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Phytochemical study and evaluation of antioxidant and antimicrobial activities of *Hedysarum naudinianum* Coss.

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Dedication

I know full well that they deserve far more than a simply humble work but I don't have something more precious to dedicate.

Dad and Mom ..just to you two ..I dedicate my work

« Aile her şeydir »

Ilhem

Dedication

With the help of almighty Allah , who marked out the path of my life for me, I was able to do this work which I dedicate:

To The light of my eyes, the shadow of my foot steps and the happiness of my life ***my mother's*** *who supported me during all my years study, for his sacrifice and his support which gave me confidence, Courage and security.*

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List of abbreviations

%: Percentage

ABTS : 2,2'-Azino-bis (3- ethylbenzthiazoline-6-sulfonic acid)

ATCC: American Type Culture Collection

A549: Human Lung Carcinoma Epithelial Cells

CaCo-2: Cancer coli-2

CT: Condensed tannins

CUPRAC: Cupric-Ion-Reducing Antioxidant Capacity

DCM: Dichloromethane

DPPH: Scavenging 1,1-diphenyl-2-picryl-hydrazyl

$FeCl_3$: Iron chloride

FRAP: Ferric reducing power

FR: Free Radicals

GAE: Galic acid equivalent

GSH: Reduced glutathione

GSHPX: Glutathion peroxydase reductases

g : Gram

HA: Hydrogen atom transfer

HAT: Hydrogen atom transfer

HCl : Hydrogen chloride

HeLa: Henrietta's cancer cells

HEK293: Human embryonic kidney 293

HEP-G2: Hepatocellular carcinoma

H2O2: Hydrogen peroxide

IC_{50}: The half maximal inhibitory concentration

Kg: Kilogram

MGC-803: Human gastric carcinoma cell

mg : Milligram

MCF-7: Michigan Cancer Foundation-7

MTT: 3-(4, 5-Dimethylthiazol-2-yl) - 2, 5-diphenyl tetrazolium bromide assay.

NH_3 : Ammonia

NO: Nitric oxide

OA: Oxidizing agents

PC3: Prostate cancer cell 3

QE: Quercetin equivalent

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SET: Single electron transfer

SOD: Superoxyde Dismutase

TCM: Traditional Chinese medicine

U-87MG: Uppsala 87 Malignant Glioma

Introduction

Throughout the ages humans have relied on nature for their basic needs for the production of foodstuffs, shelters, clothing, means of transportation, fertilizers, flavors and fragrances, and, not least, medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years (Newman *et al.*, 2000).

According to the World Health Organization (WHO) in 2008, more than 80 % of the world's population relies on traditional medicine for their primary healthcare needs. Nearly, all cultures and civilizations from ancient times to the present day have depended fully or partially on herbal medicine because of their effectiveness, affordability, availability, low toxicity and acceptability (Benmehdi *et al.*, 2012).

An imbalance between free radicals and antioxidant systems can cause oxidative stress in the human body which may result in several chronic and fatal diseases such as parkinson's, alzheimer's, cancer and cardiovascular disease.

In response, humans produce antioxidant compounds and enzymes which neutralize these free radicals, but due to the decreasing levels of these naturally occurring compounds as a result of environmental factors and other reasons, this may cause an increase in oxidative stress which can lead to cell damage and death. Supplementation containing antioxidants is required at this stage, to prevent the damage which are caused by these free radicals from natural or synthetic sources. The investigation of pharmacological active and safe antioxidants are crucial. Therefore, there is a pressing need for searching of new antimicrobial and antioxidant medications with diverse mechanisms action. Indeed, in recent years, phytochemical secondary metabolites have been extensively investigated as a source of therapeutic agents. Thus, it is anticipated that phytochemicals with potential biological properties such as the antibacterial activity will be used for the treatment of various diseases including bacterial infections. Antibiotic resistance has become a serious global concern, and the discovery of novel antimicrobial herbal constituents may provide valuable solutions to overcome the problem (Jaradat *et al.*, 2017).

The Algerian flora counts nearly 3000 species belonging to several botanical families of which 15% are endemic, remains very little explored on the phytochemical level as on pharmacologically (Kennouche, 2017). *Hedysarum,* a genus of the Fabaceae family, is represented by 300 annual and perennial species (Dong *et al.*, 2013), it includes ten species in

Algeria some of which are endemic, such as *H. naudinianum* (Torche *et al*., 2013).

