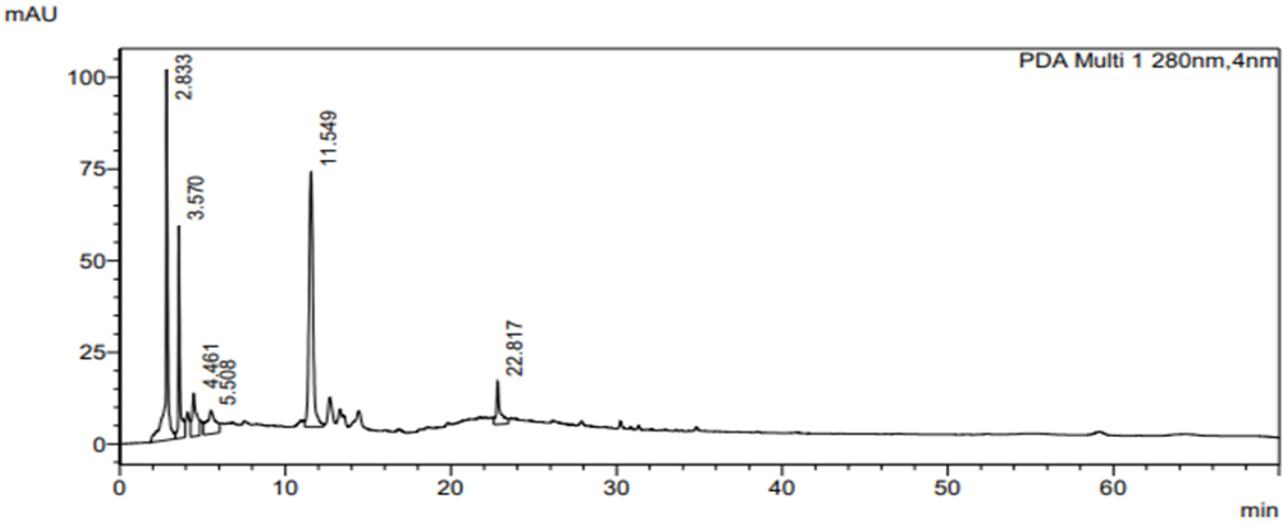


Fig. 7 HPLC chromatogram of crude *A. P*. spadices XAD aqueous extract.

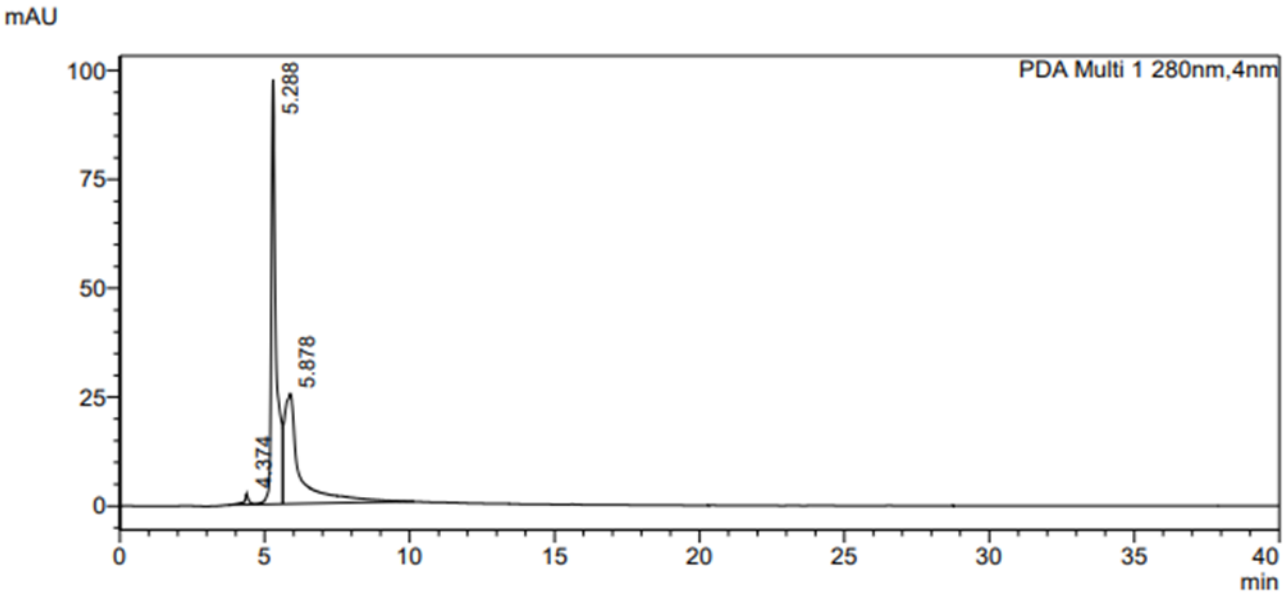


Fig. 8 HPLC chromatogram of fraction 8 (23 mg).

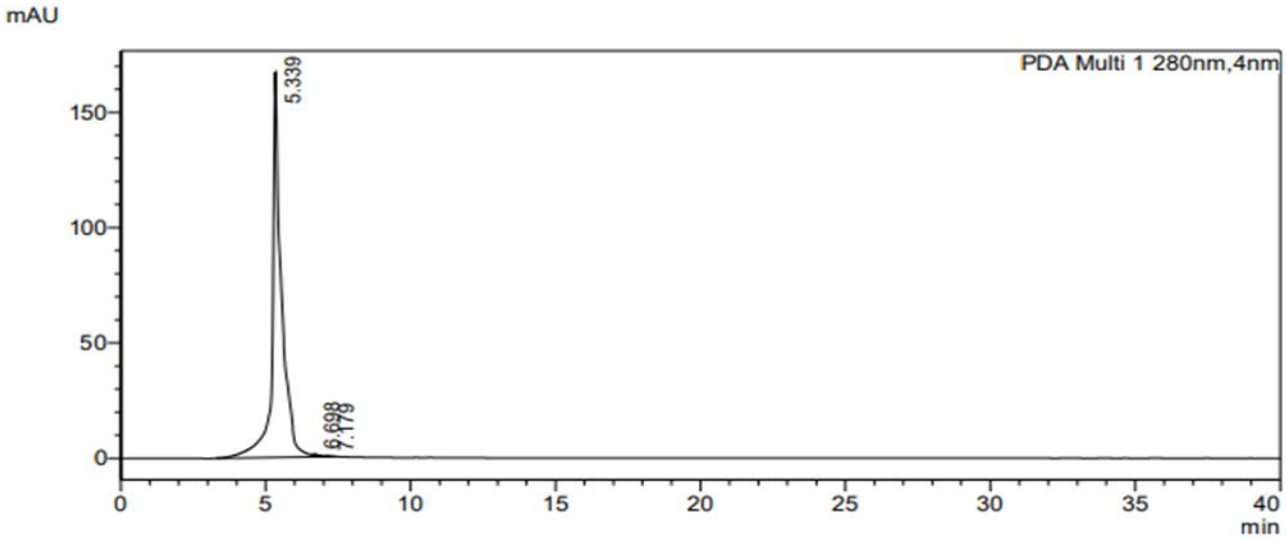


Fig. 9 HPLC chromatogram of fraction 33 (20.3 mg).

The ^{1}HNMR assignments revealed two expected glycosylated flavonoid compounds with the proposed structures as appears in the Figures 10 and 11. As it can be noted, the unknown fraction number 8, Figure. 10, reveals mixed peaks in the aromatic region (6 – 8 ppm) [114]. Depending on literature, and by recognizing the distinct peaks around 5 ppm, 6.2 ppm and 7.7 ppm that are related to H10(*q*), H8(*d*) and H7(*d*), it can be concluded that Rosmarinic acid [115] is the compound that was separated with the flavonoid compound in fraction. 8. Thus, four protons were left around 7 ppm, protons of A ring, H6(*d*) and H8(*d*) which are shifted in the up field region, and the more down field shifted B ring protons H2'(*d*) and H6'(*d*).

As it was concluded by literature reviews that had considered the ^{1}HNMR spectra of sugars, the signals belonging to the anomeric protons appear at the most downfield region of the sugar region in the spectra between 4.5 ppm and 6.0 ppm, but all of the other protons is found squeezed between 3.0 and 4.5 ppm [114, 117]. Hence, by recognizing the sugar region in the same figure, between 3 and 4.5 ppm, the number of protons pointing to the presence of 3 sugar units, if taking into consideration that the integration of one proton equal 5. It is also notable that the distinct CH_3 doublet peak between 0.8-1.3 ppm indicates the presence of rhamnose sugar unit. Additionally, that same peak around 1.3 ppm expose the presence of the disaccharide neohesperidose unit, in other words the

rahmnoglycosyl sugar unit [114]. So, one sugar unit is left and it's predicted to be glucose since it's the most common. Moreover, neither arabinose nor xylose sugars are suggested to be present since the integration of the left sugar unit does not go with the number of their protons.

