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Preliminary Phytochemical Screening and Evaluation of some biological activities of *Bituminaria bituminosa* L

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بسم الله الرحمن الرحيم

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In the name of Allah, the Most Gracious and the Most Merciful.

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Dedication

Every challenging work needs self efforts as well as guidance of elders especially those who were very close to our heart ,My humble effort go to my sweet and loving

father and mother

whose affections, love, encouragement and prays of day and night, who have been our source inspiration and gave us strength when we thought of giving up make me able to get such success and honor.

Special thanks to my husband B.HANI who shared their words of advice and encouragement to finish this study

I dedicate my dissertation work to my aunte and to my husband's family and to my friends who encouraged me and were by my side on this path until we reached its end.

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Dedication

It is simply that I dedicate this end-of-study project:

To my parents Bashir and Hamida, for their love, sacrifice and support throughout my studies,I hope that to always be a source of pride. I hope that this work is a humble testament to my eternal gratitude. God bless you.

To my three brothers: Hossam El Din, Mohamed El Amine, Raed.

To the kindest grandfather in the world, Muhammad Buhafs, may God have mercy on you, my grandfather, and put you in his vast paradise.

For all my family, without exception, uncles and aunts.

To all my colleagues at the university.

To my soulmate TOO for all the patience, support, and love to continue my university career.

M. SOUAD

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LIST OF ABREVIATIONS AND SYMBOLS

TCM : traditional Chinese medicine

HPLC : Chromatographie liquide haute performance

BuOH : n-butanol

EtOAc : Ethyl acetate

$CHCl_3$: Chloroform

GC-MS : Gas chromatography-mass spectrometry

DPPH : 2,2-diphenyl-1-picrylhydrazyl

DMSO : Dimethyl sulfoxide

PE : petroleum ether

GAE : gallic acid equivalent

IC_{50} : 50% inhibitory concentration

TFV : total flavonoids

TPP : total polyphenols

Abs : absorbance

$AlCl_3$: Aluminum trichloride

$FeCl_3$: Iron trichloride

MeOH : Methenol

EtOH : Ethanol

HCl : Hydrochloric acid

°C : Degree Celsius

Mg EAG/ g E : Milligram of gallic acid equivalent per gram of extract

Mg EQ/ g E : Milligram of quercetin equivalent per gram of extract

Na_2CO_3 : Sodium Carbonate

H_2SO_4 : Sulfuric acid

FC : Folin- Ciocalteu

µg : Microgram

µl : Microliter

nm : Nanometer

ml : Milliliter

mg : Milligram

UV : Ultraviolet

V/V : Volume/Volume

BHT : Butylated Hydroxytoluene

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Introduction

Herbs have been used in many domains including medicine, nutrition, flavoring, beverages, dyeing, repellents, fragrances, cosmetics, smoking, and other industrial purposes. Since the prehistoric era, herbs have been the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century [1,2].

The preservative effect of many plants species and herbs suggests the presence of antioxidative and antibacterial constituents in their tissues. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity [3].

Phytochemical investigations on the Fabaceae species have revealed the presence of isoflavones, prenylated flavonoids, flavanones, flavanols, saponins, glucosides, rotenoids, chalcones, alkaloids and trypsin inhibitors. Flavonoids are the most main constituents of the Fabaceae family, which has oestrogenic, antibacterial, antioxidant, antifungal, antifeedant and insecticidal activities [4].

The aim of this study is screened *Psoralea bituminosa* (syn. *Bituminaria bituminosa*) species for its phytochemicals and tested for its biological activities, including antioxidant and antibacterial powers. This plant belonging to the Fabaceae family, is commonly known as the Arabian pea or pitch trefoil. It is a very important medicinal plant, used in traditional medicine in Europe, Asia, and America, and in Africa for, its antiseptic, antihyperglycemic and anti-oxidative potentials [5]. *B. bituminosa* is a rich source of secondary metabolites with considerable pharmacologic properties. It is known for using in the treatment of skin diseases and it is reported to show cytotoxic, antibacterial, and antioxidant activities [6].

This manuscript will be divided into three distinct chapters that will highlight segments of literature review and personal work:

The first chapter provides a review of literature on the family of Fabaceae, genus of *Bituminaria* "*Psoralea*", and the species *Bituminaria bituminosa* (L.) C.H. our subject of this study as well as the biological methods.

The second chapter devoted to the experimental part which presents the mainly preliminary phytochemical tests , estimation of total phenolic and total flavoind content and the evaluation of the biological activities such as the antibacterial and the antioxidant activity of the extract prepared from our plant.

In the last chapter, we will discuss the different results obtained during this study.

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Chapter I:

Literature Review

I.1. Fabaceae family

Fabaceae (or Leguminosae) is the third-largest family of flowering plants (angiosperms), comprising a wide variety of economically and scientifically important genera and species. There are almost 770 genera and more than 19,500 species in the family, and a staggering amount of diversity therein. Legume species span the globe, with representatives in nearly every biome from deserts to tropical forests[1]. They grow as shrubs, trees, and even aquatic plants, display diverse flowering morphology, and are adapted to a wide variety of ecological and climate conditions[1].

The Fabaceae are traditionally classified into three subfamilies (sometimes treated as separate families) : Caesalpinioideae, Mimosoideae, and Faboideae (= Papilionoideae). The five largest genera of this family are *Astragalus* (over 3,000 species), *Acacia* (over 1,000 species), *Indigofera* (around 700 species), *Crotalaria* (around 700 species), and *Mimosa* (around 400 species)[2,3].

I.1.1. Systematic position

The botanical classification of Fabaceae family is presented as follows (Table I.1) :

Table.1.1 : Botanic classification of fabaceae.

Kingdom	Plantae
Sub-kingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Risidae
Ordre	Fabales
Family	Fabaceae

I.1.2. Characteristics features of fabaceae

The Fabaceae or Leguminosae, commonly known as the legume, pea, or bean family (after faba, Latin name for broad bean), consist of herbs, shrubs, trees and the climbers [4], with usually compound leaves ; flowers variable in size and shape, mostly similar to the sweet-pea or mesquite ; the fruit always a legume (a one-carpeled, beanlike, seed pod) (Figure I.1). The roots of many members have a symbiotic association with nitrogen-fixing bacteria which induce formation of root nodules (this especially common in the Faboideae)[3].

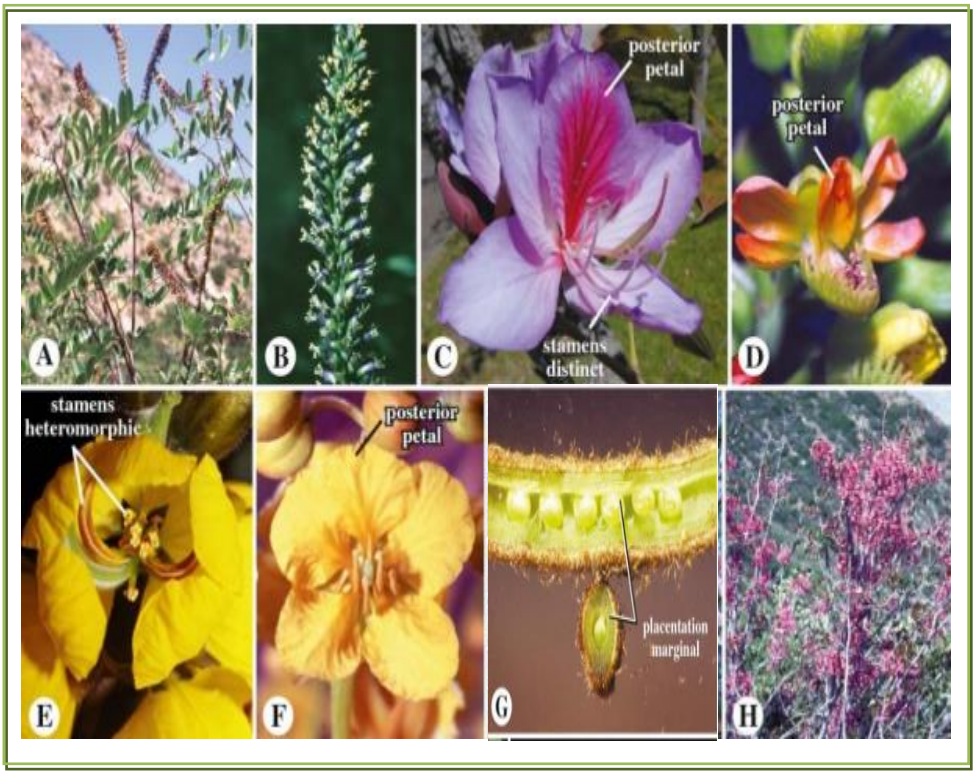


Figure.I. 1 : Morphological aspects of some fabceae.

A-H. Whole plant ; **B**. Close-up of flowers of spike ; **C-F.** Flowers ; **G**. Fruits

I.1.3. Geographical distribution of Fabaceae species

Fabaceae is one of the botanical families with the largest number of species in the world and are usually associated with seasonally dry or arid climate regions (Figure I.2) [5, 6]. The family has a variety distribution in cold mountainous regions in Europe, Asia and North America, so abundant in central Asia[7].

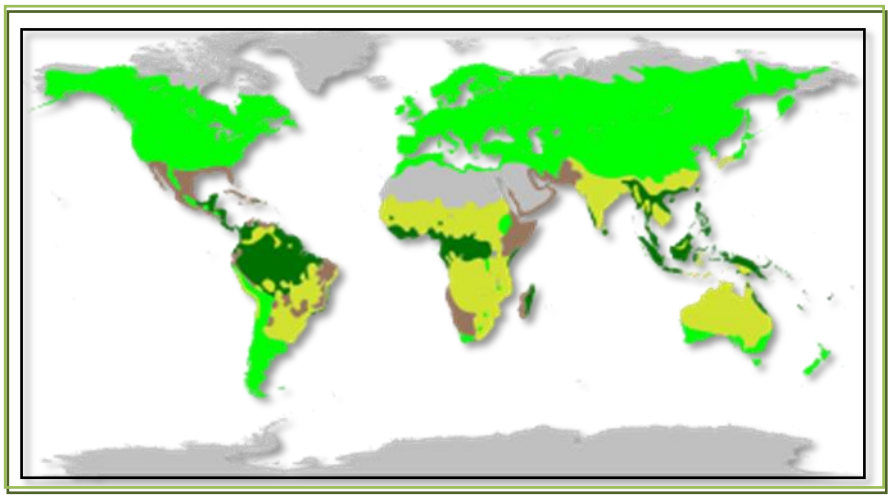


Figure.I.2 : Fabaceae disrtrubtion map. **tropical forest, temperate, grass, and succulent**

I.2. Genus *Bituminaria* (*Psoralea*)

The genus *Psoralea*, belonging to the family Fabaceae, is one of several plant genera which are reputedly known for their contribution to traditional as well as modern medicines. The name 'Psoralea' is derived from the Greek term 'Psoraleos', which means "affected with itch or with leprosy". This comprises ca. 130 species distributed all over the world, and some of the plants are used as folk medicine to treat various diseases[8].

I.2.1. Distribution of *Bituminaria* genus

The genus *Bituminaria* Heist. is mainly distributed in South Africa, North and South America, and Australia, a few of which are native to Asia and temperate Europe (Figure I.3)[9]. In fact, this diversification can be attributed to various factors, such as habitat modifications and reproductive biology. These speciation processes on the *Bituminaria* genus led to eight distinct species: ***B. bituminosa*** (L.) C.H. Stirt., ***B. morisiana*** (Pignatti & Metlesics) Greuter, ***B. flaccida*** (Náb˘elek) Greuter, ***B. basaltica*** Miniss., C. Brullo, Brullo, Giusso & Sciandr., ***B. kyreniae*** Giusso, C. Brullo, Brullo, Cambria & Miniss., ***B. palaestina*** (Bassi) Brullo, C. Brullo, Miniss., Salmeri & Giusso, ***B. plumosa*** (Rchb.) Bogdanovi´c, C. Brullo, Brullo, Ljubi˘ci´c & Giusso, and ***B. acaulis*** (Steven ex M. Bieb.) CH. Stirt [10]. In Algeria, three

species of *Bituminaria* can be found in the Tell: ***B.Bituminosa***, ***B.plicata***, and ***B. americana***[11].

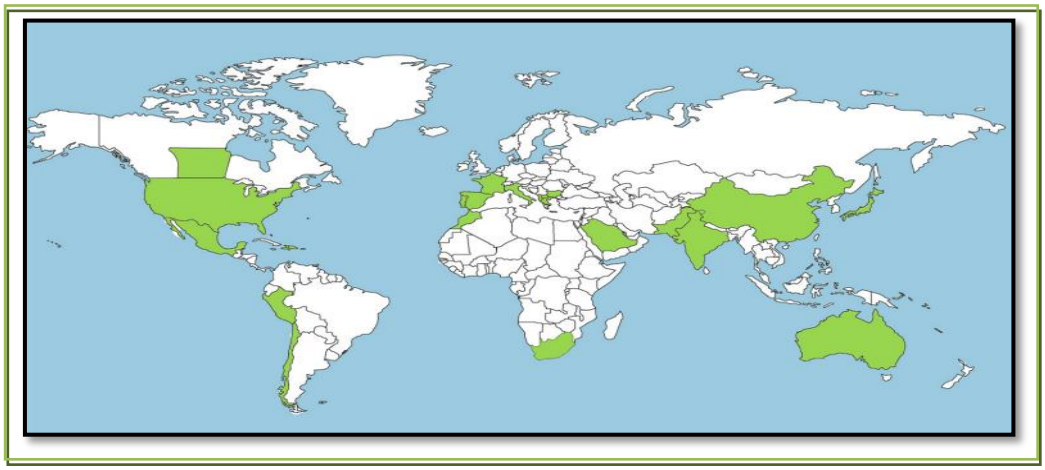


Figure.I. 3 : World map showing the distrubtion of bituminaria species (green).

I.2.2. Botanical description of *Bituminaria* species

Morphologically, the genus *Bituminaria* is differentiated by several apomorphic characters (Figure I.4). Plants of this genus are glandular, perennial, with woody stems, and long-stemmed trifoliate leaves and stipulated; the flowers, gathered in tight and rounded false heads, give pods of about 2 cm[11].

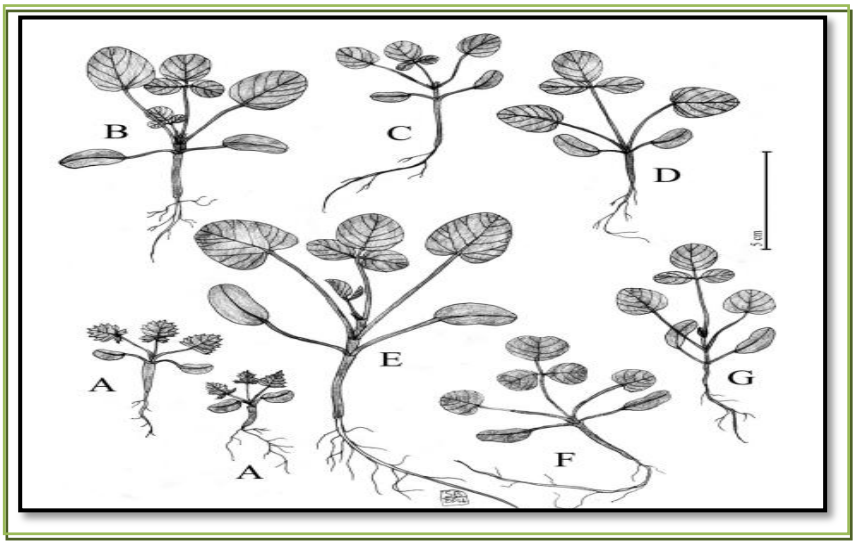


Figure.I.4 : Seedling of diffrent bituminaria.