The phytochemistry and pharmacology of *Hedysarum* species have attracted increasing worldwide attention among those involved in the research and development of new drugs. The chemical literature reveals that the various species of this genus are rich in flavonoids, triterpenes, coumarins, lignanoids, nitrogen compounds, sterols, carbohydrates, fatty compounds, benzofuran, and polysaccharides (Dong *et al.*, 2013).

The aim of present study was to investigate the chemical composition and to evaluate the antioxidant and antimicrobial activities from different extracts of *H.naudinianum* Coss. aerial parts.

Part I

Bibliographic synthesis

Chapter 01

Botanical study of *Hedysarum naudinianum* Coss.

1. The Fabaceae family

The Fabaceae (of faba meaning broad bean) or Leguminosae commonly known as the legume, pea, or bean family, are a large and economically important family of flowering plants. It includes trees, shrubs and herbaceous plants perennials or annuals, which are easily recognized by their fruits (legume) and their compound, stipulated leaves. The group is widely distributed ,is the third-largest land plant family in terms of number of species, behind only the Orchidaceae and Asteraceae, with 730 genera and over 19,400 species (Mahbubur Rahman, 2014) and the second one in terms of diversity behind the Poaceae (Gramineae) (Bourezzane, 2018).

2. The genus *Hedysarum*

2.1. Generality

Hedysarum comes from the Greek word Hedys, which means soft to graze (Bonnier, 1934). Species of this genus grow spontaneously on varied soils and in different climatic conditions, thus presenting a great diversity. This genus is represented by more than 170 species distributed across temperate Europe, the Mediterranean region, West Asia, Siberia and North America from Arizona to Canada and regions Arctic (Goudjil-benhizia, 2014).

2.2. Traditional use and biological properties of plants of the genus *Hedysarum*

2.2.1. Use in traditional medicine

In China , several species (*Hedysarum polybotrys* Hand.-Mazz., *Hedysarum limprichtii* Hlbr, *Hedysarum vicioider* Turcz.var. *Taipeicum* Hand.-Mazz. Liu, *Hedysarum smithianum*, et al) of genus *Hedysarum* have a long history of use in traditional Chinese medicine (TCM). In TCM, these plants are used to increase the energy of the body and treat infestation with gastrointestinal nematodes and may support the immune system and peripheral nervous system (Dong *et al*., 2013).

2.2.2. Biological activities

Documentary research has shown that species of the genus *Hedysarum* have not been investigated biologically. The only reference concerns the species *H. polybotrys* very rich in polysaccharides. The latter has anti-aging, antioxidant, anti-tumor, analgesic, anti-inflammatory, anti-viral and anti-diabetic effects. It also has effects against cerebral and cardiac hypoxia. It would have great potential for use in modern healthy food and vegetable cosmetics (Bourezzane, 2018).

2.3. Other interests of the genus *Hedysarum*

Hedysarum species of agronomic interest, thanks to their fodder quality and their ability

to improve soil fertility by fixing atmospheric nitrogen, can be exploited to enhance degraded regions, especially in arid and semi-arid areas (Hannachi-Salhi *et al*., 2004).

2.4. Previous phytochemical studies on the genus *Hedysarum*

Over the past few decades, 155 chemical constituents have been isolated from plants of genus *Hedysarum* through different chromatography methods, the chemical structures of these constituents include flavonoids, triterpenes and triterpenoid saponins, coumarins , lignanoids , nitrogen compounds, sterols, carbohydrates , fatty compounds and benzofuran (Nechepurenko, 2008), in addition to some polysaccharides (Dang *et al.*, 2013).

Flavonoids are usually regarded as the main groups of metabolites in genus *Hedysarum*. So far, different types (flavones, flavanones, isoflavones, chalcones, flavanols, xanthones and pterocarpanes) have been identified. Flavones are the most common and predominant in this genus (Dong *et al*., 2013).

In Algeria, the genus *Hedysarum* includes 10 annual or perennial species which are : *H. carnosum* Desf, *H. perrauderianum* Coss and Durieu, *H. humile* L, *H. pallidum* Desf., *H. spinosissimum* L., *H. glomeratum* L., *H. aculeolatum* Mumby, *H. coronarium* L, *H. flexuosum* L and *H. naudinianum* Coss.the subject of our study (Abdelguerfi-berrekia *et al*., 1991).

3. *Hedysarum naudinianum* Coss.

