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By: BENAICHE Ghania

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Extraction, separation and biological activities of some secondary metabolites from the plant *Cytisus purgans*

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Members of jury:

Chairman: MERROUCHE Abdallah	**Professor**	**University Mohamed BOUDIAF, M'sila**
Supervisor: BELATTAR Noureddine	**Professor**	**University Ferhat ABBAS, Sétif 1**
Examiner: DEHAMNA Saliha	**Professor**	**University Ferhat Abbas, Sétif 1**
Examiner: OUALI Dehimi	**Professor**	**National high school, M'sila**
Examiner: ZEID ELKHEIR Belkacem	**M C A**	**University Mohamed BOUDIAF, M'sila**
Examiner: KHENNOUF Seddik	**Professor**	**University Ferhat Abbas, Sétif 1**

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Dedicated to my parents

Abstract

In the present study the plant *Cytisus purgans* was investigated for its contents of secondary metabolites. *Cytisus* is a genus of the Leguminosae family, with six species endemic to Algeria. *Cytisus balansae Boiss*. (*Cytisus purgans*) is a rare species of Algeria (Aures, Mahdids, and Lella Khadidja).

Phytochemical study revealed the presence of two known quinolizidine alkaloids which are : sparteine and lupanine which were isolated from methanolic extract of crude mixture of alkaloids using open column chromatography and identified by their spectroscopic data (IR, MS, ^{1}H and ^{13}C NMR) and comparing with literature. In the other side, two known flavonoids were isolated from crude mixture of polyphenols which are: quercetin and sarotanoside, using open column chromatography and identified by their spectroscopic data (UV, IR, MS, ^{1}H and ^{13}C NMR) and comparing with literature.

Biological study mentioned that *Cytisus purgans* not show a significant antitumor activity against human breast cancer and human lung cancer but it shows a power antioxidant activity and a moderate antimicrobial activity.

Key words: *Cytisus purgans*, Leguminosae, quinolizidine alkaloids, polyphenols, biological activity

Résumé

Dans le travail qui suit, la plante *Cytisus purgans* a été examinée pour son contenu en métabolites secondaires. *Cytisus* est un genre de la famille des Leguminosae, il est représenté en Algérie par six espèces endémiques. *Cytisus purgans* est une espèce rare de l'Algérie (Aures , Mahdid et Lella Khadidja).

L'étude phytochimique a montré la présence de deux alcaloides quinolizidiniques connues qui sont : sparteine et lupanine, ces deux composés sont isolés à partir de l'extrait méthanolique brute des alcaloides en utilisant la chromatographie sur colonne et identifiés par leurs donnés spectroscopiques (IR, SM, ^{1}H et ^{13}C RMN) et en comparant avec la littérature. Dans l'autre coté, deux falavonoides connues qui sont : quercétine et sarotanoside sont isolés à partir de l'extrait brute des polyphenols et en utilisant la chromatographie sur colonne et identifiés par leurs donnés spectroscopiques (UV, IR, SM, ^{1}H et ^{13}C RMN) et en comparant avec la littérature.

L'étude biologique a montré que *Cytisus purgans* n'a pas une activité anti tumoral signifiante contre le cancer du sein et cancer des poumons mais a une forte activité antioxydant et a activité antimicrobienne moyenne.

Mots clés : *Cytisus purgans*, Leguminosae, alcaloides quinolizidininiques, polyphenoles, activité biologique

صخلم

نم حيث محتواها من مواد الأيض الثانوي *Cytisus purgans* في الدراسة الآتية، تمت دراسة نبتة هو جنس من عائلة البقوليات و هو ممثل بست أنواع متوطنة في الجزائر.*Cytisus*

هو نوع *نباتي نادر للجزائر (الأوراس، المعاضيد و لالة خديجة).Cytisus purgans*

الدراسة النباتية الكيميائية بينت وجود مركبين معروفين من نوع قلويد كينوليزيديني و هما: السبارتيين و الليبانين، تم فصلهما من المستخلص الميثانولي الخام باستخدام كروماتوغرافيا العمود وتم التعرف على هويتهما بواسطة طرق التحليل الطيفي (تحت الحمراء، الكتلة و الرنين النووي المغناطيسي للهيدروجين و الكربون) و بالمقارنة مع المراجع النظرية. من الناحية الأخرى، تم التعرف على وجود مركبن معروفين من نوع الفلافونويد وهما الكرسيتين و الساروتانوزيد، وتم التعرف على هويتهما طرق التحليل الطيفي (فوق البنفسجي، تحت الحمراء، الكتلة و الرنين النووي المغناطيسي للهيدروجين و الكربون) و بالمقارنة مع المراجع النظرية.

الدراسة البيولوجية أظهرت *Cytisus purgans* أن نبتة

لم تبين فعالية مضادة للأورام مميزة ضد كل من سرطان الثدي و سرطان الرئة بينما أظهرت فعالية مضادة للأكسدة قوية و فعالية مضادة للميكروبات معتدلة.

الكلمات المفتاحية: ، *البقوليات، القلويدات الكينوليزيدينية، متعددات الفينول، الفعالية البيولوجية، Cytisus purgans*

Introduction

As a most important trait, plants can produce and store secondary metabolites (of which more than 50 000 compounds have been described so far) with deterrent/repellent or toxic properties against microorganisms, viruses and/or herbivores. These products are often stored at strategically important sites: epidermal tissues or in cells adjacent to an infection, or in plant parts that are especially important for reproduction and survival (flowers, fruits, seeds, bark or roots).

In addition to defense, secondary metabolites are employed by plants to attract pollinating insects or seed-dispersing animals, e.g. by colored compounds such as betalains (within Centrospermae), anthocyanins, carotinoids, flavonoids or fragrances, such as terpens, amines and aldehydes. In this case we can consider the secondary metabolites as attracting signal substances. Also physiological roles, such as ultraviolet protection (by flavonoids or coumarins), nitrogen transport or nitrogen storage (some alkaloids, non-protein amino acids, lectins and protease inhibitors), or photosynthetic pigments (carotinoids) may be additional features.

Although the biological functions of most plant-derived secondary metabolites have not been studied experimentally in depth, it is now generally assumed that many compounds are important for the survival and fitness of a plant and that they are not useless waste products, as was suggested earlier in this century. Secondary metabolites are often not directed against a single organism, but generally against a variety of potential enemies, or they combine the roles of both deterrent and attractants (e.g. anthocyanins and many essential oils can be attractants in flowers, but are also insecticidal and antimicrobial). Thus, many natural products have dual or even multiple functions [1]**.**

The alkaloids represent the largest single class of plant secondary metabolites. They have a remarkable range of often dramatic pharmacological activity, and are also often toxic to man. Many alkaloids are used in therapeutics and as pharmacological tools. A wide range of biological effects has been reported for alkaloids, including emetic, anti-cholinergic, antitumor, diuretic, sympatho-mimetic, antiviral, antihypertensive, hypno-analgesic, antidepressant, mio-relaxant, anti-tussigen, antimicrobial and anti-inflammatory activities. However, alkaloids and other natural compounds are generally complex, making it necessary to analyze their pharmacological activities using several experimental methods and demonstrate their structure/activity correlation. It is common to find pharmacological data

where a single experimental model was used to demonstrate a biological activity. However pathological responses are extremely complex involving many biological events, so it is necessary to use different experimental models to define the exactly mechanism of action of the analyzed molecule [2].

Polyphenols have recently aroused considerable interest because of their potential beneficial biochemical and antioxidant effects on human health. Commonly referred to as antioxidants, they may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases, inflammation and others. Most of the experimental results confirmed that polyphenols have several biological activities including radical scavenging, anti-inflammatory, anti-mutagenic, anti-cancer, anti-HIV, anti-allergic, anti-platelet and antioxidant activities.

Chemically, polyphenols are diverse group of naturally occurring compounds containing multiple phenolic functionalities. They constitute a large and still expanding complex family of molecules, with diverse structures, properties and sizes ranging from monomers to polymers. Polyphenols gather a range of weakly acidic substances possessing aromatic rings bearing hydroxyl substituents and constitute one of the most numerous and widely distributed groups of substances with more than 8000 phenolic structures currently known. Polyphenols can be divided into different classes depending on their basic structure. The main classes of polyphenols are phenolic acids and flavonoids while the less common are stilbenes and lignans. Major subclasses present in food correspond to anthocyanins, flavanols, flavones, flavanones, flavonols and isoflavones [3].

In the course of our continuing search for bioactive natural plant products, this study is destined to the evaluation of some secondary metabolites of the plant *Cytisus purgans* growing in Algeria, as well as to estimate their biological activity.

Our study is divided into two parts: theoretical and practical study. The first part includes three chapters: chapter one is destined to the medicinal plants, the family of fabaceae including the studied plant, also, anterior studies concerning *Cytisus purgans*. In second and third chapters there are data, much of them tabulated, concerning quinolizidine alkaloids and polyphenols (including flavonoids), respectively. The second part is a practical divided into material and methods which describes different experiments and results obtained and their discussions, and finally, conclusion.

Part I

Theoretical study

I-Medicinal plants

I-1-The importance of medicinal plants

The term of medicinal plants include a various types of plants used in herbal therapy and some of these plants have a medicinal activities. These medicinal plants consider as a rich resources of ingredients which can be used in drug development and synthesis. Besides that these plants play a critical role in the development of human cultures around the whole world. Moreover, some plants consider as important source of nutrition and as a result of that these plants recommended for their therapeutic values. These plants include ginger, green tea, walnuts and some others plants. Other plants their derivatives consider as important source for active ingredients which are used in aspirin and toothpaste.

These days the term "Alternative Medicine" became very common in western culture, it focus on the idea of using the plants for medicinal purpose. But the current belief that medicines which come in capsules or pills are the only medicines that we can trust and use. Even so most of these pills and capsules we take and use during our daily life came from plants. Medicinal plants frequently used as raw materials for extraction of active ingredients which used in the synthesis of different drugs. Like in case of laxatives, blood thinners, antibiotics and antimalaria medications, contain ingredients from plants. Moreover the active ingredients of Taxol, vincristine, and morphine isolated from foxglove, periwinkle, yew, and opium poppy, respectively [4].

Medicinal plants have acquired increasing significance in development co-operation over the last few years. Their use and conservation are cross-sectoral concerns that embrace not only health-care but also nature conservation, biodiversity, economic assistance, trade, and legal aspects (e.g. intellectual property). Even today, the majority of the world's population is dependent upon traditional medicine, and thus also on the use of plants and plant extracts. This is particularly true of poorer sections of the population in developing countries, because natural remedies are not only cheaper than modern medicines but are often the only medicines available in remote rural regions. (According to the WHO, 80 per cent of the world's population is dependent on health-care provided by medicinal plants.)

Besides serving medical and cultural functions, medicinal plants in developing countries have an important economic role. The gathering of wild medicinal herbs frequently provides economically disadvantaged groups such as smallholders and landless herd's people with their only form of cash income.

In addition, medicinal plants are easily integrated into traditional crops such as maize, beans, and vegetables. Thanks to differences in harvest ingtimes, farmers are better able to distribute their incomes over the whole year. Small-scale traders and industries also benefit from being able to buy up dried medicinal plants and process them into teas, ointments, and tinctures for the local market.

But the economic importance of medicinal plants extends far beyond the national markets in developing countries. Germany alone imports medicinal plants worth over DM150 million each year. Most of these come from developing or transforming countries. The sustainable purchase of plant-material can therefore considerably improve trade-balances in the countries of origin and offers major potential in terms of development. The basis for this is the unique biodiversity that exists in the developing countries, where 90 per cent of the earth's genetic diversity is to be found [5].

I-2-Plants as a resource for medicinal remedies

Human survival has always depended on plants. Throughout the ages, plants have provided mankind the essentials of life, including food, raw materials for the manufacture of clothing and shelters, poison for hunting, and medical agents for relief from illness. It is estimated that plants are still a major source of health care for more than 80% of the world's population. In the industrialized world, 25% of the prescribed medicines are substances derived from higher plants, and about 120 plants derived compounds from ca. 90 plant species are used in modern therapy. Among the estimated 400000 plant species, only a small percentage have been phytochemically investigated and the fraction submitted to biological or pharmacological screening is even smaller. Based on these precedents, plants are an important source in the search for new drugs and lead compounds. Although powerful new technologies such as high-throughput screening and combinatorial chemistry increase the possibility of drug discovery dramatically, natural products still offer unmatched structural variety. The capacity of plants to synthesize very complex molecules, which are impossible to invent by any chemist, gives reason to investigate plants for bioactivity.

In the search for new natural compounds it is first of all necessary to know which plant to select and what type of biological activity to look for. The fact that many plants play important cultural roles and have already undergone a traditional "human screening", favour the ethnomedical approach, which give credence to information on the medicinal and cultural uses of the plant. Certainly, the highly biologically active arrow poisons are many times more likely to possess therapeutically valuable compounds than are extracts from higher plants

selected at random. On the other hand, random bioprospecting has been employed by the industry and is largely based on a rational "hitphilosophy" without caring much about the ecological and cultural context of the plants screened. Since indigenous plant knowledge may hold clues for curing "western diseases", it is important to regulate the access and distribution of phytogenetic resources (including secondary metabolites) as well as the corresponding intellectual property rights [6].

I-3-The Family of Fabaceae

This family is also classified together with the Mimosaceae and the Caesalpinaceae as the Leguminosae, this is a cosmopolitan family with about 700 genera and 18000 species, and is one of the most important families, is characterized by a large number of derived traits. Most taxa of this Family are herbaceous, sometimes shrubby and only very rarely trees. Typically, the leaves are pinnate and sometimes the terminal one is modified to form a tendril, used for climbing. Bipinnate leaves are not found in this family. The five sepals are at least basally united. The corolla is formed of five petals and has a very characteristic butterfly-like shape (papilionaceous), with the two lower petals fused and forming a keel-shaped structure, the two lateral ones protruding on both sides of the flower and the largest petal protruding above the flower, being particularly showy. The androecium of ten stamens generally forms a characteristic tubular structure with at least nine out of ten of the stamens forming a sheath. Normally, the fruit are pods, containing beans (technically called legumes) with two sutures, which open during the drying of the fruit [7].

This large Family is characterized by an impressive phytochemical diversity. Polyphenols (especially flavonoids and tannins) are common, but from a pharmaceutical perspective various types of alkaloids are prabably the most interesting and pharmaceutically relevant groups of compounds [8]. In the genera ***Genista*** and ***Cytisus*** (both commonly called broom) as well as ***Laburnum***, quinolizidine alkaloids, including cytisine and sparteine, are common [9]. The hepatotoxic pyrrolizidine alkaloids are found in this Family (e.g. in membres of the genus ***Crotalaria***) [10].

Other important groups of natural products are the isoflavonoids, known for their oestrogenic activity, and the coumarins used as anticoagulants. ***Glycyrrhiza glabra*** **L.** (licorice) is used because of its high content of the triterpenoid glycyrrhic acid, which, if joined to a sugar, is called glycyrrhizin (a saponin) and is used in confectionery as well as in the treatement of gastric ulcers (controversial). Last but not least, the lectins must be mentioned. These large (M_W 40,000 – 150,000), sugar-binding proteins agglutinate red blood

cells and are a common element of the seeds of many species. Some are toxic to mammals, for example phasing from the common bean (***Phaseolus* spp.**), which is the cause of the toxicity of uncooked beans [9].

In ALGERIA it is presented by 53 genera and 339 species, which are mainly distributed in ***Genista*** and ***Cassia*** [11].

I-4-Important medicinal plants from the family of fabaceae [12]

-*Cytisus scoparius* (L.) Link: common or Scotch broom, which yields sparteine (formerly used in cardiac arrhythmias, as an oxytoxic, and in hypotonia to raise blood pressure.

-*Glycyrrhiza glabra* L. (liquorice): used as an expectorant and for many other purposes.

-*Melilotus officinalis* L. (melilot or sweet clover): The anticoagulant drug warfarin was developed from dicoumarol, first isolated from spoiled hay of sweet clover.

-*Physostigma venensum* Balfour (calabar bean): a traditional West African arrow poison, which contains the cholinesterase inhibitor physostigmine, used as a myotic in glaucoma, in postoperative paralysis of the intestine and to counteract atropine poisoning.

I-5-The Genus of *Cytisus*:

Cytisus is an ornamental genus , commonly called broom , it is presented by about 50 species mainly mediterranean , is native to central and south Europe , Canaries Isles and north Africa . The common name ' broom ' may have been given to the plant because of its growth habit.

The shrubs are 1-2 meters high and deciduous. The green branches are strongly angled and appear naked or almost so. The leaves are trifoliate, the yellow flowers are usually borne solitary in axils, blooming between April and June [13].

Six species represented in ALGERIA, the more abundant is ***Cytisus trifolus* L'her.** Other rare species which are ***Cytisus balansae* Boiss.** Et **Reut.** (***Cytisus purgans***), ***Cytisus fontanesi* Spach** (Ouarsenis, Bibans, …), ***Cytisus sessilifolius* L.** (Babor), ***Cytisus boeticus* Webb.** (North of ORAN) [14].

I-6-Definition and Systematic of Studied Plant:

Cytisus purgans is a shrub always more or less ramifies, a trifoliolate and persistent leaves and a yellow flowers, native to central France, north central Spain and Portugal, Algeria and southern Morocco [15**]**.

***Cytisus purgans* (*L*.) *Benth*.** (= ***Genista linifolia L*.**) is distributed in clear forest of coast of Algiers (Algeria) which is known to name of ' **Chahmet el Atrous** ' and ' **Hisbane** ' [16].

Cytisus purgans (***L***.) ***Boiss***. (***Genista purgans L***.) , is distributed in seaside cliff of France [17]. ***Cytisus balansae Boiss***. (***Cytisus purgans***) is a rare species of Algeria (Aures, Mahdids, and Lella Khadidja) [14].

I-7-Bibliographic Information concerning ' *Cytisus purgans* ':

A few studies concerning ' ***Cytisus purgans*** ':

- A study [18] of a condensed tannin content of several shrub species from a mountain area in northern Spain , and its relationship to various indicators of nutritive value , in this study , ***Cytisus purgans*** and seven other species were studied .

- Balandrin and coll. studied a distribution of alkaloids in some species of the papilonaceous tribes Thermopsideae and Genisteae (includes ***Cytisus purgans***). Quinolizidine alkaloids were main constituents [19].

- Pascol , J. and coll in Alkaloids of Orbanche rapum-genistea [20] studied the variation of the amount of sparteine and lupanine in the host-parasite relation , in this study , the lupanine content of broomrape parasitizing providence broom (***Cytisus purgans***) was higher than the sparteine content , but the contents of both alkaloids changed similarly during growth .

- The same researchers in ' Two new ecotypes of Orbanche rapum-genistae thuill ' [21] were studied the Morphological and Biochemical characteristics of O. rapum-cytisi purgantis parasitizing ***Cytisus purgans***.

-Balandrin, R. and coll. studied the application to the measurement of in situ symbiotic fixation, ***Cytisus purgans*** includes [22].

- Aurnhammer and coll. studied the L-rhamnosyl-D-glucosides of pincombrin (I) , sarotanoside , and isosarotanoside , isolated by different workers from from ***Cytisus commutatus*** and ***Cytisus purgans*** [23].

- Adzet, T. and coll. studied the alkaloids of some Genisteae species of Catalonia (Spain), includes ***Cytisus puragns*** [24].

- Plouvier, Victor. studied the structure of three flavonosides, isosarotanosides from ***Cytisus purgans*** [25].

- Finally, Paris, R. A. and Faugeras, G. studied the use of electrophoresis in the separation of alkaloids and flavonic compounds from some legumes, includes **C*ytisus purgans***

[26].

To our knowledge, this the first report on ***Cytisus purgans*** of Algerian flora.

II-Secondary metabolites

II-1- Alkaloids

Few, if any, classes of secondary metabolites have played a greater role in the evolution of organic chemistry than the alkaloids. The biological activity exhibited by many alkaloids ensured that the plants containing them were highlighted in folklore and herbal medicine and consequently many were of importance to man before any thought was given as to what it was that caused their bioactivity.

It is therefore hardly surprising that with the advent of experimental organic chemistry a great deal of effort was expended on trying to establish what made plants like henbane, stramonium, deadly nightshade (belladonna), hemlock, aconite, the opium poppy, cinchona, ipecauanha and colchicum have the properties they did. In the last century those who made contributions to an understanding of just what alkaloids were reads like a who's who of organic chemistry. Their achievements were considerable. A number of important alkaloids were obtained in more-or-less pure state including morphine, strychnine, quinine, clochicine, nicotine and coniine.

Identification of alkaloids had, by and large, to wait until this century. Coniine was both isolated and characterized by 1886 but strychnine, while first obtained in crystalline form in 1819, did not finally have its structure established until 1946 [27].

II-1-1-Definition

Unfortunately the term alkaloid is difficult to define. The original definition of an alkaloid, first proposed in 1879 by the pharmacist W. Meissner, encompassed compounds of complex molecular structure and significant pharmacological activity confined to the plant kingdom. This definition is clearly no longer tenable, and a recent definition (1982) due to Pelletier includes cyclic nitrogen-containing molecules which are true secondary metabolites (i.e. of limited occurrence, and produced by living organism). Simple acyclic derivatives of ammonia and simple amines are thus excluded, and the additional requirement, that the nitrogen atom must have a negative oxidation state, excludes nitro and nitroso compounds. Most compounds covered by this definition derive at least part of the structure from amino acids or their derivatives [28].

II-1-2-Methods for isolation and purification

Traditionally alkaloids have been extracted from tissues by means of a relatively polar solvent such as ethanol or by the process of basifying the tissues (with ammonia) and extracting with a less polar solvent such as dichloromethane. Chloroform, which is widely used, is probably best avoided, as impurities in the solvent can lead to the formation of *N*-oxides. Dichloromethane is probably a safer choice.

The base extraction process has some advantages in terms of giving a cleaner extract from which many alkaloids can be removed by simply shaking with dilute acid (2M HCl). It can, however, lead to the intrusion of nitrogen into non-nitrogenous compounds to form ‘alkaloid artefacts’ (e. g. gentianine) while non-basic and quaternary alkaloids will fail to extract into the organic solvent. The use of acid can also lead to problems of artefact formation.

By contrast, the direct ethanolic extract of an alkaloid-containing plant will contain far more extraneous material. This can be eliminated by concentrating the extract to dryness (or at least syrup) and then washing with dilute acid to remove the water-soluble alkaloid salts [27].

II-1-3- Detection of Alkaloids

Numerous reagents are recommended for simple ' spot test ' identification of alkaloids, most working best in a dilute aqueous acid solution of the alkaloid. Pride of place among these must go to Dragendorff’s Reagent but there are many others, such as iodoplatinic acid, Mayer’s Reagent, Hagar’s Reagent and Wagner’s Reagent. Mixing a drop of extract with a drop of the Reagent will lead to the development of a precipitate if alkaloids are present.

Great care must be taken with these spot tests because, while alkaloids will generally be characterised by strong reactions, other types of natural products are able to react to some degree (flavonoids, coumarines, tannins). So-called ' false positives ' can be eliminated by taking the alkaloid-containing acid extract, making it basic and extracting the alkaloid into an organic solvent. This is followed by separation of the organic phase and its back extraction into acid again. If the new acid extract still gives a positive reaction then the presence of alkaloid base is confirmed; although if the alkaloid had been non-basic or quaternary and hydrophilic it would be lost. In addition to these general color reagents there are many others that are said to be specific to certain alkaloid types. Today, their use is very limited [27].

Table 1: Some common spray reagents for detecting alkaloids [27].

Dragendorff Reagent	**(a)** 0.85g bismuth (III) nitrate in 10ml glacial acetic acid and 40ml water. **(b)** 8g potassium iodide in 20ml water. Mix (a) and (b) in equal portions to use as spray or spot test reagent.
Cerium sulphate	0.1g cerium (IV) sulphate in 4ml water. Add 1g trichloro acetic acid, boil and add drop wise 97% sulphuric acid until solution clarifies. Heat to produce colours.
Cobalt (II) thiocyanate	Ammonium thiocyanate (3g) and cobalt (II) chloride (1g) in 20ml water.
Iodoplatinic acid	**(a)** 5% hexachloro platinic (IV) acid solution **(b)** 10% potassium iodide solution. Mix 5ml (a) with 45ml (b).
Sodium tetra phenyl boron	1% sodium tetra phenyl boron solution in ethyl methyl ketone saturated with water.

II-1-4- Preliminary investigations of alkaloid extracts

A surprising number of alkaloids are sufficiently lipophilic that they can be pre-extracted with a non-polar solvent such as diethyl ether or petroleum ether. A few others, of which nicotine is the most notable, are volatile and may be readily isolated by steam distillation. However, in most cases isolation of alkaloids is usually initiated by one of the two extraction strategies outlined above.

Thin layer chromatography (TLC), for which there are an enormous number of published systems, offers a ready, cheap and simple method to examine the complexity of an alkaloid extract and on which to base a strategy for the chromatographic separation of individual compounds like as, vacuum column chromatography, followed by isolation of individual compounds using preparative TLC, column chromatography, high performance liquid

chromatography (HPLC) or ion-exchange chromatography. Gas chromatography is only applicable to a few compounds.

Advantages can sometimes be taken of differential solubility of various alkaloid salts to give separations by fractional crystallisation. Variation in the basicity of alkaloids can be exploited in their transfer between aqueous and organic phases. One classic example is morphine which contains a basic nitrogen centre and an acidic phenolic group. Because of this it remains soluble in alkaline aqueous solutions to a higher pH than do many of the other opium alkaloids [27].

II-1-5- Structure elucidation of alkaloids

As has already been noted, the characterisation of alkaloids provided a great stimulus for organic chemistry and many synthetic methods originate from the quest for alkaloid structures. The synthetic approach is, of necessity, a relatively slow process and has been largely supplanted by the use of spectroscopic and spectrophotometric procedures in structure elucidation. This was already taking hold in the early 1960s when Sangster and Stuart (1965) produced the first major compilation of alkaloid UV spectra. Also, at this time mass spectroscopy (MS) was being explored for the identification of complex alkaloids such as benzyl tetrahydro isoquinolines. The power of nuclear magnetic resonance (NMR) spectroscopy first came to the fore in assisting with the rapid identification of such complex alkaloids as the dimmers vincristine and vinblastine from ***Catharanthus roseus***. The current situation is that powerful NMR and MS procedures will, in most cases, resolve structures with little or no ambiguity other than with respect to absolute configuration. This is a cause for some concern as the relatively simple chromatographic, spectroscopic and physical date that aid the process of establishing co-identity are no longer of importance to the structure elucidation process and, as a consequence, their measurement is often neglected [27].

II-1-6-Biosynthesis

Just the characterisation of alkaloids was a major stimulus to synthetic organic chemistry, so the study of biosynthetic routes owes much to attempts to understand the way in which alkaloids are made by the producing organism. These have largely been achieved by making radioactive (^{14}C, ^{15}N) labelled compounds thought to be precursors, and then feeding these to the organism to be metabolised into alkaloid products. These are then isolated and the position of the label determined by chemical degradation. This approach has given some outstanding successes. Just one example is the discovery of that the quinoline alkaloids of cinchona, such as quinine, originate from the tryptophan-mevalonate route, something that is not obvious at first sight.

These investigations bring the realisation that most alkaloids, at least those found in higher plants, owe the origin of their nitrogen to one of a very small number of amino acids, namely tyrosine, phenylalanine, tryptamine, (anthranillic acid),(nicotinic acid), ornithine, lysine and (histidine). Those within brackets are of lesser importance. Where fungi and other types of organism are considered then further amino acids become involved, including valine and leucine.

There are exceptions to the rule that amino acids are incorporated and form the nitrogen source of alkaloids; in some alkaloids the nitrogen apparently arises from a simple source, such as ammonia. Among these the most populous groups are the steroidal and diterpene alkaloids, in which the nitrogen is added to compounds that are otherwise of terpenoid origin. Coniine is a simple example of an alkaloid that is essentially of acetate origin, while compounds like streptomycin are amino-sugars [27].

Although most studies on quinolizidine alkaloids have been concerned with their phytochemical characterisation, the knowledge of their biological background is already substantial. The biosynthesis from lysine via cadaverine to lupanine, sparteine, matrine, lupinine and cytisine has been studied by using radioactive isotopes, or more recently by using ^{13}C- and ^{2}H-labelled precursors [29]. In addition, a number of the enzymic steps involved have been characterised [30]. But many steps are still unclear and hardly anything is konwn about the pathways leading to the many substitutions and variations of the quinolizidine ring skeletons.

Quinolizidine biosynthesis takes place in the aerial green parts of legumes. Its intracellular site is the chloroplast [31]. Biosynthesis is regulated by light; thus QA concentrations display a diurnal rhythm, with a stimulated production period during the day [32].These alkaloids are translocated by the phloem to other parts of the plant [33], and they accumulate predominantly in epidermal and subepidetrmal cell layers [34]. The subcellular site of QA storage is the vacuole, into which the alkaloids are pumped by a selective carrier system [35]. Especially rich are the seeds, which can store up to 8% of their dry weight as alkaloids. During germination the alkaloids are translocated from the cotyledons to the newly formed tissues, where they are partly degraded, obviously serving as nitrogen storage compounds [36]. They also disappear from senescing leaves during the vegetation period, indicating that they are not end products but metabolically mobile compounds [37].

Quinolizidine alkaloids metabolism has been studied in cell and organ cultures of more than 14 legume species [38]. Quinolizidine alkaloid formation is reduced in undifferentiated cells cultured *in vitro*, this seems to be caused by a reduced expression of the biosynthetic enzymes, by limited storing and transport capacity, and by an increased degradation activity of the cells [39]. Whereas the differentiated plants produce a variety of QA, the alkaloid patterns of the cell

cultures are very simple since lupanine is always the main alkaloid [40]. Callus of ***Sophora flavescens*** accmulate matrine as the main product, and those of ***Thermopsis lupinoides*** lupanine. Alkaloid formation is correlated with light and greening of the cell culture. In organised tissues QA production can be observed in shoots and leaves but not in roots, which supports the view that the genes of QA metabolism are regulated in a tissue-specific way, as is the case with many other cellular phenomena [41].

II-1-7-Distribution of alkaloids

Serious attempts to use plant secondary metabolism to aid in classification can be traced back some 35 years. One of the most notable early successes was the taxonomic realignment of the Papaveraceae and Fumariaceae. Previously these two families had been considered to be part of the order Rhoedales, which otherwise contained families such as the Capparaceae and Crucifereae. The latter are rich sources of glucosinolates while the Papaveraceae and Fumariaceae are sources of a wide range of 1-benzyltetrahydroisoquinoline alkaloids but do not produce glucosinolates. The taxonomic significance of this was recognised by Hegnauer (1958, 1961), who pointed out that chemically the alkaloids of the Papaveraceae and Fumariaceae were identical to those found in a group of families variously known as the Ranales, Polycarpicae, or Annonales plus Ranunculales (e. g. Annonaceae, Berberidaceae, Lauraceae, Magnoliaceae, Menispermaceae, Ranunculaceae, etc.).

Another taxonomic grouping with a recognised alkaloid marker is the order Caryophyllales (Mabry, 1966), including such families as the Cactaceae, Portulacaceae and Amarantaceae. It is only in this group that the betalains are produced and today decisions on whether problem taxa do or do not belong in this order will take account of the presence or absence of betalains.

While most alkaloids types do have clearly defined centres of proliferation there are often outlying occurrences that are difficult or impossible to rationalise on systematic grounds. Pyrrolizidine alkaloids are predominantly to be found in the Boraginaceae and in parts of compositae (Asteraceae) and Leguminosae (Fabaceae). There are more limited centres of distribution in other families, such as the Sapotaceae, Ranunculaceae and in the Monocotyledonae. It is probably not reasonable to try to interpret pyrrolizidine alkaloids distribution in terms of a monophyletic origin. The same is true for the tropane alkaloids, where both the Solanaceae and Eryrthroxylaceae represrent discrete centres of development.

One further point makes the problem of using alkaloids as chemotaxonomic markers more difficult. Wink and Witte (1983), using a highly sensitive detection procedure, established the presence of trace amounts of quinolizidine alkaloids in many plant species, covering a wide

taxonomic range, where they were not previously known to occur. They suggested that the genetic encoding for production of quinolizidine alkaloids was actually far more widespread than had previously been recognised and that in many potential alkaloid-producing species the pathway was simply switched off. If this can be confirmed then it has major ramification for the use of alkaloids in systematic studies and in understanding their evolution and value to the plant [27].

II-1-8-Classification of alkaloids

There are two worthwhile methods of classifying alkaloids; one that links them by some part of their chemical structure, and one based on their route of biosynthesis, emphasising the amino acid from which they are derived. Table 2 lists the major structural classes of alkaloids and their biosynthetic origins, including the cyanogenic and glucosinolate compounds [27].

II-1-8-1-Quinolizidine alkaloids

II-1-8-1-1-Structures

Quinolizidine alkaloids (QA), of which more than 170 structures have been reported (Table 3), are distinct from other alkaloids in that they contain at least one quinolizidine ring system [42]. According to the degree of substitution , at least eight groups can be distinguished (Figure 2) : (**1**) the lupinine type ; (**2**) the leontidine type ; (**3**) the sparteine /lupanine /multiflorine type ; (**4**) the α-pyridone type ; (**5**) the matrine type ; the ***Ormosia*** type (**6**), (**7**) piperidine and dipiperidines , and (**8**) miscellaneous structures , which often occur together with QA [43] .

Table 2: Typical alkaloids and allied compounds; structural classes and biosynthetic origin [27].