A. *Bituminaria acaulis* (Hoffm.) C. H. Stirt. —**B**. *Bituminaria bituminosa* (L.) C. H. Stirt. (Sicily). —C. *Bituminaria basaltica* Miniss., **C.** Brullo, Brullo, Giusso & Sciandr. —**D.** *Bituminaria kyreniae* Giusso, C. Brullo, Brullo, Cambria & Miniss. —**E**. *Bituminaria palaestina* (Bassi) Brullo, C. Brullo, Miniss., Salmeri & Giusso. —**F.** *Bituminaria flaccida* (N´abelek) Greuter. —G. *Bituminaria morisiana* (Pignatti & Metlesics) Greuter.

I.2.3. Traditional uses and pharmacological activities of the genus *Bituminaria*

Modern pharmacological researches show that the plants in *Psoralea* genus have antimicrobial, antipregnancy, estrogenic, antitumor, antioxidant, and many other pharmacological activitie (Figure I.5) [12].

Figure.I.5 : Medicinal proprities of psoralea species.

Psoralea species have been used in folklore and indigenous system of medicine for a long time [13], for exemple :

- *P. corylifolia* is the sole species of the genus distributing in China, and its seeds are used as a famous traditional Chinese medicine (TCM), having the effects of kidney importence and warming spleen and stopping diarrhea and included by Pharmacopoeia of People's Republic of China. Moreove, it is an ancient remedy for leucoderma among the traditional system of medicines in India and China and also among the people in the West. In Unani system, the plant has been effective against fever, skin

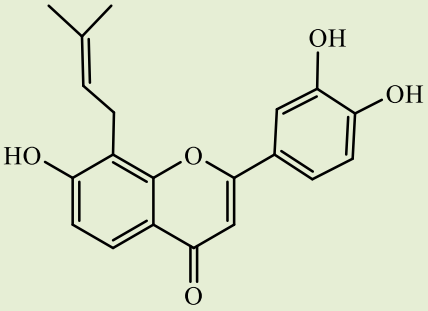
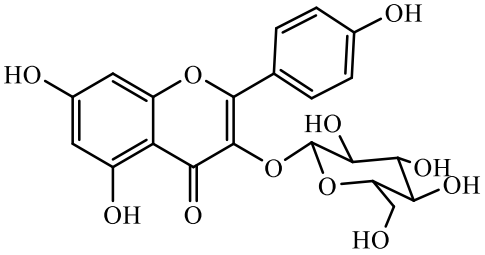
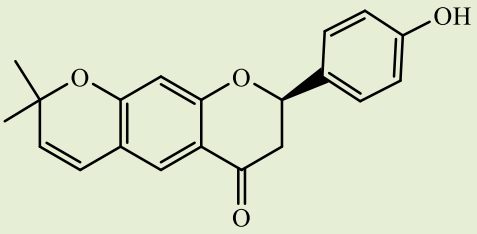
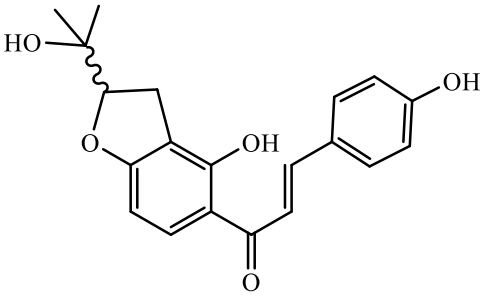
diseases and internal ulcers. It is also found to be an effective antihelmintic, sedative and it is known to improve the color of skin, hair and nails.

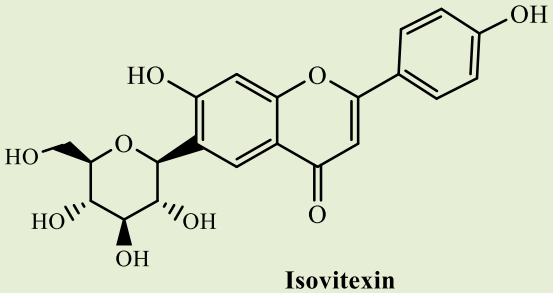
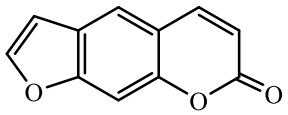
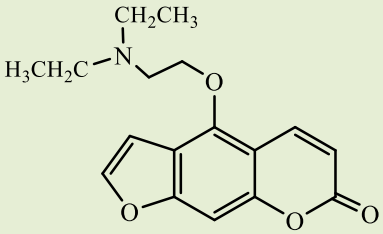
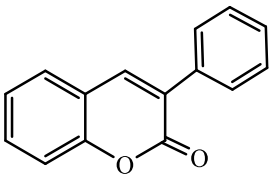
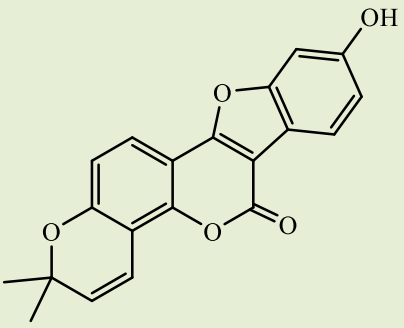
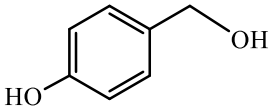
- In Japan, the ethanol extract of the seeds of *P. corylifolia* has been used as a preservative for pickles and some processed foods. The seed cake is rich in nitrogen and minerals and is used as cattle feed or manure.
- The roots of *P. argophylla* Pursh (Silver leaf Indian breadroot) are eaten raw or cooked. A tea prepared from the leaf and stem powder possess anti-pyretic properties. The decoction of plant is used as an antiseptic for wounds. The root extract is used as a remedy for chronic constipation.
- *Psoralea glandulosa* L. is cultivated in Chile for its leaves and young shoots, which are used to make a refreshing cold drink. The leaves are anti-helmintic.
- Leaves and roots of *P. hypogaea*, *P. macrostachya* and *P. orbicularis* are eaten raw or cooked.

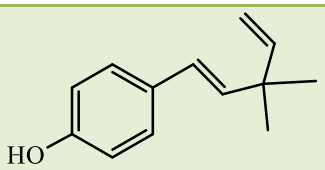
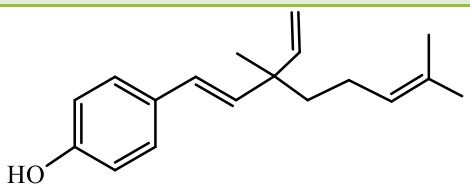
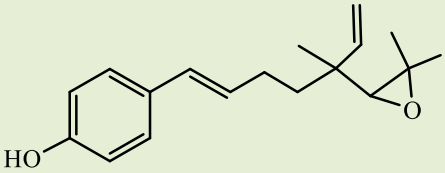
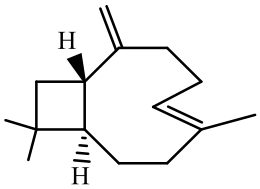
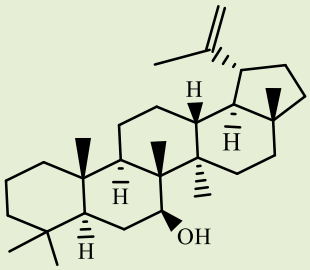
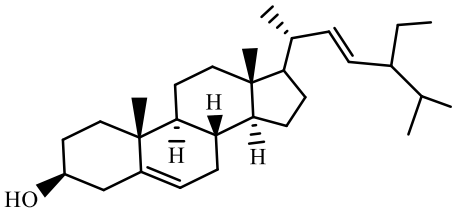
I.2.4. Phytochemistry studies of the genus *Bituminaria*

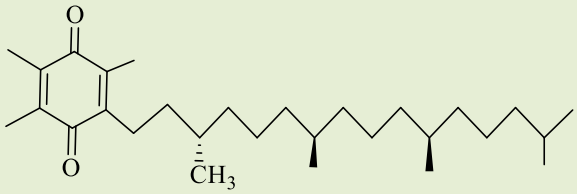
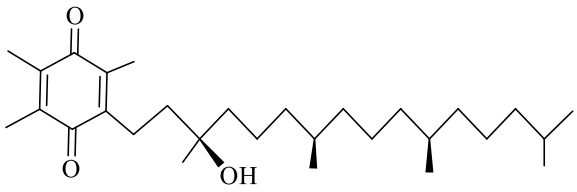
Psoralea species have been investigated since 1890s [14]. Leaf, rhizome, root, seeds, fruit and resinous extracts of *Psoralea* species have been subjected to HPLC and HPTLC analyses for producing compounds of pharmaceutical interest, such as isoflavonoids[15] and Flavonoids [8] furanocoumarins [16], pterocarpan [17] and the essential oil [18]. The table below including some secondary metabolites isolated from species of the genus *Psoralea* (Table I.2).

Table.I.2 : Compounds isolated from psoralea species.

Secondary metabolites class	Origins	Chemical constituents	References
Flavonoids	*P.corylifolia*	 **Corylifoli C**	8
		 **Astragalin**	
		 **7,8-Dihydro-8-(4-hydroxyphényl)-2,2-diméthyl-2H,6H-benzo[1,2- b :5,4- b]dipyran-6-one**	
		 **Bakuchalcon**	

	P. plicata	 **Isovitexin**	19
Coumarins	*P. corylifolia* and *P. plicata*	 **Psoralen**	8
		 **Psoralin**	
		 **C-Phenylcoumarin**	
		 **plicadin**	
Phenols	*P. corylifolia*	 **p-Hydroxybenzyl alcohol**	20

		 **Corylifolin**	21, 22
	P. glandulosa	 **3-Hydroxy bakuchiol**	23
		 **12,13-Dihydro-12,13-epoxy bakuchiol**	
Sesquiterpenoids, Triterpenes, And Steroids	*P. corylifolia* and *P. plicata*	 **β-Caryophyllene**	8
		 **Psoracinol**	
		 **Stigmasterol**	

Quinones	*P. plicata*	 **α-Tocopherol quinone methyl ether**  **α-Tocopherol quinone**	[24]

I.3. *Bituminaria bituminosa* species

Bituminaria bituminosa (L.) C.H. Stirt (*Psoralea bituminosa* L., Fabaceae), commonly known as the Arabian pea or pitch trefoil, is a perennial species of the Mediterranean area. The characteristic strong smell of bitumen (odor of asphalt) of this specices is the result of a combination of several substances such as phenolics, sulphurated compounds, sesquiterpenes and probably short-chain hydrocarbons [18].

Synonyms : *Aspalthium bituminosum* (L.) Fourr. ; 1868 ; *Psoralea bituminosa* L., 1753 ; *Psoralea palaestina* Gouan, 1773 ; *Lotodes bituminosa* (L.) Kuntze, 1891 ; *Dorychnium angustifolium* Moench, 1794 ; *Aspalthium frutescens* Medik., 1787 ; *Psoralea foetida* C. Presl, 1822 ; *Rhyncodium bituminosum* (L.) J. Presl, 1845.

Common names :

- *Aspalthium bituminosum* (L.) Fourr.
- *Bipontinia bituminosa* (L.) Alef.
- *Dorychnium angustifolium* Moench
- *Dorychnium rotundifolium* Moench

I 3.1. Distribution of *Bituminaria bituminosa*

Bituminaria bituminosa (L.) Stirt. is a pasture legume with a wide geographical distribution in the Mediterranean basin and Macaronesia islands (Figure I.6) [25]. This plant is widely distributed throughout Algeria, especially in the Tell and on the littoral [11].

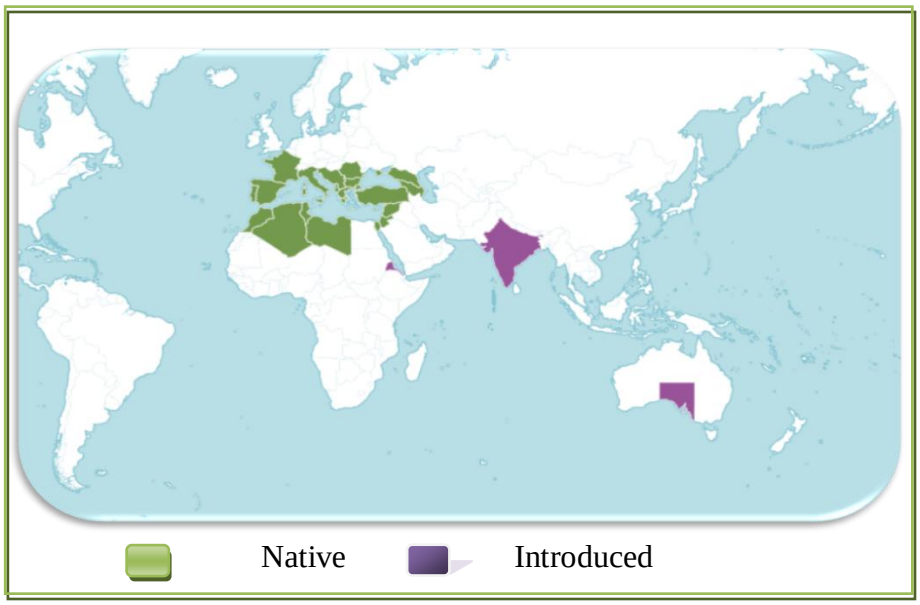


Figure.I.6 : Geographical distrubtion of B. Bituminosa species.

Native to **–**Albania, Algeria, Baleares, Bulgaria, Canary Is., Corse, Cyprus, East Aegean Is., France, Greece, Italy, Kriti, Krym, Lebanon-Syria, Libya, Madeira, Morocco, North Caucasus, Palestine, Portugal, Romania, Sardegna, Sicilia, Sinai, Spain, Transcaucasus, Tunisia, Turkey, Turkey-in-Europe, Yugoslavia. ***Introduced into–*** Eritrea, India, South Australia.

I 3.2. Botanical description of *Bituminaria bituminosa*

P. bituminosa is an herbaceous perennial and pubescent plant (50 cm to 1m). Leaves are cauline, pinnately or subdigitately 3-foliolate, linear lanceolate to broadly-ovate with entire margins. Inflorescence presents several flowers and calyx with long setaceous teeth exceeding the tube (10–15 mm) ; corolla scarcely exceeding the sharply subulate-tipped calyx-lobes, blue, violet or creamy-white (Figure I.7). It blooms between April and August.

Figure.I.7 : Leaves and flowers of *psoralea bituminosa*.

I.3.3. Traditional uses of *Bituminaria bituminosa*

This plant can grow in acidic soils with high ground water level [26]. It is mainely used to provide hay or forage for livestock. Its nitrogen fixation and drought tolerance properties making it suitable for lowinput production systems. Moreover, *B. bituminosa* colonizes heavy metal contaminated sites and so is used for the phytostabilization of contaminated or degraded soils [27]. In folk medicine, it is used :

- as a hair restoration agent in Madeira Island. Infusions prepared from the fresh leaves are used for the treatment of fever and urinary infections [28].
- to treat spasms and treat against fever and epilepsy [29].
- to treat diabetes, some population is found to use its leaf in the north-west region of Algeria (Tlemcen) [30]. This activity was confirmed by Ayoubi et al., whose study carried out on Lebanese bituminisa species revealed that the aerial parts reduced diabetic complications [31].
- against dental caries. Also, a poultice made from the fresh plant is applied externally to treat various skin problems, such as eczema, leprosy and alopecia areata.
- as emmenagogue, diuretic and astringent in the form of herbal tea (9-30g of dried herb in a cup of boiled water, 2-3 times daily). It is applied externally to the skin in the form of poultice made from the fresh plan.