3.1. Generality

H.naudinianum Coss. is a perennial, di-tetraploid and a very rare species (Quezel & Santa, 1962; Abdelguerfi-Berrekia et al., 1991;Trifi-Farah et al., 2002) ,endemic to Algeria. It is found in the tell constantinois (mount of Bibans), in Algiers (Littoral, Tell Atlas), in Boghar, and the north of Setif (Bougaa, Ain Roua) (Torche et al., 2013).

3.2. Botanical description

Perennial plant with reticulate pods smooth on the sides, shortly toothed at the periphery, very flattened, large, 8-10 mm wide. Flowers in elongated clusters, purple (Trifi-Farah *et al*., 2002; Quézel & Santa, 1963).

Figure 1.*Hedysarum naudinianum* Coss.

3.3. Systematic classification

The plant *H. naudinianum* Coss. is classified as follows:

Reign: Plantae

Class: Magnoliopsida

Family: Fabaceae

Subfamily: Papilionaceae

***Genus:** Hedysarum*

Species: *Hedysarum naudinianum* Coss. (Tela botanica, 2013).

3.4. Pedoclimatic requirements

3.4.1. Climatic requirements

H.naudinianum Coss. grows, most often, under rainfall between 360 mm and 550 mm, at an altitude of over 700 m, on steeply sloping soils, limestone to very limestone, of fine to medium texture.

3.4.2. Edaphic requirements

It grows on steeply sloping soils, limestone to very calcareous, of fine to medium texture.

3.5. Interest of *H.naudinianum* Coss.

H.naudinianum Coss. is a species of undergrowth; its role is clearly indicated in the improvement of pastoral productivity in the south of the country (Abdelguerfi-Berrekia *et al*., 1991).

4. Secondary metabolites

4.1. Definition

Secondary metabolism, designating a metabolism whose taxonomic distribution would be restricted and whose contribution to cellular functioning or the development of plants would be insignificant (Gravot, 2008). Plant organisms are the only living entities able to produce secondary metabolites.Indeed, these molecules are specifically synthesized by plant biochemical pathways which are absent in animal cells (Di Marco *et al.*, 2018).

Secondary metabolites are not vital for the body but necessarily play an important role in the complex enzymatic machinery necessary for their production. They have ecological roles (allomone, pheromone…). These molecules were selected during evolution for the interaction they have with a receptor in another organism. They therefore represent a great potential source of therapeutic agents (Thomas, 2009).

4.2. Classification of secondary metabolites

Secondary metabolites are produced in very small quantities, there are more than 200,000 secondary metabolites classified according to their chemical affiliation in this case, the terpenes, alkaloids, acetylenic compounds, waxes, and phenolic compounds (Vermerris & Nicholson, 2006).There are three main classes:

4.2.1. Phenolic compounds

The designation "polyphenols" or "phenolic compounds" brings together a vast set of more than 8,000 molecules, divided into ten chemical classes, which present all have one thing in common: the presence in their structure of at least one 6-carbon aromatic ring, itself a carrier of a variable number of hydroxyl functions (OH) (Hennebelle *et al.*,2004).

4.2.2. Phenolic acids

Phenolic acids are major components of berries. Phenolic acids are represented by cinnamic and benzoic acid derivatives (Taylor & Awika, 2017).

4.2.3. Coumarins

Coumarins, of different types, are found in many plant species and have very diverse properties. They are able to prevent the peroxidation of membrane lipids and capture hydroxyl, superoxide and peroxyl radicals (Igor Passi, 2002).

4.2.4. Quinones

They are colorful and shiny substances, usually red, yellow or orange and possessing two ketone functions. Quinones are found in plants, Fungi, bacteria. Animal organisms also contain quinones, like for example vitamin K, which is involved in blood clotting (Kansole,

2009).

4.2.5. Tannins

Tannins are naturally an occurring heterogeneous group of phenolic compounds with 49 diverse structures that share their abilities to bind and precipitate proteins (Huang *et al.*, 2018).

- **Condensed tannins**

Condensed tannins (CT) are phenolic compounds which play a determinant role in the quality of legume forages. In fact these secondary metabolites of the flavonoid pathway bind to proteins and affect enzymatic activity and protein solubility (Damiani, 2000).

- **Hydrolyzable tannins**

Hydrolyzable tannins (a group mainly responsible for the toxic effects that may appear when certain plants are consumed) (Paolini *et al.*, 2003).