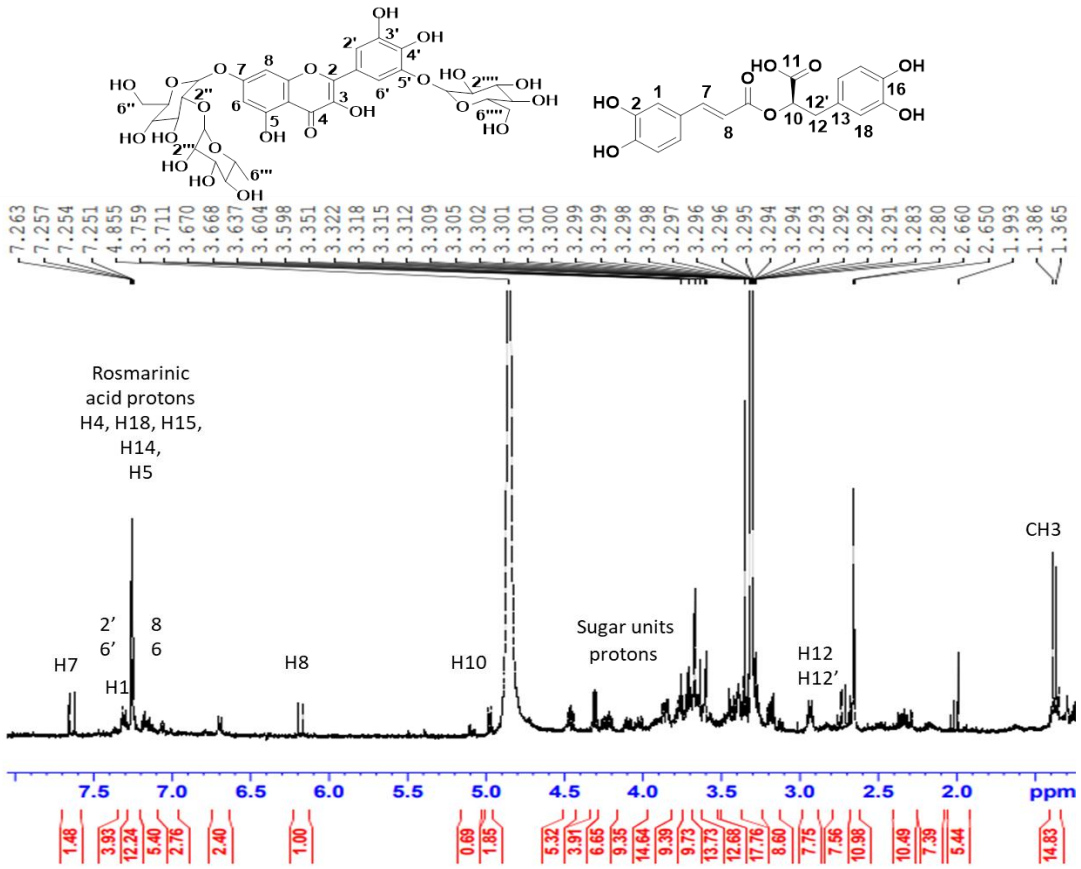


Fig. 10 The competing [1]HNMR spectra of the impure compound. 1 (expected structure on the left) and Rosmarinic acid (structure on the right) (in fraction. 8).

It can also be realized from the above figure that the missing peak at 5.7 ppm which is a characteristic peak of the sugar anomeric proton when glycosylation occur on the carbon-3 position of the aglycone flavonoid. This absence reveals that no glucose C-3 glycosidic bond in this compound. Furthermore, the peaks around 4.5 ppm, are the anomeric protons of glucose and rhamnose units, and their presence around 5 ppm or less leads to a conclusion that the disaccharide is attached to the C-7 of the aglycone [114, 116]. Consequently, the remaining glucose unit is predicted to be a substituent on the ring B due to steric hindrance effects that may forbid any bulky substitution on the ring A. It can

be concluded from all of the above that the expected compound that is contained in this fraction is Myricetin 7-O-neohesperidosid-5'-O-glucoside (Figure. 12).

Fraction. 33 was apparently more pure and its ^{1}HNMR assignment was more recognizable (Figure. 11). Apparently, the sugar units are located in the down field region of the spectra, i. e. between 3 ppm and 5 ppm, in contrast to the flavonoid protons that lie in the up field region, generally between 6 ppm and 8 ppm. As can be seen below, the B ring protons, H2'(*d*), H6'(*m*) and H5'(*d*) are around 7.5 ppm and the strong singlet peaks at 6.5 ppm and 6.7 ppm are of the A ring protons H8 and H6 respectively.

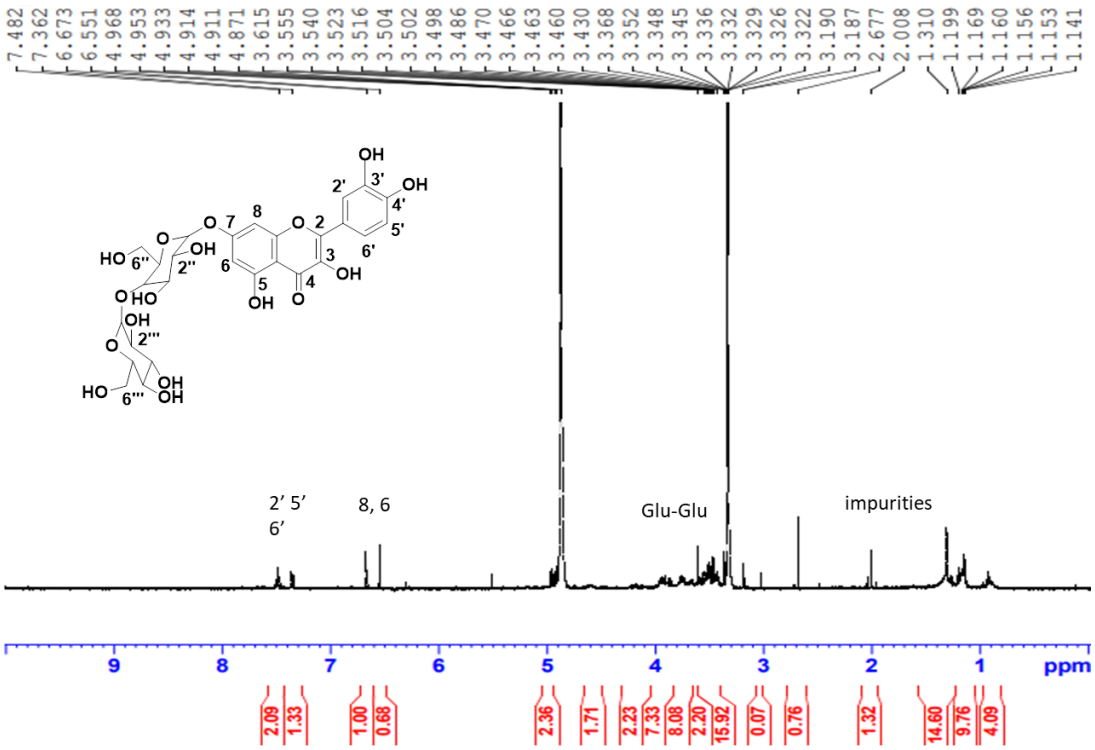


Fig. 11 The ^{1}HNMR assignments of compound. 2 (in fraction. 33) and its expected structure.

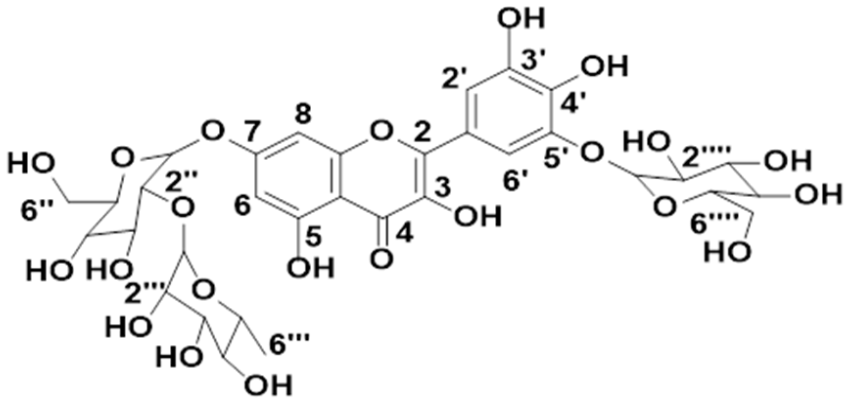
The number of protons in the sugar region in this spectra (3.3-3.9 ppm), as can be noticed from the above figure, reveals that the flavonoid compound in this fraction is connected to two sugar units which are mostly glucose, since no distinct CH3 peaks of the rhamnose sugar are detected. Also, glucose commonly forms β-linkage in glycosides,

and in this aspect, the β-linked glucose anomeric protons in general give peaks around 5 ppm when it is connected to C-7 position of the flavonoid aglycone and at 5.7 ppm when it is connected to C-3. Hence, as can be assumed from the above spectra, the two anomeric protons lie at around 4.8 ppm which implies that the aglycone is C-7 glycosylated with two β-glucose units. And this may explain the very weak splitting between A ring protons, i.e. the presence of the glucose units attached to C7 can associate with the small coupling between H6 and H8 [114]. However, the anomeric hydrogen of α-glucose locates around 5.35 ppm, and this diminishes the possibility of α-glucose to be connected to the aglycone [117]. In conclusion, it can be dedicated that the expected compound which is present in this fraction is Quercetine 7-O-sophrosied (Figure. 12).

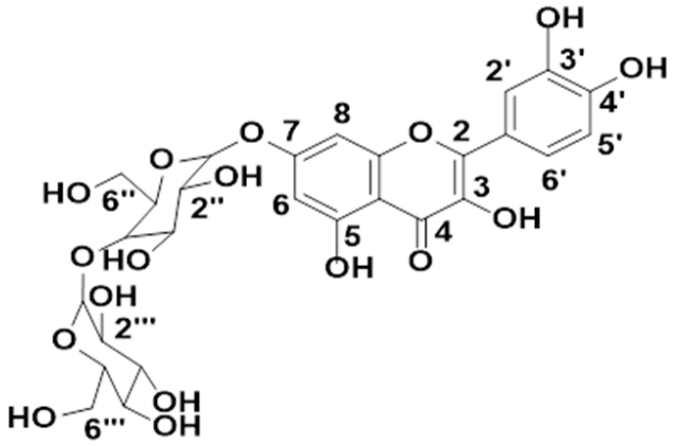
Furthermore, the absence of each of the α and β hydrogens of chalcones which appear around 8 ppm, the flavanone hydrogens (the quartet H2 between 5 ppm and 6 ppm as well as the *cis*-H3 and *trans*-H3 around 2.7 ppm and 3 ppm respectively), the hydrogens of flavanonols H2(*d*) and H3(*d*) around 4-5 ppm and 8 ppm respectively, the distinct H2 of isoflavones around 7.7 ppm and the sharp singlet H3 of flavones near 6.3 ppm, in addition to the absence of flavanononones C2 protons (the quartet around 5.2 ppm and doublet near 2.7, all are indications that the compounds in the fractions are possibly not any of the previously mentioned flavonoids [114]. Therefore, the probable compounds could be flavonols or anthocyanins, but, based on the ^{1}HNMR assignments in the literature that all are considering the H4 of anthocyanins is shifted downfield between 8 ppm and 9 ppm [114], this rise the chance that the compounds in the separated fractions are flavonol derivatives, which comes in accordance with the outcomes of this research.