Compound	Structural class	Biosynthetic origin
Cyanogens	Linamarin	Valine
	Prunasin	Phenylalanine
Glucosinolates	Sinigrin	Homoserine
	Glucotropeolin	Phenylanaline
Pyrrolidine	Hygrine	Ornithine
Tropane	Hyoscine	Ornithine
Pyrrolizidines	Heliotine	Ornithine
Piperidine	Pelletierine	Lysine
	Coniine	Acetate
Quinolizidine	Sparteine	Lysine
Lycopodium alkaloids	Annotinine	Lysine
Pyridine	Arecoline	Nicotinic acid
Quinoline	Equinopsine	Anthranilic acid
	Quinine	Tryptophan +C_{10}
	Gentianine	C_{10} – (artefact)
Quinazoline	Vasicinone	Anthranilic acid
Phenylethylamine	Mescaline	Phenylalanine/tyrosine
Isoquinoline	Lophophorine	Phenylalanine/tyrosine
	Emetine	Tyrosine + C_{10}

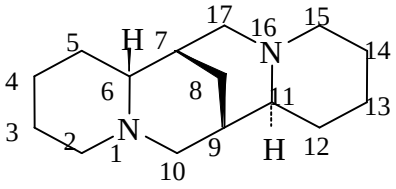
Table 2_ continued.

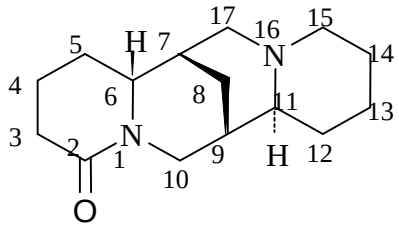
1-Benzyl(tetrahydro)iso-quinoline	Morphine Clochicine	Phenylalanine/tyrosine
Amaryllidaceae alkaloids	Lycorine	Phenylalanine/tyrosine
Mesembrine alkaloids	Mesembrine	Phenylalanine/tyrosine
Tryptamine derivates	Psylocybin	Tryptophan
	Physostigmine	Tryptophan
Diketopiperizines	Sporidesmin A	Tryptophan
Carbolines	β-Carboline	Tryptophan
Ergot alkaloids	Ergonovine	Tryptophan + C_5
Indole alkaloids	Vincristine, Vinblastine	Tryptophan + C_{10}
Imidazoles	Pilocarpine	Histidine
Indolizidines	Castanospermine	Lysine/ornithine
Terpene-derived alkaloids	Sesquiterpenes-castoramine	C_{15}
	Diterpenes-atisine	C_{20}
	Steroidal-conessine	C_{30}
Spermidines	Plaustrine	Putrescine
Purines	Caffeine	Nucleotide catabolism
Amino-sugars	Steptomycin	Phenylalanine/tyrosine
Betalains	Betanidin	Proline

II-1-8-1-2-Nomenclature

For practical purposes, QA are generally known by their trivial names, usually derived from the names of their source plants. Systematic chemical names are unwieldy and are infrequently used. A useful note on the systematic naming of the QA is given.

The sparteine is named as derived of diazocine (Dodecahydro-7, 14-methano-2H, 6H-dipyrido [1, 2-a: 1', 2'-e] diazocine, 9CI.) . The lupanine is named as derived of sparteine (2-Oxo-11α-sparteine). Systematics names can accordingly be derived for esters and hydroxylated compounds [28].

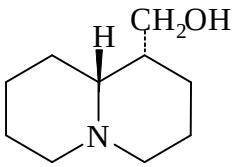




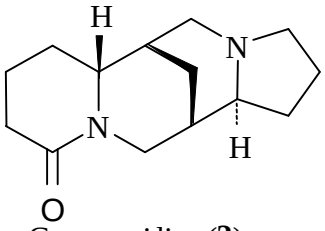
Dodecahydro-7, 14-methano-2H, 6H-dipyrido [1,2-a : 1',2'-e] diazocine, 9CI.

2-Oxo-11α-sparteine

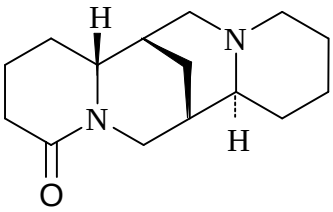
Figure 1: Nomenclature of quinolizidine alkaloids [44].



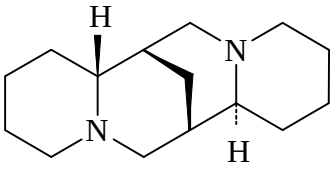
Lupinine (**1**)



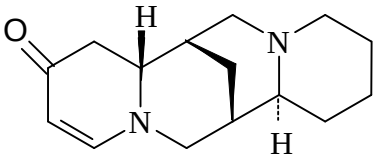
Camoensidine (**2**)



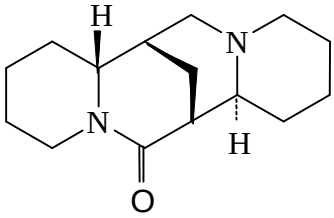
Lupanine (**3a**)



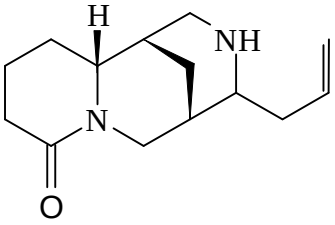
Sparteine(**3b**)



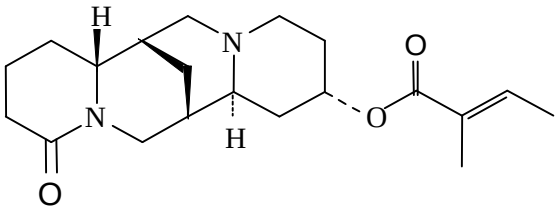
Multiflorine (**3c**)



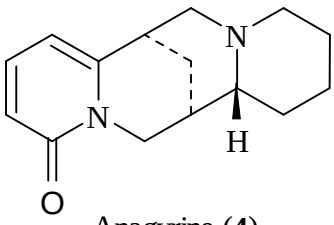
Aphylline (**3d**)



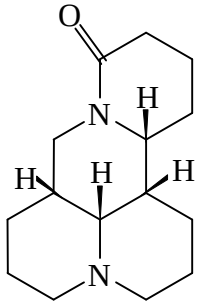
Angustifoline (**3e**)



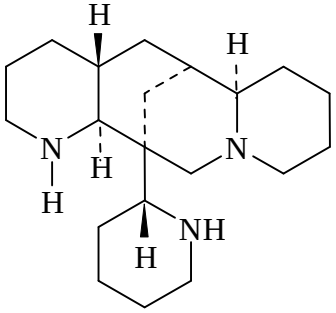
13α –Tigloyl-oxylupanine (**3f)**



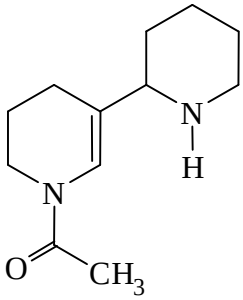
Anagyrine (**4**)



Matrine (**5**)



Piptanthine (**6**)



Ammodendrine (**7**)

Figure 2: Structural types of quinolizidine alkaloids [45].

II-1-8-1-3-Natural Distribution of quinolizidine alkaloids

Quinolizidine alkaloids are mainly distributed within the Leguminosae. Quinolizidine-accumulating tribes are considered ' primitive ' within the Leguminosae and are the Sophoreae , Dalbergieae , Euchresteae , Thermopsidae , Genisteae , Bossiaeae , Brongniartieae , Podalyrieae , Liparieae and Crotalarieae [43] . However, restricted occurrences have been reported from a number of other families which are unrelated to the Leguminosae such as Berberidaceae (***Leontice***, ***Caulophyllum***), Solanaceae, Ranunculaceae (***Cimicifuga***), Chenopodiacae (***Anabasis***) and Rubiaceae (***Readea***). Parasitic or hemiparasitic plants can tap the phloem and xylem sap of their host plants. If the host plant contains QA an alkaloid transfer is plausible, as has been observed for a number of species of the Scrophulariaceae: ***Orobanche*** [39], Castilleja [46] and ***Pedicularis*** [47], but also in other families such in ***Viscum*** (Loranthaceae) [48], Cuscuta (Cuscutaceae) [49] and ***Osyris*** (Santalaceae) [50]. Table II-3 presents an overview of the distribution of some QA on the generic level.

Plant cell suspension cultures of ***Daucus carota*** (Umbelliferae) , ***Conium maculatum*** (Umbelliferae) , ***Atropa belladonna*** (Solanaceae) , ***Chenopodium rubrum*** (Chenopodiaceae) , ***Spinacia oleracea*** (Chenopodiaceae) and ***Symphytum officinale*** (Boraginaceae) have been observed to produce minute amounts of lupanine , especially when challenged with elicitors [51] . Using a very sensitive radioimmunoassay (RIA) (see section I-2-4) for lupanine we found traces of lupanine in c. 50 out of 100 plants studied [52]. The next step will be to determine the nucleotide sequences of the corresponding genes, to see whether and how the genes and plants are related. These surprising findings suggest that the genes of QA biosynthesis are widely distributed within plant kingdom, but are generally ' silent '. Only in the Fabaceae (Leguminosae) are the genes for biosynthesis, transport and storage turnred on, since here the alkaloids serve for chemical defence.

The different structural types (Figure 3) of QA are not evenly distributed within the Leguminosae; common to all tribes are QA of the lupanine (**3a**, **3e**, **3f**) and sparteine (**3b**) type. Multiflorine (**3c**), aphylline (**3d**) and derivatives are only present in a few genera.

α-Pyridone (**4**) are also common but more restricted than type **3a** and **3b**. Lupinine and derivatives (**1**) have been detected in a comparably small number of species. Leonitidane-type alkaloids (**2**) seem to be very specialised and have as yet been found only in ***Camoensia***, ***Melolobium*** and ***Maackia*** (Table 3).

Table 3: Structures and natural distribution of quinolizidine alkaloids [45].

Alkaloid	Genus	Type
Lupinine (-)	***Lupinus , Thermopsis , Calpurnia , Virgilia , Genista , Anabasis[a] , Goebelia***	1
Camoensidine	***Maackia, Camoensia***	2
Lupanine	***Cytisus (+ , -) , Genista , Lupinus (+ , -) Diplotropis , Ormosia , Maackia (-) , Dalbergia , Templetonia (+) , Thermopsis , Baptisia (+) Laburnum , Melolobium , polhillia , Agyrolobium , Lebeckia , Dichilus , Wborgia , Rothia , Aspalathus, Rafnia, Pearsonia, Lotononis, Pedicularis[a], Pericopsis, Haplormosia. Bolusanthus, Virgilia, Cadia, Chamaecytisus, Echinospartum Ammodendron, Hovea (-), Liparia (-), Loutus , Petteria , Piptanthus (+) , Podalyria (+) Echinosophora (-) , Sophora (-) , Orobanche[a] , Spartium, Ulex , Anagyris , Retama (-) , Leontice[a] (-), Viscum[a], Thermopsis (+)***	3 a
Sparteine	***Leontice[a] (+) , Cytisus (- , +) , Genista (+ , -) , Thermopsis (+) Melolobium , Polhillia , Laburnum , Argyrolobium , Lebeckia , Spartidium , Wiborgia , Aspalathus , Rafnia , Pearsonia , Lotononis , Ormosia (-) , Pericopsis , Haplormosia , Bolusanthus , Sophora (+) , Cadia Chamaecytisus , Hovea (+) , Liparia , Piptanthus (+ , -) , Retama (+) , Adenocarpus (-) , Ammodendron (+) , Ammothamnus (+) , Anagyris (+) , Baptisia (+) , Keyserlingia (+) , Lupinus (- , +) , Thempletonia , Spartium , Chamaecytisus , Orobanche[a] .***	3b
Multiflorine	***Lupinus (-)***	3c
Aphylline	***Lupinus, Teline, Anabasis[a], Podalyria***	3d
Angustifoline	***Lupinus, Diplotropis, Ormosia, Cytisus***	3e
13 α-Tigloyloxylupanine	***Rothia , Ormosia , Calpurnia , Cadia , Lupinus, Cytisus , Orobanche***	3f
Anagyrine (-)	***Genista , Lupinus , Maackia , Sophora , Templetonia , Thermopsis , Baptisia , Teline , Ulex , Laburnum , Dichilus , Melolobium, Polhillia, Argyrolobium, Pearsonia, Castilleja[a] , Pedicularis[a] , Retama , Ormosia , Percopsis , Haplormosia , Bolusanthus , Clathrotropis , Osyris[a] , Chamaecytisus , Echinospartum , Hovea , Piptanthus , Anagyris , Cytisus , Petteria , Spartium , Ulex , Euchresta , Echinosophora , Viscum[a]***	4
Matrine	***Sophora (+) , Goebelia , Keyserlingia , Euchresta (+)***	5
Piptanthine	***Hovea (+) , Ormosia , Templetonia (+ , -) , Piptanthus***	6
Ammodendrine[b] (+)	***Lupinus , Ormosia , Maackia , Sophora , Templetonia , Cytisus , Retama , Thermopsis , Laburnum , Dichilus , Spartidium , Lebeckia , Rothia , Pearsonia , Lotononis , Pericopsis , Haplormosia , Castelleja , Genista , Echinospartum , Ammodendron , Liparia , Orobanche , Spartium , Baptisia***	7

The sign after plant refers to the optical rotation of the alkaloid. [a] not a member of the Fabaceae; [b] not a quinolizidine alkaloid , but co-occuring with QA .

Matrines (**5**) are common in the genus ***Sophora*** and ***Ormosia*** type alkaloids are especially common in the genus ***Ormosia***. Although the alkaloids from Lycopodiales (***Lycopodium***), Lythraceae (***Heimia***, ***Decodon***), Nymphaeaceae
(***Nuphar***) and from ladybird beetles (Coccinellidae) contain a quinolizidine ring system they are not reviewed here, due to lack of space [53].

A number of insects are spcialised on host plants which contain QA. Whereas some Coleoptera and Pentatomidae have been shown to excrete the dietary alkaloids [54], a number of oligophagous aphids (e.g. ***Macrosiphum albifrons***, ***Aphis genistae*** and ***A***. ***cytisorum***) actively store QA in their bodies and seem to use them for their own defence against predators [56]. Larvae of the moth ***Uresiphita reversalis*** store cystisine and N-methylcytisine, which is present in their host plant ***Teline monspessulana*** [57]. The uptake of QA seems to be specific and carrier mediated [58].

II-1-8-1-4- Extraction

It is important to ensure that, as far as possible, alkaloids isolated from plants or other sources are qualitatively and quantitatively the same as those which were present in the original material. There are several ways in which alkaloids might be altered during isolation procedures.

Alkaloids may be changed by heat or by enzymeic action when harvested plants are stored and dried. Bull and *call*. reported the loss of 50-80% of alkaloids during drying of some, but not all, ***Crotalaria*** and ***Heliotropium*** species [59]. On the other hand, Pderson [60] compared the alkaloid content of fresh leaves with that of dried leaves from several species of the Boraginaceae; allowing for the loss in weight on drying there were no changes in total alkaloid content, suggesting that enzymeic oxidation or reduction of alkaloids could take place during the early stages of drying.

If fresh green plant material cannot be extracted straight away, it should be derived as quickly as possible to minimize enzyme action and fungal decomposition. A warm environment, with plenty of circulating air, is best, direct suglight should be avoided because of possible overheating, the alkaloids are not especially light-sensitive. After drying, the plant should be stored in a cool place [10].

It is common practice to dry plant material prior to extraction. Since QA may be modified during the drying process (e.g. hydrolysis of ester alkaloids), it is recommended that material is freeze-dried (which avoids decompartmentalisation and thus enzymic breakdown processes) or that fresh material or material stored frozen at -20°C is used.

The extraction of QA is based on the fact that the free alkaloidal base does not dissolve in water but in an apolar organic solvent, such as ether or methylene chloride. In contast, the charged alkaloids (most are charged molecules under acidic conditions) are soluble in water but insoluble in apolar organic solvents. Both the free base and the salts dissolve in MeOH.

Plant powder is extracted with MeOH for several hours, which is conveniently performed with a Soxhlet apparatus. The methanolic extract is then evaporated to dryness under reduced pressure in a rotavapor. For some applications (HPLC, TLC) this extract can be used directly. Normally, these extracts need to be further purified: the dried residue is dissolved in 0.5 M HCl and extracted at least three times with methylene chloride (until a clear organic phase is obtained) in a shaking funnel to remove lipid material. Then the aqueous phase is made alkaline with 4N NH_4OH and extracted with methylene chloride using either solid-phase extraction or liquid-liquid extraction [45].

II-1-8-1-5-Separation

Since most plants contain complex mixtures of up to 30 or more individual alkaloids, Thin Layer Chromatography (TLC), with its limited resolution power, is helpful primarily for initial analysis of the major alkaloids and for preparative work. A quantitative TLC solvent systems are adequate for silica gel G plates has been described by Karlsson and Peter [61].

In Table II-4 R_f Values of some representative QA are tabulated. They can be visualised with ' Dragendorff's ' reagent as orange red spots; KI/I_2 produces dark brown stains. Plates need to be dried abd heated (20min at 110°C) prior to visualisation in order to remove the ammonia or diethyl amine which interfere with the colour reaction [45].

Table 4: Selection of TLC R_f values [45].

Alkaloid	**R_f value**		
	System 1	**System 2**	**System 3**
Lupinine	0.25	0.36	0.52
Camoensidine	0.16	0.31	0.39
Lupanine	0.30	0.69	0.51
Sparteine		0.07	0.93
Multifloine	0.27	0.35	0.26
Aphylline	0.60	0.80	0.52
Angustifoline	0.41	0.63	
$13a-$ Isolupanine	0.51	0.69	0.50
Anagyrine	0.49	0.66	0.30
Matrine	0.26	0.52	0.35
Ammodendrine	0.17	0.23	0.37
Retamine	0.25	0.18	0.65

System 1 : MeOH –28% NH_4OH (65:1) .

System 2: $CHCl_3$–MeOH–28% NH_4OH (85:15:1).

System 3: Cyclohexane –diethylamine (70:30).

For preparative work QA can be separated by liquid chromatography on silica (70-230 or 230-400 mesh, Merck) with the following solvent systems: CH_2Cl_2–MeOH –25% NH_4OH (70:30:1) [62] or CH_2Cl_2–MeOH –25% NH_4OH (90:9:1) [63] or diethyl ether–MeOH–25% NH_4OH (100:30:1) [61]. Arslanian et *al*. [64] used a basic AL_2O_3 column (Merck, Type T) with solvents of increasing polarity, starting with ether, then 10% MeOH in ether, 50% MeOH in ether, and finally MeOH.

Saito et *al.* [65] were isolated seven quinolizidine alkaloids including: (-)3β-hydroxy-l3α-tigloyloxylupanine, (-)3β,13α-dihydroxylupanine, (+)13α-tigloyloxylupanine, (+)13α-hydroxylupanine, (+)lupanine, (-)sparteine and (-)I 7-oxosparteine from the ethanolic extract of seedlings of *Cytisus scoparius* using open column chromatography with the following conditions: silica gel 60 (Merck, 230-400 mesh, 40 g) column (2 x 20 cm), solvents gradients of increasing polarities of CH_2Cl_2, MeOH-28% NH_4OH (90:9:0- 90:9: 1).

High performance liquid chromatography (HPLC) has been employed to the analysis of QA mixtures. Saito et *al*. [38] Used an Inertsil ODS (5μm) column (150 x 4.6mm) with the solvent system acetonitrile–mM potassium phosphate buffer (pH 5.5) (1:9). Murakoshi et *al*. [63] used LiChrosorb Si 100 (Merck; 10μm, 0.3 x 50cm) with the solvent system 15% MeOH in Et_2O–H_2O–28% NH_4OH (500:10:3). Saito et *al*. [66] separated 22 of these alkaloids on LiChrosorb Si 60 (5μm) (250 x 4.6mm) with the solvent systems 15% or 25 % MeOH in diethyl ether–5% NH_4OH (25:1), or 50% MeOH in diethyl ether–5% NH_4OH (25:2). R_t values are presented in [66] and [67].

Detection can be performed using refractive index (RI) or UV detectors. Since most QA have no sensitive chromopore, UV detection is performed between 205 and 220nm. α-Pyridone alkaloids (Fig. 2, type **4**), such as cytisine or anagyrine, have an aromatic ring and can be monitored at 310nm [66].

Using preparative columns, HPLC is a convenient technique for isolating sufficient amounts of individual alkaloids (only spateine, lupinine, α-isolupanine and cytisine are commercially available) for spectroscopic or biochemical purposes [67]. Wink have successfully employed a semi-preparative column (300 x 7.8mm; 10μm material, μ-Bondapak $C_{18;}$ waters), using an isocratic system with the solvent system 0.01M phosphate buffer–20% MeOH–20% isopropanol [62]. Murakoshi et *al*. [63] and Ohmiya et *al*. [67] used Lichrosorb Si 100 (Merck ; 10μm , 0.3 x 50 cm) with the solvent system 15% MeOH in Et_2O–H_2O –28% NH_4OH (500:10:3) .

Until 1980 most gas–liquid chromatography (GC) applications to separate QA mixtures involved packed columns. However , best results can be obtained using the new generation of fused silica capillary columns with bonded phases : The capillary columns (dimensions 15m x 0.23mm to 30m x 0.32mm) have a higher number of theoretical plates (> 70 000) , which allow the separation of complex mixtures (Fig. II-3) and even of enantiomers , epimeric at C-11 or C-6 , such as sparteine and *a* -isospartiene , lupanine and *a* -isolupanine ,13-hydroxylypanine and 13-epihydroxy-lupanine , anagyrine and thermopsine ,13*a* -tigloyloxylupanine and 13*b* -tigloyloxylupanine , and of *cis* and *trans* isomers , such as 13*a* -Angeloyloxylupanine and 13*a* -Tigloyloxylupanine , as well as the *trans*- and *cis*-cinnamic acid esters [68] . Furthermore, even nanogram amounts of QA can be detected by FID (flame ionisation detector) or more sensitively and specifically by a nitrogen specific detector (abbreviated PND or NPD). Therefore, for most biochemical and phytochemical applications, capillary GLC seems to be the method of choice for QA analysis. A further convenience is the possibility of performing GC-MS analyse [45] (section II-1-8-1-6-B).

As a liquid phase several silicone derivatives (0.1µm or 1µm films) are employed , good resolutions have been obtained using DB-1 or DB-5 columns (J & W Scientific) , but equivalent products of other manufacturers also work . Split injection techniques are usually appropriate. On-column injection does not provide significant advantages for most applications. Helium is routinely used as carrier gas, but hydrogen or nitrogen will also work. The injector temperature is usually set to 250°C, that of the detectors to 300°C [57].

Hydroxylated QA, sush as 13-hydroxylupanine or 4-hydroxylypanine, may be derivatised by TMS prior to injection to avoid tailing and to achieve better quantification. Care should be taken not to use the PND for these derivatives, since the PND would soon be destroyed [45].

Some authors give relative retention indices for QA (e.g. [69]). However, Kovats retention indices (RI) [70] give better comparative information and are helpful in identifying individual alkaliods in a GC profile. Some representative RI indices are given in Table 5.

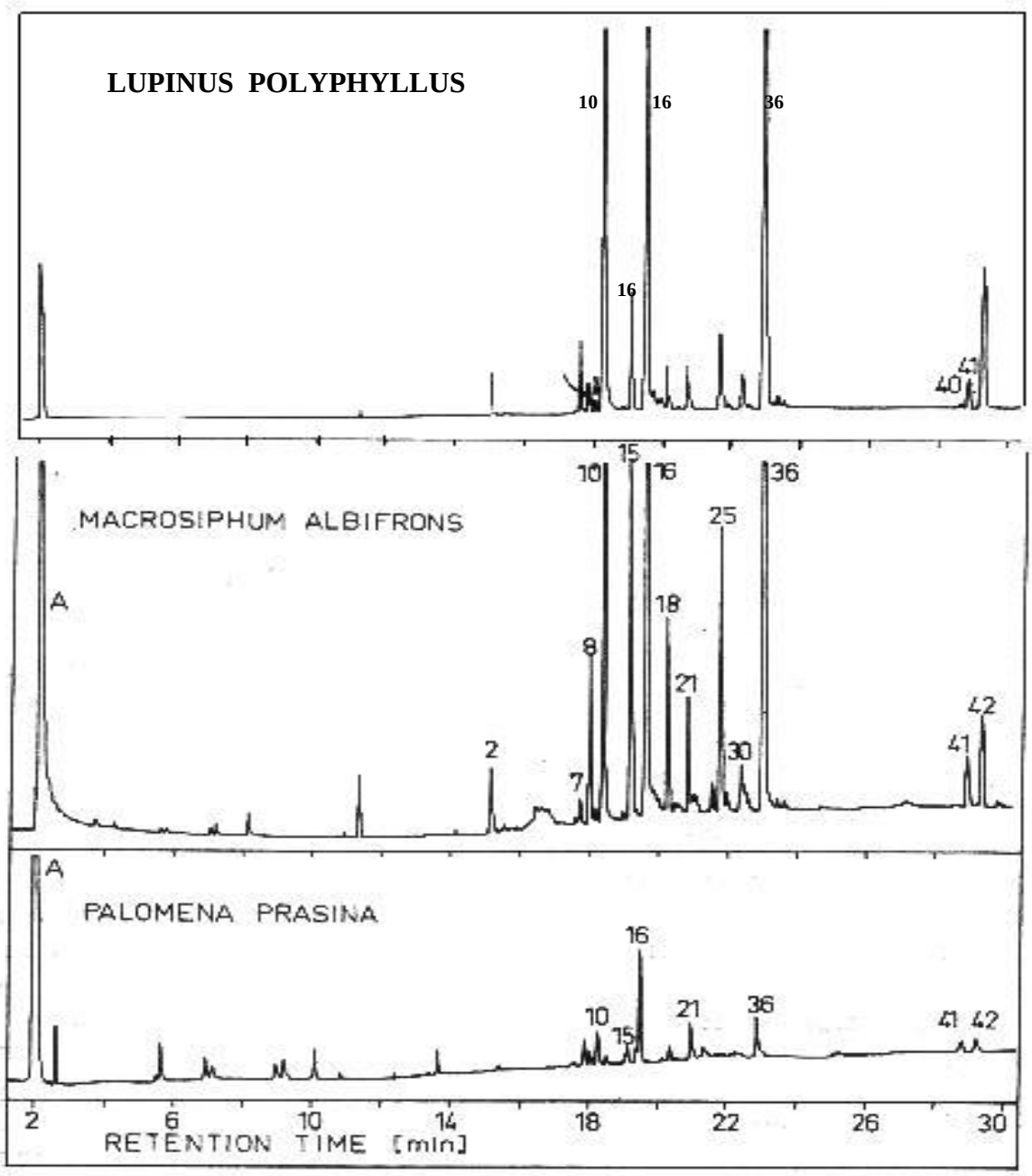


Figure 3: Separation of QA of *Lupinus polyphyllus* and two infesting insects by capillary GLC [45].

Key: 2, ammodendrine; 7, 'isoangustifoline'; 8, tetrahydrohombifoline; 9, 'dehydroangustifoline'; 10, angustifoline; 15, dehydrolupanine; 16, lupanine; 18, 11, 12-12, 13-didehydromultiflorine (*N*-methylalbine); 21, nuttaline; 25, multiflorine; 36, 13α-hydroxlupanine; 40, 13α (2-methylbutyryl) oxylupanine; 41, 13α-angeloyloxylupanine; 42, 13α-tigloyloxylupanine.

II-1-8-1-6-Characterisation

A-Ultraviolet and Infrared Spectroscopy

Only the *a* -pyridone alkaloids with an aromatic ring A and the cinnamoyl derivatives have a reasonable chromophore; thus UV spectroscopy does not normally provide much information for the identification of QA [71]. Until *c*. 1970 infrared spectroscopy was an important method for the identification of QA; such data are found in many papers [72]. Since MS and NMR are more powerful methods, infrared spectroscopy has been replaced by these to a large degree. Optical rotation measurements and circular dichroism have been employed to determine the absolute configuration of QA by, among others, [73] and [74].

B-Mass Spectroscopy

Following the pioneering work of Schumann et *al.* [75] and Neuner-Jehle et *al*. [76 , 77] who worked out the principal fragmentation patterns of the most important QA , mass spectrometry (MS) developed into a convenient method of QA characterisation [78] . Mass spectrometry is widely used today, since QA usually provide distinctive fragmentation patterns in the electron impact mode (EI-MS). Chemical ionisation (CI-MS) , field desorption (FD-MS) and fast atom bombardment (FAB-MS) are suitable for identifying molecular ions of QA esters and tricyclic alkaloids , whose molecular ions are usually obscure or absent in EI-MS spectra . A major advantage of MS is the possibility it gives of combining the high resolution power of capillary GC with sensivity and informativity of EI- or CI-MS. Work using GC-MS was very much facilitated after 1980 by the development of new GC capillary columns, the development of new methods to position the GC column outflow near the MS ion source and, most importantly, by the improved possibilities of data processing. During a GC-MS run, MS spectra are recorded continuously, thus a typical GC-MS run consists of up to 1000 spectra. These data can easily be processed by computer, with background subtraction or the profiling of individual and charateristic fragment ions (‘ion chromatography ’), which are helpful to select the relevant MS spectra. A normal plant extract contains a number of hydrocarbons and contaminants, such as phthalic acid derivatives, which can be identified because of their distinctive m/z 149 fragment [79].

In Table 5 the molecular ion and the four most informative fragments and their abundances are tabulated. For CI-MS data the molecular ion $(M+H)^+$ is given. The data for FAB-MS were reported, among others, [29]. Field desorption and chemical ionisation techniques are also helpful in the direct analysis of alkaloid mixtures [80].

Table 5: Identification of representative quinolizidine alkaloids using capillary GLC and mass spectrometry. For each compound the following information is provided (is available) : name ; Kovats retention index (RI) ; molecular ion M^+ ; EI-MS , five significant fragments and their relative abundance (%) ; CI-MS [45] .

Compound	RI		EI – MS					CI-MS
Lupinine	1422	169	152 (100)	169 (90)	148 (82)	97 (85)	136 (70)	170 (100)
Camoensidine	2080	234	122 (100)	135 (56)	234 (52)	233 (44)	84 (52)	
Lupanine	2165	248	136 (100)	149 (60)	248 (40)	150 (34)	219 (8)	249 (100)
Sparteine	1785	234	137 (100)	98 (90)	234 (44)	193 (25)	84 (10)	235 (100)
Multiflorine	2310	246	134 (100)	246 (65)	148 (20)	110 (15)	217 (5)	247 (100)
Aphylline	2180	248	136 (100)	220 (45)	124 (40)	248 (35)	191 (20)	249 (100)
Angustifoline	2083	234	193 (100)	112 (50)	150 (15)	55 (20)	134 (5)	235 (100)
13α-Acetyl-oxylupanine	2450	306	246 (100)	134 (70)	148 (30)	112 (39)	55 (30)	307 (100)
13α- Angeloyloxylupanine	2733	346	246 (100)	134 (30)	148 (15)	112 (12)	55 (10)	347 (100)
Anagyrine	2390	244	98 (100)	244 (40)	146 (20)	160 (15)	136 (15)	245 (100)
Matrine	2265	248	248 (100)	205 (80)	96 (70)	150 (60)	247 (75)	249 (100)
Ammodendrine[a]	1865	208	165 (100)	136 (60)	123 (60)	208 (55)	191 (50)	209 (100)

Laser desorption mass spectrometry (LAMMA 1000) is a soft ionisation method which produces significant $(M – H)^+$ ions and informative fragment ions. This method has been used to localise the site of QA storage in plant tissue; sparteine could be located in the epidermal and subepidermal cell layer of ***Cytisus scoparius*** stems. Lupanine is accumulated in the epidermis of petioles of ***Lupinus polyphyllus*** [81].

Alkaloid extracts of many legumes contain piperidine alkaloids such as ammodendrine, *N*–methylammodendrine, hystrine or smipine. These alkaloids also derive biogenetically from lysine via cadaverine [82]. Mass spectral information is provided in Table II-5.

C-Nuclear Magnetic Resonance Spectroscopy

Proton and carbon-13 nuclear magnetic resonance (NMR) techniques are essential and extremely powerful methods for the structure elucidation of new QA and are often used in combination with MS techniques. The only disadvantage is that some NMR techniques still require relatively large amounts of pure compounds (>10mg), which need to be first isolated from complex mixtures. In consequence , NMR techniques are usually employed for the analysis of the major alkaloids , which can be purified more easily , whereas most minor alkaloids ,

which are often very important for chemosystcmatics or other biological questions , have only been caracterised by MS , since in GC-MS studies only µg amounts are required . A representative cross-section of ^{13}C NMR data for QA is illustrated in Fig.I-3 [45].

Data for ^{2}H and ^{1}H NMR have been reported [83].

D-Ray Crystallography

In a number of recent investigations QA have been studied crystallographically by X-ray diffraction techniques [64].

II-1-8-1-7-Qantification

Many biochemical and agricultural problems (e.g. determination of QA contents in Lupin seeds, which are used for animal nutrition), require either the quantification of total alkaloids or of every single QA. For total alkaloid analysis a number of different methods are available. Prior to analysis the alkaloids need to be extracted, preferably by solid phase extraction as described above. The methods that can be used for analysis are: (1) gravimetry; (2) titrimetry with *p*-toluenesulphonic acid, and (3) photometry with bromocresol purple or I_2/KI [84].