4.2.6. Flavonoids

Flavonoids represent a class of secondary metabolites frequently described within the Fabaceae family. They are secondary metabolites of plants, of which nearly 6,500 different representatives have been identified, and their number is constantly increasing. Unlike the flavonoids present in the flowers, to which they confer their color, the flavonoids present in the leaves are masked by the presence of chlorophyll (Harborne & Williams, 2000).

Chapter 02

Antioxidants and biological activities

1. Oxidative stress

Oxidative stress is defined as the body's inability to defend itself against reactive oxygen species (ROS) due to disturbance of endogenous balance between the latter and oxidizing agents (OA). This imbalance potentially leads to damage structural and functional. (Bensakhria, 2018).The term is used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavorable (Lobo *et al*., 2010).

While the effects of ROS on cell proliferation occurred exclusively at low or transient concentrations of radicals (Valko *et al*., 2006), major ROS important in signal transduction and vascular biology are superoxide anion (O_2-ȣ), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Montezano *et al*., 2015).

1.1.Oxidative stress and redox environment of a cell

Oxidation and reduction reactions in biological systems are called redox reactions and represent the basis for numerous biochemical mechanisms. When discussing redox reactions in biological systems, instead of the terms reductant and oxidant, it is more appropriate to use an oxidant, or OA, is a substance that accepts electrons (Valko *et al*., 2006).

1.2.Free Radicals (FR)

A free radical (FR) can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. FR attack important macromolecules leading to cell damage and homeostatic disruption.

2. Antioxidants

An antioxidant is a molecule stable enough to donate an electron to a rampaging FR and neutralize it, thus reducing its capacity to damage.

2.1. Mechanism of action of antioxidants

Two principle mechanisms of action have been proposed for antioxidants.

- The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the FR present in the systems.
- The second mechanism involves removal of ROS/reactive nitrogen species (RNS) initiators (secondary antioxidants) by quenching chain-initiating catalyst.

2.2. Levels of antioxidant action

The antioxidants acting in the defense systems act at different levels

- The first line of defense is the preventive antioxidants, which suppress the formation of FR. Although the precise mechanism and site of radical formation *in vivo* are not well elucidated yet, the metal-induced decompositions of hydroperoxides and hydrogen peroxide must be one of the important sources.
- The second line of defense is the antioxidants that scavenge the active radicals to suppress chain initiation and/or break the chain propagation reactions. Various endogenous radical-scavenging antioxidants are known: some are hydrophilic and others are lipophilic. Vitamin C, uric acid, bilirubin, albumin, and thiols are hydrophilic, radical-scavenging antioxidants, while vitamin E and ubiquinol are lipophilic radical-scavenging antioxidants. Vitamin E is accepted as the most potent radical-scavenging lipophilic antioxidant.
- The third line of defense is the repair and *de novo* antioxidants. The proteolytic enzymes, proteinases, proteases, and peptidases, present in the cytosol and in the mitochondria of mammalian cells, recognize, degrade, and remove oxidatively modified proteins and prevent the accumulation of oxidized proteins (Lobo *et al.*, 2010).

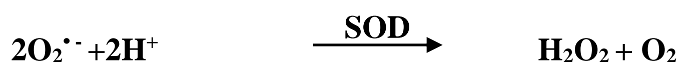
2.3. Enzymatic Antioxydants

Enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) are considered to be the body's first line of defense against ROS.

2.3.1. Superoxyde Dismutase (SOD)

One of the most effective intracellular enzymatic antioxidants is SOD (EC1.15.1.1). SOD is the antioxidant enzyme that catalyzes the dismutation of $O_2^{\bullet -}$ to O_2 and to the less-reactive species H_2O_2 (Valko *et al.*, 2006).

The role of SOD and peroxidases is complementary because good protection cannot be obtained by SOD alone (Favier, 2003).



$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

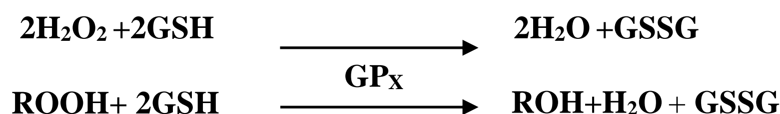
2.3.2. Catalase

Catalase (EC 1.11.1.6) is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert 6 million molecules of H2O2 to water and oxygen each minute:

The significantly decreased capacity of a variety of tumours for detoxifying H_2O_2 is linked to a decreased level of catalase.