However, by observing the spectra of the two unknowns, some sharp peaks around 2.7 ppm, 3.3 ppm and 4.8 ppm appear clearly, these are due to D. W and MeOD solvents impurities [118]. And the Hydrogens of the hydroxyl groups are solvent exchangeable protons and so unrecognizable by ^{1}HNMR. It's worth noting that the density of sugar protons are large in a small distinct area in the ^{1}HNMR spectra (from 3 ppm to 5 ppm) and, in fact, this may cause overlapping between the signals which make it hard to

resolve their peaks assignment, so, their coupling patterns are not completely understood [117]. For more details see Table. A4, appendix. 1



Compound. 1, Myricetin 7-O-neohesperidosid-5'-O-glucoside



Compound. 2, Quercetine 7-O-sophrosied

Fig. 12 The expected structures of the ^{1}HNMR characterized compounds.

4.2 Possible Nitrogenous Compounds from Rhizomes and Stems

Separation and analysis of the *A. P.* rhizomes and stems DCM and EtOAc crude extracts was mainly to examine the presence of alkaloids. GC-MS analysis (Figures. 12, 13 and 14) declared the possible existence of some compounds that are shown in Table. 4 below. And as can be noticed from the table, the stems and rhizomes of the plant have some compounds in common. But, none alkaloids were detected, except chlorophyll which is out of purpose. This study does not abolish their total absence but only proclaims that they could not be separated or recognized during the work course.

Table. 4 GC-MS analysis of *A. P.* rhizomes and stems possible compounds.

The GCMS analysis of the *A. P.* rhizomes and stems crude DCM extract			
Extract	**Part**	**The possible compounds**	**Molar mass g/mol**
DCM	**Rhizoms**	Octadecanamide	283.5
		9-z-octadecenamide	281
		Squalane	244.8
		Hexadecanamide	255.44
		Octadecanal	268.5
		(Z)-13-Docosenamide	337
		Heptacosane	380
	Stems	Isocitronellol	156
		3,5-bis(1,1-dimethylethyl)-Phenol	206
EtOAc	**Rhizomes and Stems**	Hexadecanoic acid methyl ester	270
		(E,E)-9,12-Octadecadienoic acid methyl ester	294
		Linoleic acid ethyl ester	308

As can be seen from Figure. 13, five major peakes reveal the presence of at lest five major compounds in the rhizomes DCM extract. For instance, *9-z-octadecenamide, (Z)-13-Dococenamide* and *Heptacosane* (Figure. 13) were found during the analysis. Further investigation were done and the Head-to-Tail comparison of their masses with standards revealed a good match, this rise the propability of the existance of these compounds in the rhizomes.

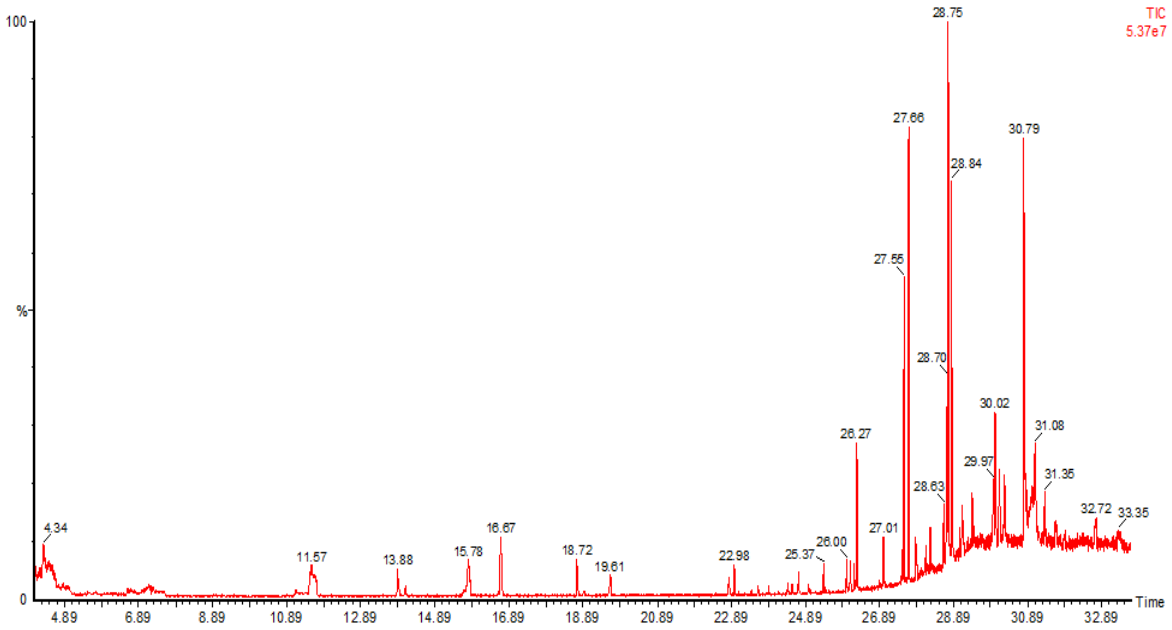


Fig. 13 The GC/MS chromatogram of *A. P.* rhizomes DCM extract.

In the same manner, the stems DCM extract were gone under GCMS Head to Tail matching analysis to examine the probability of their presence in stems, Figures. 14 and 15, *Isocitronellol* and *3,5-bis(1,1-dimethylethyl)-Phenol* were detected in this part.

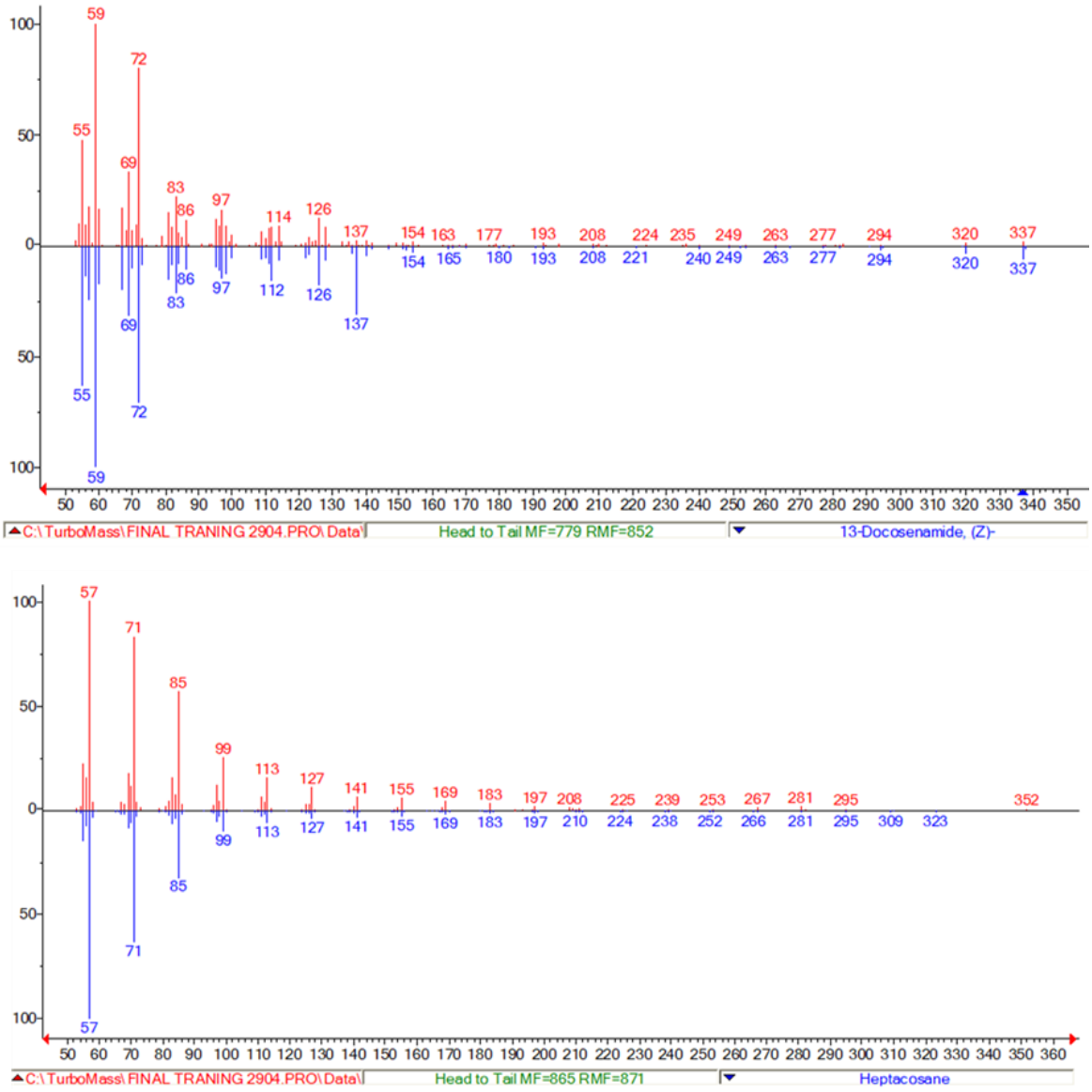


Fig. 14 The Head to Tail GCMS matching analysis of (Z)-13-Dococenamide and Heptacosane. As can be seen from the figure, the fragmentation patterns of the two compounds, a clear resemblance between the desired compounds and the standards used.