Immunological methods such as radioimmunossay (RIA) and enzyme-linked immunossay (ELISA) have been established only recently. These allow the quantification of nanogram amounts of lupanine-type alkaloids. In principle, antibodies are raised in rabbits against bovine serum albumin to which 13-hydroxylupanine is coupled over a succinic acid bridge. These antibodies are selective for lupanine-type alkaloids, but discriminate sparteine, lupinine, cytisine and tricyclic alkaloids (Fig.II-4). A respective RIA, SPA (scintillation proximity assay) and ELISA have been worked out [85]. A comparable ELISA has also been developed in Australia.

Methods (1) – (3) listed above work if the alkaloid contents in the extracts are in excess 100µg g^{-1}. Radio immune assay and ELISA techniques are especially suitable for large screenings, when only minute amounts of material are available, and if the alkaloid sample consists mainly of lupanine-type alkaloids (as is the case in many lupins) [45].

If individual alkaloids in the extracts are to be analysed, then HPLC, or preferably GC, is the method of choice. If possible, reference alkaloids should be used to work out calibration procedures [66]. For practical purposes the commercially available alkaloids (sparteine, lupinine, cytisine, and α-lupanine) are usually adequate. Quantification can either be performed by the internal or external standard procedure [45].

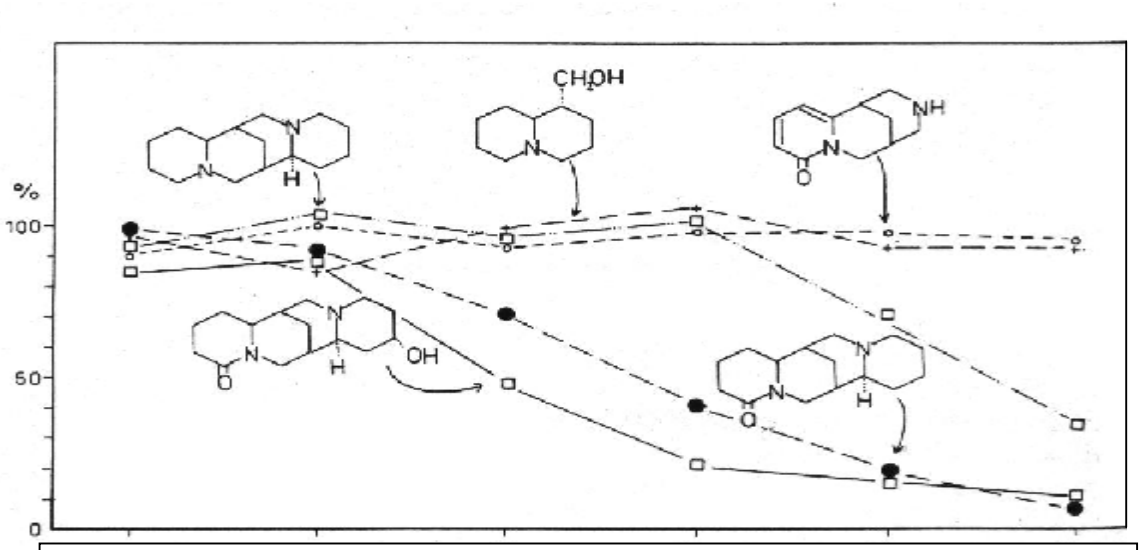


Figure 4: Selectivity and sensitivity of a lupanine RIA. Scintillation proximity assay (SPA) was performed with fluomicrospheres coupled to anti-rabbit antibodies according to the procedure of Amersham. Specific antibodies were raised in rabbits which detect lupanine and 13α-hydroxylupanine, whereas lupinine and cytisine are completely discriminated. Sparteine is recognised at higher alkaloid concentrations [45].

II-1-8-1-8-Biological activities

QA have a wide variety of biological activities. They are toxic or inhibitory for most organisms. Sparteine is used therapeutically as an antiarrythmic drug and in obstetrics for facilities birth child, whereas lupinine and matrine have been in use in folk medicine in Eastern Asia. A summary of reported pharmacological activities is given in Table 6.

Stockmen in the western U.S. have recognized the toxicity of lupines since the late 1800s, and large livestock loses, particularly in sheep, were reported in Montana and other western states. Signs include central and peripheral nervous system stimulation followed by depression, frequent urination and defecation, dilated pupils, trembling, in coordination, excessive salivation and nervousness. These progresses to muscular weakness recumbency, and eventually death from respiratory paralysis. Signs may appear as early as one hour after ingestion and progressively get worse over the course of 24 to 48 hours even if further ingestion does not occur. Generally, if death does not occur within this time frame, the animal recovers completely. The clinical signs of poisoning are presumed to be due to quinolizidine and piperidine alkaloids.

The pyridone alkaloids such as cytisine and anagyrine are more acute toxic than the corresponding saturated alkaloids such as sparteine and lupanine. Both cytisine and anagyrine have been implicated as teratogens in higher animals, and it has been shown that anagyrine has mutagenic properties and produces malformations ("crooked calf disease") in early foetal stages.

Common molecular targets for QA are nicotinic and muscarinic acetylcholine receptors and Na^+, K+ -ion channels, besides protein biosynthesis. Variation in structural diversity is thought to enhance toxicity (i.e. enabling the attack at several targets) and to reduce the chance that a herbivore will develop resistance towards alkaloids.

Little is known about individual alkaloid toxicity; however, 14 alkaloids isolated 38 from ***Lupinus albus***, ***L. mutabilis***, and ***Anagyris foetida*** were analyzed for their affinity to nicotinic and/or muscarinic acetylcholine receptors. Of the 14 compounds tested, the a-pyridones (N-methylcytisine and cytisine) showed the highest affinities at the nicotinic receptor (IC_{50}: 0.05 and 0.14 µM, respectively), as measured by displacement of radio-labeled ligand H-nicotine, while several quinolizidine alkaloid types including the teratogen anagyrine showed weak activity at the muscarinic receptor (IC_{50}: 132 µM). Lupanine which is widely distributed in legumes as a major alkaloid, displayed an IC50 of 5 µm at the nicotinic receptor and is 100 times more active than hydroxylated lupanines or alkaloids of the multiflorine series. Because nicotinic and muscarinic receptors are widely distributed within the body, a number of tissues and organs will be affected, to a certain degree, if an animal consumes these plants. Additionally, QA such as lupanine and sparteine inhibit Na^+ and K^+-channels, thus blocking the signal transduction in nerve cells at a second critical point. This might potentiate the toxicity caused by binding of the alkaloids to the AChreceptors.

Alkaloids such as sparteine have long been known to possess antiarrythmic ("cardiotonic") and uterotonic properties in both experimental animals and in humans. Experimental evidence indicates that the pharmacological actions of sparteine, especially on the utems, are mediated via prostaglandins. Its actions have also been postulated to be related to their ability to bind or chelate divalent cations such as calcium. Diamines such as sparteine and its steroisomers can act as ligands for divalent cations such as magnesium and calcium. In addition, sparteine has been implicated in membrane processes involving potassium conductance. Studies with quinolizidine alkaloids such as sparteine, multiflorine, and lupinine have established that they have a depressant effect on the central nervous system of experimental animals. In addition, human subjects who cannot rapidly metabolize sparteine show signs of CNS activity, such as diplopia, blurred vision, dizziness, and headache.

The syndrome known as "crooked calf disease" is associated with lupine ingestion and was first reported in the late 1950s and is associated with various skeletal contracture-type birth defects and occasionally cleft palate. Through epidemiologic evidence and chemical comparison of teratogenic and non-teratogenic lupines, the QA anagyrine was determined to be the teratogen. If one compares binding affinities of anagyrine in nicotinic versus muscarinic

receptors, there is 16 times greater binding affinity to muscarinic receptors. Perhaps information about the maternal or fetal mechanism of toxicity or teratogenicity of anagyrine can be applied from this comparison [6].

Table 6: Examples of biological activity of quinolizidine alkaloids [6].

Alkaloid	**Type of activity**
Sparteine	Oxytocic; uterotonic, antiarrythmic, diuretic, respiratory depressant/stimulant, hypoglycemic, inhibition of natural killer cell growth, bacteriostatic, chemical defense, inhibition of seed germination
Lupanine	Antiarrythmic, hypotensive, hypoglycemic, inhibtion of ß-glucosidase, bacteriostatic, chemical defense, inhibition of seed germination
Cytisine	Teratogenic, respiratory stimulant (nicotine-like activity), hallucinogenic, oxytocic; uterotonic, inhibition of edema, myopathy, nematocidal, chemical defense
Anagyrine	Teratogenic, inhibition of acetylcholinesterase, myopathy, nematocidal
N-Methylcytisine	Hypoglycemic, inhibition of edema, myopathy
Retamine	Uterotonic, hypotensive, diuretic
13-Hydroxylupanine	Antiarrythmic, hypotensive, bacteriostatic
Calpurine	Ichthyotoxic, antiarrythmic, hypotensive
Multiflorine	CNS depressant, antidiabetic
Matrine	Antipyretic, cardiotonic, antiulcerogenic
5,6-Dehydrolupanine	Myopathy
Thermopsine	Myopathy
Sophoridine, Allomatrine, Sophoramine	Cardiotonic

Table 6 _Continued.

Sophoramine N-oxide, Epilupinine N-oxide	Hypoglycemic
17-Oxosparteine	Inhibition of edema
Ammodendrine	Inhibition of acetylcholinesterase
Matrine N-oxide	Antiulcerogenic
Augustifoline	Bacteriostatic

II-2-Polyphenols

Polyphenols are natural products which are recognized as one of the largest and most widespread class of plant constituents occurring throughout the plant kingdom, and are also found in substantial levels in commonly consumed fruits, vegetables and beverages.

Polyphenols have recently aroused considerable interest because of their potential beneficial biochemical and antioxidant effects on human health. Commonly referred to as antioxidants, they may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases, inflammation and others. Most of the experimental results confirmed that polyphenols have several biological activities including radical scavenging, anti-inflammatory, anti-mutagenic, anti-cancer, anti-HIV, anti-allergic, anti-platelet and antioxidant activities.

Chemically, polyphenols are diverse group of naturally occurring compounds containing multiple phenolic functionalities. They constitute a large and still expanding complex family of molecules, with diverse structures, properties and sizes ranging from monomers to polymers. Polyphenols gather a range of weakly acidic substances possessing aromatic rings bearing hydroxyl substituents and constitute one of the most numerous and widely distributed groups of substances with more than 8000 phenolic structures currently known.

Polyphenols can be divided into different classes depending on their basic structure. The main classes of polyphenols are phenolic acids and flavonoids while the less common are stilbenes and lignans. Major subclasses present in food correspond to anthocyanins, flavanols, flavones, flavanones, flavonols and isoflavones [3].

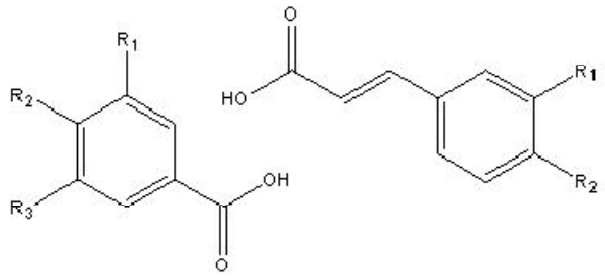
II-2-1-Definition and classification of polyphenols

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups.

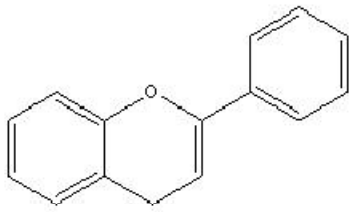
Dietary phenolics or polyphenols constitute one of the most numerous and widely distributed groups of natural products in the plant kingdom. More than 8000 phenolic structures are currently known, and among them over 4000 flavonoids have been identified . Although polyphenols are chemically characterized as compounds with phenolic structural features, this group of natural products is highly diverse and contains several sub-groups of phenolic compounds. Fruits, vegetables, whole grains and other types of foods and beverages such as tea, chocolate and wine are rich sources of polyphenols. The diversity and wide distribution of polyphenols in plants have led to different ways of categorizing these naturally occurring compounds. Polyphenols have been classified by their source of origin, biological

function, and chemical structure. Also, the majority of polyphenols in plants exist as glycosides with different sugar units and acylated sugars at different positions of the polyphenol skeletons [86].

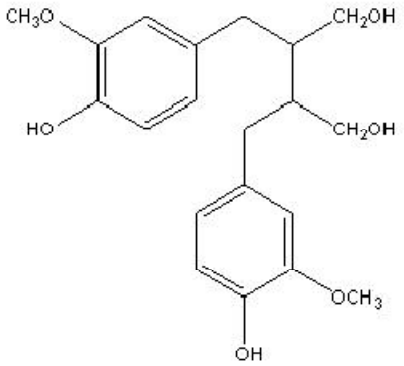
Although a large variety of plant phenols exists, most of these compounds arise from a common origin: the amino acids phenylalanine or tyrosine. These aminoacids are deaminated to cinnamic acids,which enter the phenylpropanoid pathway. A key step in this biosynthetic route is the introduction of one or more hydroxyl groups into the phenyl ring. As result, these compounds are derived from a common carbon skeleton building block: the C6-C3 phenylpropanoid unit. Biosynthesis, according to this pathway, produces the large variety of plant phenols: cinnamic acids (C6-C3), benzoic acids (C6-C1), flavonoids (C6-C3-C6), proanthocyanidins [(C6-C3-C6)*n*], coumarins (C6-C3), stilbenes (C6-C2-C6), lignans (C6-C3-C3-C6) and lignins [(C6-C3)*n*] [87]. The main phenolic structures are shown in Figure 5.



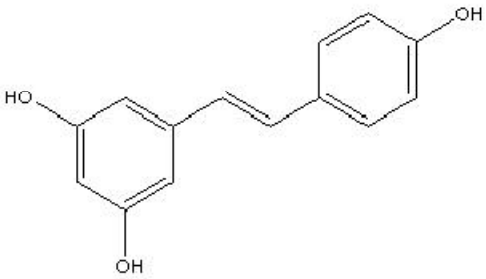
Phenolic acids (hydroxy-benzioc & cinnamic acids)



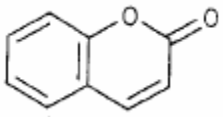
Flavonoids



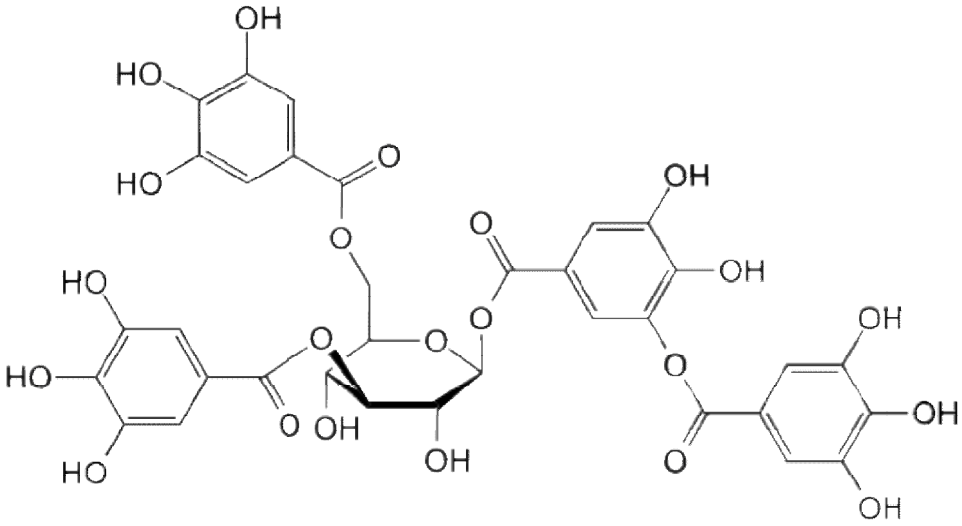
Lignans



Stilbenes



Coumarin



Tannic acid, a type of tannin

Figure 5: Main classes of polyphenols [88, 89, and 90].

II-2-2-Flavonoids

In the plant kingdom, different plant families have characteristic patterns of flavonoids and their conjugates. All these compounds play important biochemical and physiological roles in the various cell types or organs (seed, root, green part, and fruit) where they accumulate. Different classes of flavonoids and their conjugates have numerous functions during the interactions of plant with the environment, both in biotic and abiotic stress conditions. Additionally, flavonoid conjugates, because of their common presence in plants, are important components of human and animal diet.

Due to the different biological activities of plant secondary metabolites, their regular consumption may have serious consequences for health, both positive and negative.

For the mentioned reasons, methods for the efficient and reproducible analysis of flavonoids play a crucial role in research conducted in different fields of the biological and medical sciences. The identification and structural characterization of flavonoids and their conjugates isolated from plant material, as single compounds or as part of mixtures of structurally similar natural products, create some problems due to the presence of isomeric forms of flavonoid aglycones and their patterns of glycosylation. A number of analytical methods are used for the characterization of flavonoids [91].

II -2-2-1-Definition, nomenclature and structure

Flavonoids (or bioflavonoids) (from the Latin word *flavus* meaning yellow, their color in nature) and their conjugates form a very large group of natural products. They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs.

Chemically, they have the general structure of a 15-carbon skeleton, which consists of two phenyl rings (A and B) and heterocyclic ring (C) [92].

The chemical structures of this class of compounds are based on a C6-C3-C6 skeleton. They differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns (Figure 6). The flavonoids may be modified by hydroxylation, methoxylation, or *O*-glycosylation of hydroxyl groups as well as *C*-glycosylation directly to carbon atom of the flavonoid skeleton. In addition, alkyl groups (often phenyls) may be covalently attached to the flavonoid moieties, and sometimes additional rings are condensed to the basic skeleton of the flavonoid core. The last modification takes place most often in the case of isoflavonoids, where the B ring is condensed to the C-3 carbon atom of the skeleton.

Flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules. These derivatives are thermally labile and their isolation and further purification without partial degradation is difficult. The multiplicity of possible modifications of flavonoids result in more than 6,000 different compounds from this class was known in the end of the last century and this number continues to increase [91].

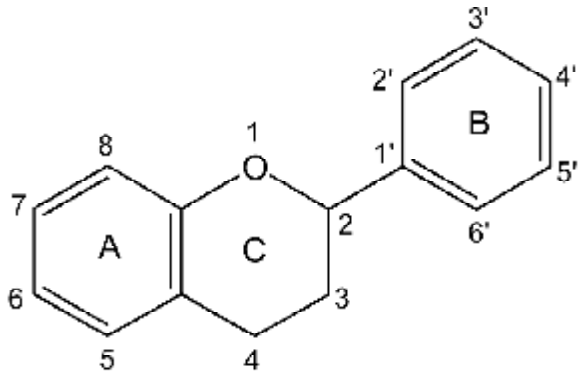
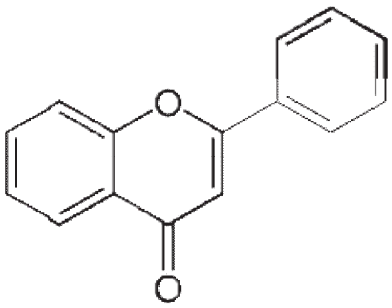


Figure 6: General structure of flavonoids [93].

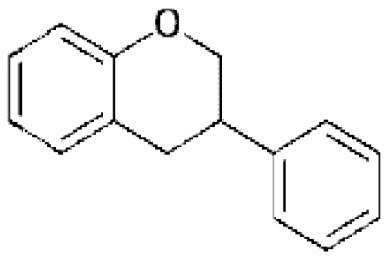
II -2-2-2-Classification of flavonoids

There are many ways to classify flavonoids, due to the position of ring B, there are: [92]

a-Flavonoids or biflavonoids derived from 2-phenylchromen-4-one

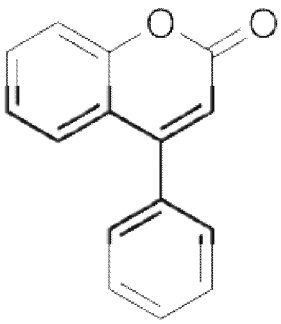


b-Isoflavonoids derived from 3-phenylchromen-4-one

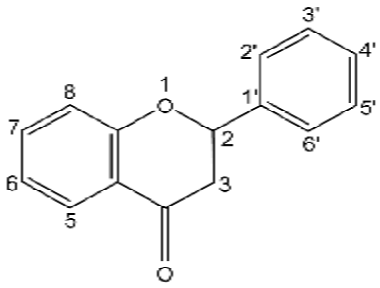


Isoflavan

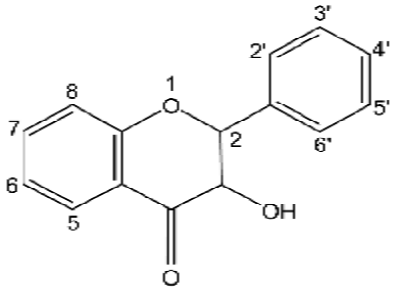
c-Neoflavonoids derived from 4-phenyl coumarin



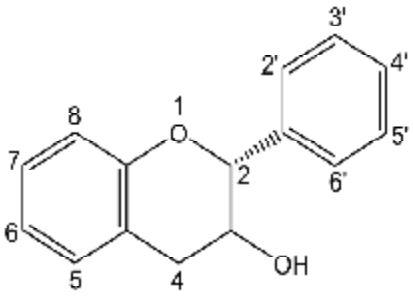
Due to their substitution, flavonoids may be classified as: [93]



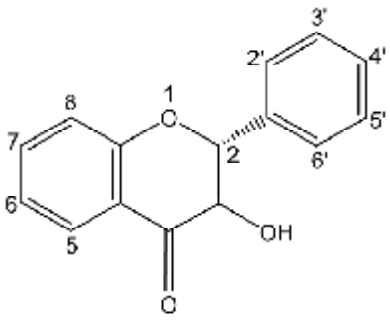
Flavones



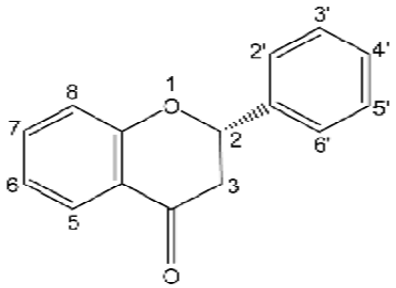
Flavonols



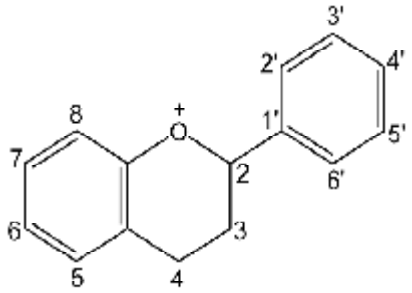
Flavan-3-ols



Flavanonol



Flavanones



Anthocyanidins

II -2-2-3-Biosynthesis of flavonoids

Flavonoids are synthesized by the phenylpropanoid metabolic pathway in which the amino acid phenylalanine is used to produce 4-coumaroyl-CoA. This can be combined with malonyl-CoA to yield the true backbone of flavonoids, a group of compounds called chalcones, which contain two phenyl rings. Conjugate ring-closure of chalcones results in the familiar form of flavonoids, the three-ringed structure of a flavone. The metabolic pathway continues through a series of enzymatic modifications to yield flavanones → dihydroflavonols → anthocyanins. Along this pathway, many products can be formed, including the flavonols, flavan-3-ols, proanthocyanidins (tannins) and a host of other various polyphenolics [94].

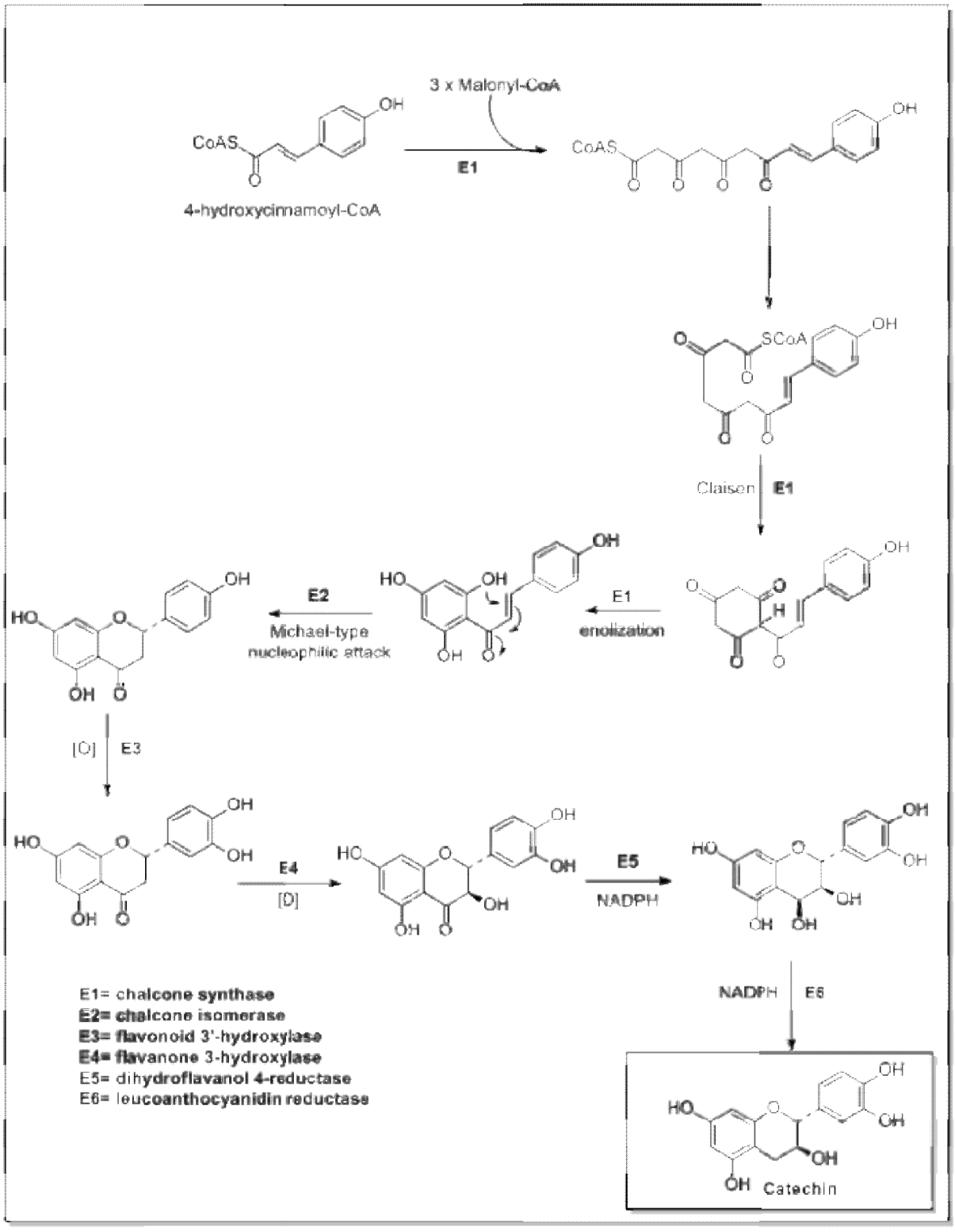


Figure 7: Possible pathway of biosynthesis of flavonoids [95].

II-2-3-Phenolic acids

Phenolic acids are non-flavonoid polyphenolic compounds which can be further divided into two main types, benzoic acid and cinnamic acid derivatives based on C1–C6 and C3–C6 backbones (Figure III-1). While fruits and vegetables contain many free phenolic acids, in grains and seeds—particularly in the bran or hull—phenolic acids are often in the bound form. These phenolic acids can only be freed or hydrolyzed upon acid or alkaline hydrolysis, or by enzymes [87].

II-2-4- Lignans

The term "Lignan" was first introduced by Haworth (1948) to describe a group of dimeric phenylpropanoids where two C6-C3 are attached by its central carbon (C8), as shown in Figure III-1. More recently, Gotlieb (1978) proposed that micromolecules with two phenylpropanoid units coupled in other manners, like C5-C5´ for example should be named "neolignans". According to Gordaliza et al (2004), lignans can be found in more than 60 families of vascular plants and have been isolated from different plant parts, exudates and resins.

Most of the known natural lignans are oxidized at C9 and C9´ and, based upon the way in which oxygen is incorporated into the skeleton and on the cyclization patterns, a wide range of lignans of very different structural types can be formed. Due to this fact, lignans are classified in eight subgroups and, among these subgroups, the furan, dibenzylbutane and dibenzocyclooctadiene lignans can be further classified in "lignans with C9 (9´)-oxygen" and "lignans without C9 (9´)-oxygen[96]

The interest in lignans and their synthetic derivatives is growing because of potential applications in cancer chemotherapy and various other pharmacological effects [97]

II-2-5-Stilbenes

The name for stilbene (1,2-diphenylethylene) was derived from the Greek word stilbos which means shining [98].

Stilbene compounds are part of a vast group of natural defense polyphenols occurring in many plant species. The name for stilbene was derived from the Greek word stilbos, which means shining. These compounds are structurally characterized by the presence of a 1,2-diphenylethylene nucleus with hydroxyls substitued on the aromatic rings, and exist in the form of monomers or oligomers[99].

Low quantities of stilbenes are present in the human diet, and the main representative is resveratrol, that exists in both *cis* and *trans* isomeric forms, mostly in glycosylated forms. It

is produced by plants in response to infection by pathogens or to a variety of stress conditions. It has been detected in more than 70 plant species, including grapes, berries and peanuts. The fresh skin of red grapes is particularly rich in resveratrol (50-100 g/kg net weight) which contribute to a relatively high concentration of resveratrol in grape juice Extensive data provide evidence for anticarcinogenic effects of resveratrol [97].

II-2-6- Coumarins

Coumarins owe their class name to 'Coumarou', the vernacular name of the tonka bean (*Dipteryx odorata* Willd, Fabaceae), from which coumarin, it was isolated in 1820.

Coumarins are classified as a member of the benzopyrone family. All of which consist of a benzene ring joined to a pyrone ring. The benzopyrones can be subdivided into the benzo-alfa-pyrones to which the coumarins belong and the benzo-gama-pyrones, of which the flavonoids are principal members. Umbelliferone, esculetin and scopoletin are the most widespread coumarins in nature. During the synthesis of these compounds ortho-hdroxylation should respectively take place on p-coumaric, caffeic and ferulic acid. The coumarins are of great interest due to their pharmacological properties. In particular, their physiological, bacteriostatic and anti-tumor activity makes these compounds attractive backbone derivatisation and screening as novel therapeutic agents [89].

To this days, around 1300 coumarins are known, with all of them being derivatives of 5,6-benzo-2-pirone (*α*-chromone) (with OH, OCH3 or CH3 substituents on the benzoic ring.). In addition to simple coumarins, *C*-prenylated and *O*-prenylated forms exist.

As derivatives of simple coumarins, other compounds are known, such as furanocoumarins, which include a furanic ring, linear pyranocoumarins, angular pyranocoumarins, dimeric coumarins, of which dicoumarol is an example and also furanochromones [100].

II-2-7-Tannins

The term tannin (from *tanna*, an Old High German word for oak or fir tree, as in Tannenbaum) refers to the use of wood tannins from oak in tanning animal hides into leather; hence the words "tan" and "tanning" for the treatment of leather. However, the term "tannin" by extension is widely applied to any large polyphenolic compound containing sufficient hydroxyls and other suitable groups (such as carboxyls) to form strong complexes with various macromolecules[101].

Tannins are another major group of polyphenols in our diets. The designation of tannin includes compounds of two distinct chemical groups: hydrolysable tannins (polymers of

ellagic acid, or of gallic and ellagic acids, with glucose) and condensed tannins, which result from the condensation of monomers of flavan-3-ol units [100].

Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. The great variety in the structure of these compounds is due to the many possibilities in forming oxidative linkage. Intermolecular oxidation reactions give rise to many oligomeric compounds having a molecular weight between 2,000 and 5,000 Daltons.

Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions [102].

II-3-Biosynthesis of polyphenols

They are mainly synthetized from cinnamic acid, which is formed from phenylalanine by the action of L-phenyloalanine ammonia-lyase PAL (EC 4.3.1.5), the branch point enzyme between primary (shikimate pathway) and secondary (phenylopropanoid) metabolism.The significance of this route can be supported by the fact that, in normal growth conditions, 20% of carbon fixed by plants flows through this pathway (figure III-4) [103].