2.3.3. Glutathion peroxydase reductases (GSHPX)

There are two forms of the enzyme glutathione peroxidase, one of which is selenium-independent (glutathione-*S*-transferase, GST, EC 2.5.1.18) while the other is selenium-dependent (GPx, EC 1.11.1.19) .These two enzymes differ in the number of subunits, the bonding nature of the selenium at the active centre and their catalytic mechanisms. Glutathione metabolism is one of the most essential of antioxidative defence mechanisms (Valko *et al.*, 2006).



2.3.4. Non Enzymatic Antioxidants

Unlike antioxidant enzymes, most of these components are not synthesized by the body and must be provided by food. In this category antioxidant we find the trace elements, reduced glutathione (GSH), vitamins E and C and polyphenols.

2.3.4.1. Vitamin E

Vitamin E has been the subject of intensive study for more than 50 years, and this enormous body of literature demonstrates conclusively that the principal role of vitamin E is to protect tissue against unwanted, destructive oxidation in fact; vitamin E is the most effective lipid- soluble antioxidant present in our cells. Because vitamin E is an antioxidant, and because it has long been known that oxidation plays an important role in carcinogenesis (Pryor, 2000).

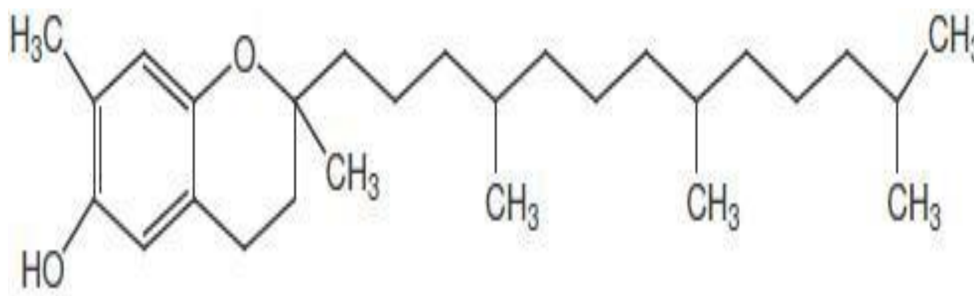


Figure 2. Structure of vitamin E

2.3.4.2. Vitamin C (Ascorbic acid)

Ascorbic acid or “vitamin C” is a monosaccharide antioxidant found in both animals and plants (Lobo *et al.*, 2010).It acts as a potent water soluble antioxidant in biological fluids by scavenging physiologically relevant ROS and RNS. It is an essential micronutrient required for normal metabolic functioning of the body .Humans and other primates have lost the ability to synthesize vitamin C as a result of a mutation in the gene coding for L-gulonolactone oxidase, an enzyme required for the biosynthesis of vitamin C via the glucuronic acid pathway (Carr & Frei, 1999).

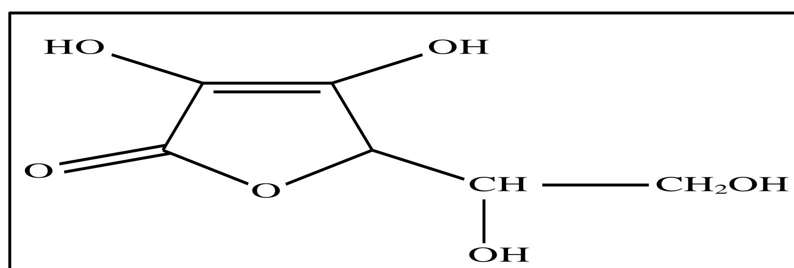


Figure 3. Structure of vitamin C

2.3.4.3. Glutathione (GSH)

Glutathione is a cysteine-containing peptide found in mostforms of aerobic life (Lobo *et al.*, 2010).The major thiol antioxidant is the tripeptide, glutathione. GSH is a multifunctional intracellular non-enzymatic antioxidant. Its reduced form is GSH, and the oxidised form is GSSG, glutathione disulphide.

2.3.4.4. Flavonoids

Polyphenolic compounds constitute one of the most commonly occuring and ubiquitous groups of plant metabolites and represent an integral part of human diet (Valko *et al.*, 2006).