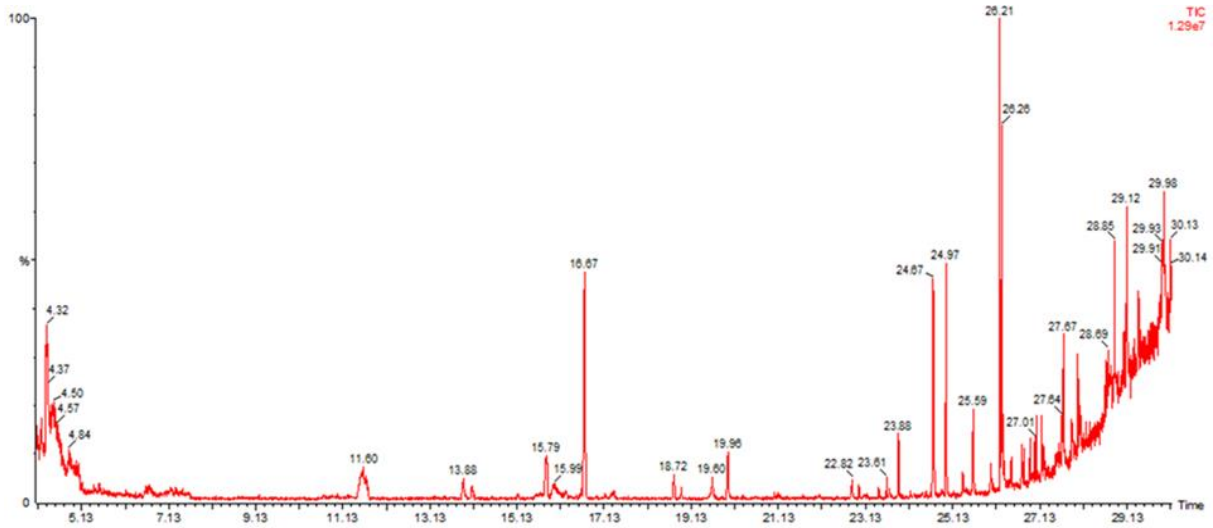


Fig. 15 The GC/MS chromatogram of *A. P.* stems DCM extract.

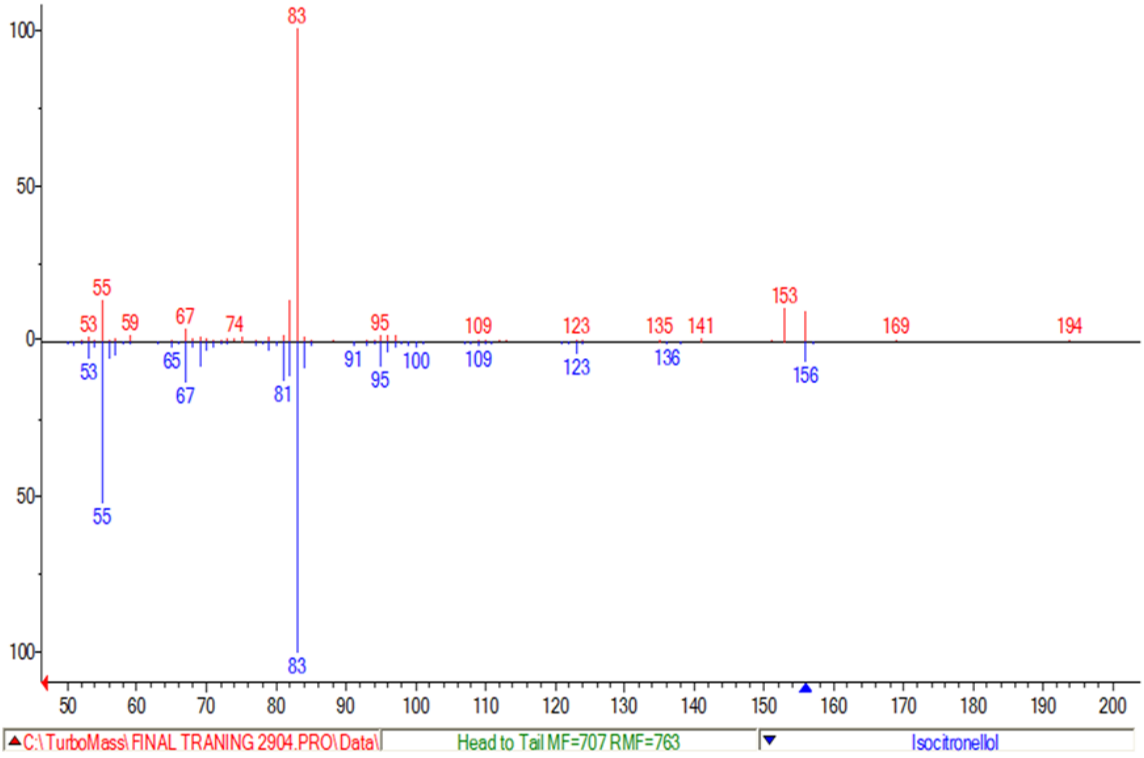


Fig. 16 The Head to Tail GCMS matching analysis of Isocitronellol.

The Figures 16 and 17 bellow describe the GCMS analysis and the mass fragmentation matching of the ethyl acetate extract of both, the stems and rhizomes. The chromatogram and Head to Tail analysis justify the presence of *Hexadecanoic acid methyl ester* and *(E,E)-9,12-Octadecadienoic acid methyl ester* among the components of the extract.

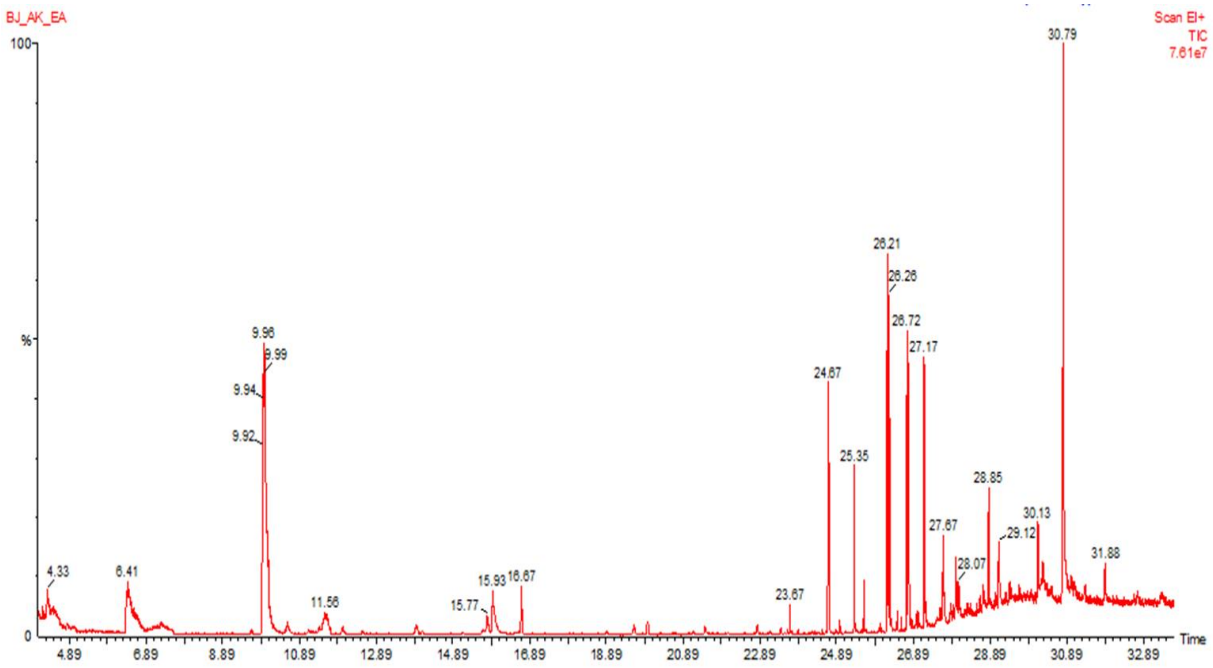


Fig. 17 The GC/MS chromatogram of *A. P.* rhizomes and stems EtOAc extract.

It's worth noting that many of these compounds that were recognized via GCMS, (their structures are shown in Figure. A4, appendix. 2) have been postulated to exert some kind of bioactivity towards pathogens. For example, *Heptacosane* were documented to play as a nontoxic candidate that modulate the activity of P-gp and exerted synergistic effects side by side to Doxorubicin drug and enhancing its cytotoxic activity against HL-60R [120]. In addition to *3,5-bis(1,1-dimethylethyl)-Phenol* which is an

anticancer agent that is still being under study [121], and *(Z)-13-Docosenamide* that was reported to exhibit anti-fungal and anti-tumor properties [122].