I.3.4. Biological effects of *Bituminaria bituminosa*

In the study of Salima Azzouzi et al. (2014), The *n*-BuOH, CH_2Cl_2 and AcOEt extracts were used to investigate the antibacterial activity of *B. bituminosa* using the disk diffusion method. The diffusion test was applied to 12 Gram-positive (*S. aureus* ATCC 29213, *S. aureus*, α *emolitic streptocoque*) and Gram-negative (*E. coliATCC* 25922, *E. coli*, *P. aeruginosa* ATCC 27853, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae*, *Enterobacter* sp. and *Serratia* sp.) microorganisms. The results of these tests showed that among the extracts tested, the CH_2Cl_2 extract was more effective than other extracts (the maximum antibacterial activity with high concentration of 2 mg/mL against *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumonia* and *Escherichia coli* ATCC 25922 (20.45 mm, 16.41 mm and 15.74 mm inhibition zone, respectively)) [32]. On the other hand, antioxidant potential of *n*-BuOH extract was evaluated through two methods: DPPH and cupric ion reducing antioxidant capacity assay. The *n*-BuOH extract showed a significant antioxidant activity (the value IC_{50} was 0.26 µg/mL using DPPH method, whereas the E% value was 0.10 L/mg every centimeter for cupric ion reducing antioxidant capacity assay) [32].

I.3.5. Chemical composition and pharmacological significance of *B. bituminosa*

Bituminosa is also a source of pharmaceutically active compounds including furanocoumarins : **psoralen** and **angelicin** (Figure I.9) and **pterocarpans** (Figure I.8) in its leaves and other organs. Furanocoumarins are used in the treatment of skin diseases, they encourage antimicrobial activity, and they have anti-HIV effects. Psoralen can be used in extracorporeal photopheresis for the prevention and treatment of rejection in solid organ transplantation. Pterocarpans have anti-proliferative, estrogenic, hepatic-protective, anti-allergy, anti-inflammatory, apoptotic, and anti-tumour activities [13].

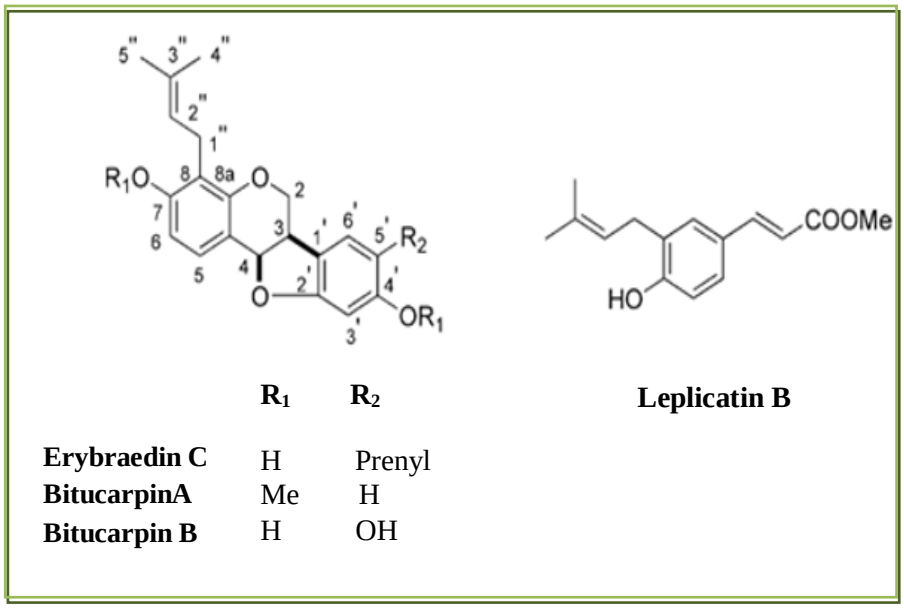


Figure.I.8 : Chemical structures of petrocarpans detected from *B.Bituminosa.*

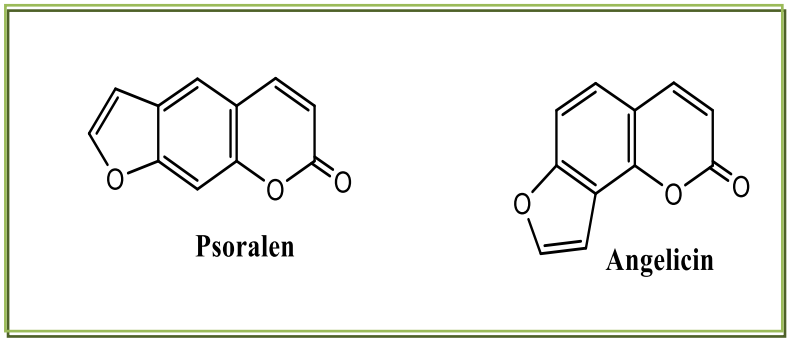


Figure.I.9 : Chemical structrues of furanocumarins from *B.Bituminosa*.

The screening of phytochemical compounds was carried out using high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MSn). More than 40 compounds were identified or tentatively characterized. A high percentage of the detected compounds corresponded to glycosylated flavonoids, especially from apigenin, although phenolic acids, lignans, and saponins were also identified (Figure I.10) [33].

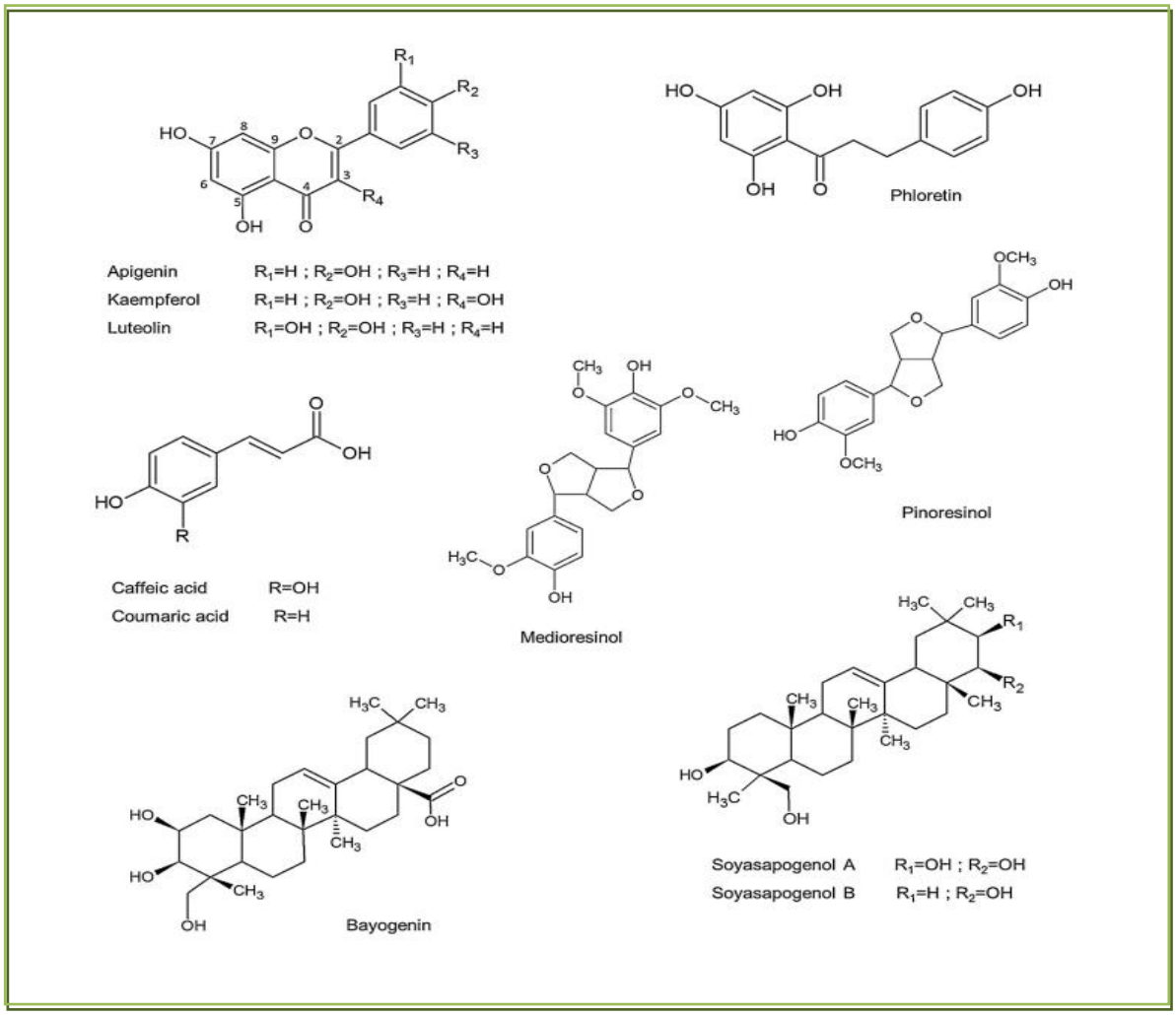


Figure.I.10 : Chemical structrues of the main compounds detected in *B.Bituminosa.*

The phytochemical study of *n*-BuOH extract of *B. bituminosa* revealed the presence of isoflavone (**daidzin**) and flavone (**isoorientin**), which identified for the first time in this species (Figure I.11) [32].

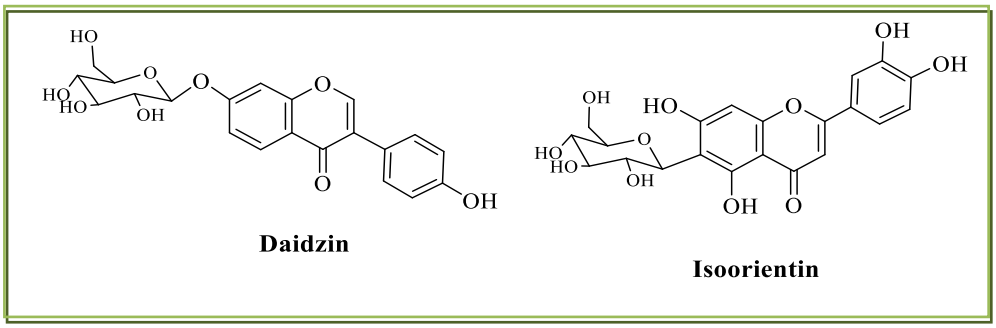


Figure.I.11 : Chemical structrues of diazin and isoorentin isolated from *B.Bituminosa.*

A new study (Ayoubi, et al. 2018) has shown that the GC-MS analysis of the aerial parts of three samples of *P. bituminosa* obtained revealed that the oil is mainly composed of monoterpenes, Sesquiterpenes represented, fatty acids and esters in different percentages relatively (Table I.3) [31].

Table.I.3 : Variation of volatile constituents of *Psoralea bituminosa* according to the physical state and method of extraction.

Physical state	**Dry**	**Dry**	**Fresh**
Method of extraction	Column	Petroleum ether	Petroleum ether
Sample No	**1**	**2**	**3**
Chemical class			
Oxygenated monoterpenes and sesquiterpenes	3.2%	12.7%	27.75%
Unsaturated monoterpene and sesquiterpenes	3.81%	24.83%	35.34%
Diterpenes	-	1.60%	-
Fatty acid and esters	37.746%	-	-
Hydrocarbons	30.131%	4.32%	21.6%
Tricyclic Amines	8.934%	2.8%	1%

I.4. Biological activities of Medicinal Plants

Medicinal plants have been used for many years for therapy and prevention of various human diseases because they have always shown many different biological activities, for exemple : antimicrobial, antioxidant, anti-inflammatory,….etc. These activities are due to the richness of plants in bioactive compounds such as : alkaloids, glycosides, saponines, polyphenols, flavonoids. The focus of this part of chapter is on interest concerning antibacterial and antioxydant activities.

I.4.1. Antibacterial activity

I.4.1.1. Bacteria definition

Bacteria are single-celled microorganisms with prokaryotic cells (Figure I.12), which are single cells that do not have organelles or a true nucleus and are less complex than eukaryotic cells[34]. The prokaryotic cells have the following characteristics such as [35] :

- No organelles, all the action takes place in the cytosol or cytoplasmic membrane.
- Most bacteria possess peptidoglycan, a unique polymer that makes its synthesis a good target for antibiotics.
- Protein synthesis takes place in the cytosol with structurally different ribosome's.

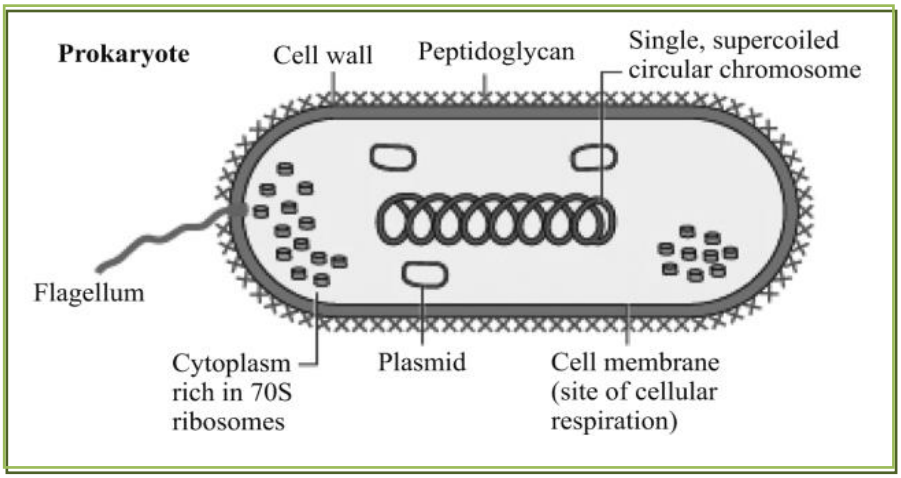


Figure.I.12 : Prokaryote cell.

I.4.1.2. Morphology and classification

Bacteria come in a wide variety of shapes and size, called **the morphology** of organism. Depending on their shape, bacteria are classified into several varieties (Figure I.13) [35].

- **Cocci** (from kokkos meaning berry) are spherical or oval cells.
- **Bacilli** (from baculus meaning rod) are rod shaped cells
- **Vibrios** are comma shaped curved rods and derive their name from their characteristics vibratory motility.
- **Spirilla** are rigid spiral forms.
- **Spirochetes** (from speira meaning coil and chaite meaning hair) are flexuous spiral forms.

- **Actinomycetes** are branching filamentous bacteria, so called because of a fancied resemblance to the radiating rays of the sun when seen in tissue lesions (from actis meaning ray and makes meaning fungus).
- **Mycoplasmas** are bacteria that are cell wall deficient and hence do not possess a stable morphology. They occur as round or oval bodies and as interlacing filaments.