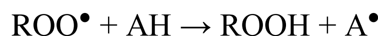
3. Biological activities

3.1. Antioxidant capacity

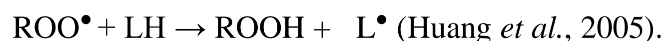
Antioxidant capacity is related with compounds capable of protecting a biological system against the potentially harmful effect of processes or reactions involving ROS and RNS. These protective effects of antioxidants have received increasing attention within biological, medical, nutritional, and agro-chemical fields (Karadag *et al.*, 2009).The antioxidant capacity of the molecules can be assessed either in vivo, on living organisms, either in vitro, using tests that mimic the phenomenon physiological. The evaluation of the in vitro antioxidant activity of natural extracts involves different methods. The latter involve the mixture of oxidizing species, such as that of FR or oxidized metal complexes, with a sample which contains antioxidants capable of inhibiting the generation of radicals (Bourezzane,

2018) .Generally, antioxidants can deactivate radicals by two major mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET). The end result is the same, regardless of mechanism, but kinetics and potential for side reactions differ (Prior *et al.*, 2005).

HAT-based methods measure the classical ability of an antioxidant to quench FR by hydrogen donation (AH = any H donor) (Prior *et al.*, 2005) in which this antioxidant and the substrate compete for peroxyl radicals $ROO^{\bullet}$:

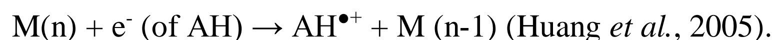


$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$



$$ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$$ (Huang *et al.*, 2005).

SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Prior *et al.*, 2005).



$$M(n) + e^{-} \text{(of AH)} \rightarrow AH^{\bullet+} + M(n-1)$$ (Huang *et al.*, 2005).

Considering that there are many methods which differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and expression of results have been developed and tested in the literature (Karadag *et al.*., 2009), it is recommended to use at least two tests with differing reaction mechanisms to confirm antioxidant activity (Prior *et al*., 2005).

3.2. Antimicrobial activity

3.2.1. Microbial infections

Microbial infections are caused by different microorganisms and are the cause of the most fatal diseases and the most widespread epidemics. The therapy of bacterial infections is mainly based on the use of antibiotics that selectively inhibit certain metabolic pathways in bacteria, without usually exert toxic effects on higher organisms (Bouhaddouda, 2016).

3.2.2. Antibiotics

Antibiotics are antimicrobial substances of biological origin, which act in low concentrations on microorganisms by blocking metabolic steps essential for their survival or growth (Michel, 1981).

3.2.3. Microbial resistance

Antibiotic resistance has become a serious public health problem affecting almost all antibacterial agents in all their fields of action. The antibiotics lose their effectiveness and diseases that were thought to be eradicated reappear. The decrease in the effectiveness of the means of control obliges to find a alternative to the use of antibiotics, by synthesizing new

compounds with virtues bactericides (Seladji, 2015).

3.2.4. Other antimicrobial products

Antimicrobial chemical agents are classified into two categories according to their effect. The first is lethal, that is, it results in the death of the individual. It is named by the suffix –CIDE: virucide, bactericide, fungicide, insecticide, such as:

- Chemically very reactive compounds: oxidants and hydrogen peroxide, halogens (chlorine and iodine) and oxide ethylene, strong acids and bases, aldehydes and phenols.
- Chemically stable compounds with more specific actions: such as quaternary ammoniums, phenolic derivatives other than phenol, chlorohexidine among others.

The second corresponds to growth inhibition in the presence of the active product. We name with the suffix –STATIC: bacteriostatic, fungistatic, essentially include metals (mercury and derivatives of copper, zinc, silver…) and dyes (Belkhiri &Baghiani, 2017).

3.2.5. The role of plants

Because soil is rich in bacteria, fungi and viruses, it is likely that plants contain chemical defence strategy to protect themselves against microbes in their environment using latent antimicrobials or synthesise them *de novo* as part of a phytoalexic response on microbial invasion (Gibbons, 2008).These antimicrobials include polyphenols which are one of the most numerous and diverse group of secondary metabolites that their antioxidant properties provide the basis for antimicrobial effects(Othman *et al.*, 2019) and flavonoids which are phenolic compounds widely distributed in the plant kingdom (Picman *et al.*, 1995).