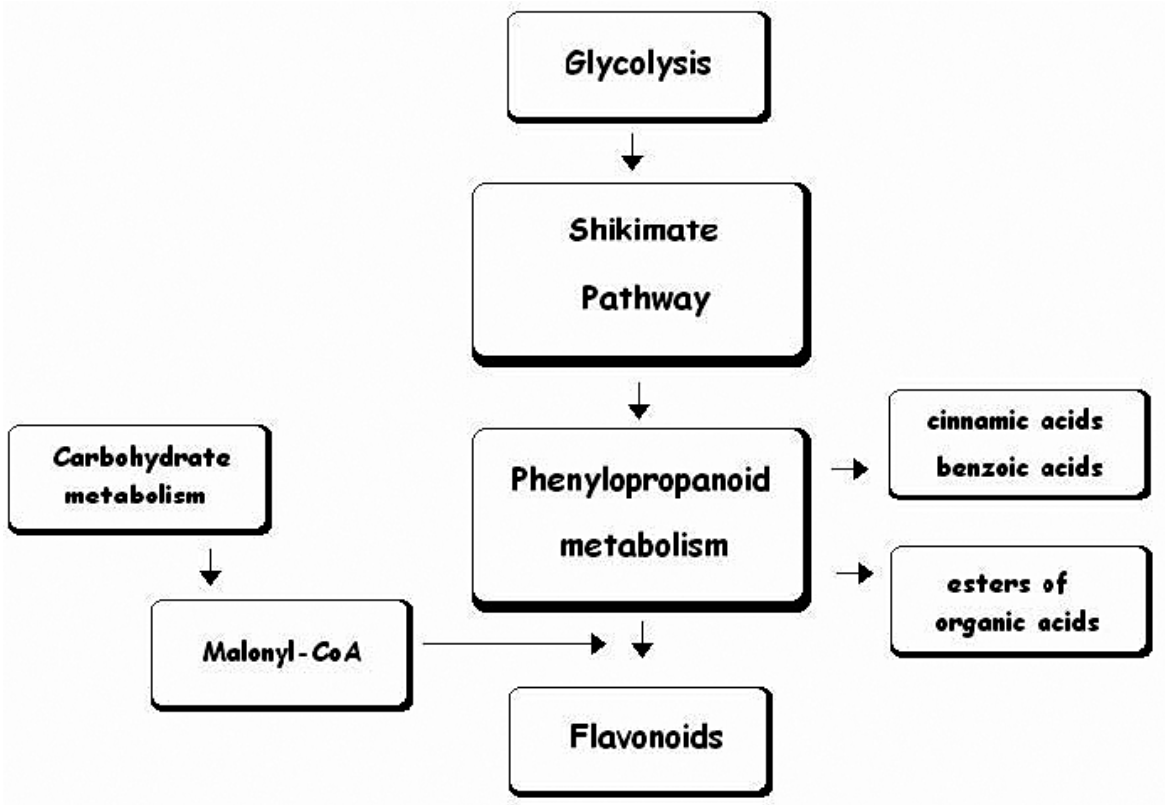


Figure 8: Biosynthesis pathways leading to formation of main groups of phenolic compounds [103].

II-4-Resources of natural polyphenols

More than 10,000 polyphenol compounds have been identified in various plants. Natural polyphenols have been found in many plants and foods, such as fruits, vegetables, tea, cereals, medical plants, microalgae, and edible and wild flowers. Vegetables are important sources of daily diets and rich in polyphenols. In addition, Mediterranean diets are associated with reduced risk of cardiovascular disease due to adequate intake, especially, of olive oil which contained high contents of polyphenols. The pigmented cereals, such as black rice, red rice and purple rice, possessed high total phenolic contents. *Salvia miltiorrhiza* Bge. (danshen), *Polygonum multiflorum* Thunb. (stem) (flowery knotweed), *Rhodiola sacra* Fu (hongjingtian), *Fraxinus rhynchophylla* Hance (ash), and *Prunus persica* (Linn) Batsch. (peach) showed high contents of natural polyphenols among medical plants. *Rosa rugosa* (rose), *Limonium sinuatum* (myosotis), *Pelargonium hortorum* (geranium), *Jatropha integerrima* (peregrina) and *Osmanthus fragrans* (osmanthe) were found to contain lots of natural polyphenols among edible and wild flowers. Furthermore, the contents of polyphenols widely fluctuate between genotype, cultivar, harvest time, and even within organic or conventional.

A number of factors could affect the content of polyphenols in daily food, such as environmental condition, storage, and food processing. Generally, phenolic acid concentrations decreased during ripening, whereas anthocyanins concentrations increased. Inappropriate cooking methods caused significant reduction in the contents of polyphenols. However, the evidence was emerging that bioavailability of many protective compounds was enhanced when the vegetables are cooked. Storage also affected the content of polyphenols. A study showed that the antioxidant activities in extra virgin olive oil would decrease after eight months of storage in closed bottles in the dark, which were from loss of phenolic compounds. On the other hand, because of the hydrolysis of complex phenols, an increase in hydroxytyrosol and tyrosol contents during storage was reported [88]. Table 7 shows the main classes of plant polyphenols, structures, sources, their specifications and biological properties [104].

Table 7: The main classes of plant polyphenols, structures, sources, their specifications and biological properties.

	Examples	**Sources**	**Specifications**	**Main biological properties**
Phenolic acids and coumarines				
Hydroxybenzoic acids C6-C1	Gallic acid, Vanillic acid, Protocatechuic acid, *p*-Hydroxybenzoic acid	Tea Red fruit (raspberry, black currant, strawberry)	Very common, in free form as well as combined, not much studied and not considered to be of great nutritional interest, sensitive to temperature, oxidation, light and pH, water soluble	Very limited therapeutic interest, antimicrobial activity and fungitoxicity, anti-inflammatory properties of salicylates
		Fruit (kiwis, blueberries, apples) Cereal grains (wheat, rice, oat flours)	Fruit (kiwis, blueberries, apples) Cereal grains (wheat, rice, oat flours)	
Coumarines	Ombelliferone Aesculetin, Scopoletin	Tonka bean, bark (chestnut), medicinal plants (*Melilotus officinalis*, *Angelica officinalis*)	Free coumarines are soluble in alcohols and organic solvents, the heterosidic forms are less soluble in water	Anti-inflammatory and antiviral activities, limited pharmacological applications: hepatotoxicity
Stilbenes C6-C2-C6	Resveratrol	Medicinal plants	Found only in low quantities in the human diet	Anticarcinogenic effects, anti-inflammatory activity
Flavonoids C6-C3-C6				

Table 7 _ continued.

Flavonols	Myricetin, Quercetin, Kaempferol and their glycosylated forms	Fruit and vegetables (Onions, curly kale, leeks, broccoli, blueberries), red wine and tea	Flavonols are the most ubiquitous flavonoids in food	
Flavones	Aspigenin, Luteolin, Tangeretin, Nobiletin, Sinensetin	Parsley, celery, cereals (millet and wheat) Skin of citrus	Flavones are much less common than flavonols in fruit and vegetables	
Flavanones	Hesperetin, Naringenin, Eriodictyol	Citrus fruit (grapefruit, orange, lemon), tomatoes and some aromatic plants (mint)	Sensitive to oxidation, light and pH, bitter taste	Vitamin P factor protecting capillaries and veins, often anti-inflammatory, antiallergenic, antiviral, anti-spasmodic, antibacterial, antioxidant and anti-carcinogenic properties, hepatoprotector, some are powerful enzymatic inhibitors
Isoflavones	Genistein, Daidzein, Glycitein	Leguminous plants (soya and its processed products)	Structural similarities with estrogens confers pseudohormonal properties	
Flavanols				

Table 7 _ continued.

Monomer form	Catechin, Epicatechin	Fruit (apricot, cherry, grape, peach, apple), green and black tea, red wine and cider	Sensitive to oxidation, light and pH, astringent and bitter taste, slightly soluble in water	
Polymer form Proanthocyanidins (C15)n	Castalin, Vescalin	Fruit (grapes, peaches, kakis, apples, berries), beverages (wine, cider, tea, beer), chocolate	Responsible for the astringent character and bitter taste, sensitive to high temperature and oxidation, water and alcohol soluble	
Anthocyanins	Cyanidin, Pelargonidin, Delphinidin, Petunidin	Red wine, some varieties of cereals, some leafy and root vegetables (aubergines, cabbage, beans, onions, radishes), flowers and most abundant in fruit	Plant pigments, highly sensitive to temperature, oxidation, pH and light, water soluble	
Lignans (C6-C3)2	Pinoresinol, Podophyllotoxin, Steganacin	Flax seed, sesame seed, cereals (rye, wheat, oat, barley), cruciferous vegetables (broccoli, cabbage), and fruit (apricots, strawberries)	One of the major classes of phytoestrogens, relatively stable under normal conditions, water soluble, unpleasant flavour	Hepatoprotector, antimitotic, antiviral, antihypertensive and cytostatic activities, inhibitors of enzymatic reactions

II-5-Extraction, separation and characterization

II-5-1-Extraction

The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals. Phenolics can be extracted from fresh, frozen or dried plant samples. Usually before extraction plant samples are treated by milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying. Generally, freeze-drying retains higher levels of phenolics content in plant samples than air-drying.

Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. It is generally known that the yield of chemical extraction depends on the type of solvents with varying polarities, extraction time and temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples. The solubility of phenolics is governed by the chemical nature of the plant sample, as well as the polarity of the solvents used.

There is no universal extraction procedure suitable for extraction of all plant phenolics. Depending on the solvent system used during exaction, a mixture of phenolics soluble in the solvent will be extracted from plant materials. It may also contain some non-phenolic substances such as sugar, organic acids and fats. As a result, additional steps may be required to remove those unwanted components.

Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted. In particular, methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone. Ethanol is another good solvent for polyphenol extraction and is safe for human consumption.

The recovery of phenolic compounds from plant materials is also influenced by the extraction time and temperature, which reflects the conflicting actions of solubilization and analyte degradation by oxidation. An increase in the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate. In addition, the viscosity and the surface tension of the solvents are decreased at higher temperature, which helps the solvents to reach the sample matrices, improving the extraction rate. However, many phenolic compounds are easily hydrolyzed and oxidized. Long extraction times and high temperature increase the chance of oxidation of phenolics which decrease the yield of

phenolics in the extracts.. Therefore, it is of critical importance to select efficient extraction procedure/method and maintain the stability of phenolic compounds.

The conventional extraction methods such as maceration and soxhlet extraction have shown low efficiency and potential environmental pollution due to large volumes of organic solvent used and long extraction time required in those methods. A number of methods have been developed in recent years such as microwave, ultrasound-assisted extractions, and techniques based on use of compressed fluids as extracting agents, such as subcritical water extraction (SWE), supercritical fluid extraction (SFE), pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE) were also applied in the extraction of phenolic compounds from plant materials.

Ultrasound-assisted extraction (UAE) is a potentially useful technology as it does not require complex instruments and is relatively low-cost. It can be used both on a small and large scale in the phytopharmaceutical extraction industry. Pressurized liquid extraction (PLE), also known under the trade name of accelerated solvent extraction (ASE), is a relative new technology for extraction of phytochemicals under high temperature and pressure.
Microwave-assisted extraction (MAE) is a process utilizing microwave energy to facilitate partition analytes from the sample matrix into the solvent. The main advantage of this technique is the reduced extraction time and solvent volume as compared to conventional extraction techniques [102].

II-5-2- Purification and fractionation

Plant crude extracts usually contain large amounts of carbohydrates and/or lipoidal material and the concentration of the phenolics in the crude extract may be low. To concentrate and obtain polyphenol rich fractions before analysis, strategies including sequential extraction or liquid-liquid partitioning and/or solid phase extraction (SPE) based on polarity and acidity have been commonly used. In general, elimination of lipoidal material can be achieved by washing the crude extract with non-polar solvents such as hexane, dichloromethane, or chloroform. To remove polar non-phenolic compounds such as sugars, organic acids, a SPE process is usually carried out. SPE is becoming popular since it is rapid, economical, and sensitive.

Column chromatography has been also employed for fractionation of phenolic extracts. Although this method is often labor-intensive and solvent-consuming, it provides greater amounts of fractions for subsequent isolation and identification of pure substances. Typically-utilized column sorbents are RP-C18, Toyopearl, LH-20 and to a less extent

polyamide resin. Ethanol, methanol, acetone, and water and their combinations are commonly used as eluents.

The classical liquid-liquid extraction procedure has been less commonly used because it is a tedious, highly time-consuming process with high solvent costs and low recoveries.

As an alternative to liquid chromatography, Countercurrent Chromatography (CCC) has been developed as an effective technique for fractionation of various classes of phenolic compounds. CCC is a preparative all-liquid chromatographic technique based on partitioning of compounds between two immiscible liquid phases, a liquid stationary phase and a liquid mobile phase. Solutes are separated according to their partition coefficients between the two solvent phases based on their hydrophobicity. The big advantage of CCC is that it uses no solid matrix and the role of two liquid phases, namely, liquid stationary phase and mobile phase, can be switched during a run. Thus, there is no irreversible sample adsorption and the recovery is 100%.

In addition, Krafczyk and Glomb employed Multilayer Countercurrent Chromatography (MLCCC) coupled with preparative High-Performance Liquid Chromatography (HPLC) to obtain pure flavonoids from Rooibos tea. This method was able to isolate up to gram of material and to verify known polyphenol structures and discover previously unpublished ones [102].

II-5-3- Quantification and analysis of phenolics

Natural phenolics are of interest from many viewpoints (antioxidants, astringency, bitterness, browning reactions, color, *etc.*). Selection of the proper analytical strategy for studying phenolics in plant materials depends on the purpose of the study as well as the nature of the sample and the analyte. The assays used for the analysis of phenolics are usually classified as either those measuring total phenolics content, or those quantifying a specific group or class of phenolic compounds. Quantification of phenolic compounds in plant extract is influenced by the chemical nature of the analyte, as well as assay method, selection of standards and presence of interfering substances.

Because of the heterogeneity of natural phenolics and the possible interference from other readily oxidized substances in the plant materials, it is not surprising that several methods have been used for determination of total phenolics and none are perfect. Among such methods are the Folin-Denis method (FD), Folin-Ciocalteu method (F-C), permanganate titration, colorimetry with iron salts, and ultraviolet absorbance. In most cases, F-C has been found preferable as compared to the other methods [90]. The F-C assay relies on the transfer

of electrons in alkaline medium from phenolic compounds to phosphomolybdic/ phosphotungstic acid complexes to form blue complexes ($(PMoW_{11}O_{40})4-$) that are determined spectroscopically at approximately 760 nm. Gallic acid is widely used as the comparison standard and values are usually compared as milligram of gallic acid equivalent per kilogram or liter of extract among samples. Owing to the general nature of the F-C chemistry, it is indeed a measure of total phenolics and other oxidation substrates. The other oxidation substrate present in a given extract sample can interfere the total phenolics measurement in an inhibitory, additive or enhancing manner. The inhibitory effects could be due to the oxidants competing with F-C reagent and/or air oxidation after the sample is made alkaline. For this reason, the FC reagent is added ahead of alkali. Additive effects occur from unanticipated phenols, aromatic amines, high sugar levels or ascorbic acid in the samples. The additive effects can be measured before adding the alkali or by a more specific assay of a known interference and then subtracted from the F-C value. Sulfites and sulfur dioxide which is a common additive for wine can cause enhancing effect. Singleton *et al.* [90] discussed the effects of potential interference compounds and methods for correcting these factors. However, despite these disadvantages, the F-C assay is simple and reproducible and has been widely used for quantification of phenolic compounds in plant materials and extracts.

In general, traditional spectrophotometric assays provide simple and fast screening methods to quantify classes of phenolic compounds in crude plant samples. However, due to the complexity of the plant phenolics and different reactivity of phenols toward assay reagents, a broad spectrum of methods is used for assay of the constituents, leading to differing and often non-comparable results. In addition to that, the methods are quite prone to interferences and consequently often result in over- or underestimation of the contents. Modern high-performance chromatographic techniques combined with instrumental analysis are the “state of art” for the profiling and quantification of phenolic compounds. Gas chromatographic (GC) techniques have been widely used especially for separation and quantification of phenolic acids and flavonoids. The major concern with this technique is the low volatility of phenolic compounds. Prior to chromatography, phenolics are usually transformed into more volatile derivatives by methylation, conversion into trimethyl silyl derivatives, *etc.*

HPLC currently represents the most popular and reliable technique for analysis of phenolic compounds. Various supports and mobile phases are available for the analysis of phenolics including anthocyanins, proanthocyanidins, hydrolysable tannins, flavonols, flavan-3-ols, flavanones, flavones, and phenolic acids in different plant extract and food samples.

Moreover, HPLC techniques offer a unique chance to analyze simultaneously all components of interest together with their possible derivatives or degradation products. The introduction of reversed-phase (RP) columns has considerably enhanced HPLC separation of different classes of phenolic compounds and RP C18 columns are almost exclusively employed.

Given the intrinsic existence of conjugated double and aromatic bonds, every phenol exhibits a higher or lower absorption in ultraviolet (UV) or ultraviolet/visible (UV/VIS) region. Thus, the most common means of detection, coupled to LC, are UV/VIS, photodiode array (PDA), and UV fluorescence detectors. PDA is the most prevalent method since it allows for scanning real time UV/VIS spectra of all solutes passing through the detector, giving more information of compounds in complex mixtures such as a plant crude extract. Other methods employed for detection of phenolic compounds include electrochemical detection (ECD), voltammetry technique, on-line connected PDA and electro-array detection (EAD), chemical reaction detection techniques, mass spectrometric (MS) and nuclear magnetic resonance (NMR) detection. MS and NMR detections are more of structure confirmation means than quantification methods.

Electromigration techniques including capillary electrophoresis (CE), capillary zone electrophoresis (CZE), and micellar electrokinetic chromatography coupled with UV, and to a less extent EC and MS detection are also employed for phenolics analysis [102].

II-5-4-Chracterisation

II-5-4-1- Ultraviolet-visible spectroscopy

All phenolic compounds contain aromatic conjugated systems that absorb light in the UV–vis spectral region enabling their detection with UV–vis detectors. A traditional UV–vis detector monitors only one or several wavelengths at the same time, whereas a diode array detector (DAD) simultaneously measures a range of wavelengths (e.g., 200–500 nm), which enables the measurement of UV–vis spectra of phenolic compounds.

The UV–vis spectra of flavonoids are comprised of absorptions arising from the benzene rings A and B and their possible conjugations to ring C. The UV–vis spectra of different subclasses of flavonoids give tentative information about the structures of flavonoid aglycones . All flavonoids have an absorption maximum at around 240–290 nm (Band II), which is mostly affected by the conjugation of ring A and its substitution pattern. Some flavonoids have another absorbance maximum at around 300–550 nm (Band I), which is detected in flavonoids where rings B and C are conjugated (via a double bond between carbons C–2 and C–3 in ring C). The absence or low absorption maximum of Band I is probably caused by the lack of conjugation between ring B and the rest of the molecule (no double bond beside the ring B).

For most flavonoids, except for anthocyanins and some aurones, the above-mentioned Band I and II absorption maxima lie in the range of UV radiation (i.e. below 400 nm), so the term UV spectrum of flavonoids is often used instead of the term UV–vis spectrum.

The locations of Band I and II maxima are affected by the number of hydroxyl groups in rings A and B as well as by the glycosylation of the flavonoid. However, the actual sugar molecule has no effect on the UV spectrum of flavonoid glycosides. Acyl substituents in glycosyls cause an additional maximum in the UV spectrum of flavonoid glycosides.

In addition to structure elucidation, UV–vis detectors are used in the quantitative analyses of flavonoids and other phenolics. The sensitivity of detection is improved by choosing a detection wavelength or wavelengths where the phenolics in question show maximum absorption [105].

II-5-4-2-Infrared spectroscopy

Near infrared (NIR) and mid infrared (MIR) spectroscopy are considered as powerful, fast, accurate and non-destructive analytical tools that can be considered as a replacement of traditional chemical analysis. Near infrared (NIR) and mid infrared (MIR) spectroscopy have been used to quantify several compounds includes polyphenols.(recent applications)

In recent years, both near infrared (NIR) and mid infrared (MIR) spectroscopy with its intrinsic benefits such as being non-invasive, rapid, almost no necessary sample preparation, on-/inline measurements, have being able to determine physical and chemical parameters simultaneously, became a widely used analytical technique in the so called field of phyto-analytics and is included in pharmacopeia to an increasing extent [106].

3- Mass spectrometry

Similarly to UV–vis detectors, mass spectrometers have a crucial role in detecting and analyzing flavonoids and other phenolic compounds. While UV–vis detectors only detect compounds with a UV–vis absorbing chromophore, mass spectrometers detect virtually all kinds of chemical compounds that are ionized in the used analytical conditions.

These two instruments are often coupled in line, so that the eluent flow from LC first passes through an UV–vis detector, after which the eluent is directed to MS (LC–DAD–MS).

In MS, analyte molecules are first ionized, after which the ions are analyzed according to their mass-to-charge ratios (*m/z*). Mass spectrometers are divided into different groups according to their ionization techniques and mass analyzers. Ionization techniques used in flavonoid analyses include, for example, fast atom bombardment (FAB) and matrix assisted laser desorption/ionization (MALDI).

In FAB, the flavonoid sample is dissolved in a liquid matrix that is bombarded with a beam consisting of fast atoms (e.g. Ar or Xe), which causes the desorption of ions from the sample matrix. In MALDI, the sample is dissolved in a matrix that absorbs energy from laser pulses, which causes the formation of plasma that produces ions from the analyte molecules.

In addition to different ionization techniques, also several mass analyzers exist, such as, ion trap (IT) and Fourier transform ion cyclotron resonance (FT–ICR) analyzers. There are also the electrospray ionization (ESI) technique which used in combination with two different mass analyzers: triple quadrupole (TQ) and quadrupole time-of-flight (QTOF) analyzers.

Both positive and negative ionization modes can be used in the analysis of flavonoids with ESI. The negative ionization often gives better sensitivity for flavonoids, probably because their ionization is enhanced by the deprotonation of the acidic hydroxyl groups. However, the positive ionization mode can sometimes give more structural and fragmentation information about the flavonoids (Cuyckens and Claeys, 2004). MS is used, first of all, to elucidate the molecular masses of flavonoids. This can be done by detecting the deprotonated or protonated molecular ions ($[M–H]^-$ or $[M+H]^+$) of the analytes. The ESI conditions may produce adducts both in the positive and negative ionization modes (e.g., a Na^+ adduct $[M+23]^+$ or a formic acid adduct $[M+46–H]^-$). In addition to molecular ions, also cluster ions (e.g., $[2M–H]^-$) can be formed, and they can be used in identifying the molecular masses of unknown compounds. Fragmentation of flavonoids in their first-order mass spectra gives further details about their structures.

The first order mass spectrum of a flavonoid normally reveals only the molecular mass of the flavonoid aglycone and possible acidic or glycosidic residues. More structural information can be gained with MS/MS analyses of flavonoids [105].

II-5-4-4- NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has an important role in the structural elucidation of flavonoids and other phenolic compounds.

In the case of flavonoid glycosides, 1H NMR spectra also show the signal (or signals) of an anomeric sugar proton whose coupling constant to the neighboring sugar proton determines whether the glycosyl is in α- or β-anomeric configuration . Further, the chemical shifts and coupling constants of anomeric and other protons help to determine the structure of the glycosyl unit. This is important, since the MS analyses of flavonoid glycosides are unable to differentiate between two different glycosyls that have same molecular masses (e.g. glucose and galactose). The position of an acyl group attached to a sugar unit can sometimes

be deduced from the 1H NMR spectrum, as the chemical shift of the sugar proton next to the acyl group often shows a marked downfield shift.

Two dimensional NMR experiments show how different atoms are bonded or spatially situated in relation to each other. For example, correlation spectroscopy (COSY) is used to determine which hydrogen atoms are coupled to each other. Heteronuclear single quantum correlation (HSQC) experiment determines which two nuclei of different atoms (most often 1H and 13C) are directly bonded to each other. Heteronuclear multiple bond correlation (HMBC) spectra show correlations between two different atoms (for example 1H and 13C) that usually are separated by 2–4 bonds. In flavonoid research, HMBC is especially useful in elucidating the attachment sites of glycosyl and acyl groups, which cannot be determined by the first-order mass spectra of flavonoids. Total correlation spectroscopy (TOCSY) experiments are useful in the assignment of glyco syl protons, especially in flavonoid oligoglycosides where the proton signals of different glycosyls are often overlapping. The positions of glycosyl units and other structural details of flavonoids can also be determined with 1H–1H experiments with nuclear overhauser enhancement spectroscopy (NOESY) and rotating frame overhauser effect spectroscopy (ROESY) that show protons that are spatially close to each other in the molecule.

An advantage of NMR is that it keeps analyte compounds intact for their possible further use, whereas in MS the analyte compounds are degraded. However, a rather high amount of an analyte (often a few milligrams) is needed for NMR experiments, and the NMR spectra of mixtures can be difficult to interpret. Eluent flow from LC can be coupled to NMR (LC–NMR). This technique could be used to analyze mixtures of phenolic compounds without the time-consuming purification of individual compounds, but the instrumentation and the use of deuterated solventscan be too expensive for routine use. As noted above, the need for rather large sample sizes in NMR is another limiting factor for the use of LC–NMR as compared to LC–MS [105].

II-6- Bioactivities of Polyphenols

Natural polyphenols have shown numerous biological activities and health benefits for prevention and treatment of age-related diseases, cancers, heart diseases …etc [88].

II-6-1- Antioxidant Activity

Among the notable bioactivities of phenolic compounds, the antioxidant activities have been widely studied, including scavenging of free radicals, inhibition of lipid oxidation, reduction of hydroperoxide formation, and so on.

There were many experiments *in vitro* that proved that phenolic compounds were usually major contributors of antioxidant capacities of plants. Polyphenols may also function as antioxidants through their effects on plasma, membranes, transcription factors and enzyme activities *in vivo*. Natural polyphenols may also function as antioxidants through their effects on membranes. Furthermore, the working mechanism of natural polyphenolic antioxidants has been summarized in two papers, that is, H atom transfer, electron transfer and metal ions chelation action. Many polyphenols scavenge free radicals through the hydrogen atom transfer mechanism because higher energies are involved in the single electron transfer process. Polyphenols could chelate transition metals through their multiple OH groups and the carbonyl moiety, when present.

Polyphenols could be used not only as antioxidant for human but also food preservatives in food industry.

II-6-2- Cardioprotection Activity

Postprandial hyperlipemia and oxidative stress, a well-defined risk factor for atherosclerosis, could be reduced by polyphenols. Atherosclerosis develops in lesion-prone regions of medium-sized arteries. Atherosclerosis might be present or clinically-silent for decades before becoming active and producing pathological conditions such as acute myocardial infarction, unstable angina, and sudden cardiac death. The rate of myocardial infarction is continually increasing. Numerous studies have shown that dietary polyphenols could reduce the risk of thrombosis, which is one of the leading causes for myocardial infarction, ischemic heart disease, *etc*.. In addition, epidemiologic studies provided strong evidence that the incidence of cardiovascular disease was low in the Mediterranean area. The consumption of Mediterranean diet which enriched in green vegetables, fruits, fish, and grape wine, particularly red wine, imparted a greater benefit in the prevention of coronary heart disease than the consumption of other diets.

II-6-3-Anticancer Activity

Cancer is a major cause of death across the world. Polyphenols could play an important role in anticancer. The anticancer effects of polyphenols have been observed at mouth, stomach, duodenum, colon, liver, lung, mammary gland or skin. Many polyphenols, such as proanthocyanidins, flavonoid, resveratrol, tannins, epigallocatechin-3-gallate, gallic acid and anthocyanin, have been tested; all of them showed protective effects in some models although their mechanisms of action were found to be different.

Natural polyphenols might have a better protective effect on metastatic breast cancer.

Polyphenols influence the metabolism of pro-carcinogens by modulating the expression of cytochrome P450 enzymes involved in their activation to carcinogens. Polyphenols could also protect genomic DNA by mobilizing of endogenous copper possibly chromatin bound copper. Many mechanisms of action have been demonstrated for chemopreventive effect of polyphenols, such as antiproliferation, antioxidation, estrogenic/antiestrogenic activity, induction of cell cycle arrest or apoptosis, induction of detoxification enzymes, regulation of the host immune system and changes in cellular signaling.

II-6-4- Anti-Inflammation Activity

Excessive inflammation is considered as a critical factor in many human diseases, including obesity, type II diabetes, cardiovascular diseases, neurodegenerative diseases and ageing. Polyphenols have shown significant anti-inflammation effects *in vivo* and *in vitro.*

There have been numerous topical applications of plant extracts having flavonoids known as anti-inflammatory compounds. Quercetin, a kind of flavonoids, could play a critical role in reducing the risk of atherosclerosis through activation of inflammatory signaling. Consumption of polyphenol-rich foods like grapes or their by-products, which have anti-inflammatory properties, could reduce inflammation. Resveratrol commonly found in grape products has been reported to reduce inflammation in many mechanisms. For example, it could block proinflammatory cytokines, suppress inflammatory gene expression and activate transcription factors that antagonize chronic inflammation. Thus, polyphenol-rich grape products reduce obesity-mediated chronic inflammation.

In recent years, we were allowed to understand interactions of different polyphenols with basic mechanisms of inflammatory response by the modern methods in cell and molecular biology. Polyphenols could affect vascular inflammation and injury not only as antioxidants but also as modulators of inflammatory redox signaling pathways. There are several anti-inflammatory mechanisms of polyphenols *in vivo*. One of the important anti-inflammatory mechanisms is the inhibition of eicosanoids generating enzymes including phospholipase A2 and cyclooxygenase. Polyphenols were able to modulate cyclooxygenase-2 activity and gene expression in different cell types.

These studies strongly proved the idea that polyphenols had the capacity to modulate the immune response and had a potential anti-inflammatory activity.

II-6-5- Antimicrobial Effect

Polyphenols have been demonstrated potential antibacterial, antifungal and antiviral activities.The stilbenes pinosylvin, pinosylvin monomethyl ether and piceatannol were

demonstrated to have clear antimicrobial activities. The destabilization of the outer membrane of Gram-negative microorganisms, as well as interactions with the cell membrane might be one of the specific mechanisms behind the antibacterial action.

Polyphenols were able to suppress a number of microbial virulence factors, such as reduction of host ligands adhesion, inhibition of biofilm formation, neutralization of bacterial toxins, and show synergism with antibiotics. Polyphenols were proved to have conjunctive use with antibiotics in order to potentiate their efficacy, to lower antibiotic dose, and therefore to reduce antibiotic adverse reactions.

Despite the use of polyphenols in treatment, the antimicrobial property of polyphenols was also proposed to develop new food antimicrobial and preservatives for increasing consumer pressure on the food industry to avoid synthetic preservatives.

II-6-6- Anti-Ageing effect

Due to notable antioxidant activity of polyphenols, they might be beneficial in reversing the course of neuronal and behavioral ageing. The researchers evaluated the role of grape seed extract on antioxidant status in discrete regions of the central nervous system of young and aged rats Age-associated increase in lipid peroxidation was observed in the spinal cord, cerebral cortex, striatum and the hippocampus regions of aged rats. Activities of antioxidant enzymes and levels of non-enzymic antioxidants were found to be significantly decreased in all the brain regions studied in aged rats when compared to young rats. However, normalized lipid peroxidation and antioxidant defenses were reported in the grape seed extract-supplemented aged rats. These findings demonstrated that grape seed extract decreased the incidence of free radical-induced lipid peroxidation in the central nervous system of aged rats. Latter, they discovered that polyphenols inhibited the accumulation of age-related oxidative DNA damages in neural tissue. In addition, the risk of Parkinson's disease, a neurological disorder characterized by degeneration of dopaminergic neurons, could be reduced by polyphenols.

The results demonstrated that orally administered rutin significantly attenuated memory deficits in Alzheimer's disease transgenic mice, decreased oligomeric Aβ level, increased super oxide dismutase activity and glutathione/glutathione disulfide ratio, reduced glutathione disulfide and MDA levels, downregulated microgliosis and astrocytosis, and decreased interleukin IL-1β and IL-6 levels in the brain. These results indicated that rutin was a promising agent for Alzheimer's disease treatment because of its antioxidant, anti-inflammatory, and reducing Aβ oligomer activities. Because of strong antioxidant and anti-

inflammatory properties, maize bran polyphenol and ferulic acid were also reported to be beneficial in Alzheimer's disease.

II-6-7- Other Bioactivities of Polyphenols

Except for the above introduced bioactivities, polyphenols have shown several other health beneficial effects. There were evidences showing that polyphenols had a protective response to skin damage, erythema and lipid peroxidation from UV exposure.

The protection of kidney by polyphenols has also been reported. Polyphenols could protect the kidney against rhabdomyolysis following glycerol injection.

Except for kidney, polyphenols might protect against obstructive lung disease. Insulin resistance, a hallmark of metabolic disorders, is a risk factor for diabetes and cardiovascular disease. Concordant results were reported that polyphenols, such as resveratrol and epigallocatechin-3-gallate, *in vivo* had a beneficial effect on energy metabolism in diseases such as diet-induced obesity and insulin resistance.

Part II

Practical study

I-Material and Methods

I-1-Material

I-1-1-Chemicals and general material

Toluene, ethyl acetate, acetone, diethylamine, dimethyl sulfoxide, methanol, ethanol, hexane, n-butanol, diethyl ether, chloroform, hydrochloric acid, ammonium hydroxide, anhydrous sodium sulfate, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, L-glutamine, trypsin and ethylene diamine tetra acetic acid (EDTA) were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany, Gallic acid,Trichloro acetic acid (TCA), 2,2`-diphenylpicrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT), were purchased from Sigma (Germany), and all other reagents salts and solvents were obtained from Prolabo, Aldrich, and Organics. All common chemicals and solvents used were of analytical grade. .

Silica gel 60 (Merck, 70-230 mesh) was used for column chromatography. Preparative TLC was performed by using silica gel P/UV_{254} with $CaSO_4$ (Machery-Nagel, Germany, 2 mm layer of adsorbent). Analytical TLC was performed on silica gel (Silica gel 60, layer 0.20 mm, Alugram Sil G, Mashery-Nagel, Germany). Visualization of TLC plates was performed by using UV lamp at 254 nm and 365 nm (VL-4.LC, 365/254, Vilber Lourmat, France), as well as in iodine vapors.

I-1-2-Biological material

I-1-2-1-Plant collection and identification

Cytisus purgans was collected on June 2013 at Aures Mountains (2328m of altitude) at Khenchela province in the north east of Algeria, and identified by Professor Mohamed Kaabech, Laboratory of biodiversity and phytogenetic resources, University of Ferhat Abbas, Sétif, Algeria. A voucher specimen is deposited at the herbarium of laboratory of organic chemistry and phytochemistry of University Mohamed Boudiaf of M'sila, Algeria.