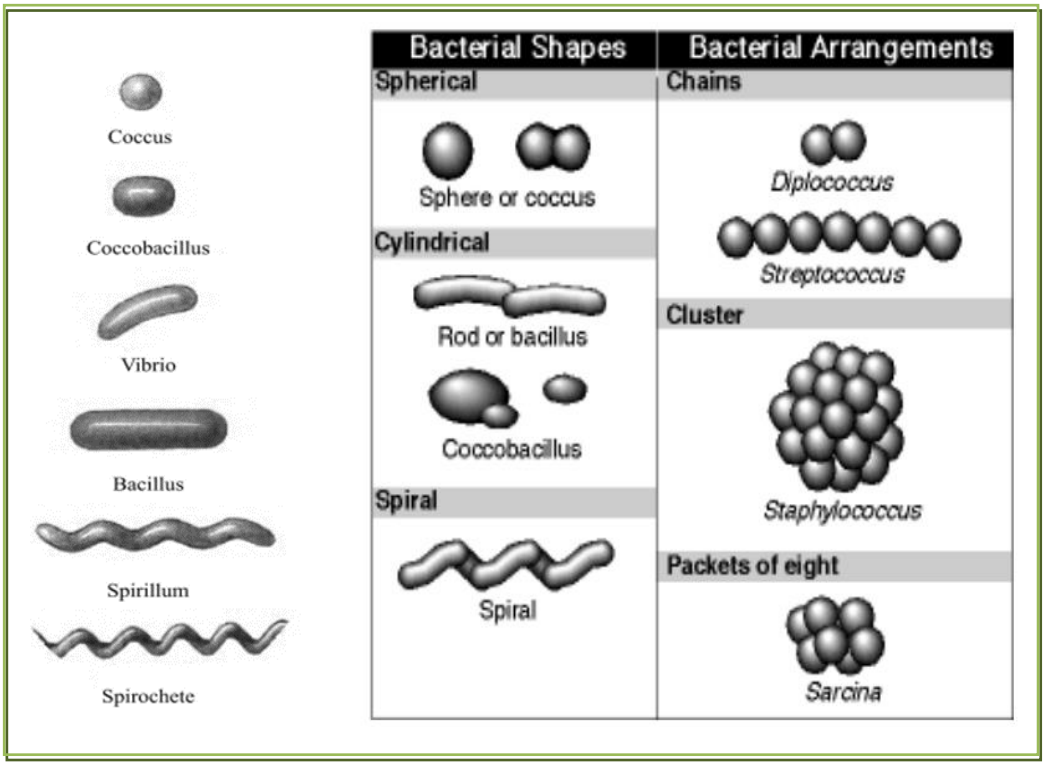
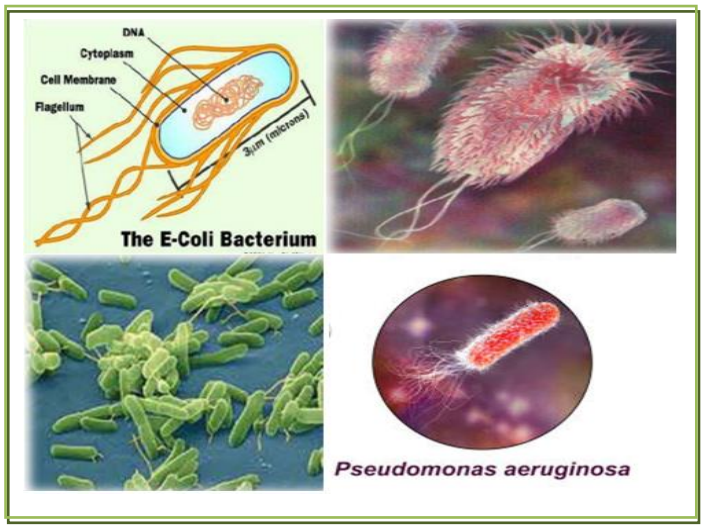


Figure.I.13 : Shapensed arrangements of bacteria.

A second major criterion or distinguishing bacteria is based on the cell wall structure. There are types of cells wall that give different staining characteristics with a series of stains and reagents called the Gram stain [36]. The Gram stain was devised by histologist Christian Gram as a method of stain bacteria in tissues (Figure I.14), which are classified into:

- Bacteria with a thin wall layer and an outer membrane stain red with this protocol and are called **Gram negative**. Gram negative bacteria are more resistance to antibiotics. But, they are susceptible to Streptomycin, Chloramphenicol, and Tetracycline. For exemple, we cite here : *Escherichia coli* and *Pseudomonas aeruginosa*.



- Bacteria with a thicker wall layer, lacking the outer membrane, stain violet and are called **Gram positive**. For exemple : *Staphylococcus aureus*.

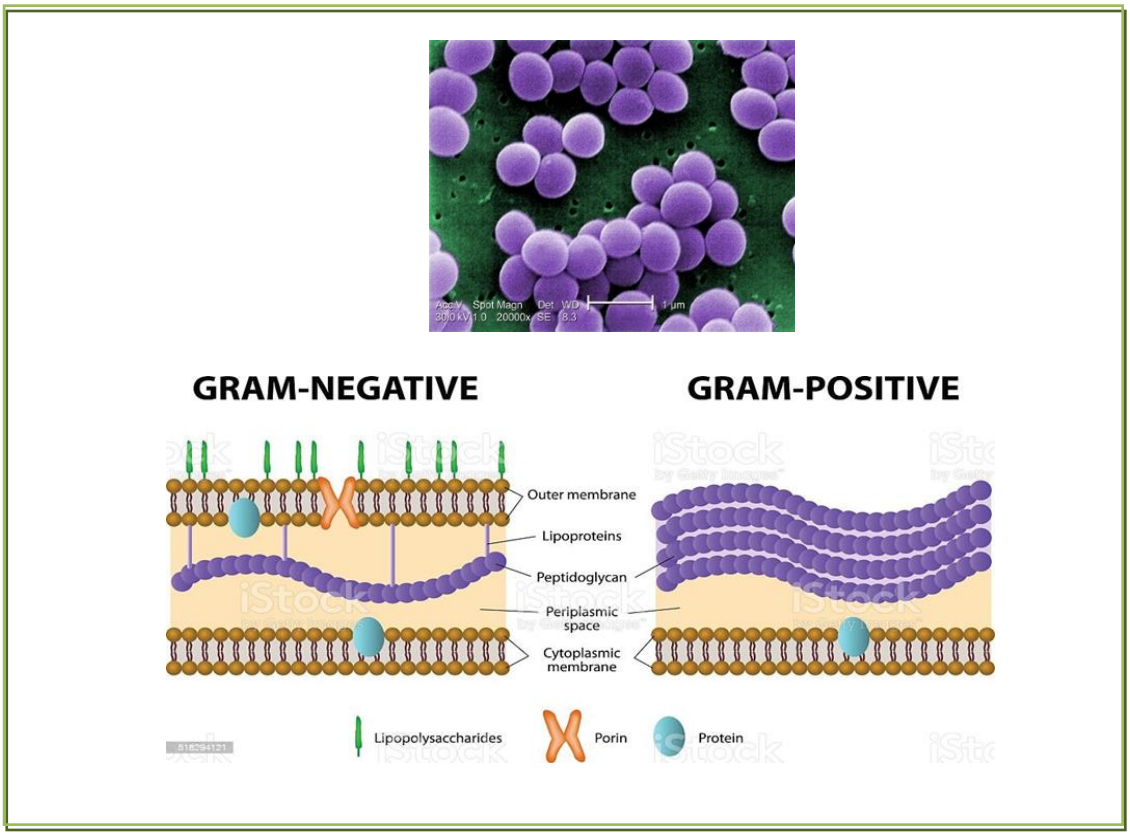


Figure.I.14 : Gram positive and gram negative cell wall.

I.4.1.3. Methods for *in vitro* evaluating antimicrobial activity

In recent years, there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance. Therefore, a greater attention has been paid to antimicrobial activity screening and evaluating methods. Several bioassays such as **disk-diffusion**, **well diffusion** and **broth or agar dilution** are well known and commonly used, but others such as **flow cytofluorometric** and **bioluminescent** methods are not widely used because they require specified equipment and further evaluation for reproducibility and standardization, even if they can provide rapid results of the antimicrobial agent's effects and a better understanding of their impact on the viability and cell damage inflicted to the tested microorganism [37].

- **Agar disk-diffusion method**

Agar disk-diffusion testing developed in 1940, is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured (Figure I.15).

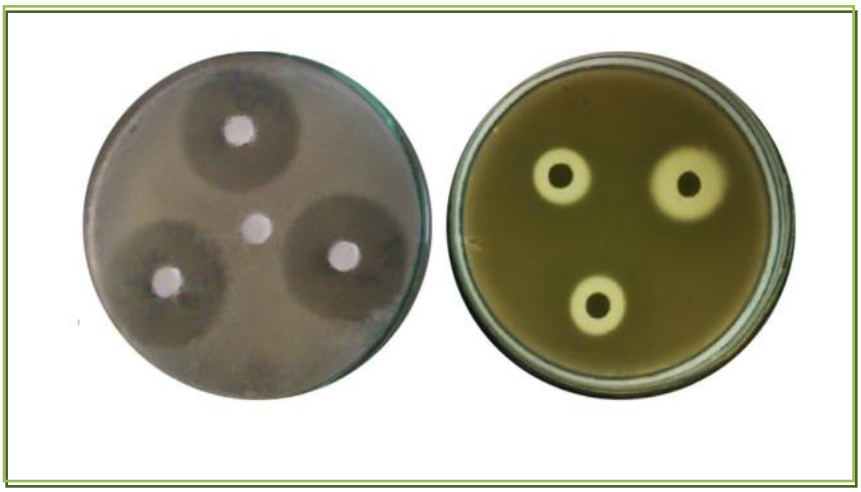


Figure.I.15 : Agar difussion methods.

I.4.2. Antioxidant activity

The production of oxidants is a typical event associated with aerobic metabolism. When oxygen is supplied in excess or its reduction is insufficient, reactive oxygen species or free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are generated Accumulation of the free radicals in body organs or tissues can cause oxidative damage to biomolecules and membranes of cell, eventually leading to many chronic diseases, such as inflammatory, cancer, diabetes, aging, cardiac dysfunction and other degenerative diseases [38].

The antioxidant agents may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress. In fact, antioxidants are capable of stabilizing or deactivating, free radicals before they attack cells Reactive oxygen species can be eliminated by a number of enzymatic and non-enzymatic antioxidant mechanisms. Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, and catalase. Non-enzymatic antioxidants include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione, carotenoids, flavonoids, and other antioxidants. However, under oxidative stress conditions, enzymatic antioxidants may not be sufficient, and non-enzymatic antioxidants (dietary antioxidants) may be required to maintain optimal cellular functions [39].

A great number of medicinal plants contain compounds exhibiting antioxidant properties as phenolic compounds (eg. quercetin), which possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals (Figure I.16) [40].

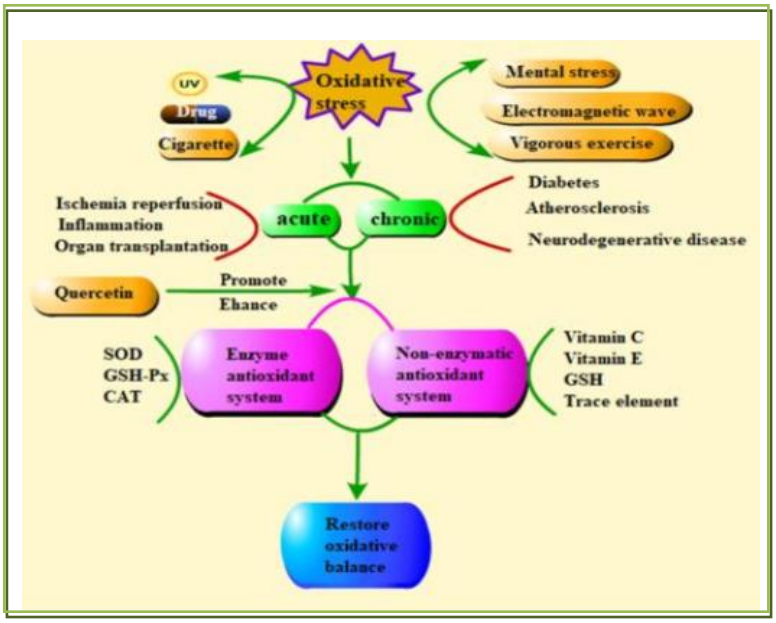


Figure.I.16 : Basic principale of antioxidant activity of quercetin.

I.4.2. Methods for *in vitro* evaluating antioxidant activity

The antioxidant activity can be evaluated *in vitro* or *in vivo* by means of simple experiments. Antioxidant activity cannot be measured directly but is determined by the effects of the antioxidant to control the degree of oxidation. There are a variety of methods to evaluate antioxidant activity present in a variety of matrices (plant extracts, blood serum, etc.) (Table I.4 and Figure I.17). Some methods involve a different oxidation step followed by the measurement of the response, which depends on the method used to evaluate the activity. When the antioxidant activity of a sample is studied, it is necessary to consider the source of ROS as well as the target substrate [41].

Table.I..4 : Methods most commonly used to evaluate antioxidant activity in vitro.

Method	Characteristics
Total radical-trapping antioxidant parameter (TRAP)	TRAP assay involves the initiation of lipid peroxidation by generating water-soluble ROO• and is sensitive to all known chain-breaking antioxidants[42]
Ferric-reducing antioxidant power (FRAP)	Colorimetric method that evaluates the reduction of Fe3+ -tripyridyltriazine complex (Fe3+-TPTZ) by turning it into a ferrous form (Fe2+-TPTZ)[43]
Total antioxidant capacity (TAC)	This method is used to measure the peroxide level during the initial stage of lipid oxidation. Peroxides are formed during the linoleic acid oxidation, which reacts with Fe 2+to form Fe3+and later these ions form a complex with thiocyanate[44]
Inhibition of 2,2-diphenyl-1-picrylhydracyl radical (DPPH•)	Colorimetric method based on the measurement of the scavenging capacity of antioxidants towards DPPH•[45]

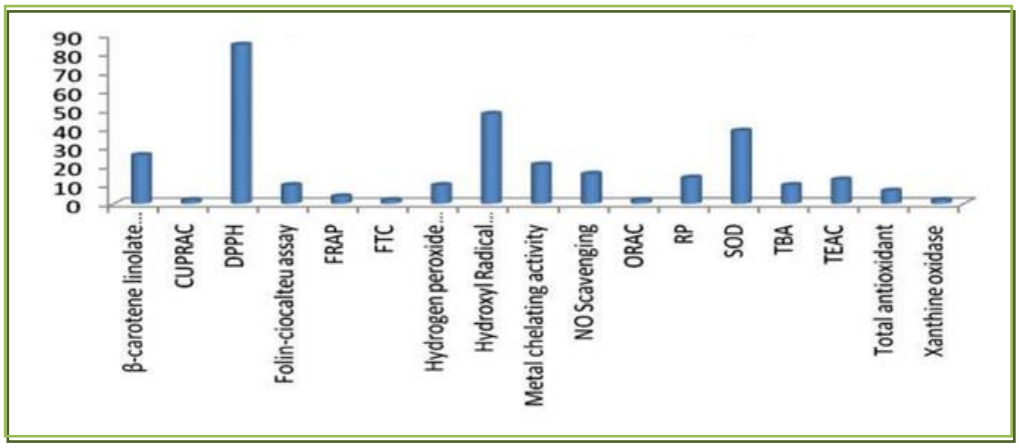


Figure.I.17 : Frequency methods used to evaluation antioxidant activity in vitro.

- **Radical scavenging capacity DPPH• method**

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Figure I.18) is characterized as a stable free radical because π electrons of the aromatic systems present in the molecule can compensate for the lack of an electron. DPPH• does not dimerize, as most other free radicals do. The delocalization of the electron also gives rise to a deep violet color, characterized by absorption in solution at around 517 nm [45].

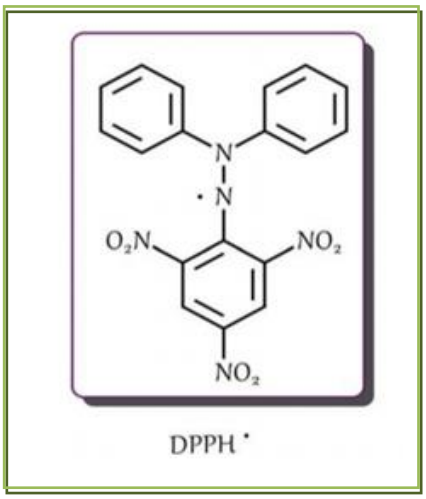


Figure.I.18 :1,1Diphenyl-2-picryphydrazyl radical DPPH.

When a solution of DPPH• is in contact with a substance that can donate a hydrogen atom or with another radical (R•), the reduced form DPPH-H or DPPH-R is produced with the consequent loss of color (to yellow) and therefore the decrease or loss of absorbance (Figure I.19). Consequently, the reduction of DPPH• provides an index to estimate the ability of the

test compound to trap radicals and in this case the law of Lambert-Beer is fulfilled in the useful absorption interval [46].