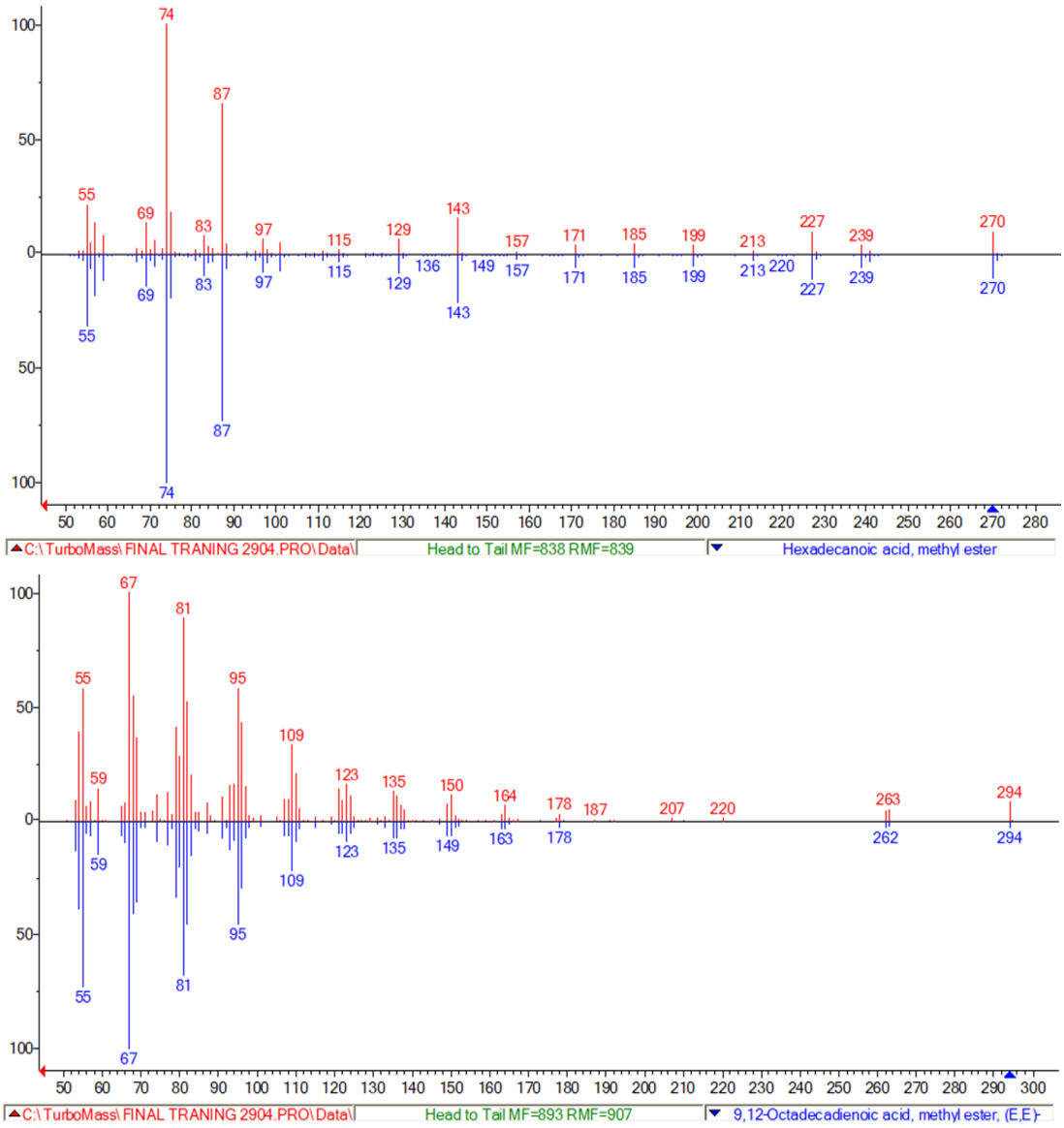


Fig. 18 The Head to Tail GCMS matching analysis of Hexadecanoic acid methyl ester and (E,E)-9,12-Octadecadienoic acid methyl ester. Check Figure. A3, appendix. 2 for more analysis.

4.3 Bioactivity Assays

It was established that the biological beneficial properties of flavonoids were referred in the first place to the hydroxyl groups functionalities on the B ring; which can be either the catechol moiety (1,2-Benzenediol), hydroquinone moiety (1,4-Benzenediol) or galloyl moiety (1,2,3-Benzenetriol) that due to their lower redox potentials (0.23 V-0.75 V) are able to donate electrons and hydrogen atoms to highly oxidizing free radical species (2.13 V-1.0 V) via redox reaction and form a stable phenoxyl radical, and side by side with the C2-C3 double bond on the C ring in conjugation with the 4-oxo group that provides planar structure which contributes to electron shifts via resonance effects which induces delocalization across the whole molecule the thing that influences the dissociation constants of phenolic OH groups, i.e. electrophilic ortho or para quinones, in order to enable the molecule to bind to relevance nucleophilic molecular targets such as thiols and amino groups of proteins and glutathione efficiently through electrophilic coupling reactions, so that, functioning its anti-inflammatory, antioxidant, anti-tumorous, anti-diabetic and anti-aging activity [23, 24, 26].

4.3.1 Anti-bacterial assay

The work that was done in collaboration with a research group from department of pharmacy at Birzeit university on antibacterial and anti-inflammatory activity of *A. P.* spadices and spathes has stated that, first, the extracts at all concentrations, 100 mg/mL, 50 mg/mL and 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.563 mg/mL and 0.78 mg/mL respectively, didn't show any growth inhibition when applied on both Gram-negative bacteria *E. Coli* and *P. Aeruginosa* as well as the Gram-positive bacteria *S. Aureus*. Secondly, it exhibited no inhibition activity on the fungi *Candida albicans* at the same aforementioned concentrations. As can be seen in Figure. 19, no zone of inhibition in all bacteria and fungi. These outcomes come in contrary with the obvious results that was concluded by the same group which stated that the first two concentrations 100 mg/ml and 50 mg/ml of the spadices extract showed moderate growth

inhibition when applied on the *Methicillin Resistance Staphylococcus Aureus* (*MRSA*-gram positive bacteria). This contradiction in results can mainly be regarded to the variance in the phytochemical components that are contained within the two spadices extracts that were used. This ,of course, comes as a consequent of the difference in their cultivation areas. It should be considered that the extract from the first run was collected from Ramallah mountains 2020 and the second extract was collected from Bitunya 2021. However, in agreement with the previous point, the outcomes that was reported by Dr. Hani research group are similar to the results that were reported by Naseef, and co-researchers (2017) which demonstrated that *A. P.* leaves extracts showed neither inhibitory effects on the bacterial species *E. Coli, S. Aureus and P. Aeruginosa*, nor antifungal effects on the *C. Albicans* fungal isolate[77]. Probably, this may be an indication that the extract of the *A. P.* spadix plant does not contain sapaonin compounds, since the former were confirmed to have strong antibacterial activity[86], or contain them in very small and negligible amounts that are not enough to exert any activity. These results may suggest that the flavonoid compounds that are exist in both *A. P.* spathes and spadices alcoholic extracts lack to C-5 and C-4' hydroxylation which is documented to improve the antibacterial inhibition properties of flavonoids. On the other side, they may have hydroxyl groups on C-6, C-3' or C-5' the thing that reduce the bacterial inhibitor effects of flavonoids[123]. In other explanations, and according to Structure Activity Relationship (SAR) studies which previously documented that the O-methylation and O-glycosylation of the OH groups of B ring alters the planarity and exerts steric hindrance and leads to reducing the anti-oxidant ability of the flavonoid while it improves the antiviral and antibacterial effects[23]. This may lead to a conclusion that the compounds in the two tested extracts may not have methylated or glycosylated units in their structure.

It was also reported by the same group that *A. P.* spadices extract at concentration range (25-1.56 mg/mL) prevent the hemolysis and protect the RBC membrane against lysis which induced by hypotonic solution, meanwhile, the maximum anti-inflammatory effect was significant at 25 mg/mL concentration which induced 72.3% inhibition. And this can be correlated to a possible presence of flavonoids with catechol moiety, i. e. C-3' and C-4' hydroxylation pattern [28]. But, this does not diminishes the possibility of

presence of compounds other than flavonoids responsible for this inhibition. It's important to note that catechol group is recognized by scientific researches as the most significant responsible pharmacophore, producing stable ortho semi-Quinone radical via electron delocalization providing high activity through intra-molecular hydrogen bonding between catechol OH groups, which leads to high scavenging capacity therefore, strong pharmacological activity. Conversely, the existence of only one hydroxyl in ring B diminishes the biological activity [23,26].

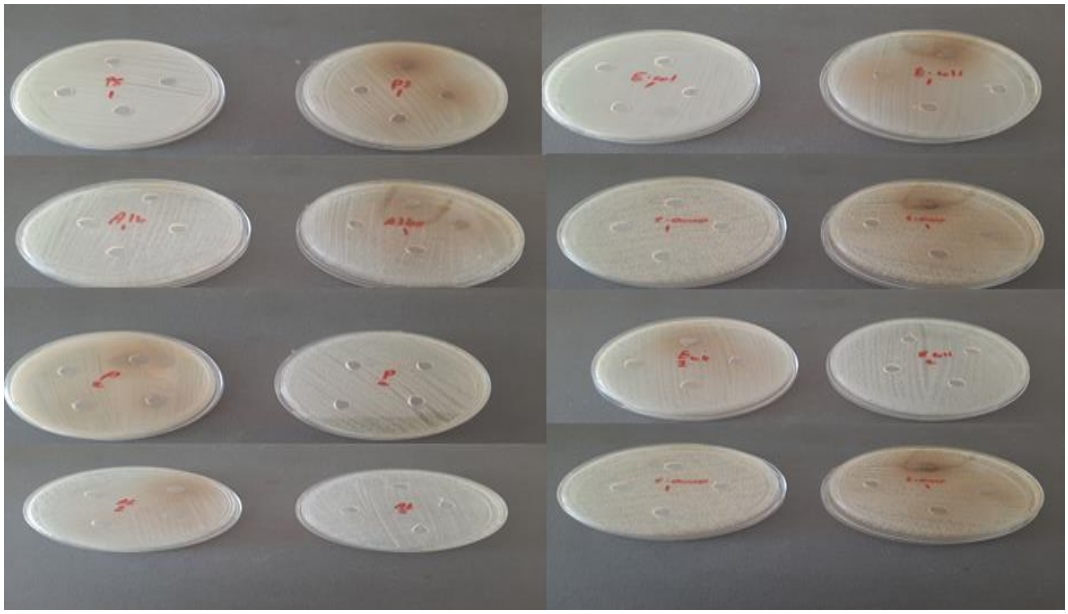


Fig. 19 *P. Auroginosa, S. Aureus, E. Coli* and *C. Albicans* treated with *A. P.* spadices and spathes extracts.

4.3.2 Anti-cancer assay

Based on the positive outcomes of the previous anticancer assays that were carried on *A. P.* leaves alcoholic extracts, a preliminary investigations concerning the anticancer activity of the spadix and spathes polar extracts of the plant. In collaboration with Dr. Stiban / Department of biology, HEK932T and HeLa cancer cells (Figure. 20) were treated with different concentrations of *A. P.* spadices crude methanol extract and its aqueous and butanol sub-extracts as well as *A. P.* spathes crude methanol extract.

As can be noticed from Figures. 21 and 22, less absorbance of the formazan dye was obtained when HeLa cells treated with all of the extracts comparing with its absorbance when HEK932T cells treated at the same concentrations. In short, formazan is a colored reduction product of tetrazolium compound that biologically reduced in the presence of metabolic enzymes of the living cells.