I-1-2-2-Biologicals

Human breast cancer (MDA-MB-231) and human lung cancer (A549) cell lines, obtained from Human Tumor Cell Bank (HTB) of the American Type Culture Collection (ATCC). Microbial strains used were: *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Micrococcus lauteus, Pseudomonas aeruginosa* and *Bacillus cereus,as bacteria, and: Candida albicans, Fusarium oxysporum and Alternaria alternat*, as fungi, obtained from the laboratory of the department of SNV, university Mohamed BOUDIAF, M'sila, Algeria.

I-2-Methods

I-2-1-Phytochemical study

I-2-1-1-Extraction of crude extracts

For alkaloids, a specific alkaloid extraction was carried out of the plant *cytisus purgans*, as the literature had revealed alkaloids from this genus.

The air-dried aerial parts of plant were cut into small pieces and were macerated to exhaustion with 99 % methanol three times (24h x 3) at room temperature. The combined extracts were concentrated, acidified with 5 % hydrochloric acid and then, extracted with diethyl ether (three times). The aqueous layer was made alkaline with 25 % ammonium hydroxide to pH (9-10) and extracted with chloroform (six times). The chloroform extracts were combined and dried over anhydrous sodium sulfate (Na_2SO_4) and evaporated to dryness *in vacuo* to give crude alkaloid mixture [63] (figure 9).

For polyphenols, the fresh, whole plant was collected and shade dried to obtain 100 g dry sample which was later coarsely powdered and used for solvent extraction. The sample was extracted at room temperature for 3 × 24 hours. The extraction was performed with 70% aqueous methanol solution (70 methanol/30 water, v/v) and 70% aqueous ethanol solution, respectively. The extracts were filtered through a Buchner funnel, The aqueous solution was extracted with hexane several times to eliminate lipids and the solvent was removed on rotary evaporator (BÜCHI, 461, Germany) to dryness under reduced pressure at 40° to give crude extract of polyphenols [107].

The crude extract of polyphenols was partitioned into methanol, diethyl ether and n-butanol, respectively.

I-2-1-2-Determination of total phenolic content

The total phenolic content was determined by using the Folin-Ciocalteu assay. This phenol reagent consists of a mixture of the heteropoly acids, phosphomolybdic and hosphotungstic acids in which the molybdenum and the tungsten are in the 6+ oxidation state. On reaction with a reductant, the molybdenum blue and the tungsten blue are formed and the mean oxidation state of the metals is between 5 and 6 [108].

In brief, standard curve using gallic acid (7.81 and 250 µg/ml) was made with following steps:

0.2 mL of the diluted sample extract was transferred in tubes containing 1.0 mL of a 1/10 dilution of Folin-Ciocalteu's reagent in water. After waiting for 04 minutes, 0.8mL of a sodium carbonate ($NaCO_3$) solution (7.5% w/v) was added to the sample. The tubes were then

allowed to stand at room temperature for 30 min before absorbance at 765 nm was measured. The TPC was expressed as gallic acid equivalents (GAE) in mg/100 mL of plant extract. The concentration of polyphenols in samples was derived from a standard curve of gallic acid using regression equation. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained [109].

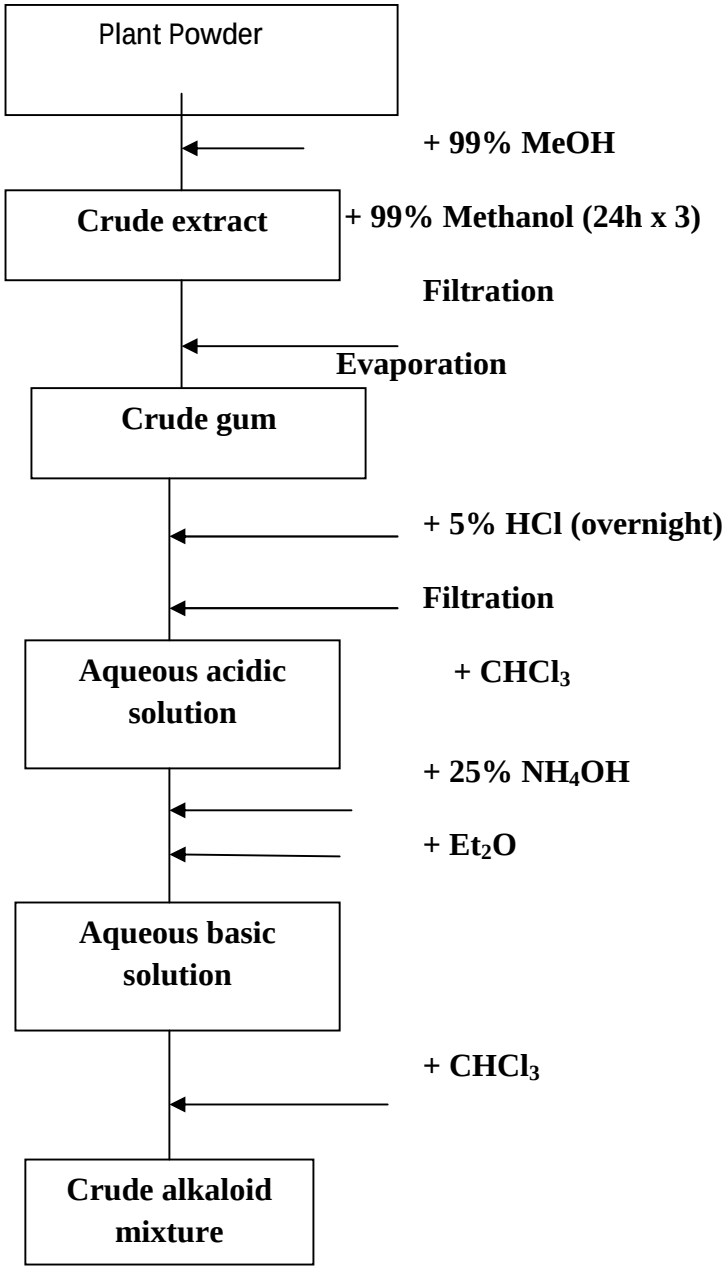


Figure 9: Different steps of quinolizidine alkaloids extraction.

I-2-1-3-Determination of total flavonoid content

The total flavonoid content was determined according to the aluminium chloride colorimetric method. The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples

were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax = 430 nm. The same procedure was repeated for the standard solution of rutin (2-64 µg/ml) and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained [109].

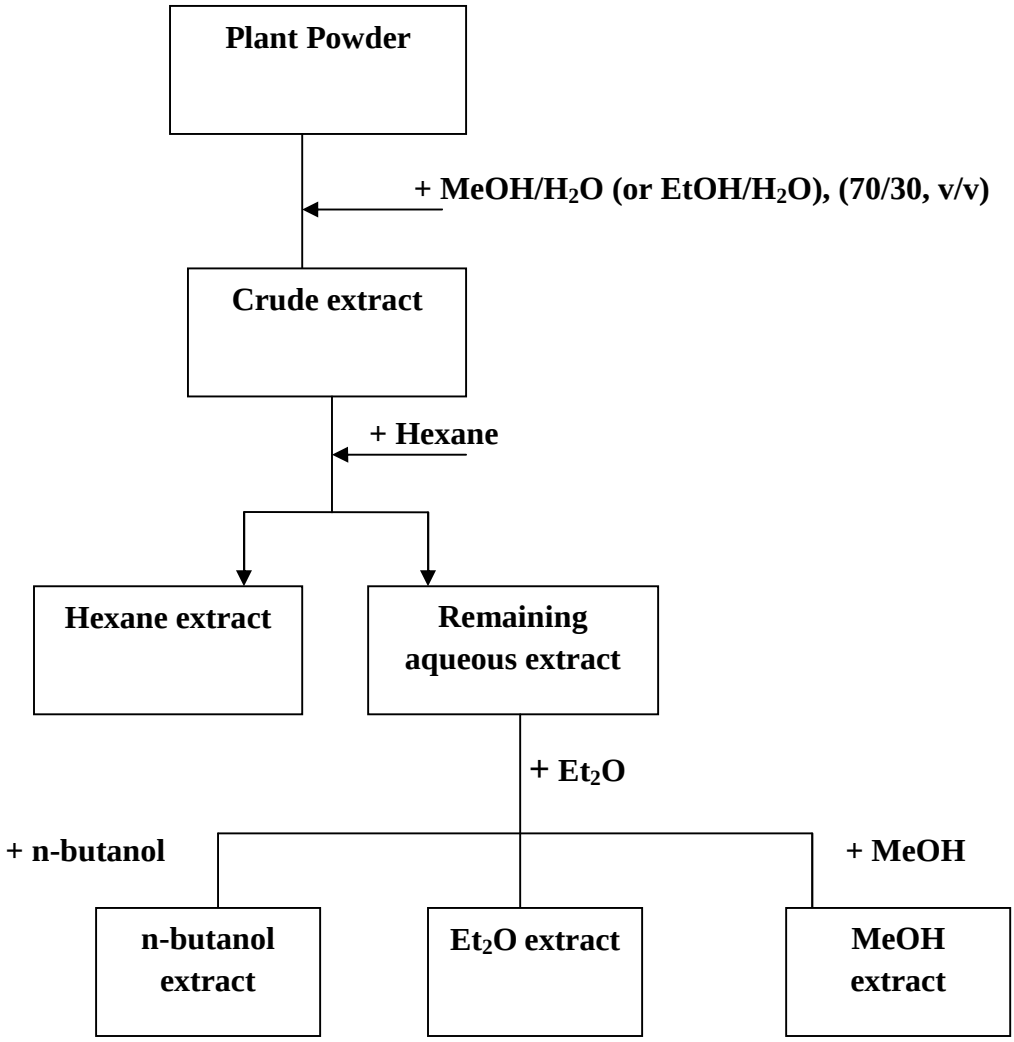


Figure 10: Different steps of phenolic compounds extraction.

I-2-1-4-Fractionation and isolation of pure compounds

Fractionation and isolation were carried out from the crude extracts of alkaloids and polyphenols: chloroform, methanol and diethyl ether with open column chromatography (CC). The phytochemical investigation of these extracts yielded 04 known natural products.

Pure compounds ***1***, ***2***, ***3*** and ***4*** (sparteine, lupanine, quercetin and sarotanoside) were isolated using open column chromatography. Fractionation processes were conducted by the help of analytical and preparative thin layer chromatography (TLC) and GC/MS spectroscopy.

For the isolation of compounds ***1*** and ***2*** , open column chromatography was performed on a classic 30 cm long x 4 cm diameter glass column packed with 130 g of silica gel 60 (70-230 mesh). The ethyl acetate solution of 1 g of alkaloid fraction was applied to the top of column by use of a pipette. Elution was started with toluene, then with solvent mixtures with increasing polarities: toluene:ethyl acetate:acetone and toluene:acetone:diethylamine. Finally, all 145 fractions (obtained by column chromatography) were monitored by analytical TLC (above mentioned solvent mixtures).

Fraction 141 was rechromatographed on preparative TLC. Mobile phase used for elution was chloroform:methanol:acetone=85:10:5 w/w. After elution and drying of preparative TLC plate, by scraping the layers and additional desorption from methanol, sparteine and lupanine were isolated. Purity of compounds was confirmed on analytical TLC with the same mobile phase mentioned above. Also, gas chromatographic/mass spectrometric analyses on Agilent 6890/5973 GC/MS system confirmed purity of isolated compounds.

In the other hand, for the isolation of compounds ***3*** and ***4***, the crude mixture of polyphenols was dissolved in methanol, after, this methanol extract was treated with diethyl ether, and obtained solid which removed from solution by filtration. Solid was subjected to column chromatography (silica gel 75-200 mesh) (eluent chloroform: methanol= 4:6). Obtained solid was sarotanoside.

One part of crude methanol extract was subjected to column chromatography (silica gel, 75-200 mesh). Mobile phase was chloroform with increasing amount of methanol. Obtained fraction was subjected to TLC (mobile phase Chloroform:Methanol=8:2, 7:3 and 6:4). Fraction with similar Rf values (spots were observed under UV light at 354 nm) were combined, and again subjected to CC. On the basis of MS, IR, 1H and 13C NMR data, isolated compounds was assigned as quercetin.

I-2-1-5-Structural characterization

UV spectra were recorded in methanol on Agilent Cary 300 (190–900 nm) spectrophotometer (Canada).

Infrared spectra were run on Thermo Scientific Nicolet 6700 FT-IR spectrometer (4000-400 cm^{-1}) (United States of America). Infrared spectra (KBr technique).

The NMR spectra were recorded on a Bruker Avance III 500 spectrometer, ^{1}H NMR at 500.26 MHz and ^{13}C NMR at 125.80 MHz, solvent DMSO d_6, TMS internal standard. Chemical shifts were given in (ppm). The samples were prepared by the dissolution in DMSO (signal for^{1}H- at 2.5 ppm and at 39.5 ppm for ^{13}C-NMR).

Mass spectra were recorded on a Agilent 6890/5973 GC/MS system (Agilent, Santa Clara, Canada) using HP-5ms column (30 m, id 0.25 mm, film thickness 0.25 μm); split mode of injection (split ratio:40.8:1); injector temperature 270 ºC; pressure 17.18 psi, MSD Transfer Line Heater 280 ºC; flow rate of He 1.3 mL/min, temperature program: initial temperature 150 ºC with increasing of temperature at 10 ºC/min to 290 ºC. Temperature of: MS quadruple detector 150 ºC, ion source 230 ºC. Mass scan range 35-700 amu at 70 eV. Multiplate reader used for MTT cell viability assay, Zenith 3100 (Anthos Labtec Instruments GmbH, Austria).

I-2-2-Biological study

I-2-2-1-Antitumor activity of plant extracts

Stock solutions of the both plant extracts (alkaloids and polyphenols) were made in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL, filtered through a 0.22 mm Millipore filter before use, and diluted by a nutrient medium to various working concentrations, so the final concentration of DMSO in culture medium never exceeded 0.5 % (v/v).

Human breast cancer (MDA-MB-231) and human lung cancer (A549) cell lines, obtained from Human Tumor Cell Bank (HTB) of the American Type Culture Collection (ATCC), were used in this study. MDA-MB-231 cells were grown in RPMI1640 and A549 cells were grown in DMEM medium. Both media were supplemented with 10 % fetal bovine serum, penicillin/streptomycin and L-glutamine. Cells were maintained in a monolayer culture into the 25 cm^2 tissue culture flasks (Nunclon^{TM}). The cells were prepared for experiments using trypsinization procedure with trypsin/EDTA [110].

-Methyl tetrazolium-MTT cell viability assay

Plant extracts were tested for their effect on viability of two different human tumor cell lines. MDA-MB-231 and A549 cells were seeded in 96-well plates (Sarstedt^{TM}) at $3x10^3$ cells in 200 µL of appropriate tissue culture medium per well. Every concentration of examined

compounds was tested in triplicate, and incubated at 37 ºC in a humidified 5 % CO_2 atmosphere. Control cells contained the appropriated amount of DMSO. Culture medium with corresponding concentration of investigated plant extract, but without cells, was used as a blank.

The viability of cultured cells was determined by assaying the reduction of MTT to formazan. In brief, cells were treated with different dilutions of alkaloids or poly-phenols (0.001, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 mg/mL) or cultivated in the cell culture medium containing the appropriate amount of DMSO (control). After 6h, 24 h and 48h of incubation, the culture medium was removed, and MTT solution was added. After additional 4 h of incubation, medium with MTT was removed and DMSO (150 µl/well) was added to dissolve the formazan crystals. The plates were shaken for 10 minutes. Absorbance was measured at 595 nm with a multiplate reader. The results are presented as relative to the control value (untreated cells).

I-2-2-2-Antioxidant activities

1-Radical scavenging activity test (DPPH assay)

The antioxidant activity of the plant extracts and standard (BHT) was determined by the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) as, previously described by Topcu et al. Briefly, 50 µl of of methanolic solutions of polyphenolic extract or standard (BHT) at various concentration- were added to 1950 µl of a DPPH methanolic solution (2,5 mg/100 ml) . After a 30 min incubation period at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows:

$$\mathbf{I\ \% = [(Abs_0 - Abs_1) / Abs_0] \times 100}$$

Abs_0 was the absorbance of the blank

Abs_1 was the absorbance in the presence of the test compound.

The antioxidant activity was estimated by the IC_{50} (concentration providing 50% inhibition of DPPH radicals) graphically using a calibration curve in the linear range by plotting the extract concentration *vs* the corresponding scavenging effect [111].

2-Reducing power

The reducing power of each extract (crude mixture of polyphenols) and BHT was determined according to the Topcu et al. method. The capacity of the extracts to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. For this purposes, 250 µl

of extracts prepared at different concentrations were mixed with 250 µl of phosphate buffer (0.2 M, pH 6.6) and 250 µl of potassium ferricyanide . The mixture was incubated at 50^{o}C for 20 minutes. 250 µl of Trichloro acetic acid (10%) was added. The supernatant was mixed with 1 ml of distilled water and 0.2 ml of ferric chloride was added, and then the solution's absorbance of the incident radiation was determined spectrophotometrically at 700 nm. The absorbance at 700 nm was used as the indicator of the reducing power. Increased absorbance of the reaction mixture indicated increased reduction capability. BHT was used as standard antioxidant [112].

1-2-2-3-Antimicrobial activity

1-Preparation of Inoculum and culture media

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi that were incubated without agitation for 24 h at 37°C and 25°C respectively. The cultures were diluted with fresh Muelle D Ainton and Sabouraud dextrose broth to achieve optical densities corresponding to 106 colony forming units (CFU/ml) for bacteria and 10^5 spore/ml for fungal strains [113].

One liter of MHA medium was prepared by dissolving 33.9 g of the commercially available Mueller Hinton Agar Medium (HiMedia) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 min. The autoclaved medium was mixed well and poured onto 100 mm petriplates (25-30ml/plate) while still molten.

One liter of nutrient broth was prepared by dissolving 13 g of commercially available nutrientmedium (HiMedia) in 1000ml distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 min.

Potato infusion was made by boiling 300 g of sliced potatoes in approximately 1 liter water for 30 min and then decantingor straining the broth through cheesecloth. Distilled water is added such that the total volume of the suspension is 1 liter. 20 g dextrose and 20 g agar powder are then added and the medium is sterilized by autoclaving at 15lbs for 15 min.

One liter of Sabouraud Dextrose Agar Medium was prepared by dissolving 65 g of Sabouraud dextrose agar in 1000ml of distilled water. The medium was sterilized by autoclaving at 121oC for 15 min at 15 lbs.

2- Antimicrobial activity

For the evaluation of the antimicrobial activity of the samples, agar disc diffusion method was used. The strains were grown on Mueller-Hinton agar slants at 37°C for 24 h and checked for purity. After the incubation, the cells were washed off the surface of agar and suspended in sterile physiological solution. The number of cells in 1 ml of suspension for inoculation measured by McFarland nefelometer was 5 × 107 CFU/ml. 1 ml of these suspensions was homogenized with 9 ml of melted (45°C) Mueller-Hinton agar and poured into Petri dishes. On the surface of the agar, 6 mm diameter paper discs were applied and impregnated with 15 µl of samples. The final concentrations of the extracts investigated were 10, 20, and 50 mg/ml. The plates were incubated 48 h at 37°C and the diameters of the resulting zones of inhibition (ZI) were measured and expressed in mm. The evaluation of the antimicrobial activities of the samples was carried out in triplicates [114].

Part II

Practical study

II-Results and discussions

In the present study, we aimed to investigate the alkaloid and polyphenol profile of the aerial parts of the plant C*ytisus purgans* growing in Algeria, as well as to evaluate antitumor, antioxidant and antimicrobial activity of both extracts.

Open column chromatography was used for the isolation of two known quinolizidine alkaloids which are: sparteine and lupanine and two known flavonoids which are: sarotanoside and quercetin. In the other hand, the Folin-ciocalteu and aluminium chloride colorimetric methods were used for the investigations of polyphenolic and flavonoids content, respectively.

The evaluation of biological activity of both extract shows a significant antioxidant power and a moderate antimicrobial activity, while the antitumor activity of both extracts was not significant.

Our results is divided into two great parts, the first one is destined to the phytochemical study which is divided, also, into quinolizidine alkaloids and polyphenols, the second one was destined to the biological study which divided into three parts: antitumor, antioxidant, and antimicrobial activity.

There have been several studies on the secondary metabolites profiles of *Cytisus* species [63, 115, 116] . However, this the first study for the *Cytisus purgans* of Algerian flora.

II-1-Phytochemical study

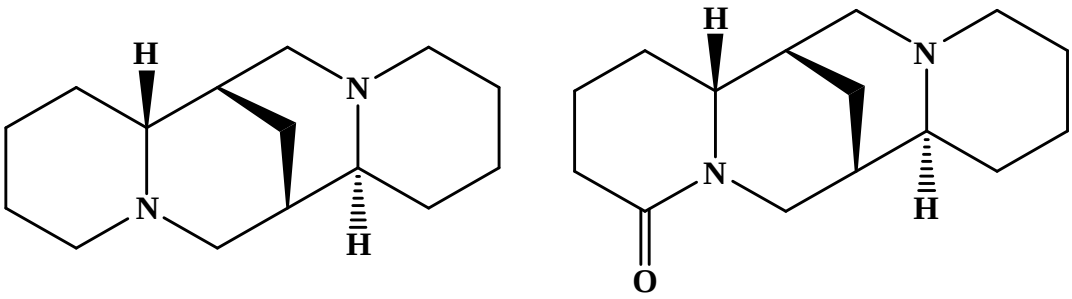
II-1-1-TLC screening

The crude mixture of alkaloids was analyzed by thin layer chromatography (TLC) in order to get more information about the chemical composition of the extract. By observing the TLC plates under UV 254 nm and 366 nm, extract showed many bands.

After spraying with iodine reagent, many brown bands were observed indicating the presence of alkaloids.

II-1-2-Quinolizidine alkaloids

Fractionation and isolation of quinolizidine alkaloids were carried out with open column chromatography to yield two known compounds which are sparteine ***1*** and lupanine ***2***. Fractionation processes were conducted by the help of TLC and GC/MS analysis. Compounds ***1*** and ***2*** were identified using different spectral data and comparing with literature.



1 ***2***

Figure 11: Structures of sparteine *1* and lupanine *2*.

The mass spectrum of compound ***1*** (M_w = 234g/mol) showed a molecular ion $[M]^+$ at m/z 234 (23.77 % rel. int.). A base peak at m/z 193 (22.13%) corresponding to $[M-41]^+$ indicating the loss of C_3H_5 which also showed at m/z 41 (10.65 %) (allylic cation) [117].

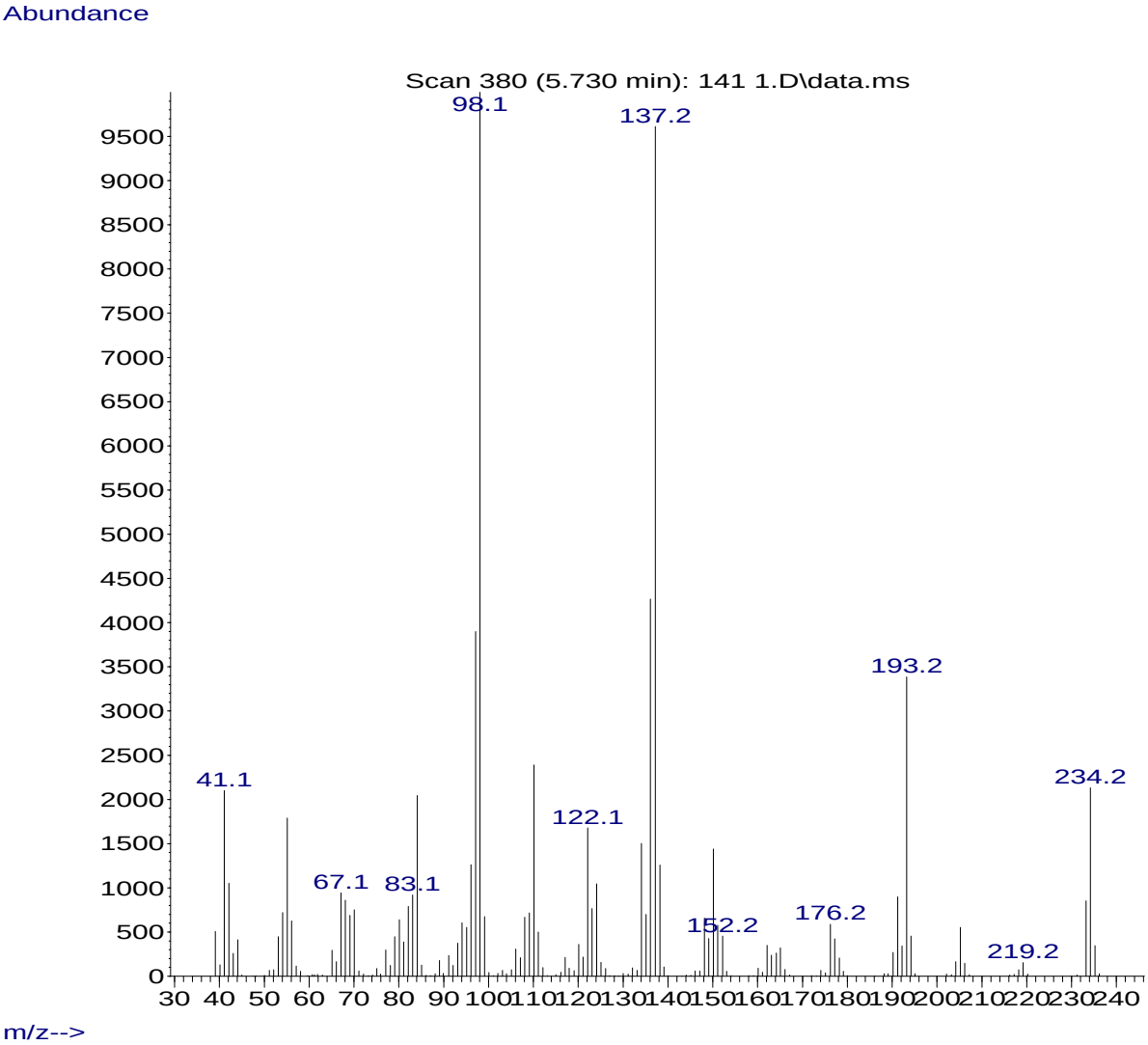


Figure 12: The mass spectrum of sparteine.

Another base peak at m/z 137 (100%) indicate the cleavage of the compound. The peaks at m/z 110 (18.85 %), m/z 98 (64.75 %) and m/z 55 (9.01 %) corresponding to fragmentation of lupinine ion respectively [117].

It is known also that the fragment at m/z 98 (64.75 %) arises from ring A following the fragmentation pattern of the sparteine-type alkaloids[118]

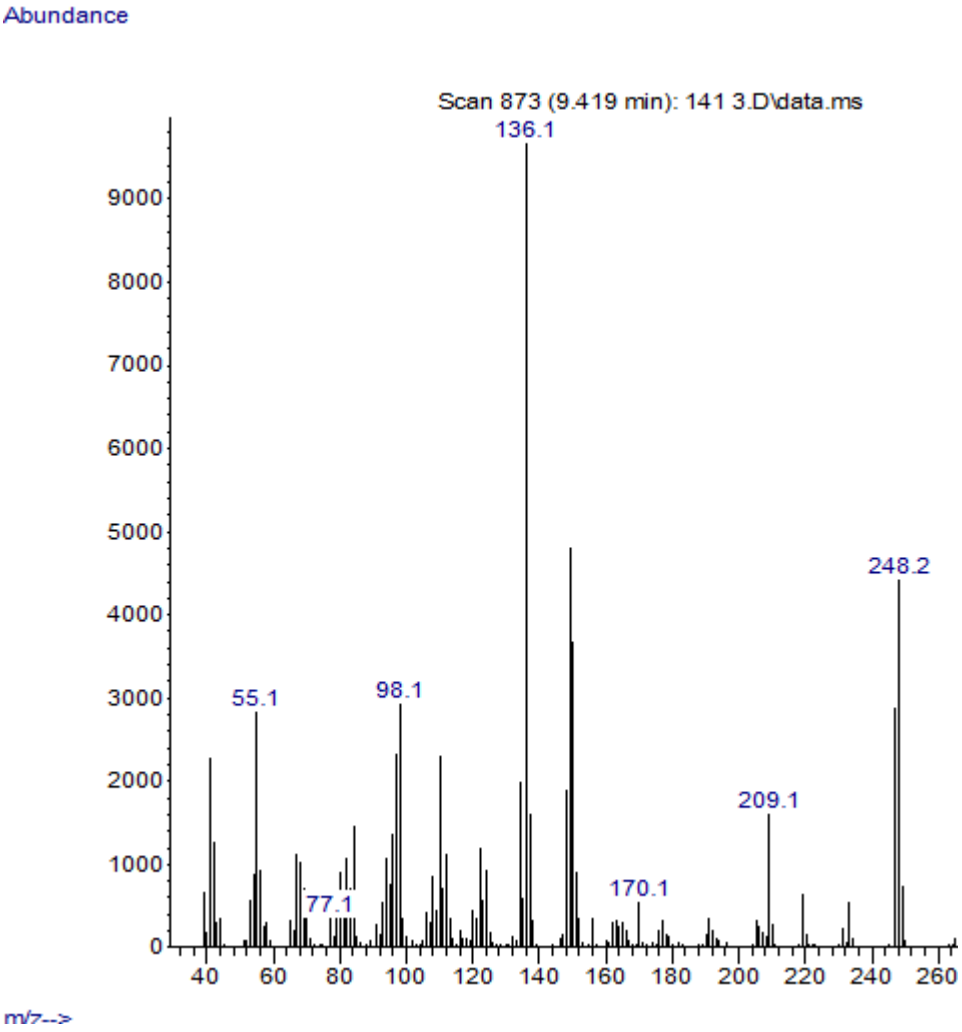


Figure 13: The mass spectrum of lupanine.

The mass spectrum of compound ***2*** (Figure 13), (M_w = 248g/mol) revealed a fragmentation pattern typical for a lupanine-type alkaloid, with fragments : $[M]^+$ at m/z 248 (69.67 %). A base at m/z 247 (50.81 %) corresponding of [M - H] +, another peak at m/z 219 (11.47 %) indicating the loss of 28 units of mass $[M - CO]^+$. The predominant ions at m/z 149 (57.37 %), 136 (100 %), 110 (19.67 %), 98 (20.49 %), 84 (10.65 %), 55 (16.39 %) and m/z 41 (9.83 %) are characteristic of lupanine [45,119 and 120].

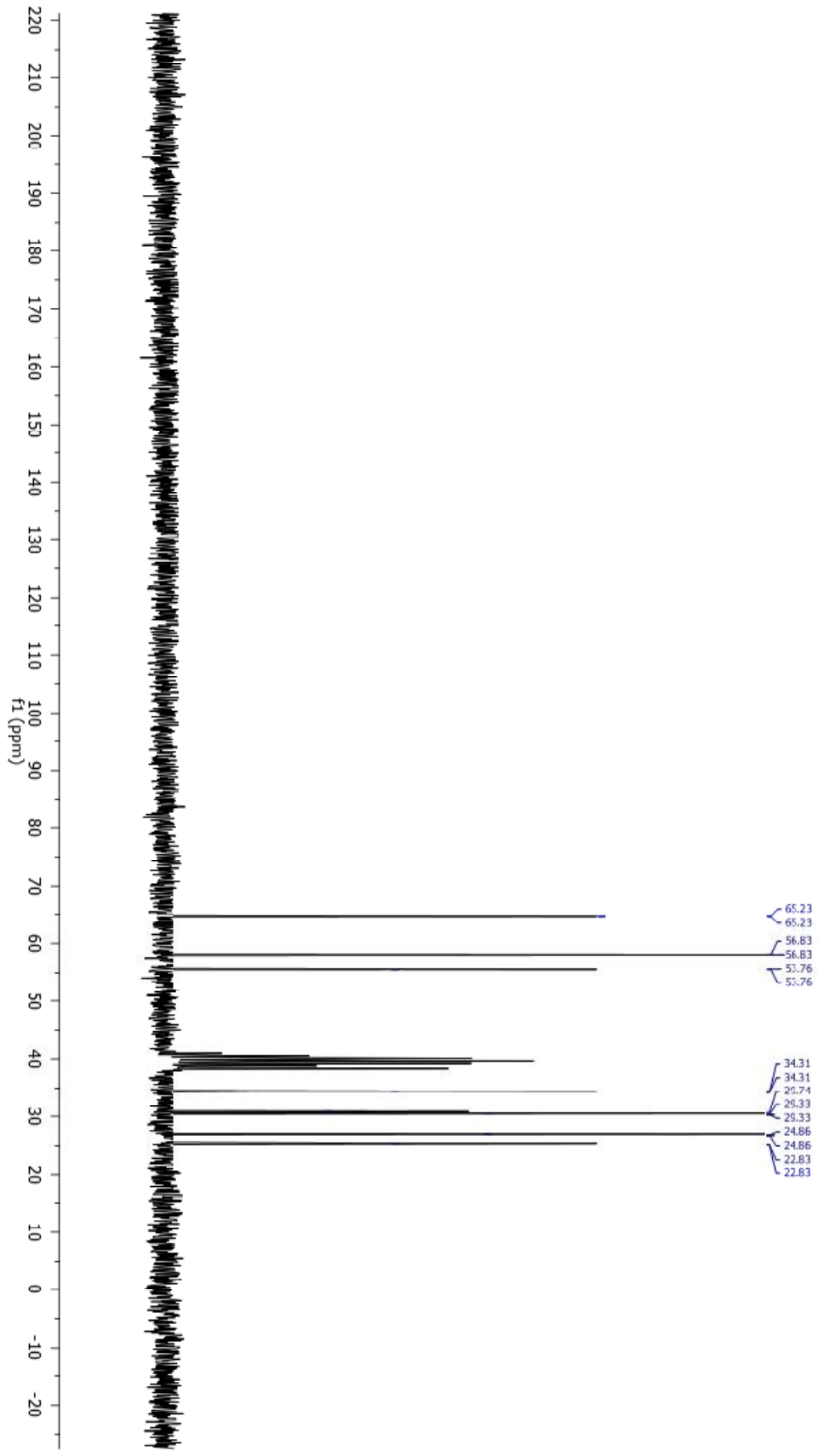


Figure 14: The ^{13}C spectrum of sparteine.