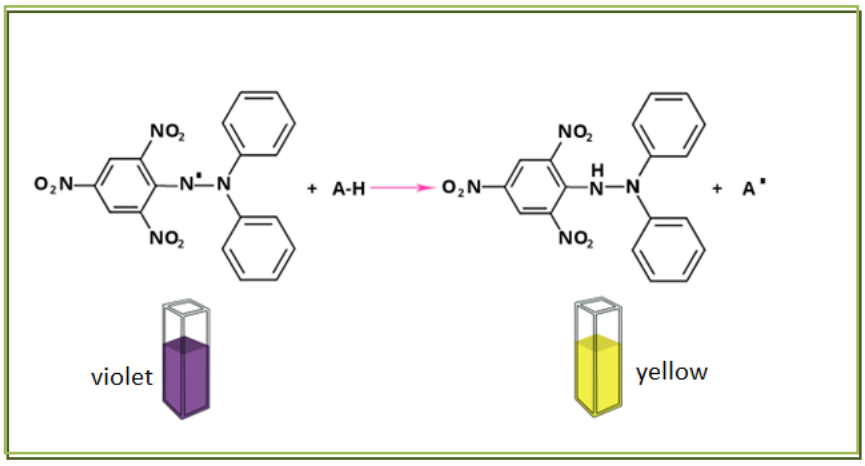


Figure.I.19 : DPPH reduced by antioxidant.

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Chapter II :

Materials and Methods

II.1. Plant material

The present work deals with the study of *Bituminaria bituminosa called "adna "or "Mentia"* (Figure II.1). collected in April 2022 in Batna area (Djerma) and was identified by Dr Bachir Oudjehih (Department of agronomy, University of Batna).

As any plant is not usually used immediately after picking, it is necessary to know the best methods to preserve its active ingredients and therefore its therapeutic properties. In fact, after harvest, the plant material was cleaned and left to dry in the dark. the dry plant has been crushed after (Figure II.1).

Figure.II.1 : Plant materiel *Bituminaria Bituminosa L* "*psoralea bituminosa*".

II.2. Phytochemical screening

The aim of this study is to evaluate the chemical composition of the plant in order to assess their biological power. In fact, standard methods were used for the identification of the existence of the phytochemical compounds in plant such as: alkaloids, polyphenols, flavonoids, tannins, saponins,...., The different steps followed are shown in the following flowcharts:

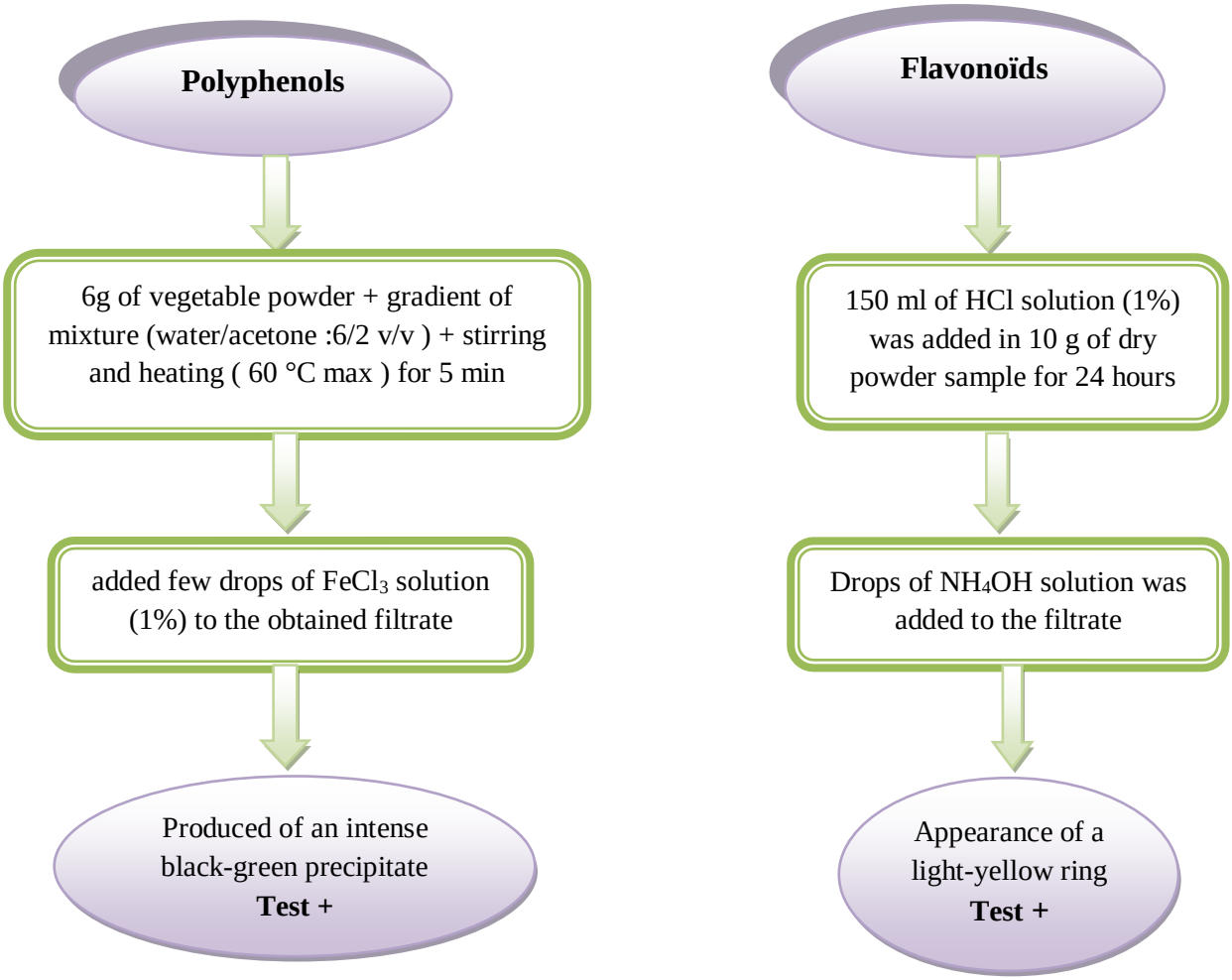


Figure .II.2 : Preliminary assays for polyphenols and flavonoid recongnition.

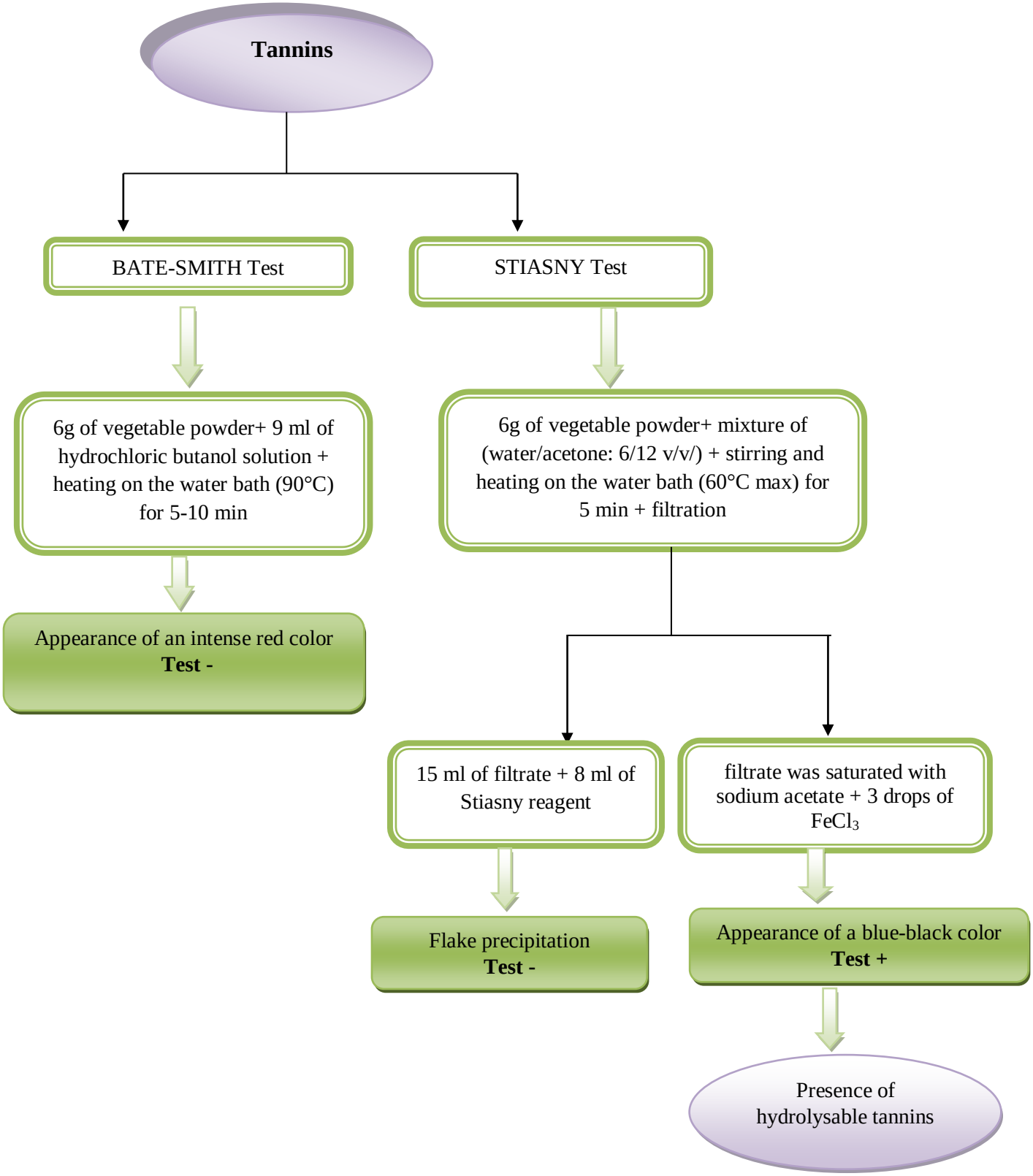


Figure.II.3 : Preliminary assays for tannins (catechic and hydrolysable) recongnition.

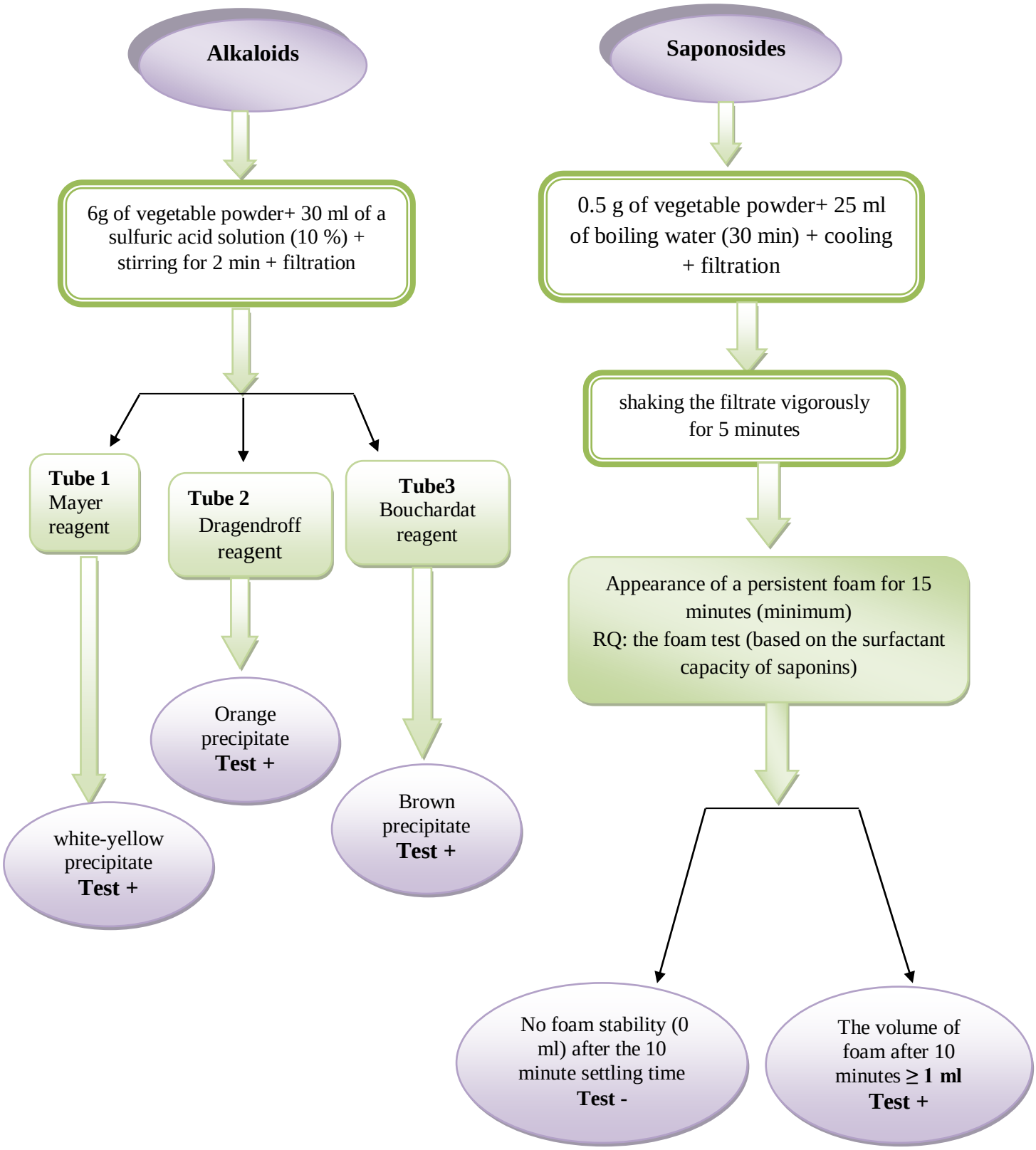


Figure.II.4 : Preliminary assays for alcaloids and saponins recongnition.

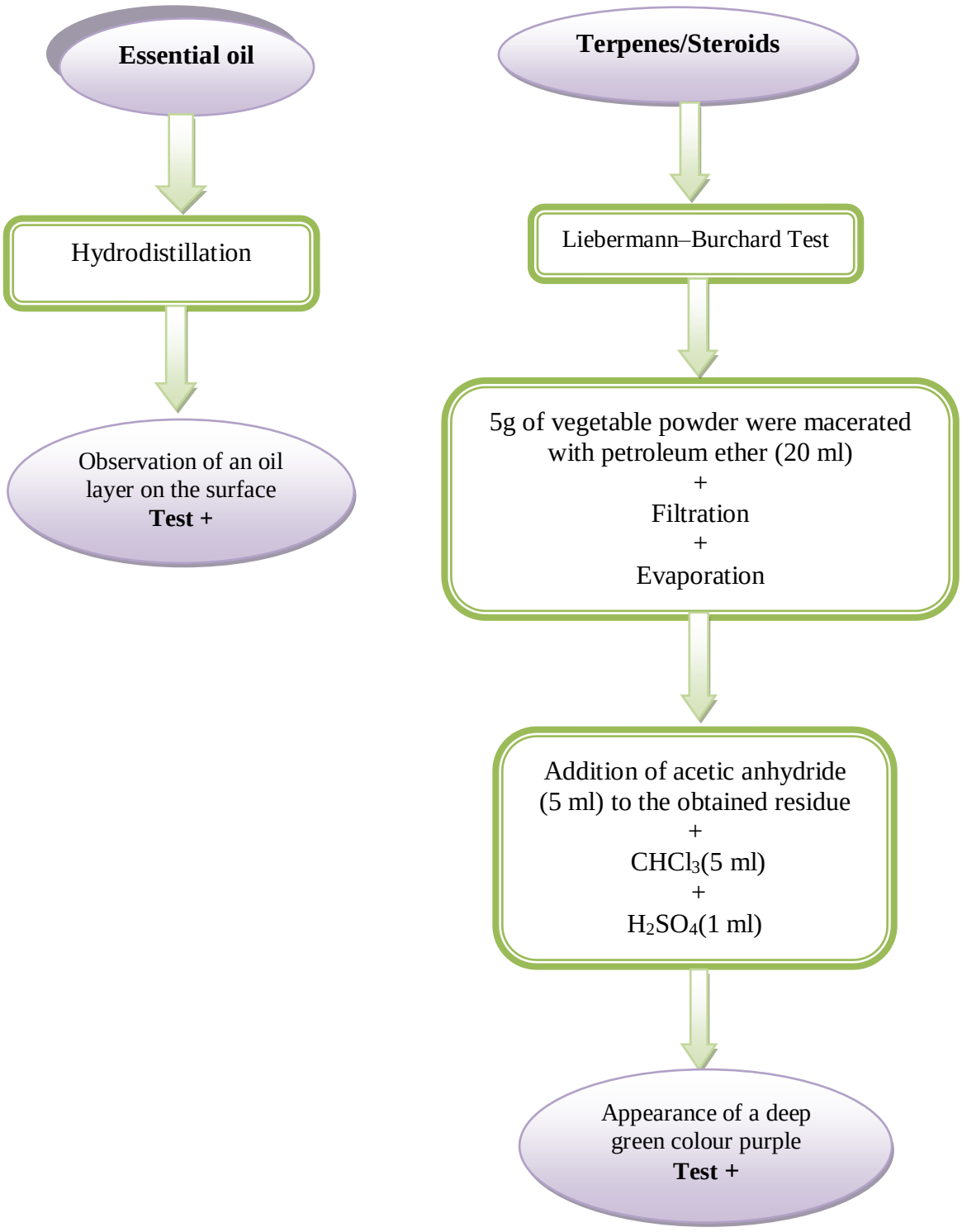


Figure.II.5 : Preliminary assays for terpens /steroids and essential oil recongniton.