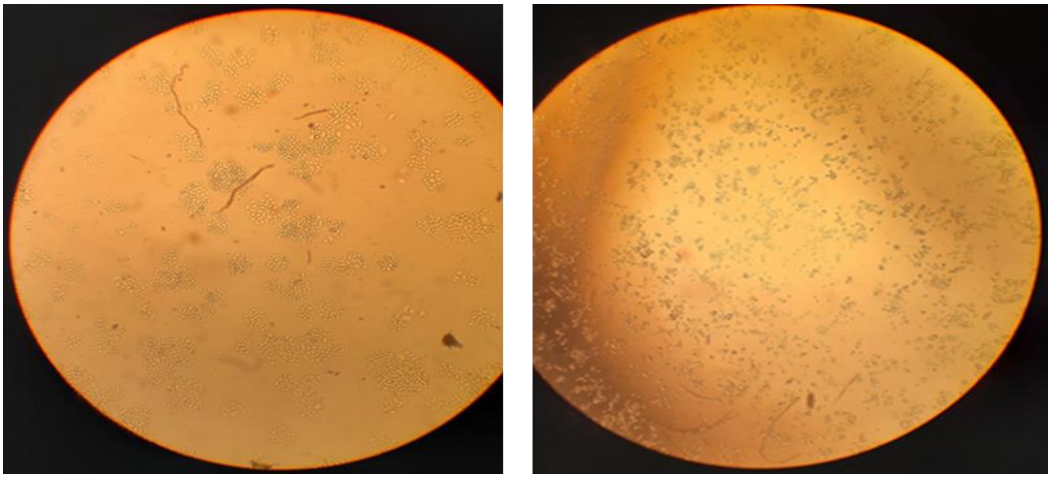


Fig. 20 HeLa (on the left) and Hek-293T (right) cancer cells.

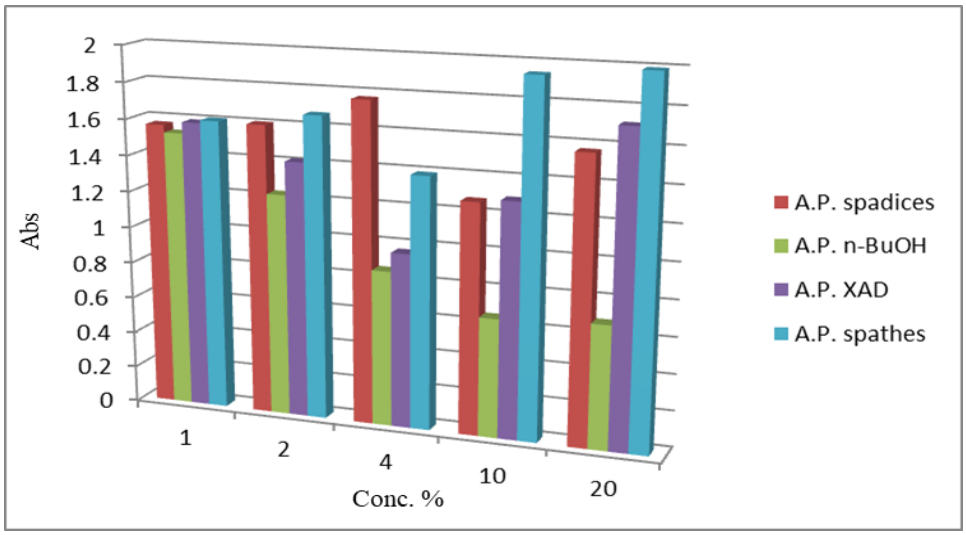


Fig. 21 Cytotoxic effects of *A. P.* polar extracts on HEK-293T cells after 48hrs.

At 1% and 2% concentrations, a recognizable HEK-232T cells viability was recorded, and it's noticeable that the *A. P.* spadices and spathes methanolic crude extracts had the least inhibition activity against the aforementioned cell line when they are compared with

the XAD aqueous and n. BuOH extracts, and this is reasonable and can be related to the increase of the concentration of the active compounds in the XAD and n. BuOH extracts. But, a strong fluctuation can be indicated at 10% and 20% concentration of the XAD extract, this raises a question about what could be the reason behind this increase in the cells viability at those high concentrations after the significant decrease in the cell viability at lowest concentrations. At the first glance, this may attributed to the possible not equal distribution of the HEK-232T cancer cells in the 691-plate. On the contrary, the n. BuOH extract were recorded to decrease cells proliferation at all concentrations from 4% to 20%, since the cell viability were in continues decrease proportionally to the concentration.

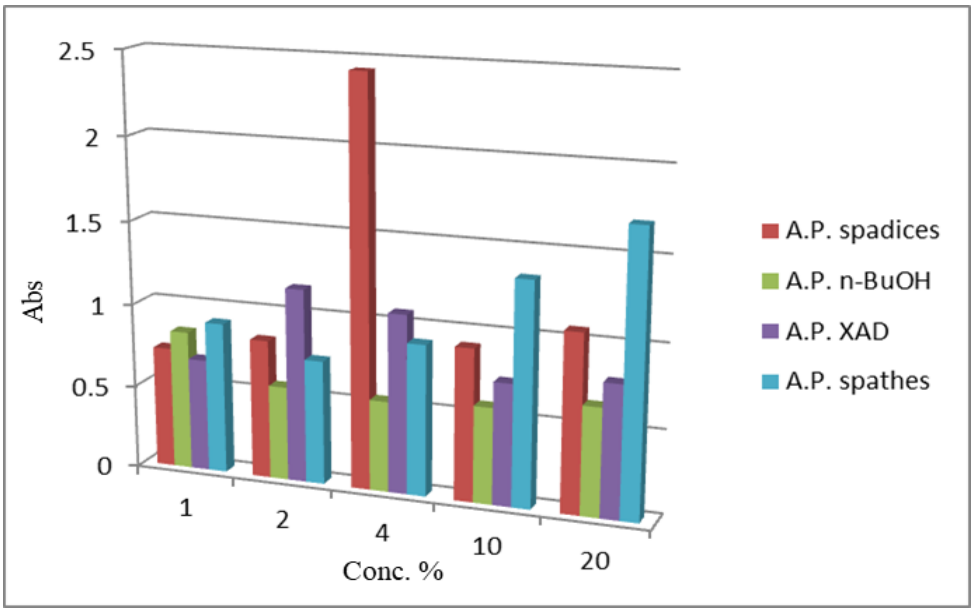


Fig. 22 Cytotoxic effects of *A. P.* polar extracts on HeLa cells after 48hrs.

On the other side, it's obvious that the HeLa cancer cells marked a considerable decrease in the cells viability at all concentrations of all the extracts with some fluctuations in both spadices, spadix and XAD extracts. These fluctuations can be attributed to the distribution of cells as previously mentioned. On the positive side, all of the concentrations of aqueous XAD and n. BuOH extracts obtained a lesser cell viability of HeLa cells if compared to DMSO and MeOH controls (Abs_{avg} of the controls are 1.06

and 1.01 respectively) which, if fluctuations were excluded, gives an indication that these two polar extracts may have anti cancerous properties.

It's important to point out that the butanol solvent that was used has a very high boiling point which may leave traces of the solvent in the dried extract, so, it is uncertain whether the inhibition activity comes from the active compounds in the butanol extract or from the butanol itself or some kind of synergism occur and both, the solvent traces and the compounds in the extract, affect the cells viability.

Another assay was done to compare between the activity of the *A. P.* extracts of different polarity (Figure. 23), all of the concentrations used of all the extracts (*A. P.* spathes and spadices MeOH extracts, *A. P*. spadices n. BuOH and aqueous XAD extracts, *A. P*. spadices DEE extract, and finally *A. P*. rhizomes and stems DCM extracts) resulted in absorbance lesser than the DMSO control ($Abs_{avg,}$ 0.9) which generally indicated that there is an inhibition activity exerted from all the extracts against the HEK932T cancer cell line.

Spadices MeOH and DEE extracts recorded the lowest inhibition of cell proliferation at almost all the concentrations that was used with some fluctuations that may be related to the reason that was described before. According to the figure below, XAD and n. BuOH spadices extracts marked the best inhibition except at the concentration 40*M*g/ml which recorded the best inhibiting effects of crude spadices and spathes MeOH extracts as well as stems DCM crude extracts. If the results at concentration 40*M*g/ml were excluded based on the possibility that the amount of cancer cells in the wells of 691-plate that were dedicated to this specific concentration was less than the amount of cells in the other wells, it can be concluded that the crude spadices MeOH and the spadices DEE extracts exerted the weakest inhibition among the other extracts. Conversely, the XAD and n. BuOH extracts in addition to the rhizomes DCM crude extract were reported to have the best anti-proliferation activity. While, the inhibition ability of spathes crude MeOH extract remained almost unchanged at the three concentrations

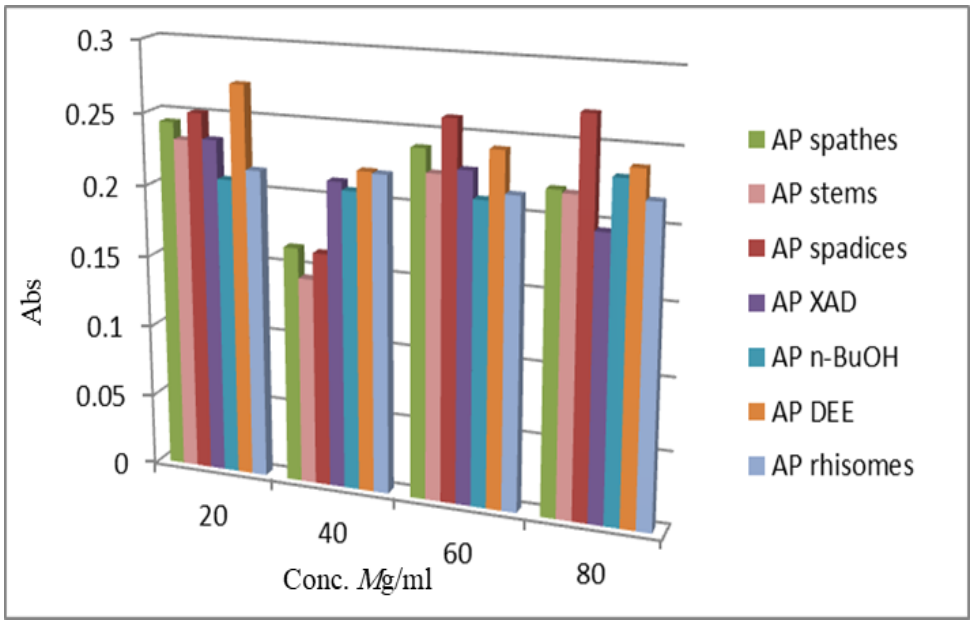


Fig. 23 Cytotoxic effects of *A. P.* extracts of different polarity on HEK932T cells after 48hrs.