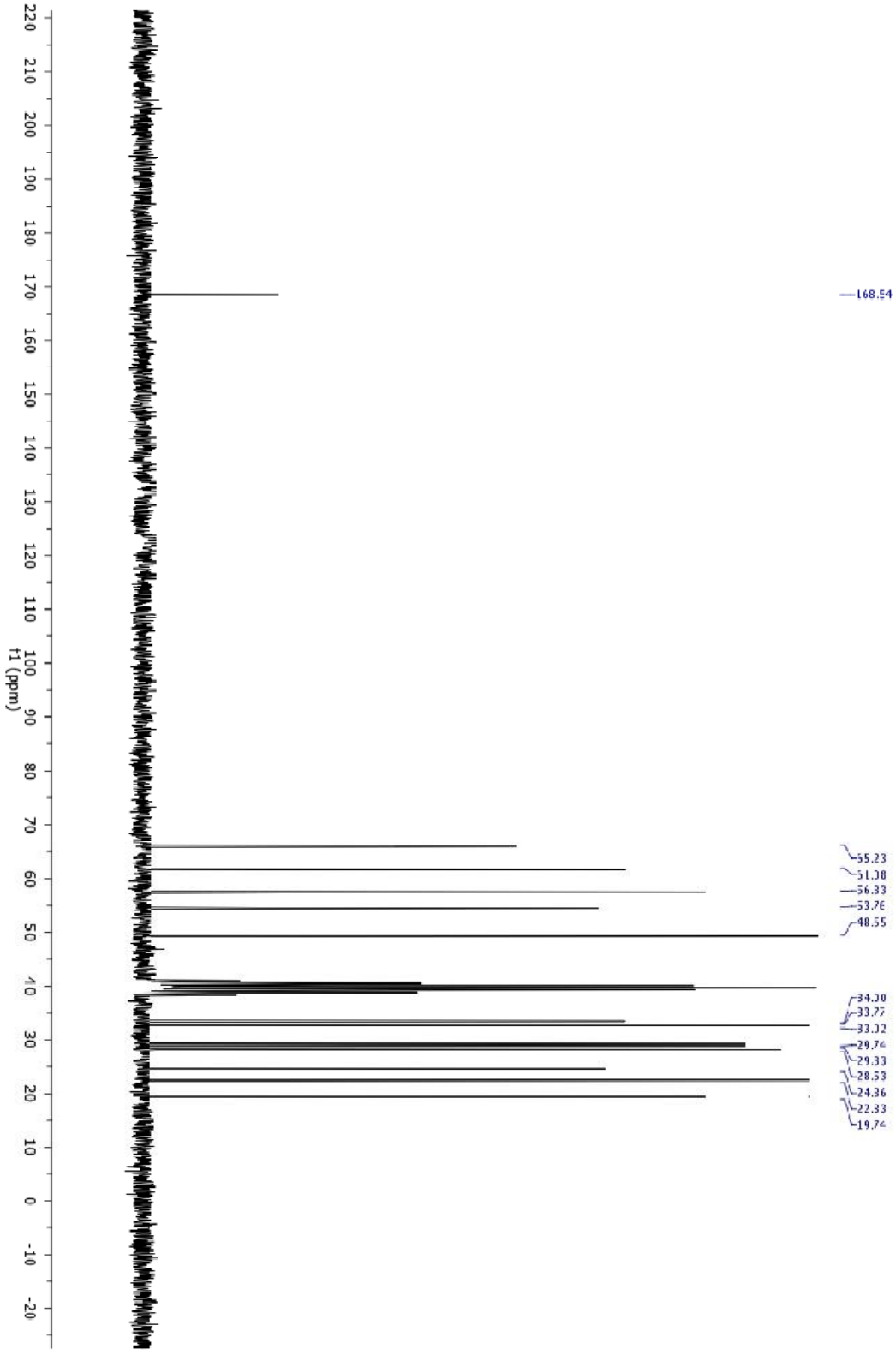


Figure 15: The ^{13}C spectrum of lupanine.

The ^{13}C NMR spectra (Figures 14 and 15) of compounds ***1*** and ***2*** revealed the signals for 15 carbon atoms for both compounds. The chemical shift of C-2 (168.54 ppm) in the ^{13}C spectrum of lupanine indicated a non conjugated carbonyl group [6]. Based on this information, the molecular formula of lupanine was established as $C_{15}H_{24}N_20$.

Table 8: ^{13}C NMR spectral data of sparteine and lupanine.

Carbon	**Sparteine δ ppm DMSO d_6**	**Lit. values δ ppm [121] $CDCl_3$**	**Lupanine δ ppm DMSO d_6**	**Lit. values δ ppm [6] CD_3Cl**
2(CH_2) , C=O	56.81	56.0	168.54	174.1
3 (CH_2)	29.33	25.6	33.02	33.7
4(CH_2)	22.88	24.5	19.74	20.2
5 (CH_2)	36.21	29.1	28.53	28.1
6(CH)	65.23	66.3	61.08	62.2
7(CH)	36.97	32.9	29.74	33.3
8 (CH_2)	34.31	27.4	38.11	27.1
9(CH)	37.25	35.9	34.00	36.1
10(CH_2)	61.9	61.8	48.52	47.9
11 (CH)	65.21	64.2	65.23	65.7
12(CH_2)	37.11	34.5	33.77	34.0
13 (CH_2)	24.86	24.6	22.83	25.4
14(CH_2)	29.74	25.8	24.86	25.6
15(CH_2)	53.78	55.2	56.83	56.7
17(CH_2)	53.74	53.4	53.76	53.7

The ^{1}H NMR spectra of compounds ***1*** and ***2*** revealed six multiplets in the region between δ 1.31 ppm and δ2.36 ppm, which were all integrating for more than one proton, this is indication that the ring A is aliphatic[6]. .

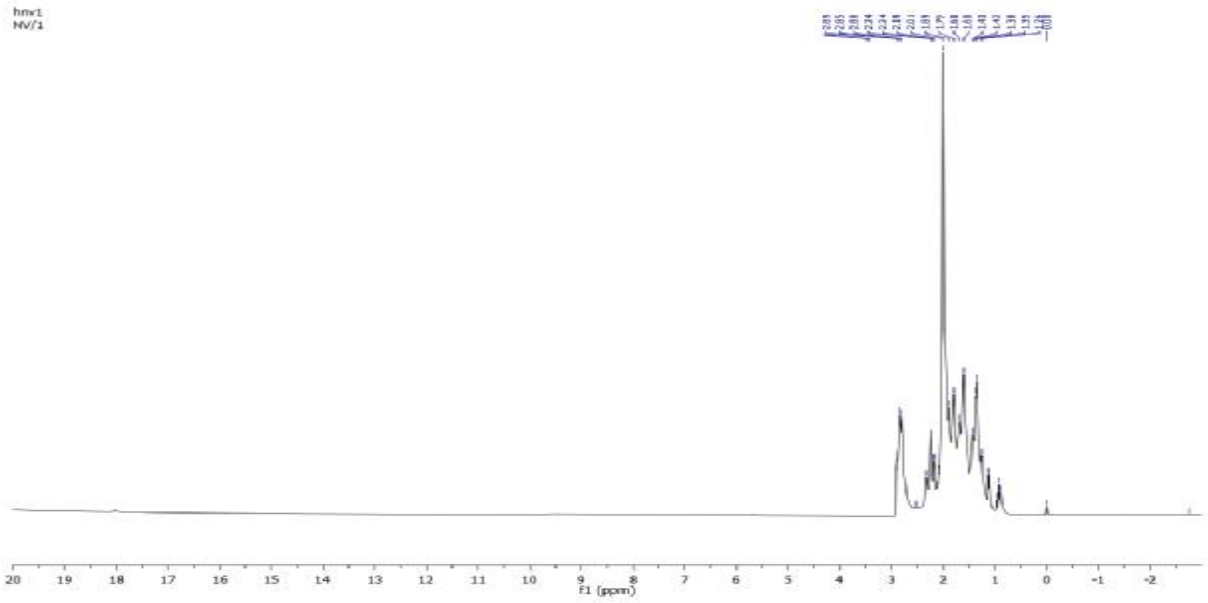


Figure 16: The ^{1}H spectrum of sparteine.

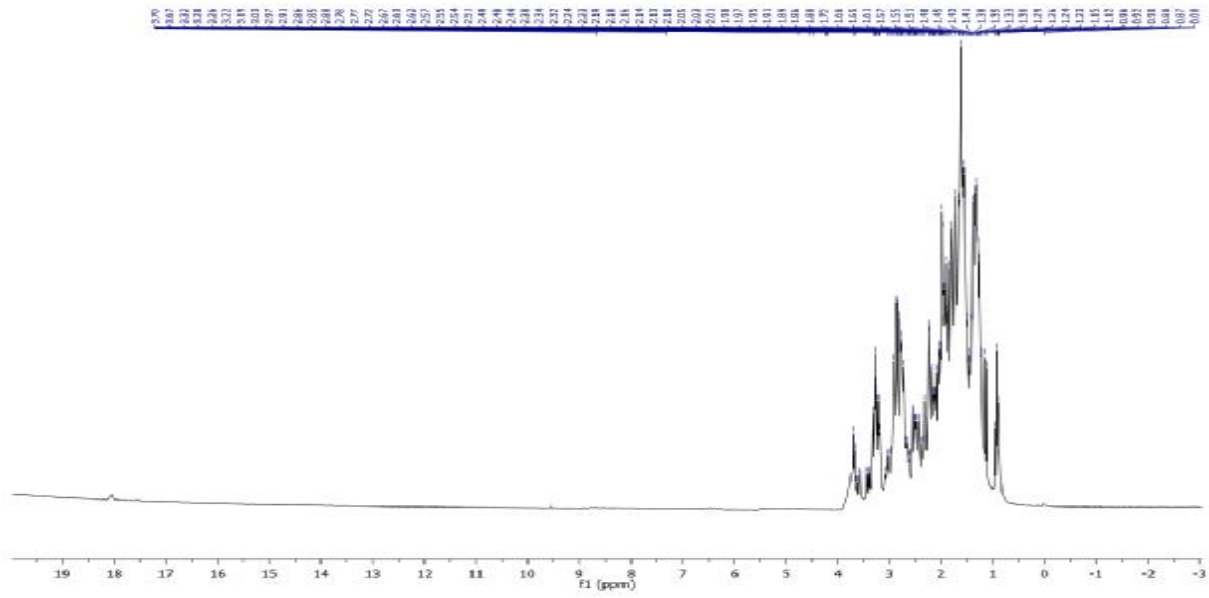


Figure 17: The ^{1}H spectrum of lupanine.

Table 9: ^{1}H NMR spectral data of sparteine and lupanine.

Proton	**Sparteine δ ppm DMSO d_6**	**Lit. Values δ ppm [121] $CDCl_3$**	**Lupanine δ ppm DMSO d_6**	**Lit. values δ ppm [6] CD_3OD**
2a	2.19-2.89	1.79		
2b	1.80	2.53		
3a	2.01	1.38	2.20	2.36
3b	2.01	1.38	2.20	2.36
4a	1.68	1.05	1.51	1.84
4b	1.60	1.55	1.70	1.63
5a	1.38	1.24	1.45	1.83
5b	1.26	1.12	1.72	1.59
6	1.68	1.58	3.32	3.43
7	1.80	1.69	1.97	2.12
8a	2.01	0.90	2.03	1.37
8ß	1.26	1.91	1.15	2.15
9	1.43	1.32	1.55	1.66
10a	2.19-2.89	1.84	3.70	2.58 (dd, 7 =2.5,13.1)
10ß	1.80	2.38	2.44	4.43 (td, y =2.3,13.1)
11	2.01	11.83	1.47	1.63
12a	1.35	1.35	1.48	1.59
12b	1.43	1.21	1.26	1.41
13a	1.68	1.15	1.12	1.75
13b	1.68	1.55	1.64	1.31
14a	1.60	1.43	1.43	1.58
14b	1.60	1.43	1.43	1.58
15a	1.80	1.86	1.80	2.81
15b	2.19-2.89	2.63	2.68	1.95
17a	2.19-2.89	2.20	1.86	2.88 (dd,y= 10.0,11.9)
17b	2.19-2.89	2.54	2.72	1.96

The IR spectra of compound ***1*** and ***2*** (Figure 18 and 19) showed multiple bands at ~2960 cm^{-1} and at ~ 2845 cm^{-1} suggested quaternary nitrogen and combination overtones, embodying the C-H stretching the Bohlman's bands characteristic of quinolizidine-type alkaloids [122].

The peaks at ~1650 cm^{-1} and at ~1400 cm^{-1} indicating the presence of a conjugated carbonyl group and C-N stretching band, respectively[123].

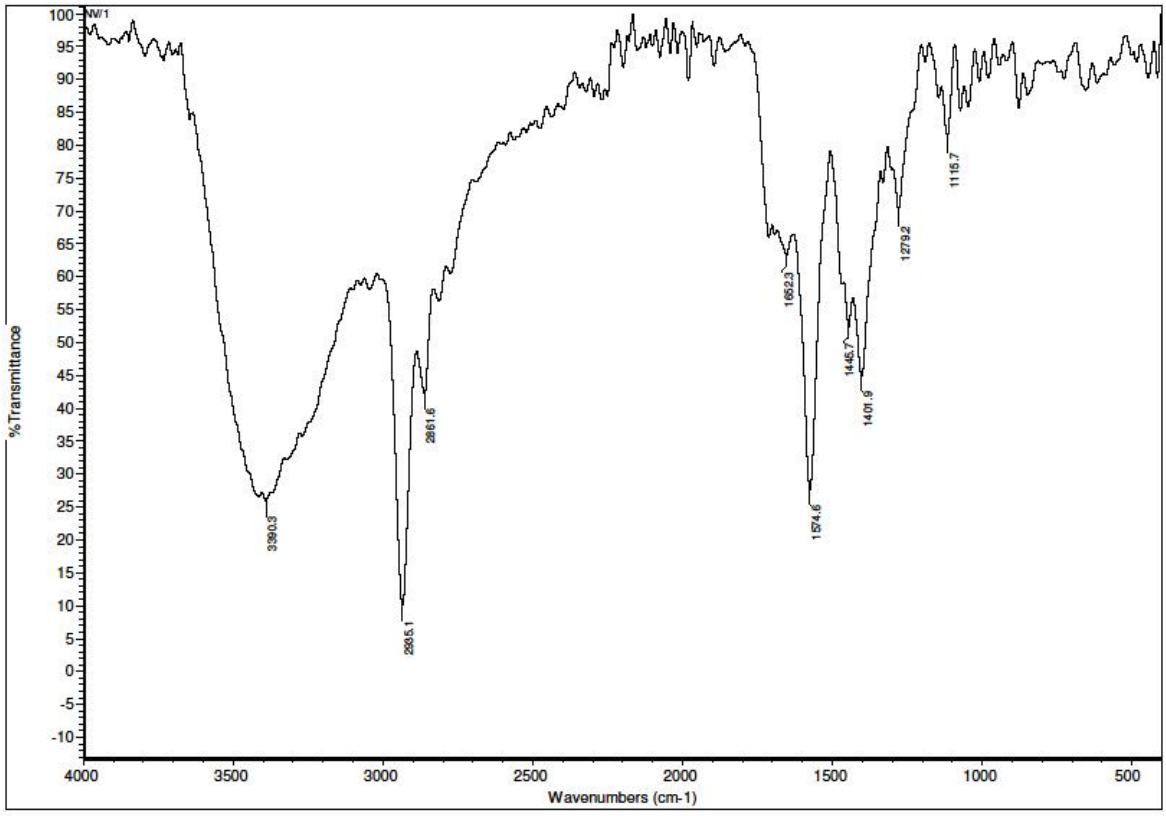


Figure 18: The IR spectrum of sparteine.

The IR spectrum of compound ***2*** (Figure **1**9) revealed the presence of an intense peak at 1022 cm^{-1} due to the C-O stretching band.

Sparteine and lupanine have a number of carbon-hydrogen vibrational bands in the 2800 – 3000 cm^{-1} region, and of the low frequency C–H stretching bands extending down to 2500 cm^{-1}, the so-called Bohlman "*trans*-band". It is well known that the nature of the "*trans*-band" depends on the conformational and configurational arrangement of sparteine and its derivatives [124]. Broadened band in IR spectra with maxima at 3399 cm^{-1} are from solvent.

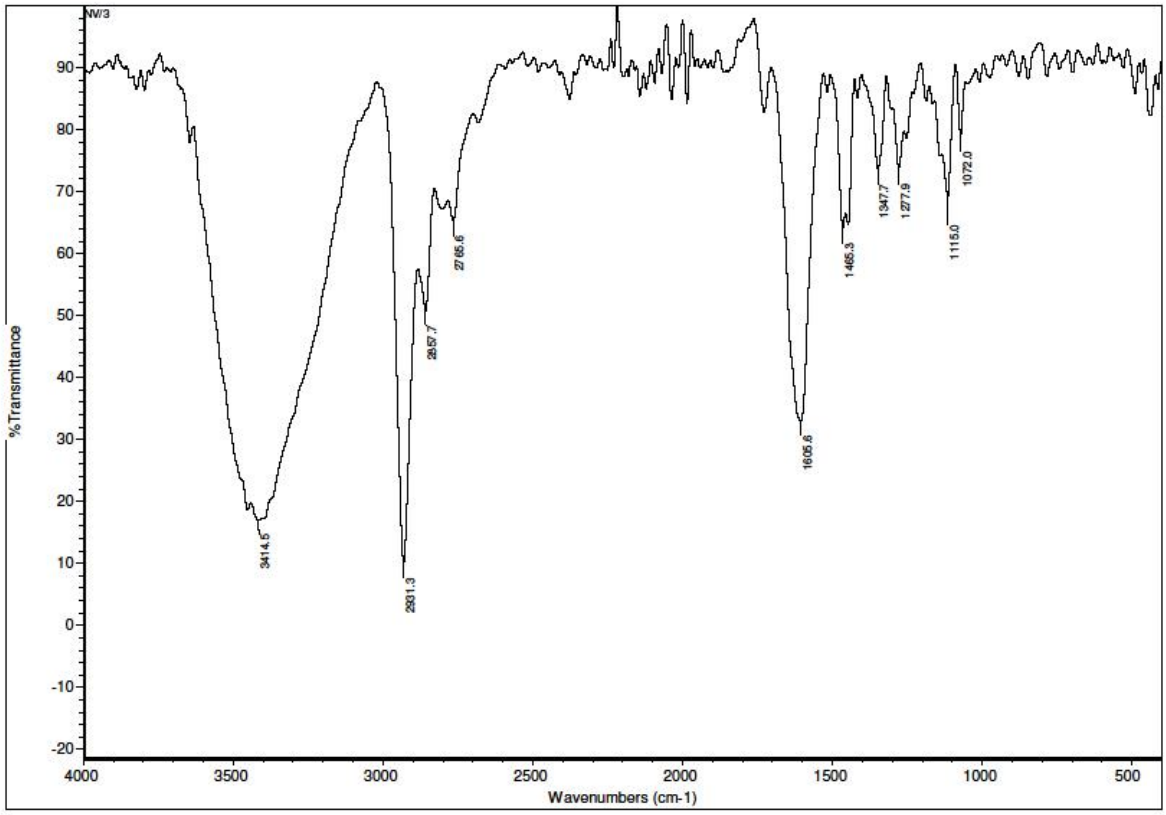


Figure 19: The IR spectrum of lupanine.

II-1-3-Polyphenols

Cytisus purgans was investigated regarding their polyphenolic and flavonoids content using the Folin–Ciocalteu and aluminum chloride colorimetric methods, respectively. In this section, there are a comparison between extractions' yield including70% aqueous methanol extract and 70% aqueous ethanol extract.

II-1-3-1-Extraction yield and total polyphenols and flavonoids content

In the leaves of *Cytisus purgans* the maximum extraction yield was obtained with 70% aqueous methanol solvent (13.75%), while the 70% aqueous ethanol extraction gave the lowest yield (11. 32%) which is similar to the yield of *Cytisus scoparius* (11.76%) [111].

The total phenolic content (TPC) of the plant extracts was measured using the Folin–Ciocalteu colorimetric method, based on the principle of reduction of phosphomolybdic acid by phenols in the presence of aqueous alkali [125]. *Cytisus purgans* have TPC range between 1.82% and 1.39% for methanolic and ethanolic extract, respectively. This result is, for ethanolic extract, inferior to that of *cytisus scoparius* (2.25%) and superior of that of *cytisus multiflorus* (1.3%) [126,111]. This can be explained probably because of the difference between species and other factors like culture conditions.

The total flavonoidic content (TFC) of the plant extracts was determined by using the aluminum chloride colorimetric method, based on the reaction of aluminum ion with flavonoids at alkaline medium forming red chelates[king]. For *cytisus purgans*, TFC range from 0.12 and 0.10 for methanolic and ethanolic extract respectively [125].

Table 10: Extraction yield and total polyphenols and flavonoids content in the leaves of *Cytisus purgans*.

Extract	**Yield %**	**TPC mg GAE/g extract**	**TFC mg RE/g extract**
Methanolic	13.75	1.82	0.12
Ethalonic	11.32	1.39	0.10

The total phenolic content in plant extracts of the species *Cytisus purgans* depends on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction.

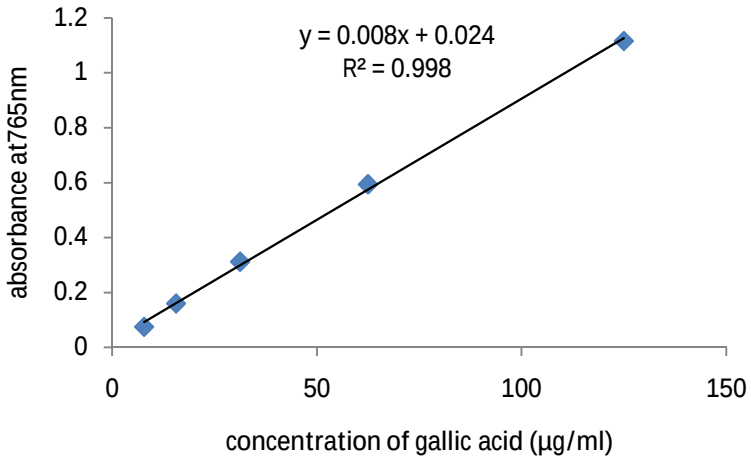


Figure 20: Calibration curve of standard gallic acid for determination of total phenolics content.

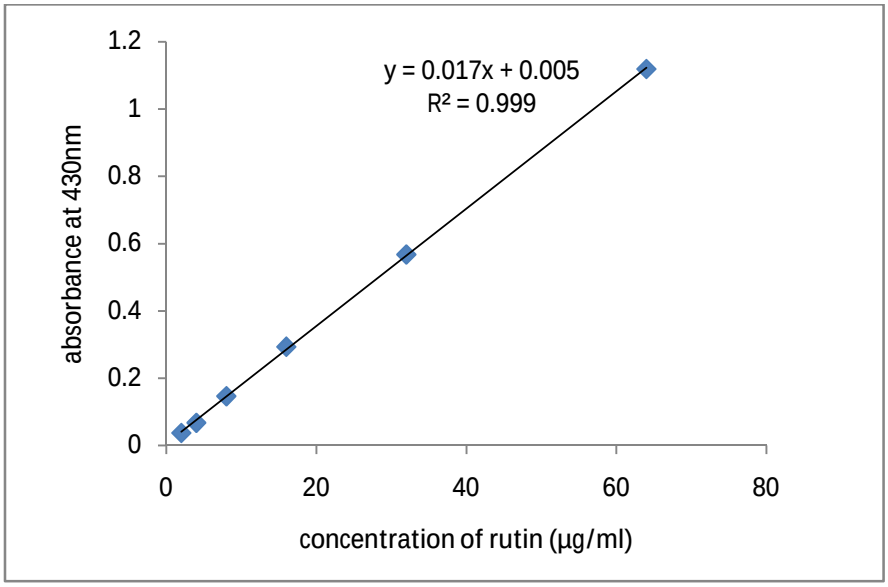


Figure 21: Calibration curve of standard rutin for determination of total flavonoids content.

The regression equations and correlation coefficient (*R*2) were obtained directly from the Microsoft Excel 2003 software data management system.

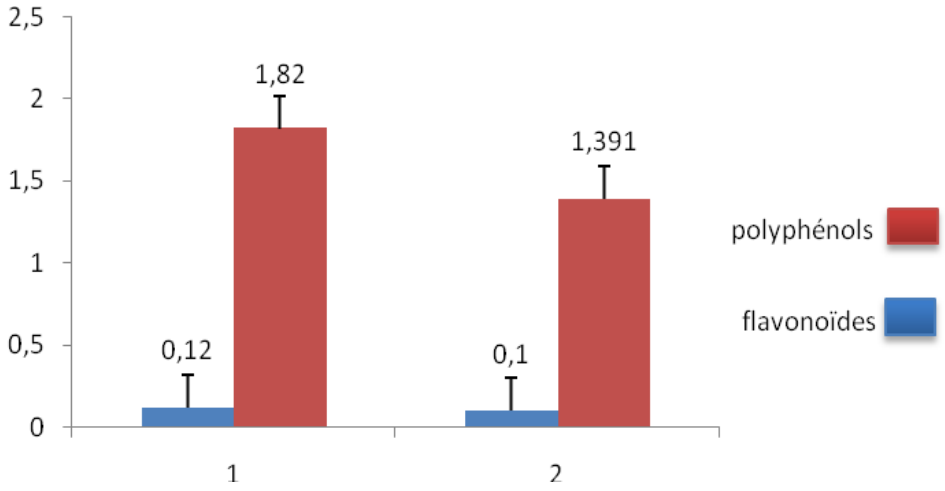
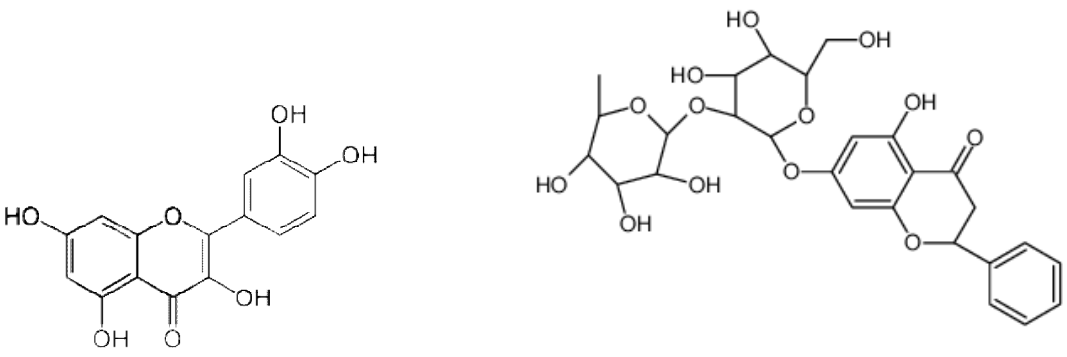


Figure 22: Content of polyphenols and flavonoides in both extracts of the leaves of cytisus purgans. 1: Methanolic extract, 2: Ethanolic extract.

II-1-3-2- Isolation of flavonoids

Phytochemical investigation of the polyphenolic extract of the plant *Cytisus purgans* revealed the presence of two flavonoids (Figure 23), which one of them is a flavonol-type which is quercetin (compound ***3***) and the other is a flavonoid glycosie-type which is sarotanoside (compound ***4***). These two compounds were identified using UV, IR, MS and NMR spectral analysis and comparing with literature data.



3 ***4***

Figure 23: Structures of quercetin *3* and sarotanoside *4*.

Compound ***3*** was isolated as a yellow amorphous powder. Its UV spectrum in MeOH (Figure 24) give absorptions at 209, 254 and 370 nm which are consistent with the presence of a 3, 5, 7, 3`, 4`- pentahydroxyflavone structure[127].

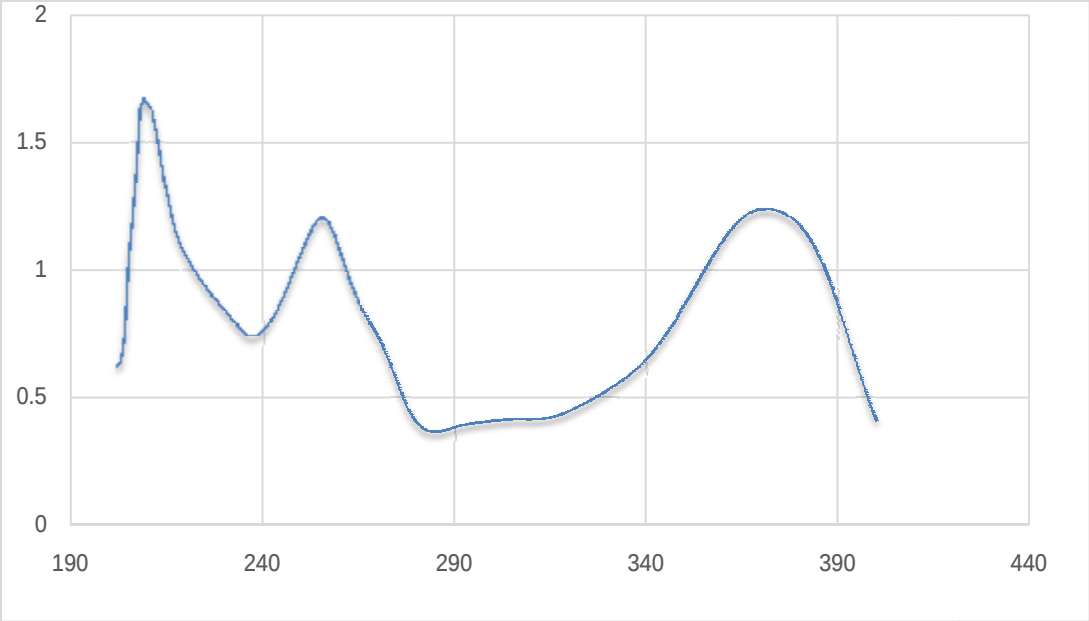


Figure 24: The UV spectrum of quercetin.

The mass spectrum of compound ***3*** (Figure two-15) show a principal fragment at 302 m/z indicating $M^{+\cdot}$ a peack at 301 m/z ($[M-H]^{+}$), another mass pick at 241 m/z indicating the loss of 60 units ($CH_3C{=}O\ OH$).The loss of $CH_2{=}CH_2$ is indicated by a pick at 213 m/z.

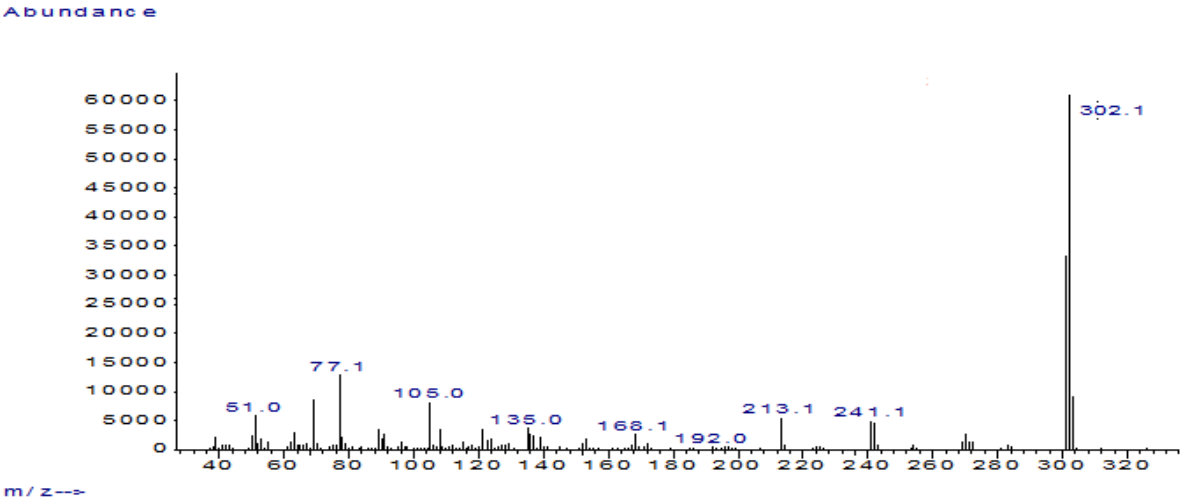


Figure 25: The MS spectrum of quercetin.

The reference [Tsimogiannis [1],*, Martina Samiotaki [2], George Panayotou [2] and Vassiliki Oreopoulou [1],*] are proposed a possible pathway of fragmentation in mass spectroscopy which is illustrated in the figure **26.**

The ^{13}C NMR spectrum of compound ***3*** (Figure 27) showed the presence of 15 carbon signals, we mention here that DMSO gives signals at almost 40 ppm.