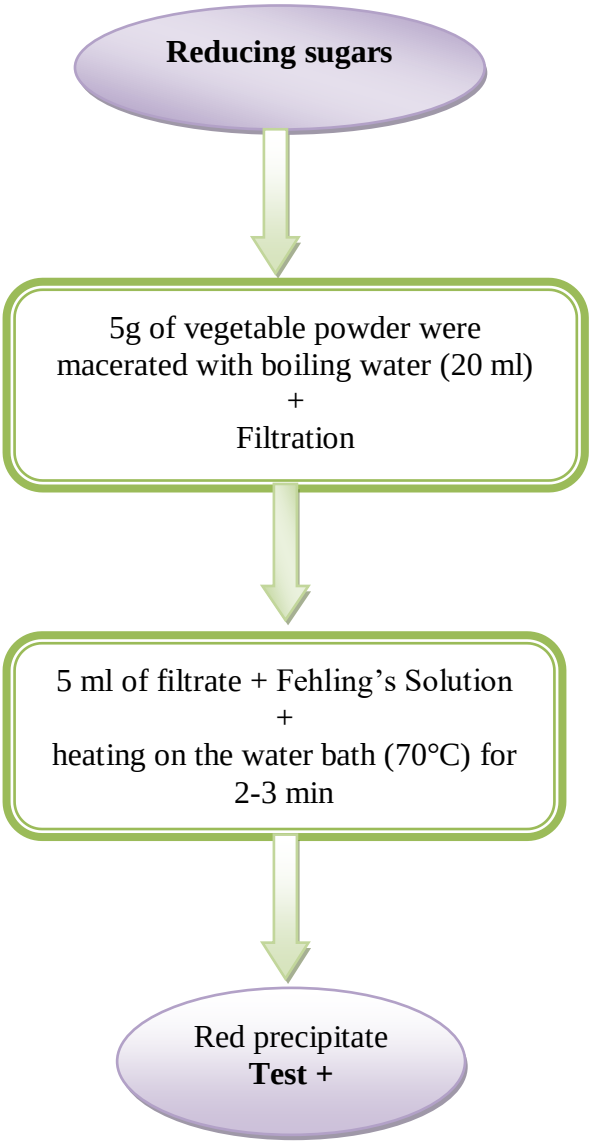


Figure II.6: Preliminary assays for reducing sugars recognition.

II.3.Extraction techniques

II.3.1.Maceration

The dried plant, *B. bituminosa* (50 g), was extracted three times with mexture of EtOH: H_2O (70:30, V/V) for 48 h. The mixture must be vigorously stirred in the room temperature(Figure II.7) .

Figure.II.7: Maceration process.

II.3.2. Filtration and evaporation

After sedimentation, the hydroalcoholic solution was filtered through filter paper.The obtained filtrate was evaporated in a vacuum rotary evaporator under reduced pressure at 40 °C until the filtrate was concentrated (Figure II.8).

Figure.II.8: Filtration and evaporation process.

II.3.3. Liquide–liquide extraction (LLE)

The suspension was extracted successively with petroleum ether, ethyl acetate and *n*-butanol (Figure II.9). The organic phases were dried with Na_2SO_4, filtered and concentrated to obtain the following extracts: PE (1.5g), EtOAc (1g) and *n*-BuOH (2g) (Figure II.10).

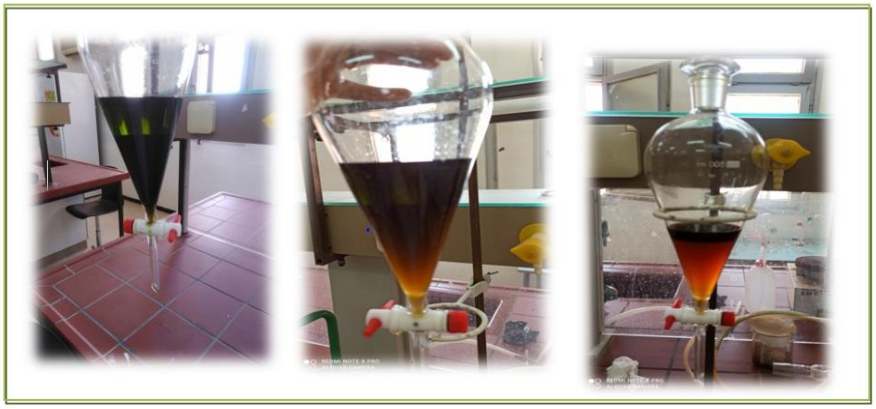


Figure.II.9: Liquide-liquide extraction process.

The obtained extracts were collected in stopper glass bottles and stored at 0 °C.

Figure.II.10: The obtained extracts (PE/EtOAc and n-BuOH).

II.4.Thin layer chromatography analysis (TLC)

Thin layer chromatography (TLC) is a method for analyzing mixtures. it can be used to help determine the number of components in a mixture, the identity of compounds, and the purity of a compound. The extracts (PE, EtOAc and *n*-BuOH) obtained were tested for Thin Layer Chromatography (TLC), while the best ones are shown in **figures** (**II.11** to **II.13**). Analytical TLC was performed in silica gel plates Kieselgel 60 F_{254} as stationary phase. To visualize the spots, plates are puted under uv light.

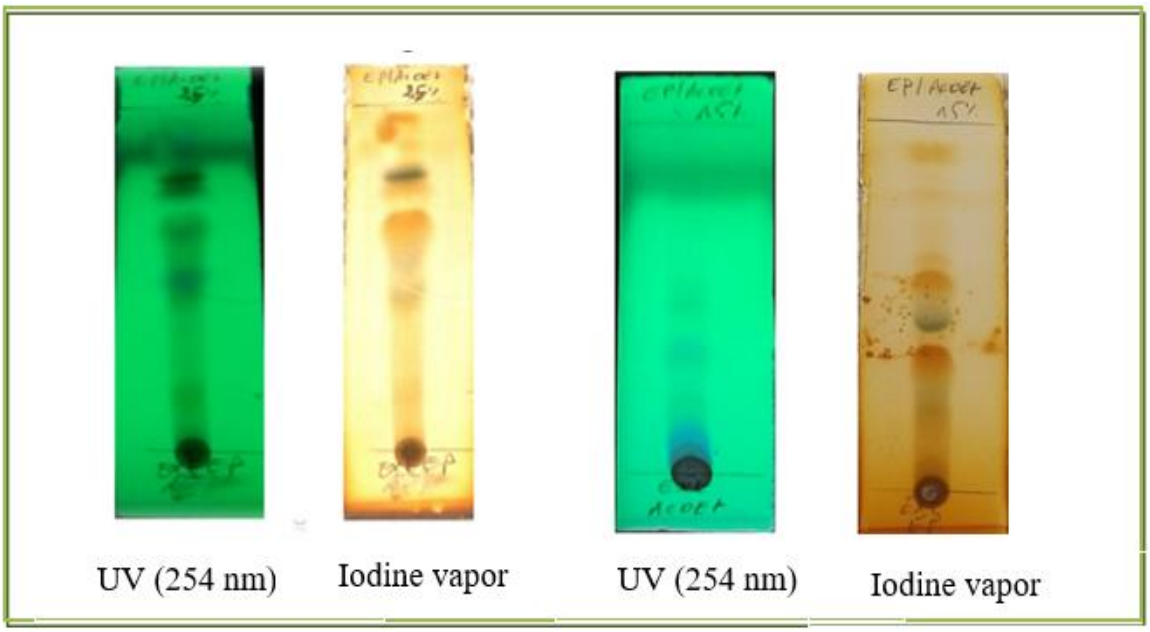


Figure.II.11 : TLC of petroleum extract of *B.Bituminosa.*

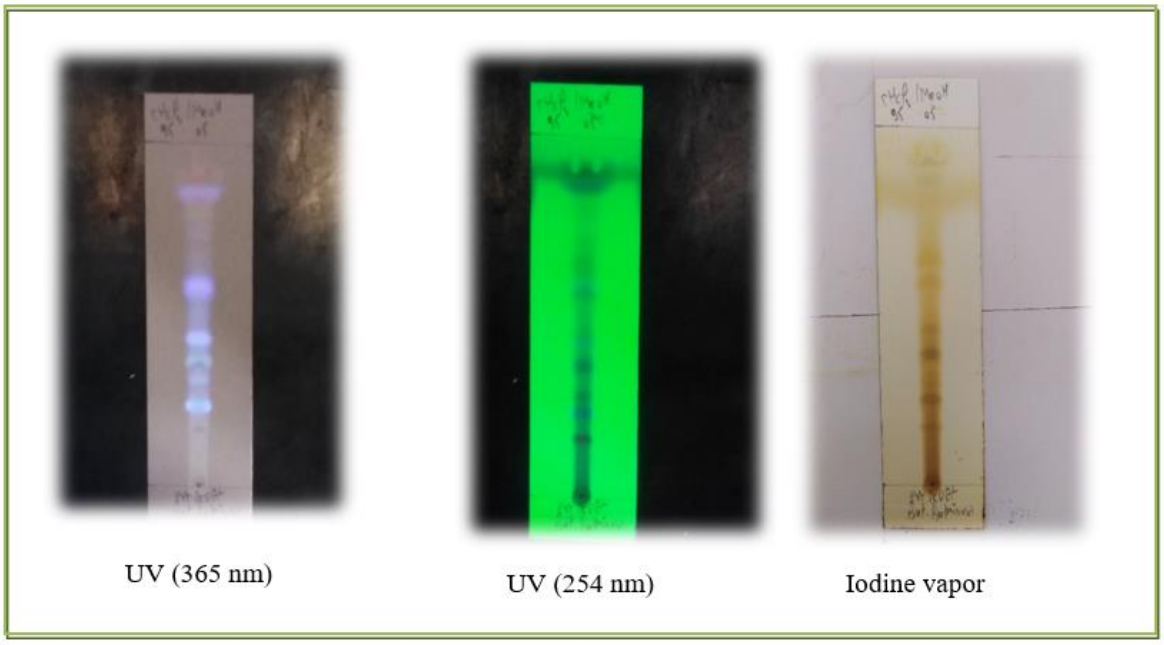


Figure.II.12 : TLC of ethyl acetate extract of *B.Bituminosa.*

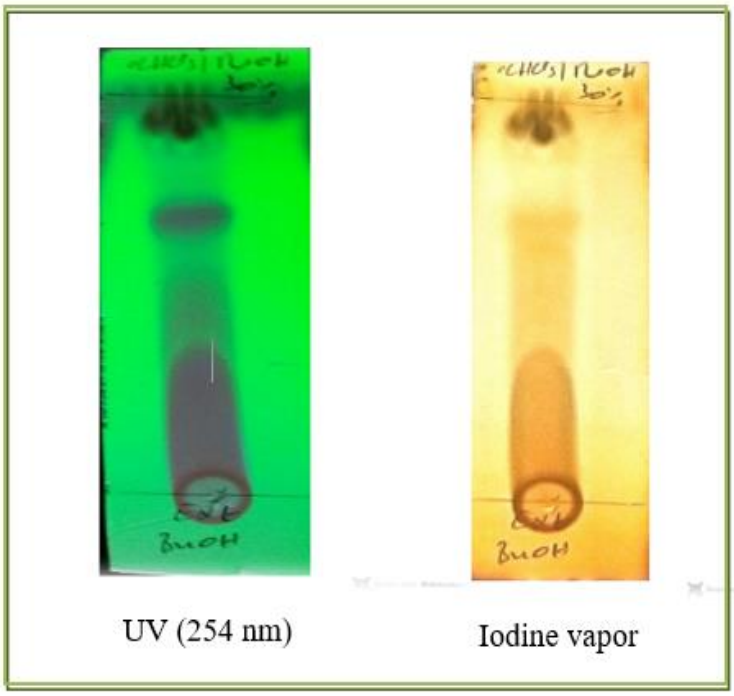


Figure.II.13 : TLC of *n*-BuOH of extract *B. Bituminosa*.

II.5.Determination of total phenolic content in EtOAc extract

The total phenolic content in plant extract (EtOAc) was determined by using Folin-Ciocalteu colourimetric method described by Singleton & Rossi (1965) [1], based on oxidation-reduction reaction. 200 µL of diluted sample (1 mg/1mL or 0.5 mg/1 mL) were added to 1 mL of diluted Folin–Ciocalteu reagent (1/10). After 4 min, 800 µL of saturated sodium carbonate (75 g/l) were added. After 2 hours of incubation at room temperature, the absorbance at 765 nm was measured by UV–Vis spectrophotometer (UviLine 9400) (Figure II.14). Gallic acid (0–175 µg/ml) was used for the standard calibration curve (Figure II.15). The results were expressed as Gallic acid equivalent (GAE) per mg of crude extract (µg EAG/ mg of extract) and calculated as mean value ± SD (n = 3).

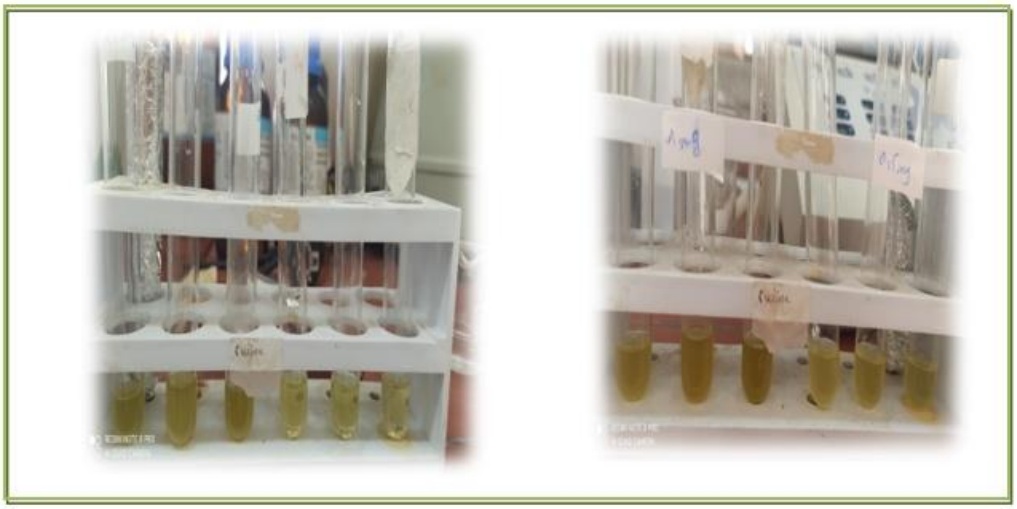


Figure20 : Samples (1 mg and 0.5 mg)before and after incubation for determination of the total phenolic content in plant extract (EtOAc).