In conclusion, n. BuOH and aqueous XAD spadices extracts were documented to have the best cancer inhibition effects at most of the concentrations used against both HEK-232T and HeLa cancer cell lines. The two extracts are expected to have flavonoids among their components due to their suitable polarity to these compounds. However, there is a probability that these two extracts have C-glycosylation patterns in their compounds that improved their anticancer properties if taking into consideration that SAR and other studies had confirmed that C-glycosylation increase the activity toward radical scavenging and antioxidant ability. Concomitantly, the presence of 3-OH on the C ring besides the B functionalities works as an additional value that confers the resonance stability and increases the antioxidant and radical-scavenging activity as well as cytotoxicity [23, 24, 26]. Obviously this comes in agreement with the results of this research since the antioxidant as well as radical scavenging ability are directly related to the anti-cancerous activity.

Moreover, in contrast to ring B, ring A hydroxylation has less contribution to anti-oxidant activity owing to the fact that it's meta OH groups are less oxidizable than ortho B groups, it's 5 and 7 OH groups impair the activity of the 3-OH on the ring C [23, 26].

Chapter. 5 Conclusion And Recommendations

5.1 Conclusion

In this work, *Arum Palaestinum* spadix with its surrounding parts were separated, analyzed, characterized and studied for their bioactive ability towards some kinds of bacteria, fungi, and some cancerous cell lines as well.

Two possible compounds were separated and their ^{1}HNMR spectra were studied and analyzed to come up with their expected structures that, in fact, need to be confirmed by ^{13}CNMR and 2D-NMR to be justified. GC-MS analysis revealed that about twelve compounds are exist in the rhizomes and stems of the plant.

Paradoxical antibacterial results were reported, and encouraging anticancer records were obtained. Actually, more investigations need to be done in order to confirm the accurate results of both sides.

5.2 Recommendations

The possible structures that were suggested based on their ^{1}HNMR, are not solid enough to be declared as the actual compounds in the *Arum Palaestinum* XAD aqueous extract and consequently in the Spadix part of the plant. So that, further and repeatable separation must be done to go through further characterization via ^{13}CNMR and 2D-NMR for more justification of the nature of the two compounds. Furthermore, it's preferable to go further during separation process of flavonoids and examine the biological activity of the most pure and fully characterized fractions of the aqueous extract. This would determine the exact bioactive compound that may have the anti-bacterial, anti-fungal and anti-cancerous properties.

Extended anti-bacterial investigations are needed in order to double check the contradictory between the results that were obtained, in order to confirm the outcomes. The results of the preliminary anticancer examinations are a spot light in this field, but, it's better to go on further investigations to determine the exact activity of the spadices, rhizomes and stems parts of the *Arum Palaestinum* plant. It's possible to study the synergistic effects of the previously mentioned extracts to examine their enhancing/ inhibiting ability on the action other drugs against cancer cells or the other pathogens.

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Appendices

Appendix. 1

Table. A1 Polar separation of *A. P* spadices aqueous extract filtrated by XAD / Sephadex LH-20 (500g).

Eluent mixture % + 0.50ml TFA						
Fraction #	**Volume ml**	**MeOH:**	**DW:**	**TFA ml**	**Mass g**	**Color**
1	150	20	80	0.5	0.0255	Red
2	130	20	80	0.5	0.0442	Red
3	150	20	80	0.5	0.0367	Red
4	150	20	80	0.5	0.0131	Pale red
5	155	20	80	0.5	0.0132	Pale red
6	150	20	80	0.5	0.0163	Strong red
7	140	20	80	0.5	0.0062	Purple
8	148	20	80	0.5	0.023	Red
9	150	20	80	0.5	0.0051	Purple
10	148	20	80	0.5	0.0048	Red
11	130	20	80	0.5	0.0041	Purple
12	200	20	80	0.5	0.0119	Red
13	150	30	70	0.5	0.0025	Black
14	150	30	70	0.5	0.0447	Black
15	150	30	70	0.5	0.0029	Pale black
16	160	30	70	0.5	0.0032	Purple
17	240	30	70	0.5	0.0024	Purple
18	130	30	70	0.5	0.0059	Brown
19	150	40	60	0.5	0.0059	Purple
20	150	40	60	0.5	0.0067	Purple

21	150	40	60	0.5	0.0169	Purple
22	150	40	60	0.5	0.0041	Strong brown
23	150	40	60	0.5	……	Pink
24	125	40	60	0.5	0.0046	Pale black
25	150	40	60	0.5	0.0128	Pale pink
26	150	40	60	0.5	0.0009	Purple
27	150	40	60	0.5	0.0075	Purple
28	150	40	60	0.5	0.0008	Brown
29	150	40	60	0.5	0.0059	Brown
30	150	40	60	0.5	0.0043	Pale black
31	148	50	50	0.5	……	Pale pink
32	150	50	50	0.5	0.0013	Brown
33	150	50	50	0.5	0.0203	Brown
34	150	50	50	0.5	0.0038	Brown
35	148	50	50	0.5	0.0023	Brown
36	150	50	50	0.5	0.0062	Pale brown
37	135	60	40	0.5	0.0026	Pale brown
38	150	60	40	0.5	0.0048	Pale brown
39	150	60	40	0.5	0.0172	Brown
40	148	60	40	0.5	0.001	Pale brown
41	130	60	40	0.5	0.0035	Pale brown
42	150	70	30	0.5	0.0024	Pale brown
43	130	70	30	0.5	0.003	Pale brown
44	140	70	30	0.5	0.0018	Reddish brown
45	148	70	30	0.5	0.0037	Pale brown
46	150	70	30	0.5	0.0014	Pale brown
47	160	80	20	0.5	0.0025	Pale brown
48	175	80	20	0.5	0.0127	Reddish brown
49	175	80	20	0.5	0.0052	Yellowish brown

50	230	80	20	0.5	0.0038	Pale brown
51	150	80	20	0.5	……	Yellow

Table. A2 Polar separation of *A. P*. spadices and its XAD aqueous extract via HPLC run.

Time	**% conc. Of D. w**	**% conc. Of ACN**
10	86	14
14	86	14
32	60	40
43	60	40
65	60	40
69	90	10
70	Stop	Stop

Table. A3 Polar separation of *A. P*. spadices XAD aqueous extract via programmed HPLC run.

Time, min	**A %**	**B %**
0	95%	5%
50	80%	20%
65	65%	35%
70	40%	60%
75	10%	90%
78	95%	5%
80	95%	5%

Table. A4 ^{1}HNMR spectral details.

Fraction. 8			
Rosmarinic acid	Protons	Chemical shifts/ ppm (J)	Multiplicity
	H1	7.317, 7.321 (*J*=2)	*d*
	H4	7.058, 7.075 (*J*=8.5)	*d*
	H5	7.228, 7.240 (*J*=6)	*dd*
	H7	7.624, 7.655 (*J*=15.5)	*d*
	H8	6.165, 6.196 (*J*=15.5)	*d*
	H10	4.971, 4.985	*q*
	H12	2.650, 2.660	*dd*
	H12'	2.900, 2.910	*dd*
	H14	6.684, 6.701	*dd*
	H15	6.684, 6.701	*d*
	H18	7.125, 7.131 (*J*=3)	*d*
Compound. 1	H6	7.139, 7.145 (*J*= 3)	*d*
	H8	7.257, 7.263 (*J*= 3)	*d*
	H2'	7.254, 7.257 (*J*= 1.5)	*d*
	H6'	7.251, 7.254 (*J*= 1.5)	*d*
Sugars		(3.280-4.692)	
Fraction. 33			
Compound. 2	H6	6.551	*s*
	H8	6.673	*s*
	H2'	7.505, 7.509 (*J*=2)	*d*
	H5'	7.435, 7.451 (*J*=8)	*d*
	H6'	7.482, 7.488,7.492 (*J*=2, 3)	*m*
Sugars		(3.463-4.980)	

Appendix. 2

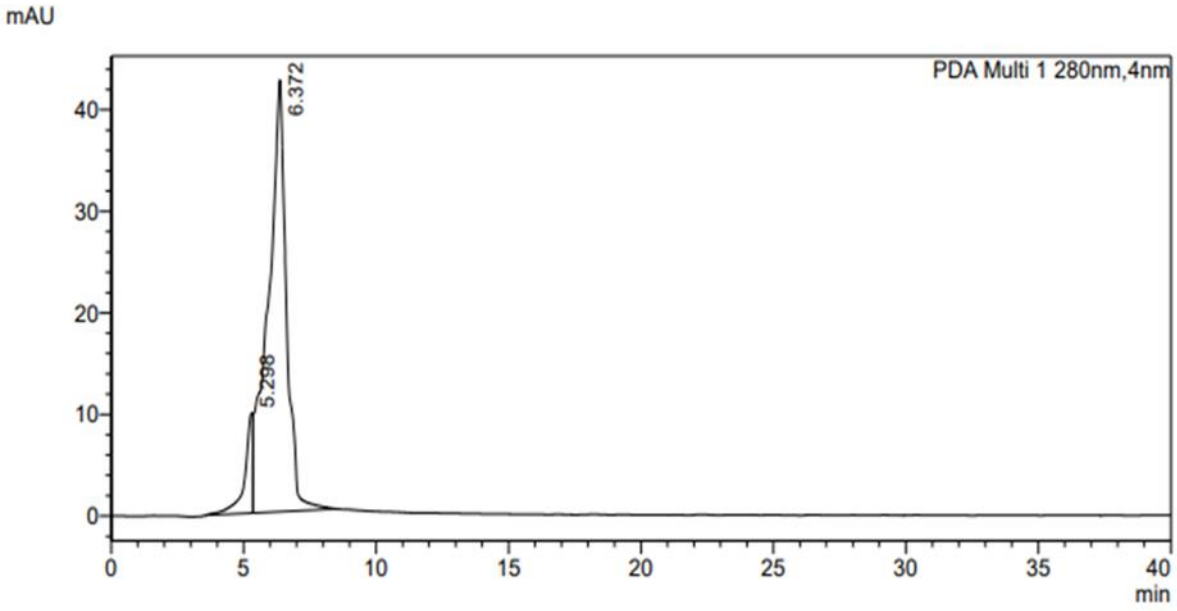

Figure. A1 HPLC chromatogram of fraction. 1 that possibly contains the 3rd major compound.