In the ^{1}H-NMR spectrum of compound ***3***, the aromatic region exhibited an ABX system at 7.73 (1H, *d*, *J* = 2.0 Hz, H-2`), 7.62 (1H, *dd*, *J* = 2.0 and 7.5 Hz, H-6`), and 6.85 (1H, *d*, *J* = 8.0 Hz, H-5`) due to a 3`, 4` disubstitution of ring B and a typical *meta*-coupled pattern for H-6 and H-8 protons (6.15 and 6.35 ppm, *d*, *J* = 2.0 Hz). Broad singulet at 9.40 ppm was attributed to 3-OH of 3, 3` and 4` respectively. OH of 7 and 5 were at 10.55 ppm and 12.50 ,respectively. Based on the NMR data and comparison of the data given in the literature, the structure of compound ***3*** was identified as quercetin.[127].

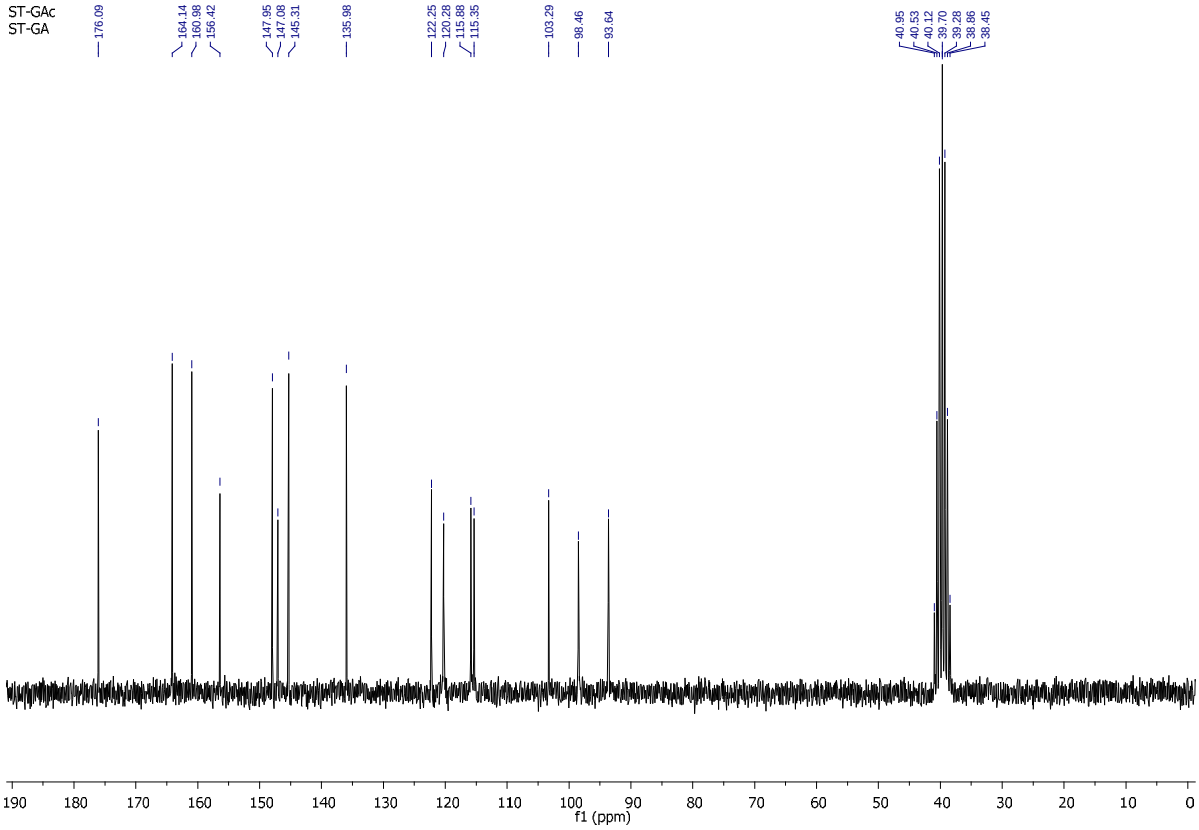


Figure 27: The ^{13}C spectrum of quercetin.

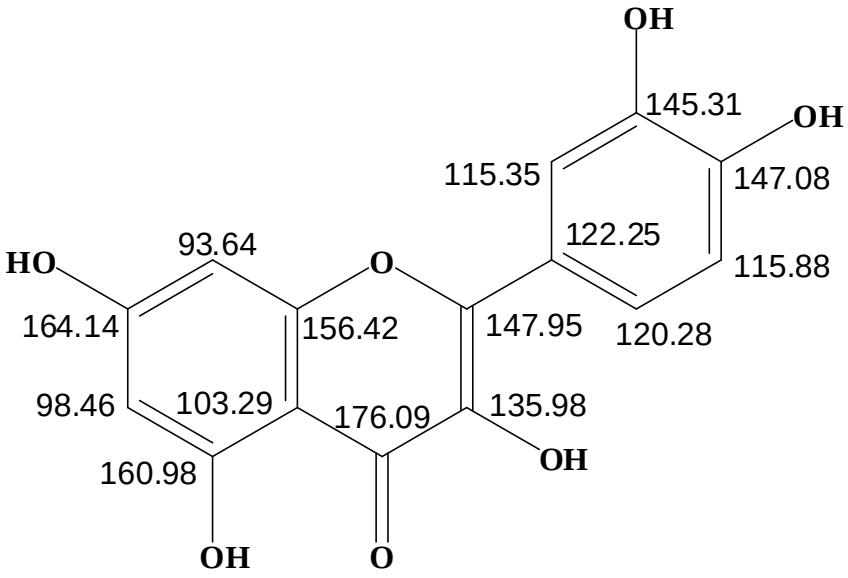


Figure 28: The configuration of ^{13}C NMR values of quercetin.

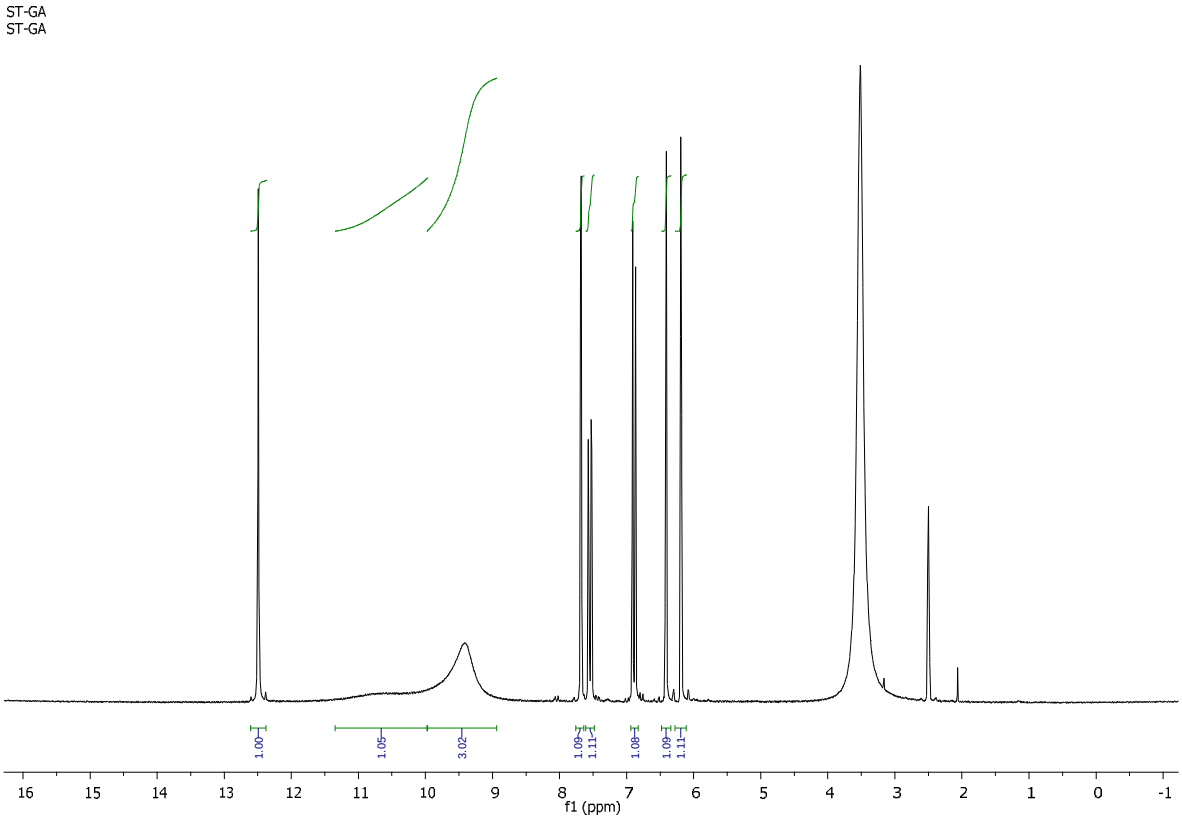


Figure 29: The ^{1}H spectrum of quercetin.

The Ft-IR spectrum revealed seven peaks confirmed the presence of functional groups to quercetin. It includes 3404.40 cm^{-1} (polymeric OH stretch), 2851.88 cm^{-1} (C-H group in aromatic ring), 1664.22 cm^{-1}(C=O stretch), 1382.92 cm^{-1}(phenol (or) tertiary alcohol, OH bend), 1262.89 cm^{-1} (C-O stretch), 1131.55 cm^{-1} (C=C stretch) and 1015.10 cm^{-1} (C-C stretch). The above infrared functional group characteristics were cited in literature [128].

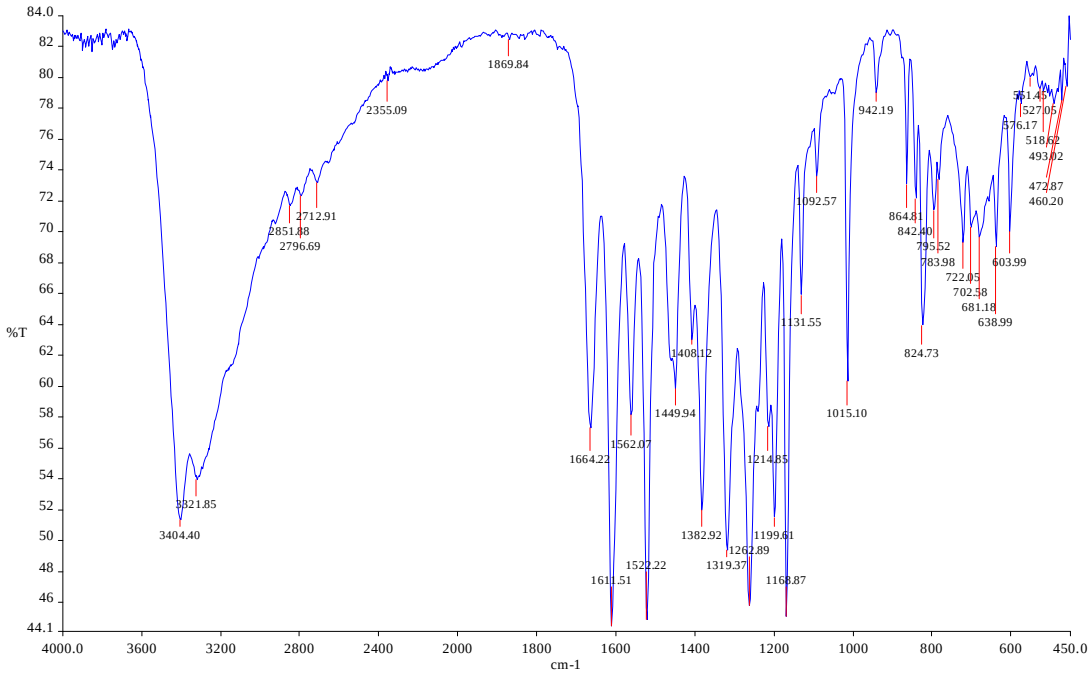


Figure 30: The IR spectrum of quercetin.

Compound ***4*** is a flavonoid glycoside which is sarotanoside, other synonyms of this compound are: Pinocembrin 7-rhamnosylglucoside, (2S)-7-[(2S,3R,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-2-yl]oxy-5-hydroxy-2-phenyl-2,3-dihydrochromen-4-one.

MS spectrum was recorded on Agilent Tof MS who gives high resolution of molecular ion. It is instrument from 2007 year so fragment ions (MSn ions) are not good. Agilent have one specific format for this, raw file, and software that opened this ms spectra are only on computer who is directly linked to the instrument (analyst 2).

ESI way for ionization sometimes gives problems, especially for compounds that have sugar units. Previous degradation of this part by nebulizer gas at 350° C sometimes gives problems.

For all above reasons, we will use only molecular ion and the loss of sugar unit for the identification of compound *4*.

Mass spectra of compound ***4*** show a peak at 563 m/z indicating of $[M-H]^+$, the peak at m/z 255 indicating the loss of sugar unit [129].

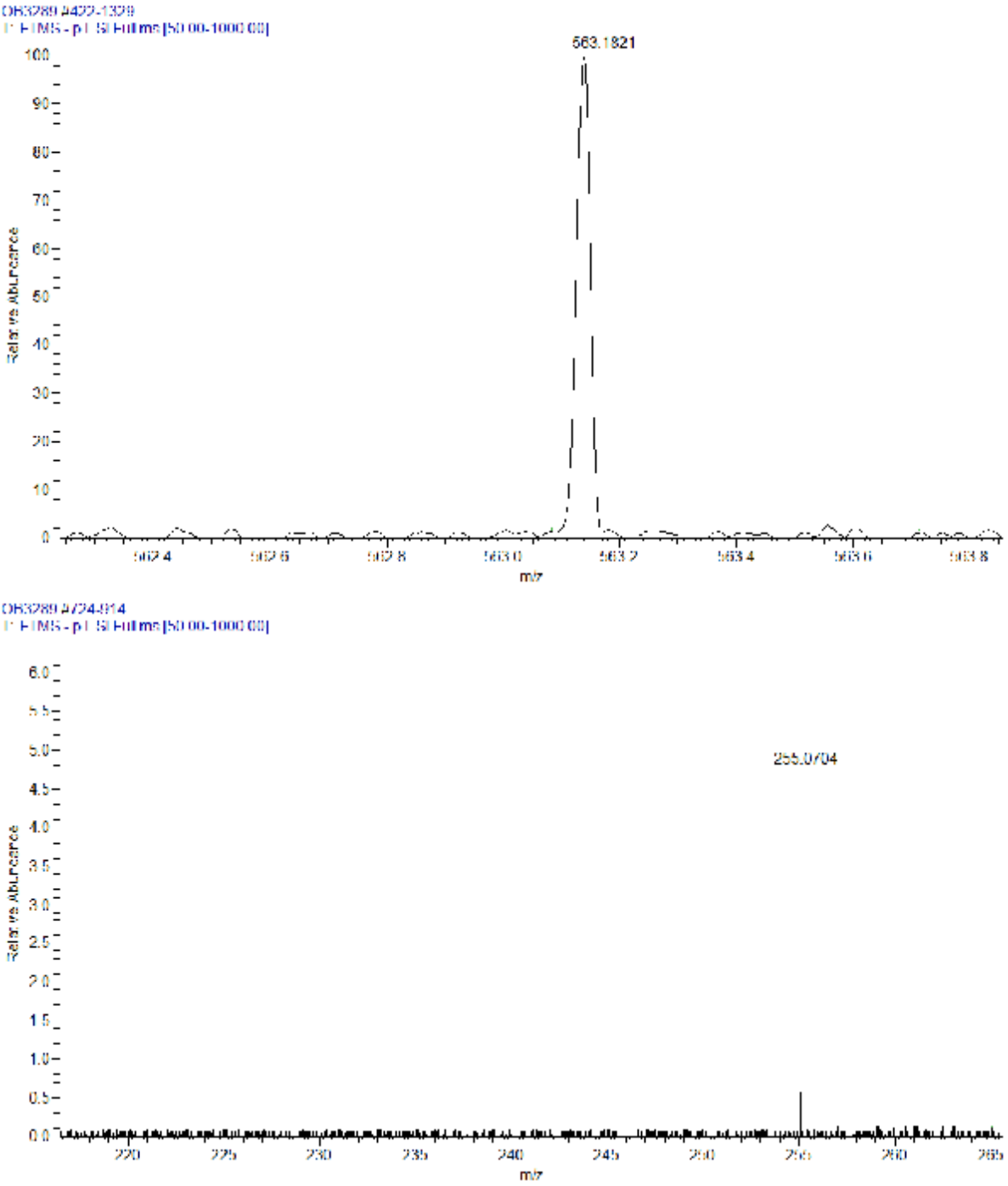


Figure 31: The mass spectra of sarotanoside.

The ^{13}C NMR spectrum of compound ***4*** mentions the presence of 27 carbons (figure two-19 and table two-4).

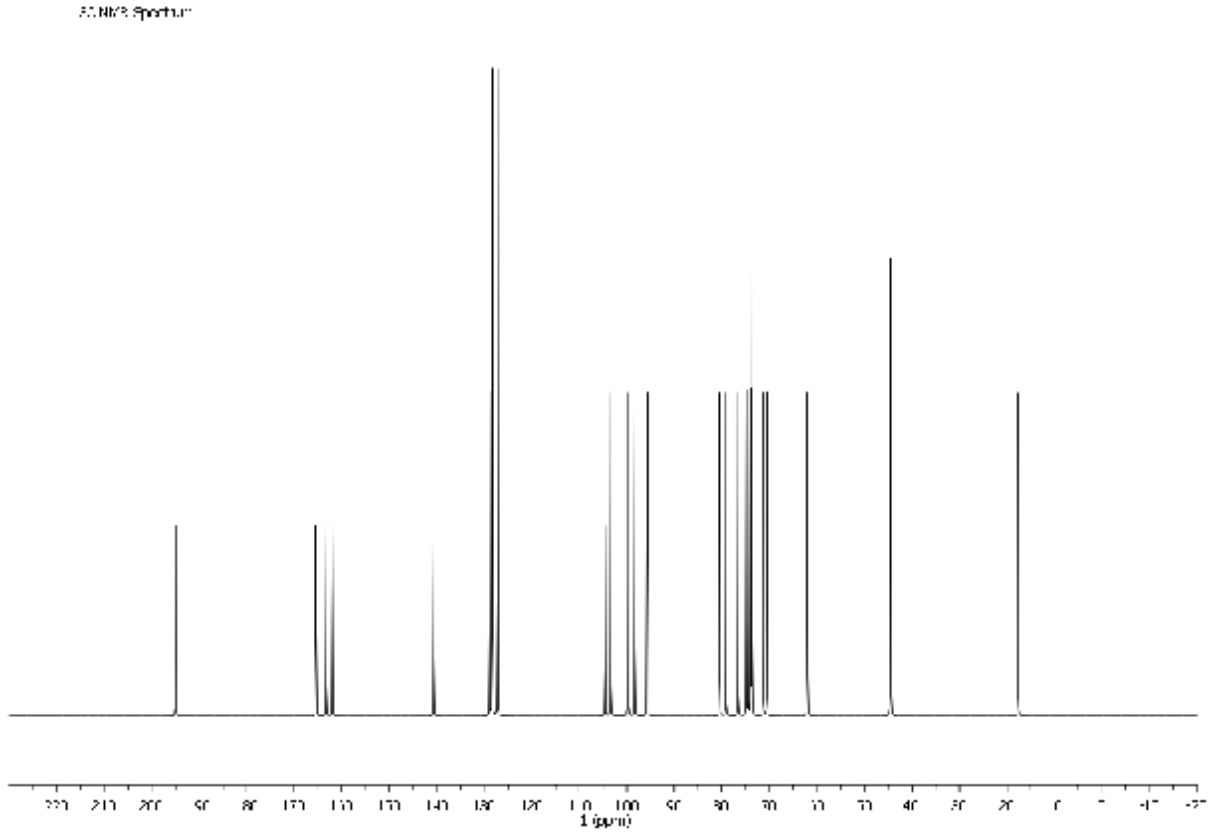


Figure 32: The ^{13}C NMR spectrum of sarotanoside.

From the ^{1}H spectrum of compound ***4*** (Figure 33), it was clear that there were two sugar residues. In addition to the sugar proton peaks, peaks with shifts characteristic of aromatic protons were observed and the compound appeared to contain two aromatic rings. These deductions were confirmed by the appearance of carbon peaks in the aromatic region of the carbon spectrum. An additional signal at δ_C 79.0 (not assigned to the sugar carbons) indicated that the non-aromatic aglycone portion of the compound contained an oxygenated carbon. Two other carbon peaks, a methylene and a carbonyl were observed at δ_C 44.3 and δ_C 197.4, respectively. The compound was identified to be a flavanone by comparison with spectroscopic data given in the literature.

Two doublets observed at δ_H 6.17 (dd, J= 2.1 Hz) and δ_H 6.20 (dd, J= 2.1 Hz) are characteristic of a phloroglucinol-type A-ring in flavonoids. The B-ring protons appeared 30 as a five proton multiplet from δ_H 7.54 -7.35 and, therefore, this ring does not contain any substituents. The remaining non-sugar proton signals (C-ring) at δH 5.50 (dd, J= 3.0 & 12.6

Hz),δ_H 3.14 (dd,J= 12.6 & 17.4 Hz) and δH 2.83 (dd, J= 3.0 & 17.4 Hz) appeared to be coupled to one another and were assigned as H-2β(δ_C 79.0), H-3α and H-3β(δ_C 44.3), respectively[130].

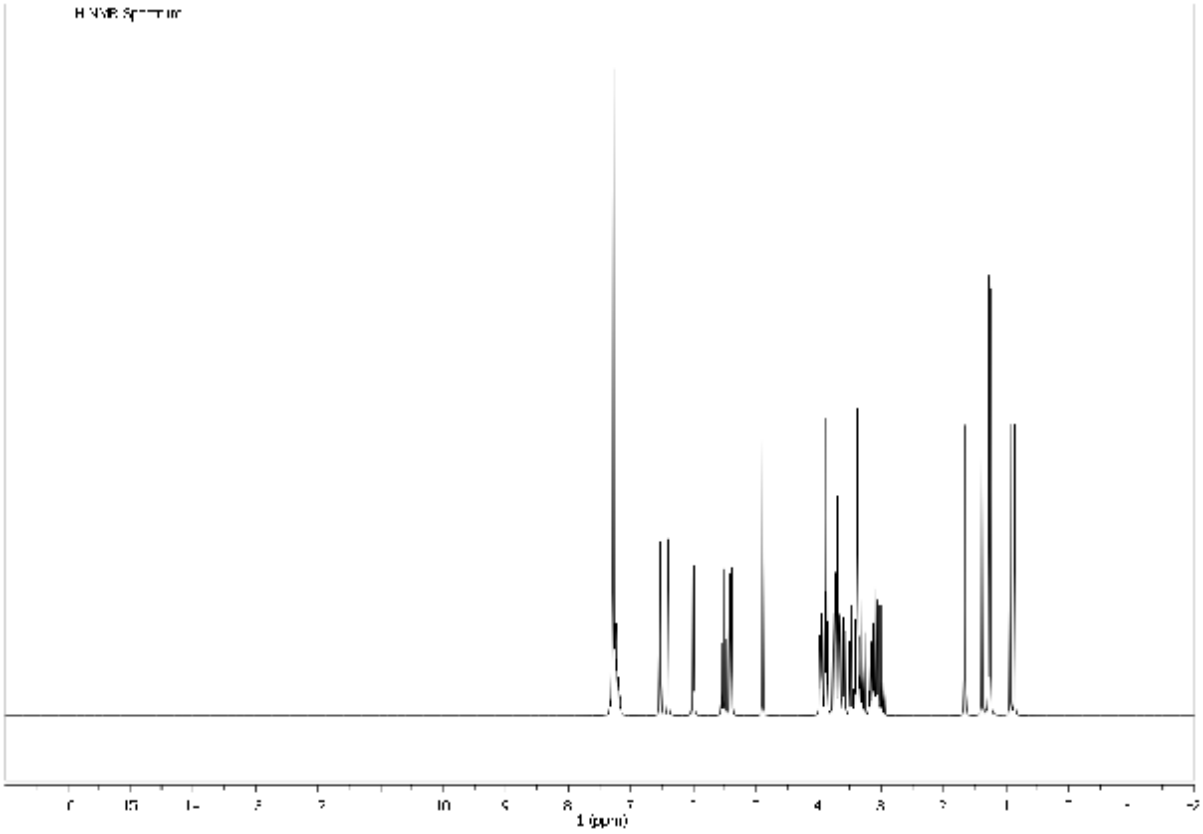


Figure 33: The ^{1}H NMR spectrum of sarotanoside.

The presence of a methyl doublet at δ_H1.28 (d, J= 6.3 Hz), characteristic of a rhamnose methyl group, was observed and one of the sugar residues was thus identified as rhamnose. Naturally occurring rhamnosyl moieties normally have an α-linkage in which the C-1 proton has an equatorial-equatorial coupling, which gives rise to a doublet with a small coupling constant. In this compound the rhamnose H-1 proton was observed at δ_H 5.24 (d, J = 1.5 Hz). The other sugar moiety was identified as glucose with the characteristic C-1 glucose proton observed at δ_H 5.10 (d, J= 7.2 Hz). The large coupling constant is indicative of axial axial coupling suggesting that the glucose was β linked. Due to extensive overlapping of the sugar signals, the individual protons were not distinguished from one another. These protons were found in the region between δ_H 3.93 to δH 3.40. Comparison with spectroscopic data reported in literature, compounds 4 was identified as: pinocembrin 7-β-neohesperidoside (sarotanoside) [130].

Table 11: ^{13}C NMR spectral data of sarotanoside .

Carbon	**δ (ppm)**	**Carbon**	**δ (ppm)**	**Carbon**	**δ (ppm)**
2	77.1	10	103.2	4``	69.65
3	42.2	1`	138.5	5```	76.91
4	196.7	2`	126.7	6``	60.4
5	162.9	3`	128.6	1```	100.4
6	96.4	4`	128.6	2```	70.4
7	164.9	1``	97.4	3```	70.5
8	95.2	2``	78.6	4```	71.8
9	162.5	3``	76.11	5```	68.2
				6```	18.0

The UV spectrum of compound ***4*** revealed absorption at λ_{max} 227, 285 and 327 nm, respectively, which are characteristic of flavonoids glycosides [129].

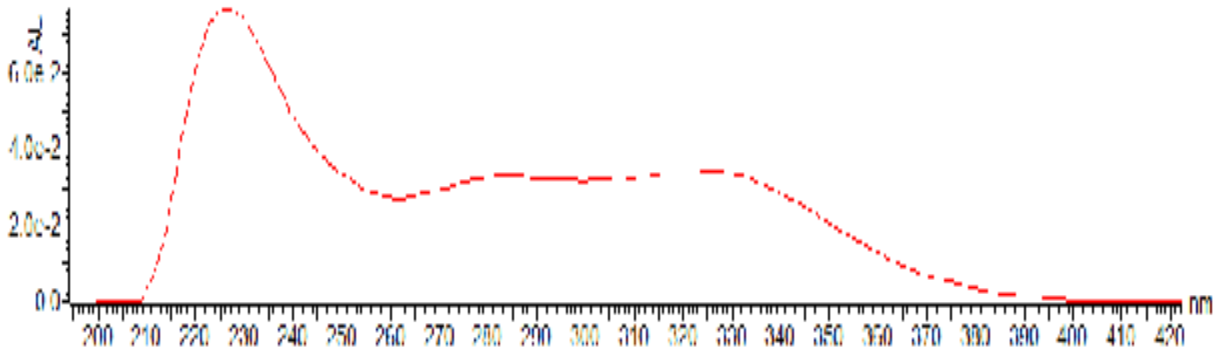


Figure 34: The UV spectrum of sarotanoside.

The IR spectrum of compound ***4*** showed hydroxyl absorption at 3700-3300 cm^{-1}, bands at 1600 and 1660 cm^{-1} due to the presence of an unsaturated function (C-4 of flavonoid), other peaks at 2930 cm^{-1} due to methylene groups and peaks at 150&1600 cm-’ due to an aromatic ring. A broad carbonyl stretching band in the region 110&1600 cm-‘, suggested a glycosidic nature [131].

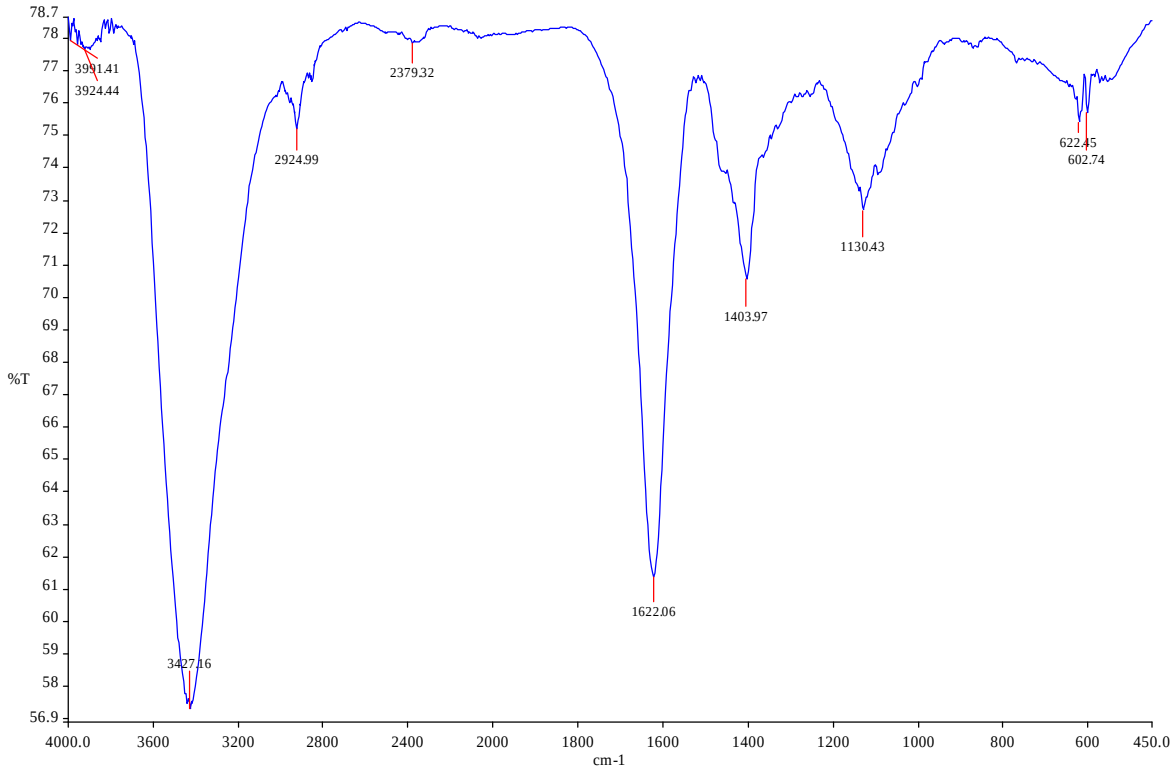


Figure 35: The IR spectrum of sarotanoside.

II-2-Biological study

Humans have used medicinal plants throughout their history life, and long time before, good records were kept about herbs use. Texts from ancient China and India provide a good example of very early use of medicinal herbs. They contain prescriptions of countless plant-derived medicines. In modern times, natural products from plants have been isolated for drug discovery and development. During the last 20 to 30 years, the analysis of secondary plant products has progressed a lot. The use of modern analytical techniques has succeeded in the elucidation of exact structural formulas and the most important biosynthetic pathways.

Secondary metabolites, also known as phytochemicals, natural products or plant constituents are responsible for medicinal properties of plants to which they belong. The role they play in the plant is not, to date, well known or understood, but it may be beyond the protection.

Natural products are an important source of drug candidates in pharmaceutical industry, more deeply we understand them, the easier it is for scientists to intervene in alleviating different kind of diseases. The recent references have been consulted for presenting updated information, but also showing the new potentialities of plant second metabolites in drug research and development [132].

In this study, we highlight the biological activities of the plant *Cytisus purgans*: antitumor, antioxidant and antimicrobial activity.

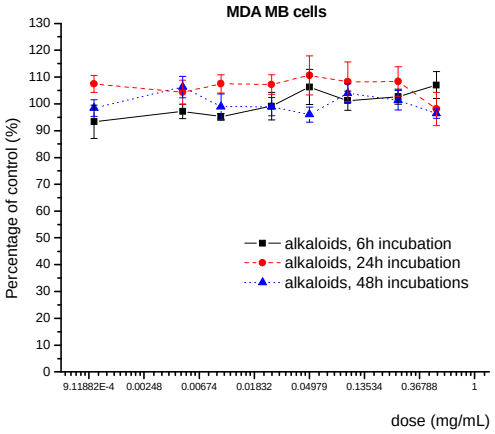
I1-2-1-Antitumor activity

Cancer is a multi-step disease incorporating environmental, chemical, physical, metabolic, and genetic factors which play a direct and/or indirect role in the induction and deterioration of cancers. Strong and consistent epidemiology evidence indicates a diet with high consumption of antioxidant rich fruits and vegetables significantly reduces the risk of many cancers, suggesting that certain dietary antioxidants could be effective agents for the prevention of cancer incidence and mortality. These agents present in the diet are a very promising group of compounds because of their safety, low toxicity, and general acceptance. Consequently, in the last few years, the identification and development of such agents has become a major area of experimental cancer research. Phenolic compounds constitute one of the most numerous and ubiquitous group of plant metabolites, and are an integral part of the human diet. It was found that in addition to their primary antioxidant activity, this group of compounds displays a wide variety of biological functions which are mainly related to modulation of carcinogenesis. Various *in vitro* and *in vivo* systems have been employed to determine the anticarcinogenic and anticancer potential of these natural phenolic compounds

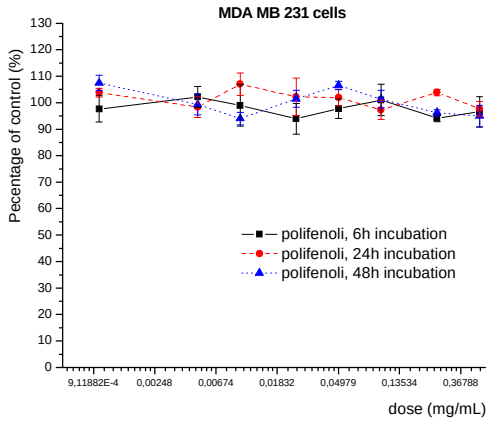
or extracts [133]. For this approach we used the MTT Assay (Mosmann, 1983) which is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells being dead following a toxic damage, cannot transform MTT. This formation production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity. The reaction is mediated by dehydrogenases enzymes associated with the endoplasmatic reticulum and the mitochondria [134].