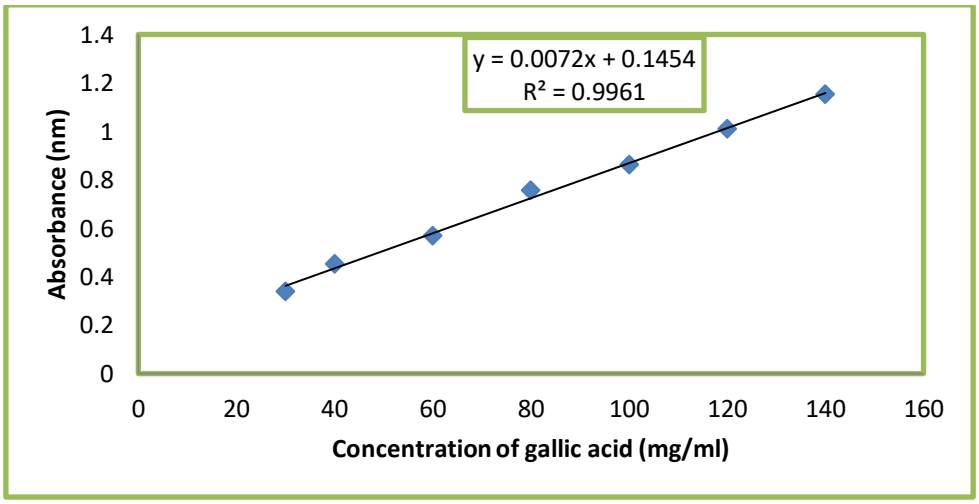


Figure.II.15: Standard calibration curve of gallic acid.

II.6. Determination of total flavonoid content in EtOAc extract

Total flavonoid content was quantified by the trichloroaluminum method [2]. 1 mL of $AlCl_3$ (2%) solution was added to 1 mL of the EtOAc extract(1 mg/1mL and 0.5 mg/1 mL). The mixture was vigorously agitated and after 10 min of incubation at room temperature, the absorbance was read at 430 nm against the suitable blank (Figure II.16). Quercetin (1.25–25 µg/mL) was used to establish the calibration curve to estimate the concentration of flavonoids (Figure II.17). The results were expressed in micrograms of equivalent of quercetin per milligram of extract (µg EQ/mg of extract).

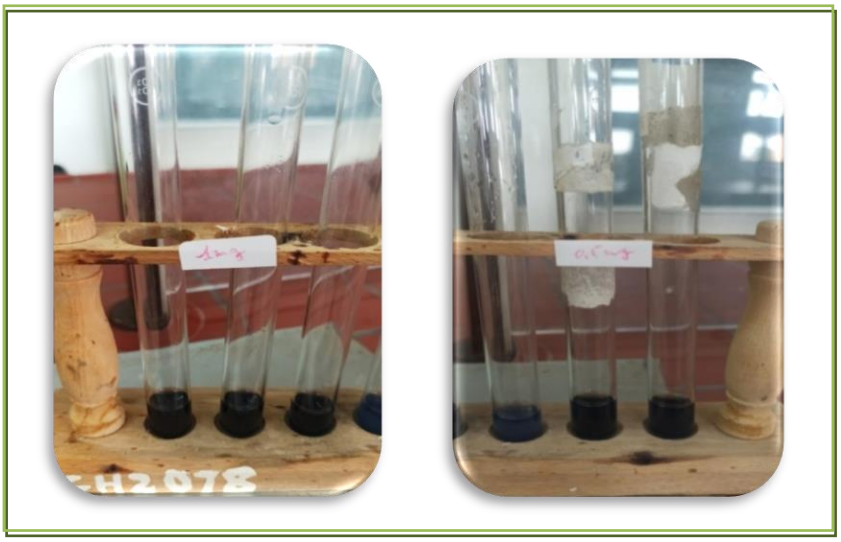


Figure .II.16: Samples (0.5 mg and 0.25 mg) before and after incubation for determination of the total flavonoid content in plant extract (EtOAc).

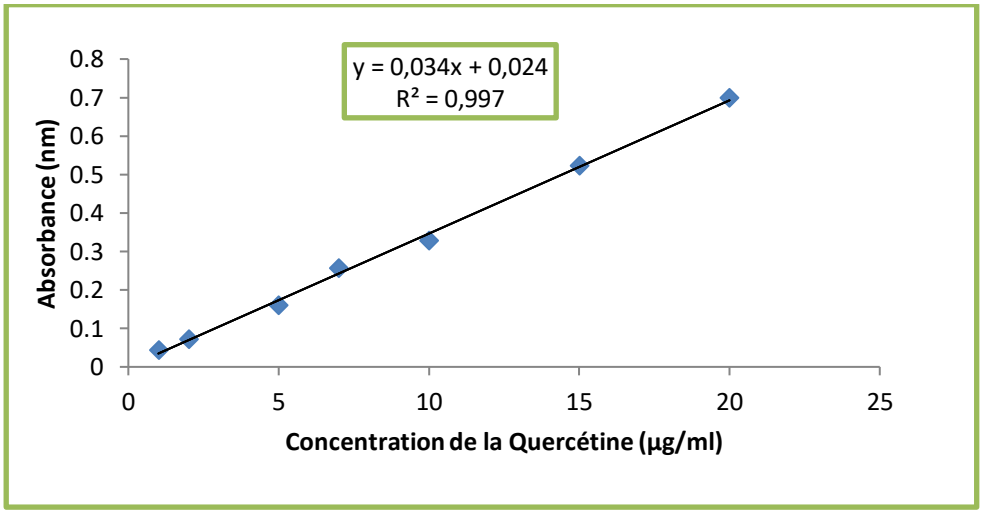


Figure.II.17 : Standard calibration curve of quercetin.

II.7. Biological Activities

In this part of work, we focused on the study of the antimicrobial effect and antioxidant power by the *in vitro* investigation of EtOAc extract of *Bituminaria bituminosa*. Given the means available, we have chosen the simplest methods used to evaluate these two biological activities: DPPH free radical-scavenging assay and disk diffusion method against bacterial strains, respectively.

II.7.1. Antioxidant activity of EtOAc extract

Free radical scavenging capacity of the ethyl acetate extract was determined with the 1,1-diphenyl-2- picrylhydrazyl (DPPH) assay, according to the method described by Menaceur and his collaborators (2014)[3]. 25µl of different dilutions of the extract or standard BHT were added to 975 µl of DPPH (0.025 mg/ mL), the mixture was kept in the dark place at room temperature for 30 minutes. The absorbance was measured at 517 nm (Figure II.18). The percentage of DPPH radical-scavenging activity of each extract was calculated as follows:

$$I\% = [(A_{Blank} - A_{Sample})/ A_{Blank}] \times 100$$

A_{Blank} is the absorbance of blank and A_{Sample} is the absorbance of positive control or sample. The experiments were performed in triplicate and the results were transmitted as mean (values ± SD).

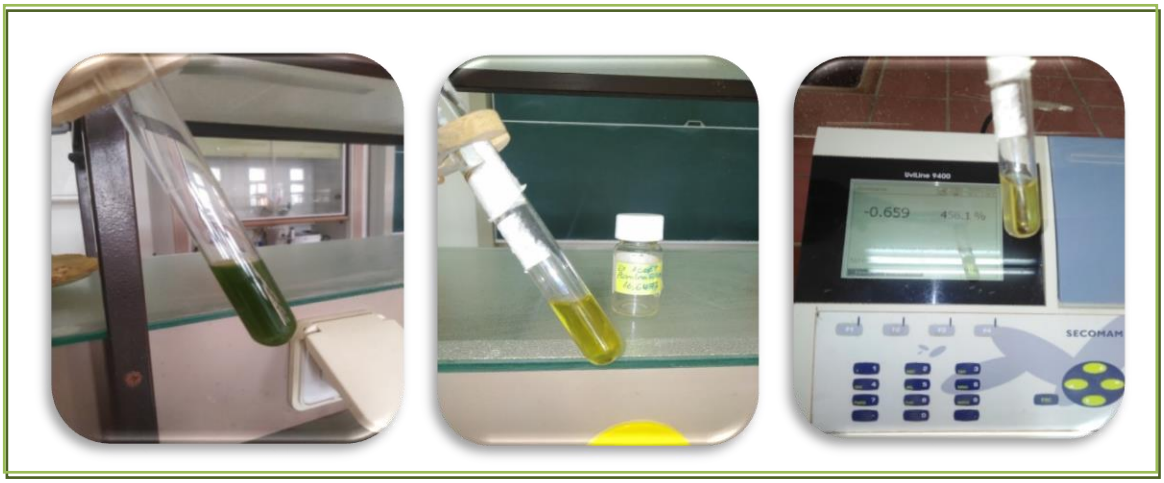
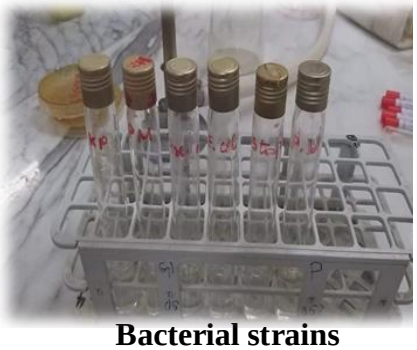


Figure .II.18 : Samples before and after incubation for determination of anti oxidant activity of EtOAc extract of *B.Bituminosa.*

II.7.2. Antibacterial activity of EtOAc extract

The antibacterial activity of the crude extract (EtOAc) obtained from the plant *B. bituminosa* was estimated by agar disk diffusion assay [4], against six bacterial strains, including one Gram-positive (Staphylococcus aureus) and five Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*,*poteurs mirabilis*,*proteurs vulgaris*).The bacterial strains obtained from the laboratory of bacteriology, Department of Pharmacy, faculty of Medicine, University of Batna, were initially isolated by the method of streaking the four quadrants in sterile conditions and at optimum temperatures according to the strain concerned for 24h. the bacterial suspension was adjusted to a density of 1.0×10^7 UFC mL^{-1} from an overnight culture and poured into sterile petri dishes containing Mueller Hinton agar (MHA) using a cotton swab. EtOAc was dissolved in DMSO at a concentration of 10µg/mL and 500 µg/mL, then 10 µl of these solutions were spotted onto sterile filter paper disks (6 mm in diameter,Whatman no 1) and deposited in the center of the petri dish. After 24 h of incubation at 37 °C, the inhibition zones were measured in mm. The antibiotics (Chlorophenicol, Cefotaxine, Tobromycine, 10 µg/disk) were used as a positive control for the tested bacteria, and dimethyl sulfoxide (DMSO) as negative control. The tests were performed in triplicate (three boxes for each concentration of extract, antibiotic and for each strain). The results were expressed by the diameters of zones of inhibition around the discs produced.

All steps of evaluated antibacterial activity of the crude extract (EtOAc) were shown in figure.II.19 :



Bacterial strains

Mueller Hinton agar (MHA) culture

The bacterial suspension poured into sterile petri dishes containing (MHA)

Placed antibiotic disks and spotted extract solution onto sterile filter paper disks and deposited in the center of the petri dish

Incubation at 37 °C for 24 h

Figure.II.19: Antibacterial activity of EtOAc extract of the plant *B.Bituminosa* against six bacterial strains.

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chapter III

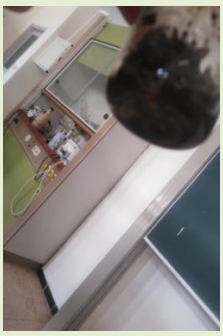
Results and Discussion

III.1.Phytochemical screening

The plant *Bituminaria bituminosa* was screened for its phytochemicals to reveal the presence of many secondary metabolites including: reducing sugars, steroids, flavonoids, saponins, tannins, alkaloids, polyphenols and essential oil [1]. The results were presented in the table below (Table III.1).

Table.III.1 : Pytochemical screening of *B.Bituminosa*.

Class of chemical compounds	Presence / Absence	Results
Reducing sugars	++	**Before** **After** 
Polyphenols	++	
Flavonoids	++	

Sterols and terpenes		++	
Condensed Tannins	**BATE-SMITH Test**	-	
	STAINSY Test	-	
Hydrolysable tannins	**STIASNY Test**	+	
Saponosides		-	
Essential oil		+	

Alcaloïds	**++**	**Before** **After** 

(-) Absence, **(+)** Presence in small quantity, **(++)** Presence in significant quantity

According to the results of the phytochemical tests obtained, we detect different families of chemical compounds. we can conclude that:

- The presence of polyphenols, flavonoids is in high quantity which are recognized for their antioxidant properties. In some cases, they are known for their antiviral, antimicrobial and antitumor activity.
- The presence of alkaloid can display a wide range of biological activities[2]
- These results confirme the richness of plant *Bituminaria bituminosa* in active chemical compounds which may explain its use in traditional medicine to treat many diseases.
- The majority detected secondary metabolites in our plant were previously isolated from species of the same genus (*Bituminaria*, Chapter 1).

III.2.Chromatographic (TLC) analysis of the different obtained extracts

The crude extracts (PE, EtOAc and *n*-BuOH) were obtained from the extraction process of *Butiminaria bituminosa* (Figure III.1).A chromatographic analysis by using TLC was carried out on these three extracts. Consequently, the chromatograms (Figures II.11 to II.13, in chapter 2) were showed a crucial richness of the ethyl acetate extract in secondary metabolites, and above all, in polyphenolic compounds, in particular coumarins (pterocarpans; fluorescent spots under UV light 365 nm). Through these results, we decided to study the chemical and biological properties of this extract (EtOAc).

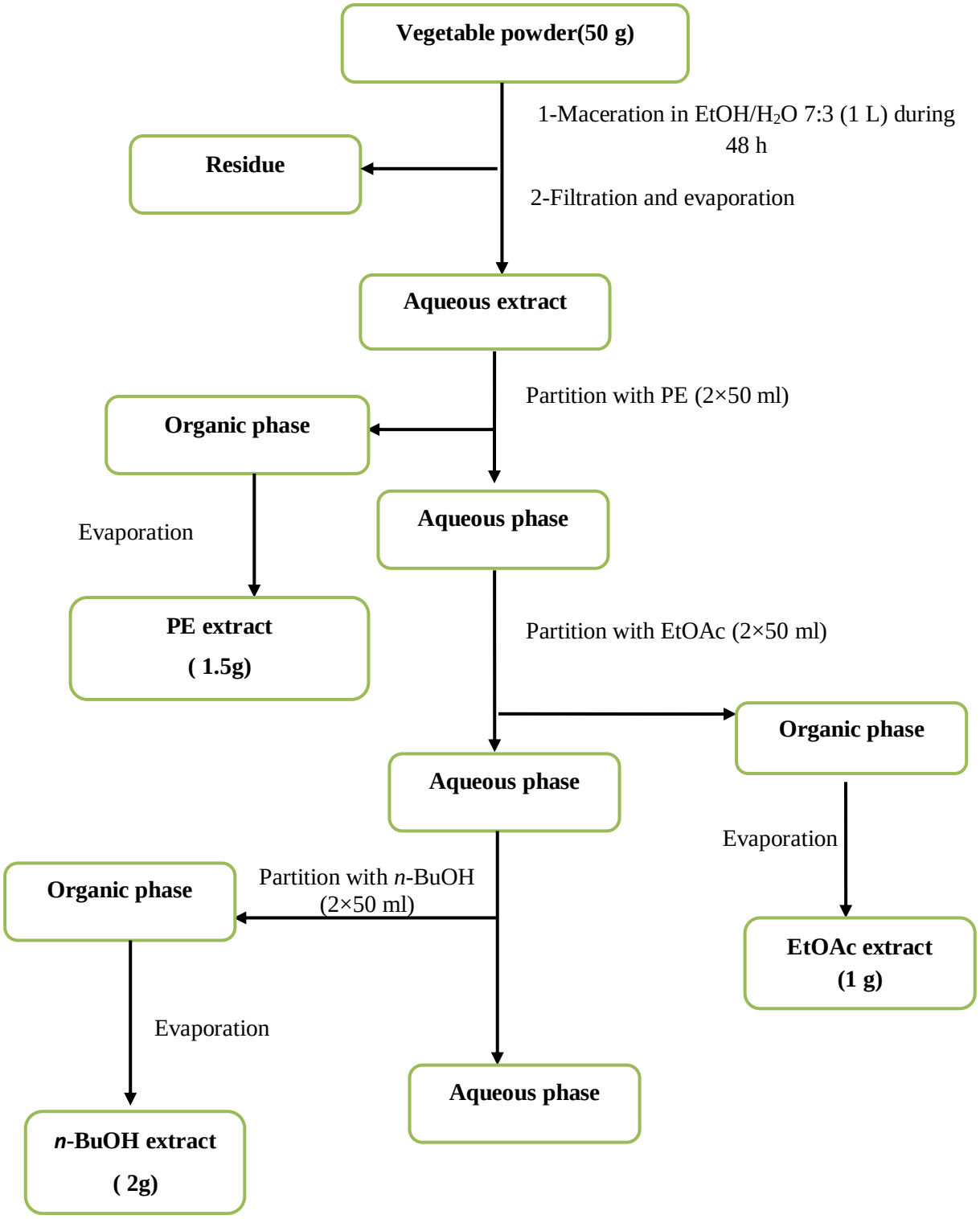


Figure.III.1 : Extract sheme of *B.Bituminosa*.