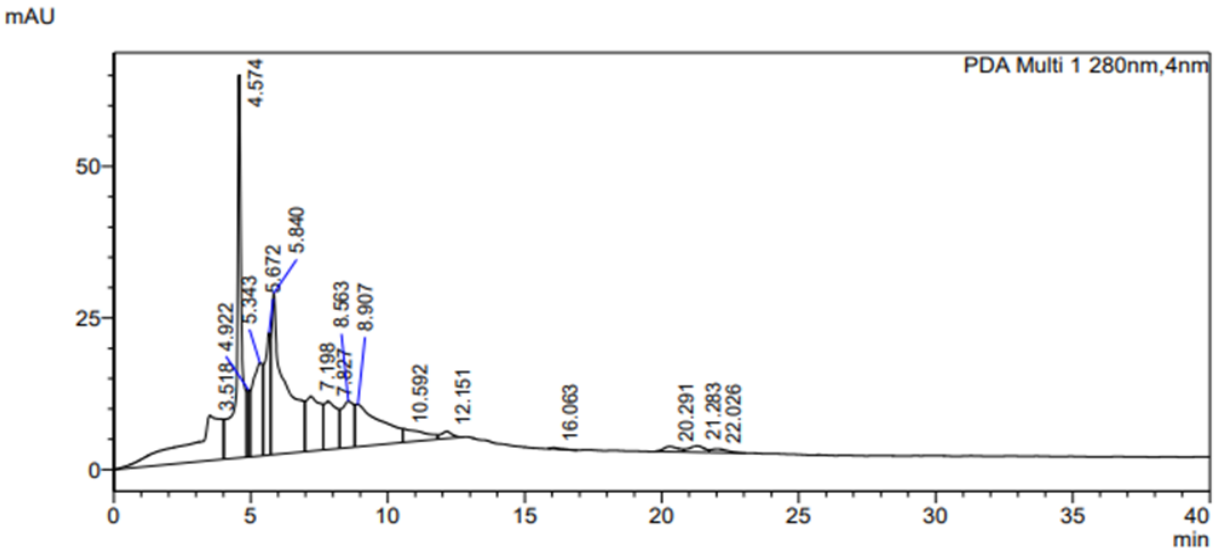

Figure. A2 HPLC chromatogram of fraction. 40 that possibly contains the 4th major compound.

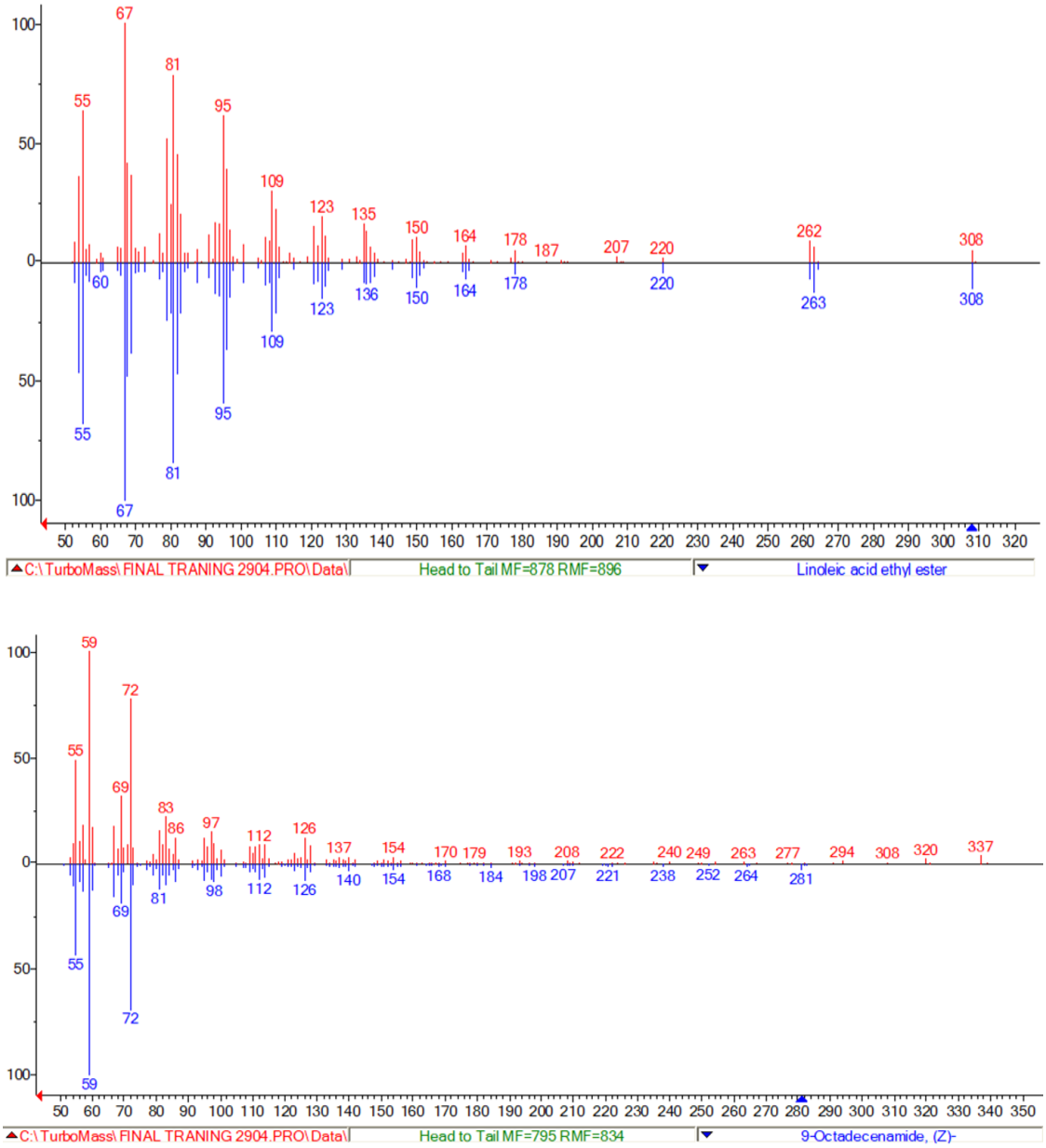

Figure. A3 The Head to Tail GCMS matching analysis of 9-z-octadecenamide and Linoliec acid ethyl ester.

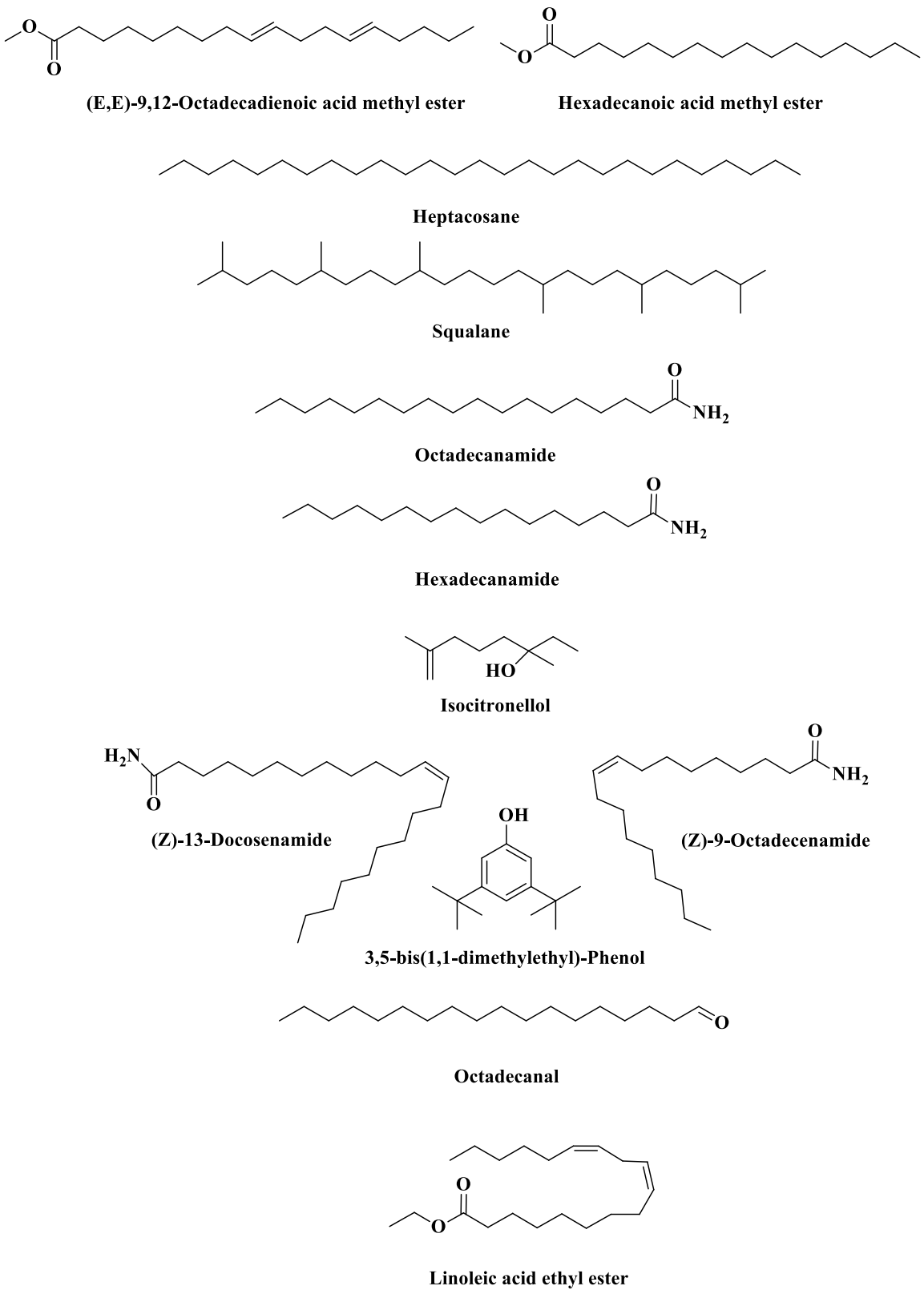

Figure. A4 Structures of the compounds analyzed by GC-MS.