In this context, the *in vitro* antitumor activity of plant extracts (alkaloids and polyphenols) was assayed on two different cell lines (human breast cancer, MDA-MB-231 and human lung cancer, A549 cell lines). Human cancer cell lines have been a useful tool for the study of the genetics, molecular biology, biology, and therapy of cancer in many tumor types [135]. The cell viability of studied human cancer cells, 6h, 24h and 48h after treatment with increasing concentrations of the plant extracts is presented in Figure two-25. Numerical data illustrating cell viability were obtained from MTT assays and presented as percentage of control. Tested plant extracts are showed no antitumor effects toward of both human cancer cell lines. Neither increasing the dose as well as prolongation of the incubation time with the tested plant extracts did not lead to a statistically significant increase of antitumor effect.

In fact, Anne-lise SAGEN had tested lupanine for cytotoxicity against KB cells, but didn't show any activity [6].



A



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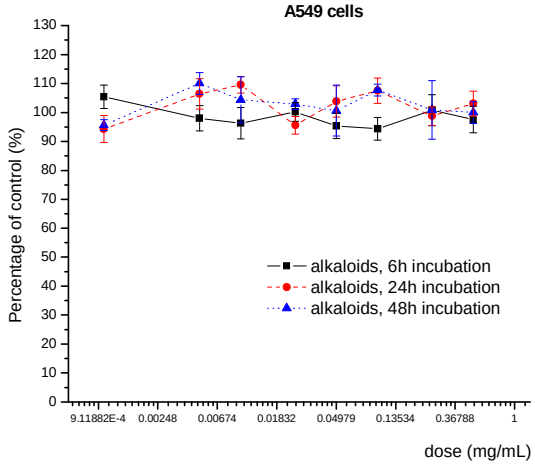

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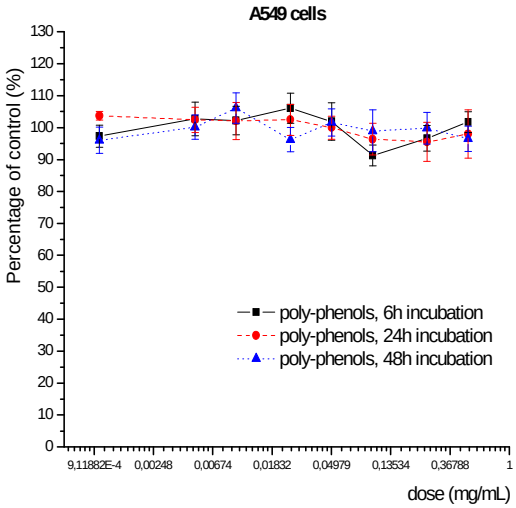


D

Figure 36: Cell viability of MDA-MB 231 (panel A and B) and A549 cell lines (panel C and D), 6h, 24h and 48h after treatment different concentrations of plant extracts (alkaloids, panel A and C, and poly-phenols, panel B and D) estimated by MTT assay. Results from three separate experiments are presented as percentage of control (Mean+S.E.).

The efficacy of cancer chemotherapy is limited by cellular mechanisms of resistance that result in the increased efflux of chemotherapeutic agents, thereby reducing intracellular drug levels. The ability of cells to acquire resistance to multiple compounds, termed multidrug resistance (MDR), is often mediated by the over expression of ATP-binding cassette (ABC) transporters, which move substrates out of the cell against a concentration gradient [136].

However, many chemotherapeutic drugs are presently placed in a predicament of reduced therapeutic effect due to the problem of drug-resistance. Chemotherapeutic drugs also

exert toxicity to normal cells, which in turn causes the unpleasant side effects to the patients. For these reasons, research and development of new classes of anticancer agents which exhibit efficient and selective toxicity in tumor cells is enticing increased attention [134].

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide [136]. In fact, lung cancer is highly lethal and resistant to most anticancer interventions. Treatment resistance is mediated, in part, by enhanced expression of cell survival proteins that help facilitate tumor progression.

Lung cancer is the leading cause of death from malignancy in both males and females with more than 200,000 new cases diagnosed in North America on 2003. Over 85% of these patients eventually die from disseminated disease accounting for 29% of all cancer deaths, more than prostate, colorectal, and breast cancers combined. Sixty percent of patients are considered inoperable at the time of diagnosis and 20% are poor surgical candidates. The overall 5-year survival rate of 10–13% has not changed appreciably over the last two decades [137]. Nucleotide-based therapies targeting clusterin chemosensitize human lung adenocarcinoma cells both *in vitro* and *in vivo.*

Triple-negative breast cancer (TNBC) is aggressive and lacks targeted therapies.

In the other hand, breast cancer is the most frequently diagnosed cancer in women and one of the leading causes of cancer death for women. Worldwide, over 1.3 million cases of invasive breast cancer are diagnosed, and more than 450,000 women die from breast cancer annually. In the US, approximately 200,000 cases of invasive breast cancer and 50,000 cases of in situ breast cancer will be diagnosed annually, and more than 40,000 women die from breast cancer each year – second only to lung cancer. The mortality due to breast cancer has been declining in the US since 1990. The continuing decrease in mortality from breast cancer has been attributed to early detection due to screening, improved adjuvant therapy, and more recently to decreases in the incidence due to lowered rates of usage of hormone replacement therapy. Despite the decreased incidence and mortality, breast cancer remains a major cause of cancer mortality for women and accounts for 15% of all cancer deaths in women in the US.

Clinically, breast cancer can be divided into distinct subtypes that have prognostic and therapeutic implications. Breast cancer patients routinely have the expression of estrogen receptor (ER), progesterone receptor (PR), and amplification of HER-2/Neu evaluated. These markers allow classification of breast cancer tumors as hormone receptor positive tumors, HER-2/Neu amplified tumors, and those tumors which do not express ER, PR, and do not have HER-2/Neu amplification. The latter group is referred to as triple-negative breast cancer (TNBC) based on the lack of these three molecular markers.

TNBC represents approximately 10–15% of all breast cancers and patients with TNBC have a poor outcome compared to the other subtypes of breast cancer.

Interestingly, work with cancer cell lines grown in 2D culture found that MDA-MB-231 cell line was relatively resistant [135].

II-2-2-Antioxidant activity

II-2-2-1-Antioxidant and antiradical activity

The general methods of determination of antioxidant activity are summarized in many reviews. Due to their practical significance much attention is paid to studies of natural products and food supplements. Numerous studies have demonstrated that the antioxidant activity measured depends substantially on the test system used and recommended to base any conclusions on at least two different test systems.

Most of the methods of determination of total antioxidant activity characterize the ability of the tested compound or product to scavenge free radicals and/or to complex metal ions driving the oxidation process.

It should be emphasized that there is a great difference between "antiradical" and "antioxidant" activity and that they do not necessarily coincide. According to Burlakova and coworkers (1975) the antiradical activity characterizes the ability of compounds to react with free radicals (in a single free radical reaction), but antioxidant activity represents the ability to inhibit the process of oxidation (which usually, at least in the case of lipids, involves a set of different reactions). Consequently, all test systems using a stable free radical (for example, DPPH, ABTS, etc) give information on the radical scavenging or antiradical activity, although in many cases this activity does not correspond to the antioxidant activity [138].

Antioxidants play an important role as health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties.3 A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity [139].

II-2-2-2-The DPPH scavenging activity

The DPPH assay method is based on the reduction of DPPH, a stable free radical.5 The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple color). When Antioxidants react with DPPH, which is a stable free radical becomes paired off

in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH.6 Radical to the DPPH-H form, results in decolorization (yellow color) with respect to the number of electrons captured.7 More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug.8 When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenylpicrylhydrazine; non radical) with the loss of this violet color (although there would be expected to be a residual pale yellow color from the picryl group still present) [138].

II-2-2-3-Reducing power

The reducing power (RP) of an extract is determined by direct electron donation in the reduction of ferri cyanide [Fe(CN)6]3- to ferro cyanide [Fe(CN)6]4-. The product was visualized by addition of free Fe3+ ions after the reduction reaction, by forming the intense Prussian blue colour complex, (Fe3+)4[Fe2+(CN-)6]3, and quantified by absorbance measurement at 700 nm [140].

II-2-2-4-Evaluation of antioxidant activity by DPPH radical scavenging method

DPPH has been widely used for free radical-scavenging assessments due to its ease and convenience [141]. In the present study, all extracts were found to be effective scavengers against DPPH radical, as it shown in figures. They were superior to BHT and their activities increased in a concentration dependent manner.

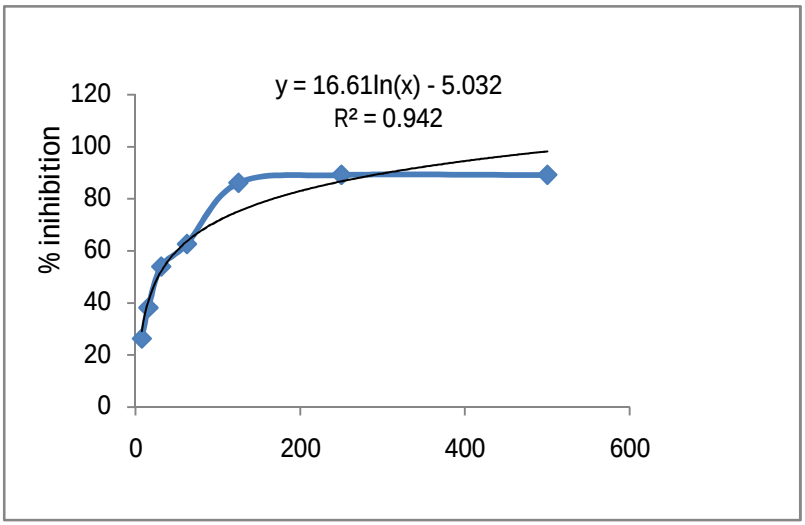
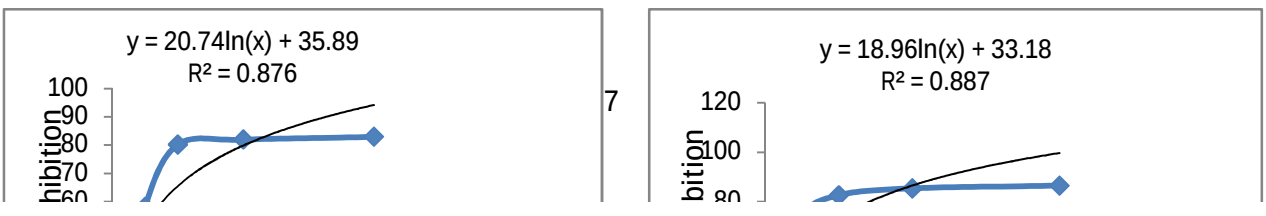


Figure 37: DPPH radical scavenging activity (%) versus concentration (µg/mL) of BHT.



Methanolic extract in MeOH

Methanolic extract in distilled water

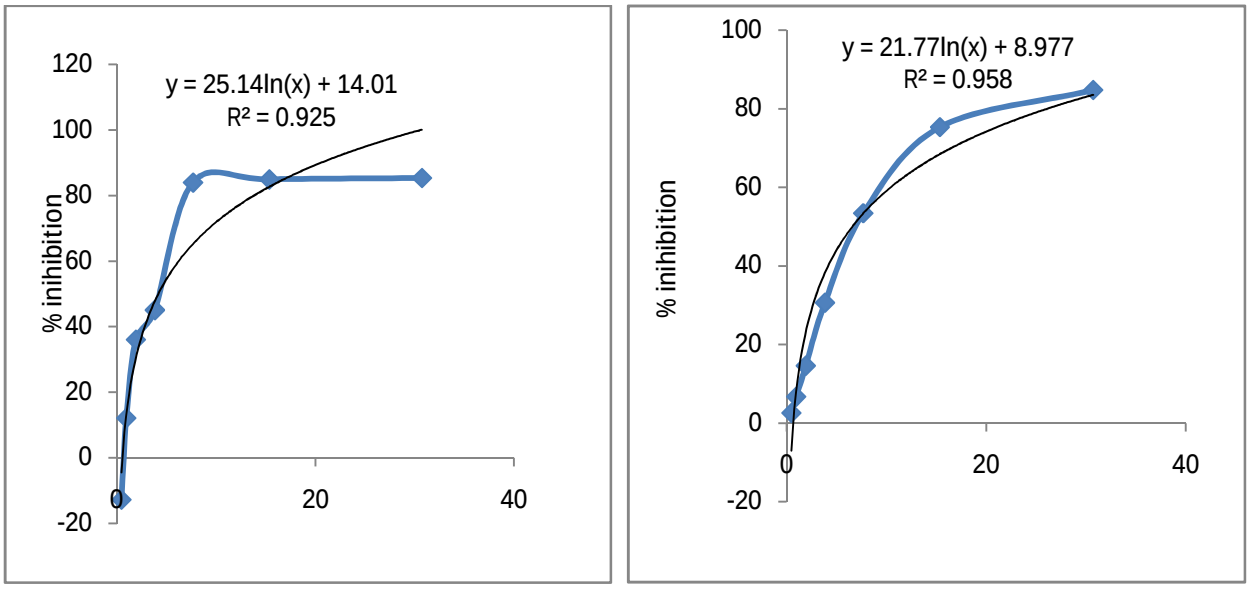


Figure 38: DPPH radical scavenging activity (%) versus concentration of polyphenols (µg/mL) of methanolic extract in MeOH and in distilled water.

Figure 39: DPPH radical scavenging activity (%) versus concentration of polyphenols (µg/mL) of ethanolic extract in MeOH and in distilled water.

It is well known that phenolic compounds are potential antioxidants and free radical-scavengers; hence, there should be a close correlation between the content of phenolic compounds and antioxidant activity [141].

The IC_{50} value, defined as the concentration of the extract required for 50% scavenging of radicals under the experimental condition employed, is a parameter widely used to measure the free radical scavenging activity; a smaller IC_{50} value corresponds to a higher antioxidant activity.

Table 12: Concentration of extracts at DPPH radical scavenging activity 50% (IC_{50}).

		DPPH IC_{50} (µg/mL)
Control	BHT	27.38
Methanolic extract	In MeOH	1.97
	In distilled water	2.41
Ethanolic extract	In MeOH	4.17
	In distilled water	6.55

Butylated hydroxyl toluene (BHT), have long been widely used as antioxidant additives to preserve and stabilize the freshness, nutritive value, flavor and color of foods, and animal feed products. However, at least one study has revealed that BHT could be toxic, especially at high doses. Nowadays, there is an increasing interest in the substitution of synthetic food antioxidants by natural ones. The antioxidant compounds from waste products of food industry could be used for protecting the oxidative damage in living systems by scavenging oxygen free radicals, and also for increasing the stability of foods by preventing lipid peroxidation [114].

II-2-2-5- Evaluation of reducing power

In the reducing power assay, the more antioxidant compounds convert the oxidation form of iron (Fe^{+3}) in ferric chloride to ferrous (Fe^{+2}) [142]. The results of this research showed that the plant *Cytisus purgans* exhibited a significant reducing power.

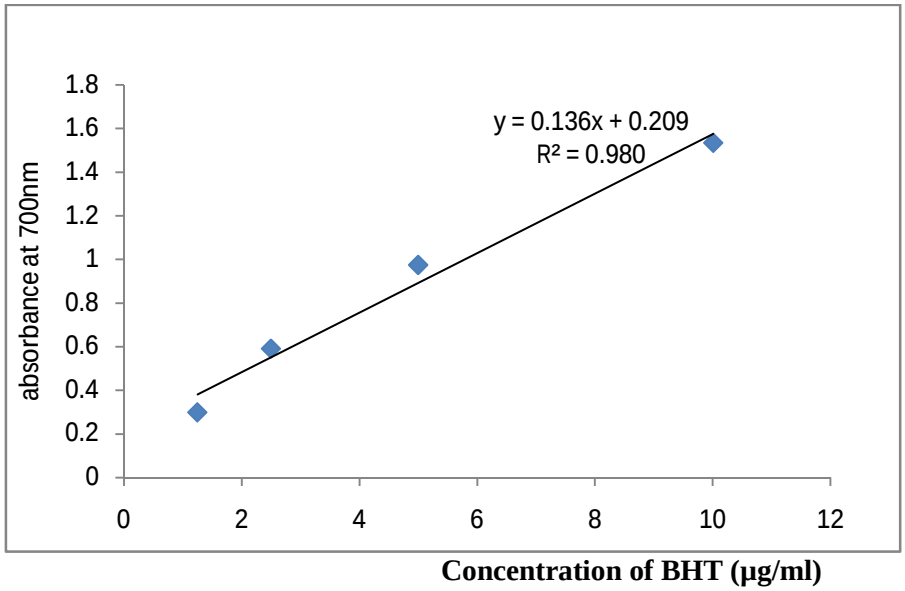
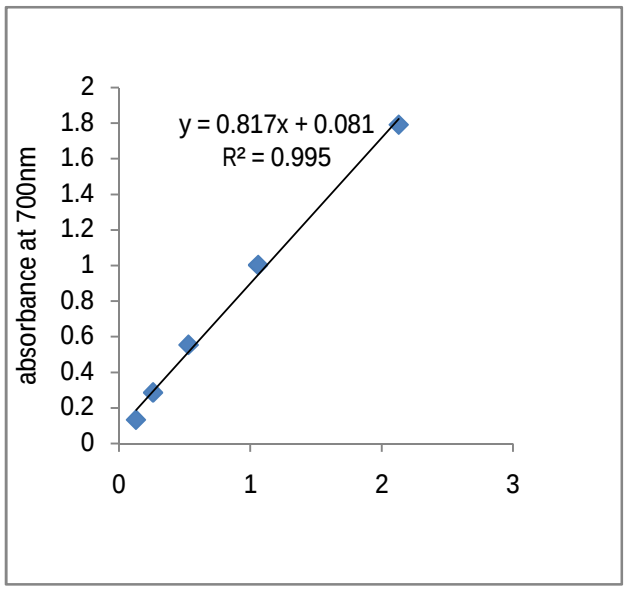


Figure 40: Reducing power of BHT.



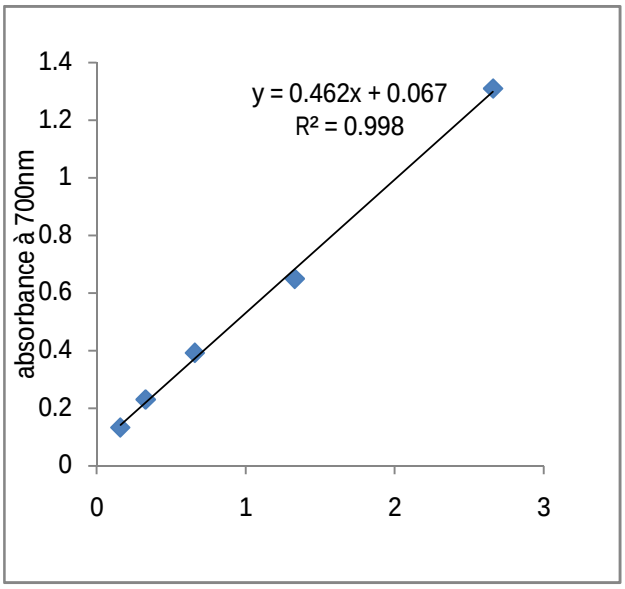


Figure 41: Reducing power of methanolic extract in MeOH and in distilled water, respectively.

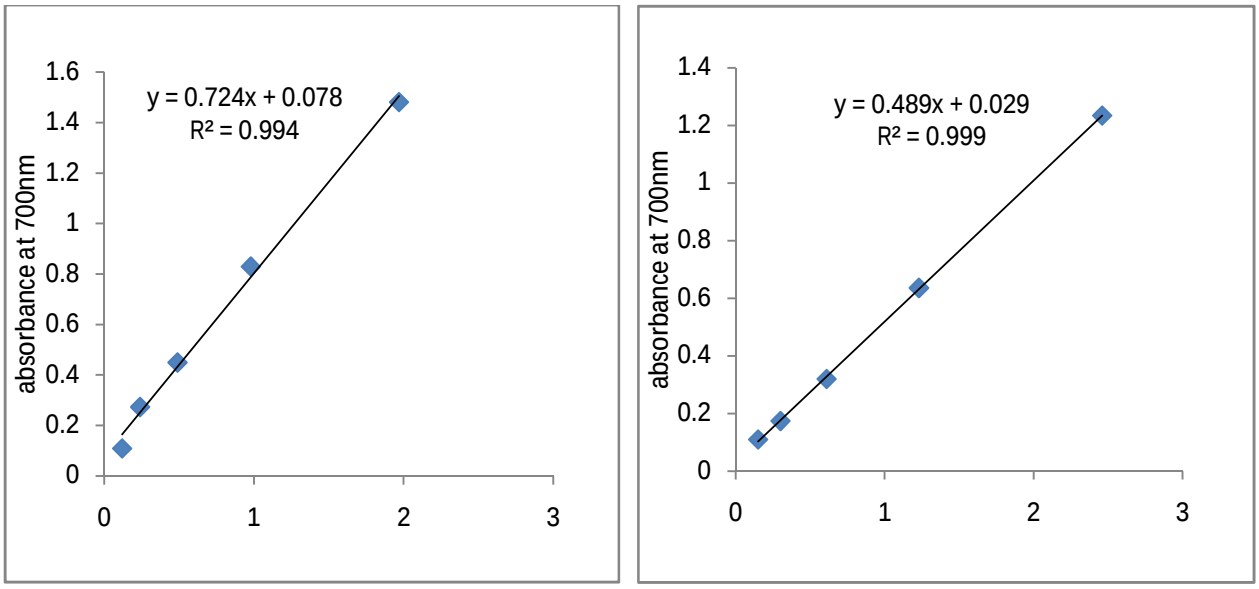


Figure 42: Reducing power of ethanolic extract in MeOH and in distilled water, respectively.

The CE_{50} of both methanolic and ethanolic extracts of polyphenols diluted in MeOH or in distilled water of the leaves of the plant *Cytisus purgans* were of 0.51 and 0.93 µg/ml and 0.58 and 0.95 µg /ml respectively.

Table 13: Reducing power of polyphenols extracts of the leaves of the plant *Cytisus purgans* and of BHT.

		EC_{50} (µg /ml)
Control	BHT	2.13
Methanolic extract	In MeOH	0.51
	In distilled water	0.93
Ethanolic extract	In MeOH	0 .58
	In distilled water	0.95

These results are clearly more important than positive control BHT (EC_{50}=2.13 µg/ml).

Potent reductive ability of the extracts implies its capability of donating hydrogen atom in a dose dependent manner. The high phenolic and flavonoid content of the extracts may be the contributing factor towards its antioxidant activity. Because of the presence of the hydroxyl groups phenolic constituents can function as hydrogen donor. Antioxidant potential of flavonoids against oxidative stress is the interesting subject of many investigations [143].

In fact, kæmpferol, rutin and quercetin (three flavonoids) have an important reducing power with CE_{50} of 5.70**,** 6.15 and 1.48 µg/ml, respectively [144].

Cytisus purgans show reducing power more than *Cytisus multiflorus* which have an CE_{50}=95.7 µg/ml (8). In the other side, Louis et *al.* [111] mention that the ethanolic extract of the plant *Cytisus scoparius* have an important reducing power with CE_{50}=0.86 µg/ml.(34)

II-2-3-Antimicrobial activity

Different methods have been employed in laboratories to determine the susceptibility of microorganisms to antimicrobial agents. Two main methods for the *in vitro* determination of the susceptibility of microorganisms against antimicrobials are the disk agar diffusion test, wherein antibiotic-impregnated disks are used with an agar medium, and the dilution techniques, wherein the test microorganism is exposed to increasing concentration of an antibiotic either in broth or agar.

The disk agar diffusion method is the most widely used laboratory technique for antimicrobial susceptibility test. This method is simple, requires a short duration, and easy to perform. It also provides an accurate determination of the susceptibility of an organism to a measured amount of test antibiotics [145].

The *in vitro* antimicrobial activity of *Cytisus purgans* extracts was evaluated by disc diffusion method using selected Gram-positive and Gram negative bacteria and some fungi strains. The diameters of the inhibition zone (ZI) are shown in Table 14 and in figure 43.

Table 14: Diameter of the inhibition zone (ZI) of crude mixture of flavonoids and alkaloids of *Cytisus purgans*.

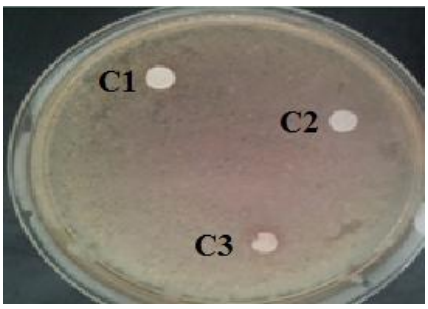
Microbial strains	**Concentration (mg/ml)**	**Diameter of zone of inhibition (mm)**	
		Crude mixture of flavonoids	**Crude mixture of alkaloids**
Echerichia coli	C_1		
	C_2		
	C_3	**7**	
Staphylococcus aureus	C_1		
	C_2	15	
	C_3	14	
Staphylococcus epidermidis	C_1		
	C_2	9	
	C_3	10	
Klebsiella pneumoniae	C_1		7.5
	C_2	7	8
	C_3	7	9
Micrococcus luteus	C_1		8.5
	C_2	8	10
		9	11.5
Pseudomonas aeruginosa	C_1		
	C_2	12	
	C_3	11	
Bacillus cerreus	C_1		
	C_2	10	
	C_3	8	

Table 14_ continued.

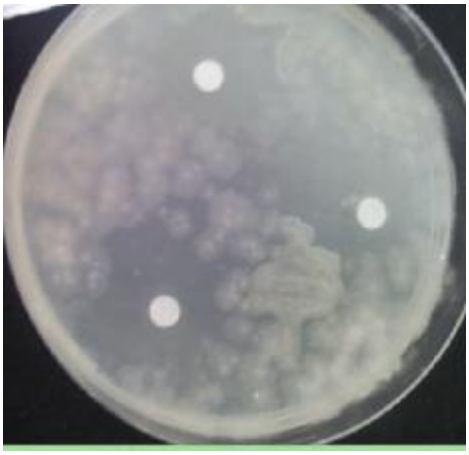
Bacillus cerreus	C_1	-	
	C_2	10	
	C_3	8	
Candida albicans	C_1	-	7
	C_2	11	8
	C_3	7	9.5
Fusarium oxysporum	C_3		16
Alternaria alternat	C_3		13

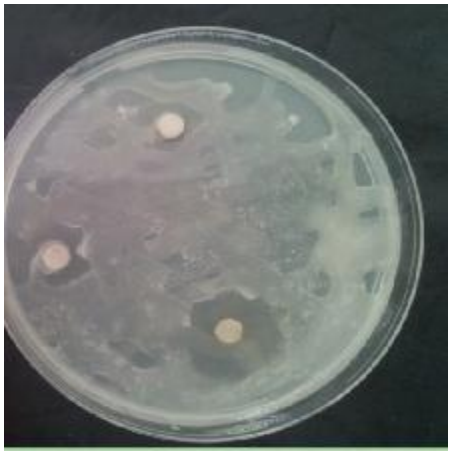
The data indicate that the crude mixture of flavonoids exhibited the activity against the investigated food pathogens. Gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* demonstrated higher susceptibility than Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*.

An important fact is that exhibited by the crude mixture of alkaloids against fungal strains which are *Fusarium oxysporum* and *Alternaria alternat*, respectively. The crude mixture of flavonoids showed antibacterial activity against *Staphylococcus aureus*, one of the most common gram-positive bacterium causing food poisoning. On the other hand, a weak antimicrobial activity was found against *Escherichia coli* and *Pseudomonas aeruginosa*. It is also important to note that this typical soil and water bacterium *Pseudomonas aeruginosa,* showed the lowest sensibility. Phenolic compounds possess high levels of antimicrobial activity, e.g. carvacrol, oxygenated derivatives (thymol methyl ether) and its precursor's *p*-cymene and γ-terpinene. Most of the studies on the mechanism of phenolic compounds focused on their effects on cellular membranes, altering their function and in some instances their structure, causing swelling and increasing their permeability [114].



Klebsiella pneumoniae





Fusarium oxysporum ***Alternaria alternat***

Figure 43: Antimicrobial effect of crude mixture of alkaloids.

These results suggest that *Cytisus purgans* is a potential source of broad-spectrum antimicrobial agents. The antimicrobial activity of the crude mixture of flavonoids may be attributed to that these compounds have been reported to be involved in inhibition of nucleic acid biosynthesis and other metabolic processes [146]. Alkaloids have been characterized to inhibit spore germination of plant pathogens. Moreover, flavonoids and alkaloids are synthesized by plants in response to microbial infection [147]. Phenolic compounds with a C_3 side chain at a lower level of oxidation and containing no oxygen have often been reported to be antimicrobials. The partially hydrophobic nature of their phenolic compounds has also been reported to be responsible for their antimicrobial activity. The mechanism of the toxicity of polyphenols against microbes maybe related to inhibition of hydrolytic enzymes (proteases) or other interactions that inactivate microbial adhesins, cell envelope transport

proteins and non-specific inter-actions with carbohydrates . The antifungal and antimicrobial activity of phenolic and flavonoid com-pounds has been reported previously. Isolation of the responsible elements is necessary for fully elucidating the antibacterial activity of these crude extracts. This might also provide insight about their possible use in food and non-food systems [146].

Conclusion

In conclusion, our research on secondary metabolites found in the plant *Cytisus purgans*, this study which is divided into two great parts: phytochemical study and biological study, the first is divided also into two parts: isolation and identification of alkaloids and isolation and identification of polyphenols, the biological study is divided also into three parts: antitumor, antioxidant and antimicrobial activities of both crude mixture of alkaloids and ploy phenols.

In the chemical study we are used different methods for extraction, separation and identification. Using open column chromatography revealed the presence of two known quinolizidine alkaloids which are sparteine and lupanine and two known flavonoids which are quercetin and sarotanoside, their structures were confirmed by the use of spectral basis (UV,IR, MS, NMR and GC/MS spectra) and comparing with literature data.

The maximum extraction yield of polyphenols was obtained with 70% aqueous methanol solvent (13.75%), while the 70% aqueous ethanol extraction gave the lowest yield (11. 32%). The total phenolic content (TPC) of the plant extracts was measured using the Folin–Ciocalteu colorimetric method. TPC range between 1.82% and 1.39% for methanolic and ethanolic extract, respectively. The total flavonoidic content (TFC) of the plant extracts was determined by using the aluminum chloride colorimetric method. TFC range from 0.12 and 0.10 for methanolic and ethanolic extract, respectively.

In the biological study, we investigated *in vitro* antitumor activity of plant extracts (alkaloids and polyphenols) on two different cell lines (human breast cancer, MDA-MB-231 and human lung cancer, A549 cell lines). Tested plant extracts are showed no cytotoxic effects toward of both human cancer cell lines. Neither increasing the dose as well as prolongation of the incubation time with the tested plant extracts did not lead to a statistically significant increase of antitumor effect.

The crude mixture extracts of polyphenols were shown significant antioxidant activity. In the present study, all extracts were found to be effective scavengers against DPPH radical with IC_{50} values of 1.97 and 2.41 for methanolic extract in methanol and in distilled water, respectively, and IC_{50} values of 4.17 and 6.55 for ethanolic extract in methanol and in distilled water, respectively. On the other side, The CE_{50} of both methanolic and ethanolic extracts of

polyphenols diluted in MeOH or in distilled water of the leaves of the plant *Cytisus purgans* were of 0.51 and 0.93 μg/ml and 0.58 and 0.95 μg /ml, respectively.

The *in vitro* antimicrobial activity of *Cytisus purgans* extracts was evaluated by disc diffusion method using selected Gram-positive and Gram negative bacteria and some fungi strains. Both extracts of flavonoids and alkaloids showed antimicrobial activity, an important fact is that exhibited by the crude mixture of alkaloids against fungal strains which are *Fusarium oxysporum* and *Alternaria alternat*, respectively.

Our results suggest that *Cytisus purgans* is a potential source of antioxidant and antimicrobial agents and could be used as a natural antioxidant and preservative in food and non-food systems. Further phtyochemical analysisis required to isolate the elements of the plant that show a broad spectrum of pharmacological activity.

In summary, these results demonstrate the high antioxidant activity of the enriched phenolic extract of *Cytisus purgans*, suggesting the importance of polyphenols in this capacity. Use of crude extracts can be pharmacologically useful. However, isolation and characterization of phytochemicals will help to establish structure-activity relationships in the discovery of new drugs.

More studies are now being undertaken in order to analyze the individual contribution of polyphenols in the *Cytisus purgans* antioxidant ability.

These *in vitro* experiments support the importance of polyphenol content in the antioxidant activity of this plant. Since antioxidant activity is crucial in many health disorders, these results might support some of the traditional uses of *Cytisus purgans*. Future studies will be *in vivo*.

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