III.3. Total phenolic content(TPP)

The results of the total phenolic content of EtOAc extract of *B. bituminosa* are presented in Table III.2 below.

Table.III.2 : Total phenolic content of EtOAc of B.Bituminosa.

Extract	Total phenolic content (µg GAE/ mg of plant extract
EtOAc	133 ± 0.014

The total phenolic content was carried out according the calibration curves established by gallic acid: $y = 0,0072x + 0,1454$, $R^2 = 0,996$.In our study, the result of TPP was found in EtOAc extract of *Bituminaria bituminosa* showed (133 ± 0.014GAE/mg). This good result was compared with those previously studied, on Lebanese *B. bituminosa* (aerial parts) exhibited the presence of phenolic content with 217.48 mg/g Gallic acid equivalent[3], which means a higher level of polyphenols compared to the results of the present study .The variation in total phenolic content between species of the same genus could be due to extrinsic factors (environment, stage of maturation and storage period) and intrinsic factors (genetic potential of the individual species for the biosynthesis of polyphenols)[4].

III.4. Total flavonoid content(TFV)

The results of the total flavonoid content of EtOAc extract of *B. bituminosa* are presented in Table III.3 below.

TableIII.3 : Total flavonoid content of EtOAc extract of *B.Bituminosa*.

Extract	Total flavonoid content (µg QE/ mg of plant extract
EtOAc	92.20± 0.039

The total flavonoid content was carried out according the calibration curves established by quercetin: $y = 0,034x + 0,024$, $R^2 = 0,997$.The result obtained from present study showed that

the EtOAc extract contains a good amount of flavonoid (92.20± 0.039µg QE/mg). Although, previous study, on the aerial parts of *B.bituminosa* found in Lebanon was exhibited a better flavonoidal content with 135.83 µg QE /mg of plant extract [2].

III.5. Antioxidant activity

The antioxidant activities of the EtOAc extract of *B. bituminosa* and BHT as standard were evaluated through the ability to scavenge DPPH. The results are presented in Table III.4.

Table.III.4 : Antioxidant activity of EtOAc extract of *B. Bituminosa* by the DPPH assay.

Extract/Standard	**IC_{50} (mg/mL)**
EtOAc	0.19± 0.011
BHT	0.03 ± 0.02

The EtOAc extract has relatively a weak radical scavenging activity toward DPPH (0.19 ± 0.011mg/mL) comparing to BHT (0.03 ± 0.02mg/mL). Therefore, the EtOAc extract of *B. bituminosa* was showed a moderate antioxidant activity.

The comparison of the obtained result with previous studies showes that same species has almost the same activity as scavenger agents against DPPH free radical [5].

The antioxidant activity observed in the tested extract can be attributed to the presence of several types of secondary metabolites, known for their antioxidant potential.

III.6. Antibacterial activity

The diffusion test was applied to one Gram-positive (*Staphylococcus aureus*) and five Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*) microorganisms. The control treatment (DMSO had no inhibitory effect on any of the tested microorganisms. The results of these tests are presented in Figure III.2 below.

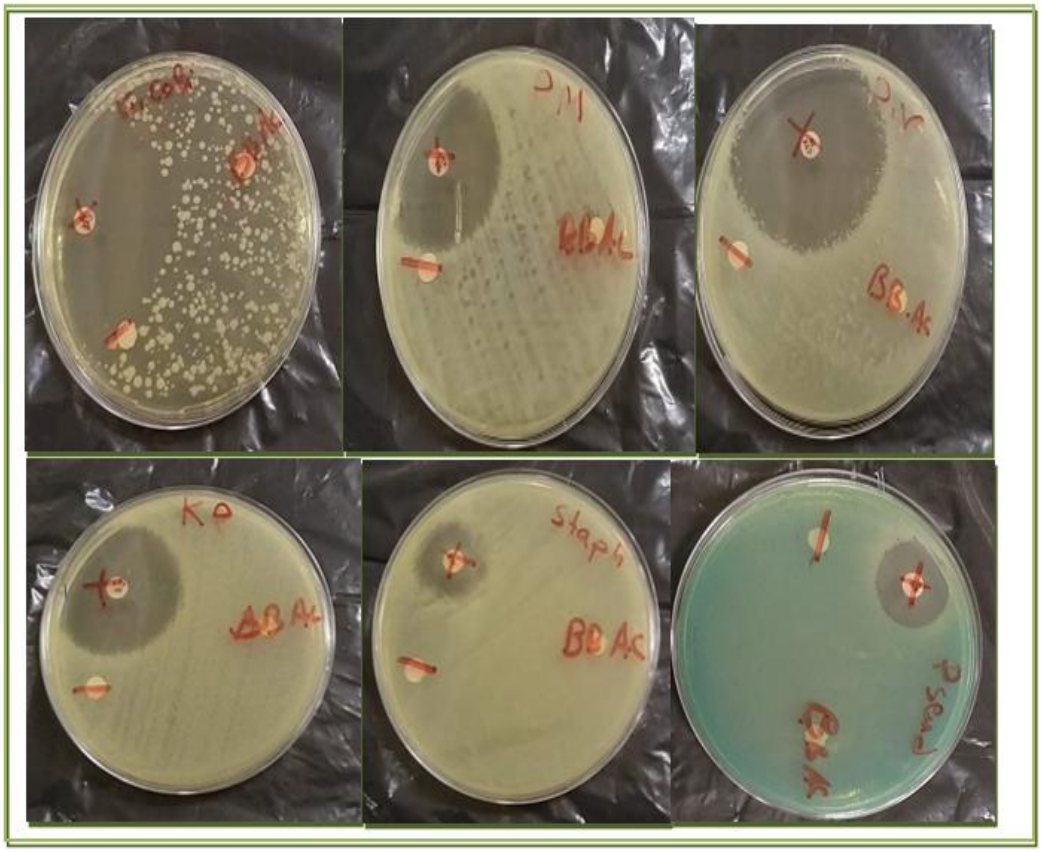


Figure.III.2 : Antibacterial activity of EtOAc extract of the plant *B. Bituminosa* against six bacterial strains.

Unfortunately, the crude extract ethyl acetate (EtOAc) did not display any antibacterial effects against all the tested strains .The comparison of the obtained result with previous studies showed that same species had a good bacterial activity [5].

The absence of the antibacterial activity in our study could be explained by:

- The nature and the amount of the bioactive compounds with antibacterial proprieties, which could be totally absent or present in a very small amount in this extract EtOAc.

- The addition of DMSO to plant extracts decreases their antibacterial activities [6].

- The loss of sensitive compounds during the grinding or the conservation of the vegetal material [7].

- The diffusion capacity of substances (present in the extract) in the agar medium, antimicrobial power of the diffused substances, the growth and metabolic activity of the microorganisms in environment [8]. In addition depending on the date of harvest there will be very significant variations in chemical composition and activity [9].

- The difference in the structure of the bacterial wall plays an important role in the susceptibility of bacteria[10]. Several studies have highlighted the great sensitivity of Gram-positive bacteria to plant extracts compared to Gram-negative bacteria[11]. In Gram-negative bacteria, the lipopolysaccharide-rich outer membrane constitutes an effective barrier of permeability whose negative surface charges prevent the diffusion of hydrophobic molecules (non-polar compounds).

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Conclusion

The present work focused on the preliminary phytochemical screening and the evaluation of *in vitro* antioxidant and antibacterial activities of crude extract (ethyl acetate EtOAc) obtained from the species *Bituminaria bituminosa* L.This plant,called «Adna » or «Menitna », was collected in April 2022 in Djerma area. It is a perennial wild legume plant characterized by the strong smell of bitumenand widely distributed throughout the Mediterranean basin. It is used to provide hay or forage for livestock and in folk medicine as vulnerary, cicatrizing and disinfectant agent.

From our study it can be concluded that various phytochemicals, including phenols and flavonoids, are present in *B. bituminosa* with good amounts (TPP; 133 ± 0.014 GAE/mg of plant extract) and (TFV; 92.20± 0.039 µg QE/mg of plant extract).

Our study confirmed that *B. bituminosa* has an antioxidant effect. Indeed, the ethyl acetate extract showed moderate anti-radical activity against the free radical DPPH with IC_{50} value: 0.19 ± 0.011 mg/mL.

On the other hand, the antibacterial activity of the extract(EtOAc) has been determined *in vitro* using disk diffusion method against standards and six clinical strains (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*). The results showed that the extract does not have any activity against all strains. This negative result may be due to the method used which must be changed to others such as dilution methods.

For the continuity of the work started with more efficiency, many perspectives can be envisaged in particular the isolation and characterization of the secondary metabolites of this plant and then testing them for their antibacterial, anti-inflammatory, anti-fungal and anti-carcinogenic power.

Abstract

This study is devoted to the estimation of total phenolic content, flavonoid content, and the evaluation of *in vitro* antioxidant and antibacterial activities of crude extract (ethyl acetate EtOAc) obtained from the species *Bituminaria bituminosa L*. The phytochemical analysis of this plant by using preliminary tests and TLC technic, revealed the presence of many secondary metabolites including: reducing sugars, steroids, flavonoids, tannins, alkaloids, polyphenols and essential oil. In further, the total phenolic and flavonoid contents were determined spectrophotometrically by the Folin–Ciocalteu and trichloroalluminium technics. Indeed, the ethyl acetate extract recorded a good content of polyphenols (133 ± 0.014 GAE/mg of plant extract) and flavonoids (92.20 ± 0.039 µg QE/mg of plant extract). Additionally, the antioxidant activity of crude extract (EtOAc) was evaluated by using DPPH$^{\bullet}$ assay, where the results showed that the tested extract has the ability to scavenge the free radical DPPH with IC_{50} value: 0.19 ± 0.011 mg/mL. Antibacterial activity was performed according to disc diffusion method. Unfortunately, the crude extract (EtOAc) has no antibacterial effect against all the tested strains compared to Chloramphenicol, Cefotaxime and Tobramycin as positive controls.

Key words : *Bituminaria bituminosa*, phenolic, flavonoid content, antimicrobial, antioxidant activity .

ملخص

هذه الدراسة خصصت لتقدير المحتوى الفينولي الكلي ، ومحتوى الفلافونويد الكلي ، وتقييم الأنشطة المضادة للأكسدة والبكتيريا في المختبر للمستخلص الخام (أسيتات الايثيل) الذي تم الحصول عليه من النبتة Bituminaria bituminosa . أظهر التحليل الكيميائي النباتي لهذا النبات باستخدام الاختبارات الأولية و تقنية TLC ، وجود العديد من المستقلبات الثانوية بما في ذلك: السكريات المختزلة ، المنشطات ، الفلافونويد ، التانين ، القلويدات ، البوليفينول والزيوت الأساسية , علاوة على ذلك ، تم تحديد إجمالي محتويات الفينول والفلافونويد باستخدام تقنية Folin-Ciocalteu و trichloroalluminium. سجل مستخلص أسيتات الإيثيل محتوى جيد من البوليفينول (133 ± 0.014 GAE / ملجم من المستخلصات النباتية) والفلافونويدات (92.20 ± 0.039 ميكروغرام / ملجم من المستخلص النباتي)

بالإضافة إلى ذلك ، تم تقييم النشاط المضاد للأكسدة للمستخلص الخام (EtOAc) باستخدام مقايسة DPPH ، حيث أظهرت النتائج أن المستخلص المختبَر لديه القدرة على اختزال الجذور الحرة بقيمة IC50: 0.19 ± 0.011 مجم / مل. تم إجراء النشاط المضاد للبكتيريا وفقًا لطريقة انتشار القرص. لسوء الحظ ، المستخلص الخام (EtOAc) لم يظهراي تاثير مضاد للبكتريا ضد جميع السلالات المختبرة مقارنة بالكلورامفينيكول والسيفوتاكسيم و التوبراميسين كعناصر تحكم إيجابية.

الكلمات الأساسية : Bituminaria bituminosa L، محتوى الفلافونويد و الفينول مضادات البكتريا ، نشاط مضادات الأكسدة

Appendices

Appendix1 : *Reagents and solutions*

1- MAYER's test
Mercuric chloride…….....1.35g+ Potassium iodide…...5 g Distilled water……30mL Shake until dissolved then add: distilled water……...q.s 100 ml
2 - Dragendorff's test
Bismuth subnitrate ……. ……0.85 g + Glacial acetic acid……..10 mL Potassium iodide……...8 g Distilled water……..70mL
3- BOUCHARDAT test
Iodine……...….2 g Potassium iodin ….. …….. 2 g Distilled water ……...…….. .q.s 100 ml
4- STIANSY test
Formaldehyde ……..….6 ml Hydrochloric acid…….3mL
5- FERRIC CHLORIDE solution
Crystallized ferric chloride……1 g Distilled water ……..…….…….. q.s 10 mL
6 - Folin Ciocalteu reagent
Sodium tungstate ……100g + Sodium molybdate……..25g dissolved in 700 mL of distilled water 85% phosphoric acid…….100g +Concentrated hydrochloric acid……100g Boil under reflux for 10 hours Lithium sulphate….150g + a few drops of bromine and boil again for 15 minutes + cool + distilled water ……q.s 1L

Appendix 2 : Total phenolic content of EtOAc extract of *B. bituminosa* extract(three trials + Blank)

	EtOAc(1mg)	**EtOAc (0,5mg)**	**blank**
Absorbance at (765nm)	1.093	0.908	1.783
	1.093	1.642	1.792
	1.062	0.924	1.781

Appendix 3 : Total flavonoid content of EtOAc extract of *B. bituminosa* extract(three trials + Blank)

	AcOEt (0.5mg)	**AcOEt (0,25mg)**	**blank**
Absorbance at (430nm)	2.185	1.223	1.196
	1.937	1.112	1.197
	2.28	1.126	1.196

Appendix 4 : DPPH radical scavenging activity of EtOAc extract (three trials + Blank)

Concentration mg/ml	**Absorbance at 517nm**			
	R1	R2	R3	Blank
0.8	0.2387	0.2619	0.2543	0.357995
0.4	0.2830	0.2210	0.2035	0.357995
0.2	0.2116	0.2093	0.1927	0.357995
0.1	0.6431	0.5574	0.5604	0.357995
0.05	0.8854	0.8472	0.7313	0.357995
0.025	1.0391	1.0281	0.9996	0.357995

Appendix 5: Antioxidant activity of EtOAc extract of B. bituminosa

Concentration mg/ml	%inhibition
0,8	29.7113594
0,4	34.12476723
0,2	42.86778399
0,1	63.95716946
0,05	129.4134078
0,025	185.5493482