Part I
Experimental study

Chapter 03
Materials and methods

1. Plant material

Hedysarum naudinianum Coss. was harvested from El Outaya (34°55'36.3''N, 005°38'54.0''E, arid climate), northwest of Biskra (Figure 4) in February 2020 and identified by reading assistant Mokrani D, the plant material consists of the aerial parts (stems , leaves, flowers) , These various organs were dried at shade and protected from humidity at room temperature for a few days. Once dried the three parts of the plant were reduced to powder and then subjected to extraction.

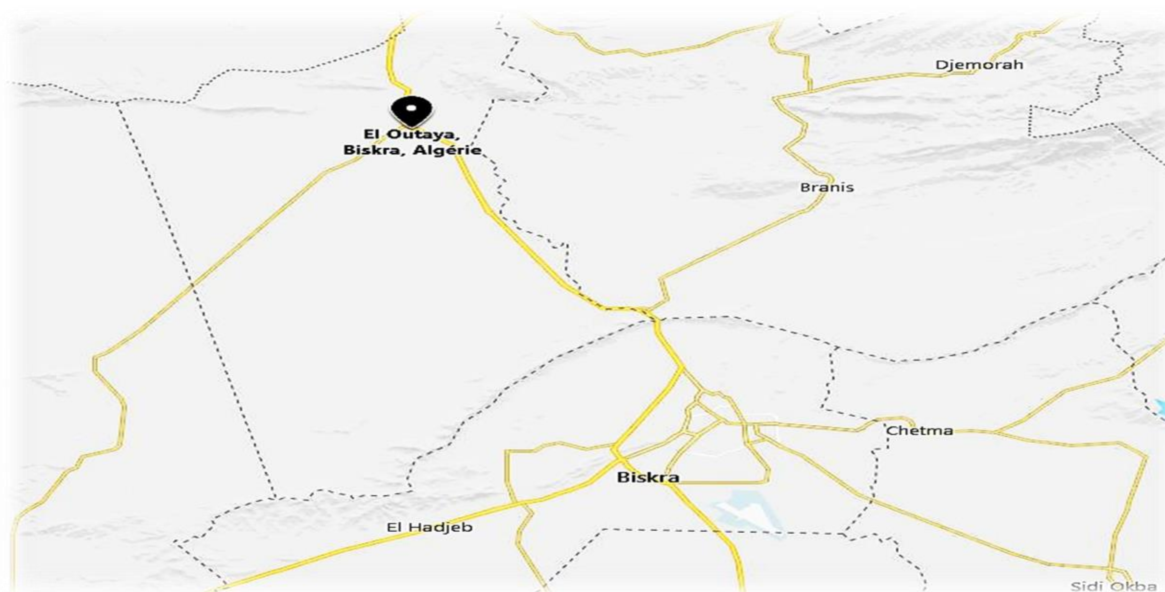


Figure 4. Geographic location of the El Outaya region (Bing, n.d.)

2. Phytochemical tests

2.1. Preparation of aqueous extract

For conventional extraction (refluxing), 10g of powdered plant material was mixed with 170 ml of distilled water in a round bottom flask and refluxed for about 30 min. The temperature was kept between 20-40ºC. The mixture was filtered and the aqueous extract was subjected to the following tests:

2.1.1. Starch

5 mL of aqueous extract was added 10 mL of NaCl saturated solution. After heating, starch reagent (Appendix 1) was added, a blue-purplish colour is a positive test for the presence of starch (Benmehdi *et al* ., 2012).

2.1.2. Saponosides

The detection of saponosides was carried out by adding a little water to 2 ml of the

extract aqueous, after two vigorous shakes, the tube was left to stand for 15 min and the height of foam was measured. The height of the foam at least 1 cm, showed the presence of saponosides. However, the height of the foam indicated the value of the foam index (Feuya tchouya & Nantia, 2015).

2.1.3. Tannins

1 ml of the aqueous extract was added to 1 ml of water, a 1 to 2 drops of diluted ferric chloride solution was added. A dark green or blue green coloration indicated the presence of tannins (Sabri *et al*., 2012).

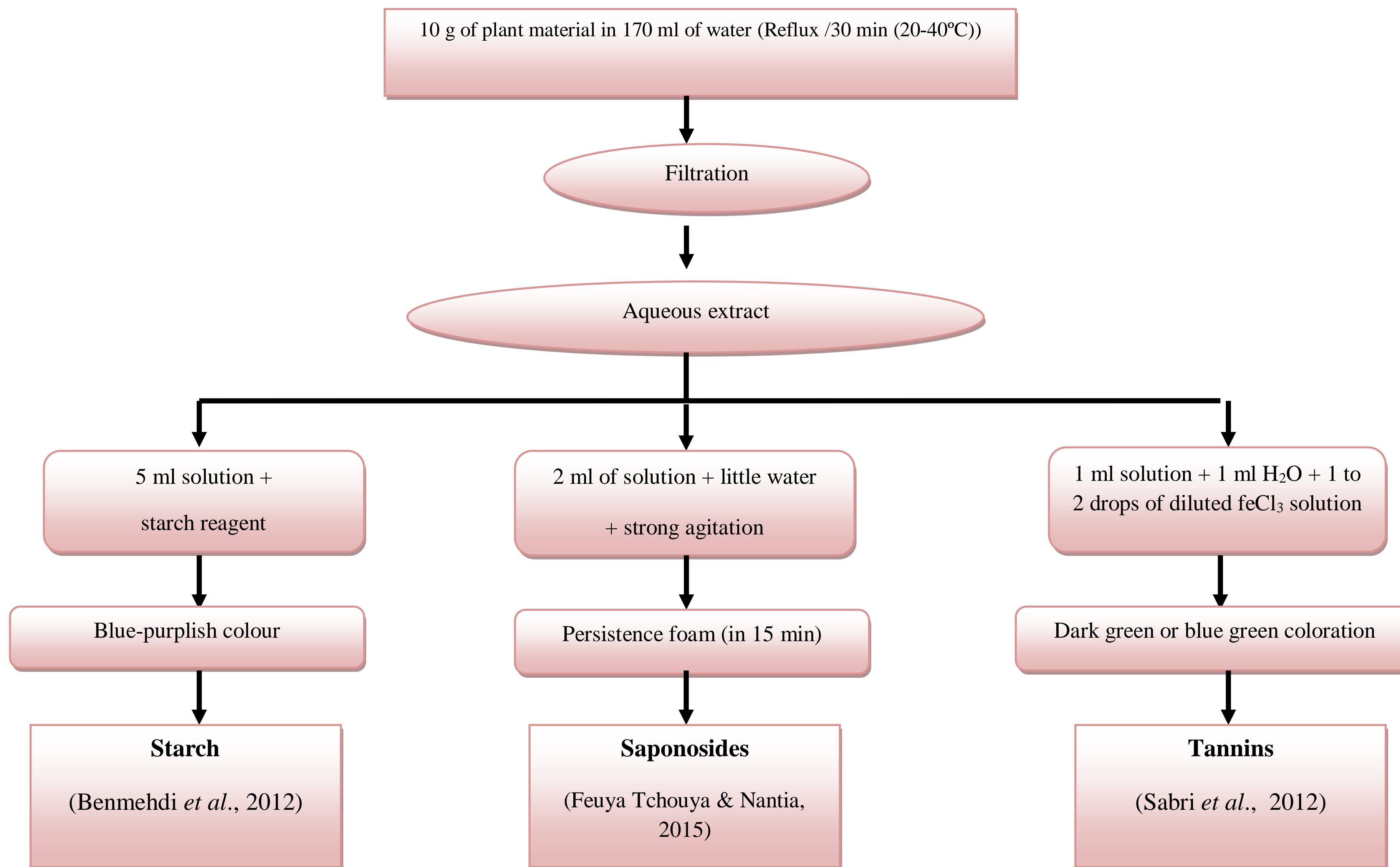


Figure 5. Phytochemical tests 1

2.2. Preparation of ethanol extract

10 g of powder was mixed with 130 ml of ethanol in a round bottom flask and refluxed for about 30 min. The temperature was kept between 20-30ºC. The mixture was filtered and the ethanolic extract was subjected to the following tests:

2.2.1. Flavonoids

The presence of flavonoids was detected by treating 1ml of the ethanolic extract with 10 % NaOH solution, formation of intense yellow colour indicated presence of Flavonoid (Sawant & Godghate , 2013).

2.2.2. Tannins

1 ml of the ethanol extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride solution was added and observed for green to blue-green (cathechic tannins) or a blue-black (gallic tannins) coloration.

2.2.3. Reducing compounds

The ethanol extract (1 ml) was added to 1ml of water and 20 drops of boiling Fehling's solution (A and B) in a test tube was added too. The formation of a precipitate red-brick in the bottom of the tube indicates the presence of reducing compounds (Sabri *et al* ., 2012).

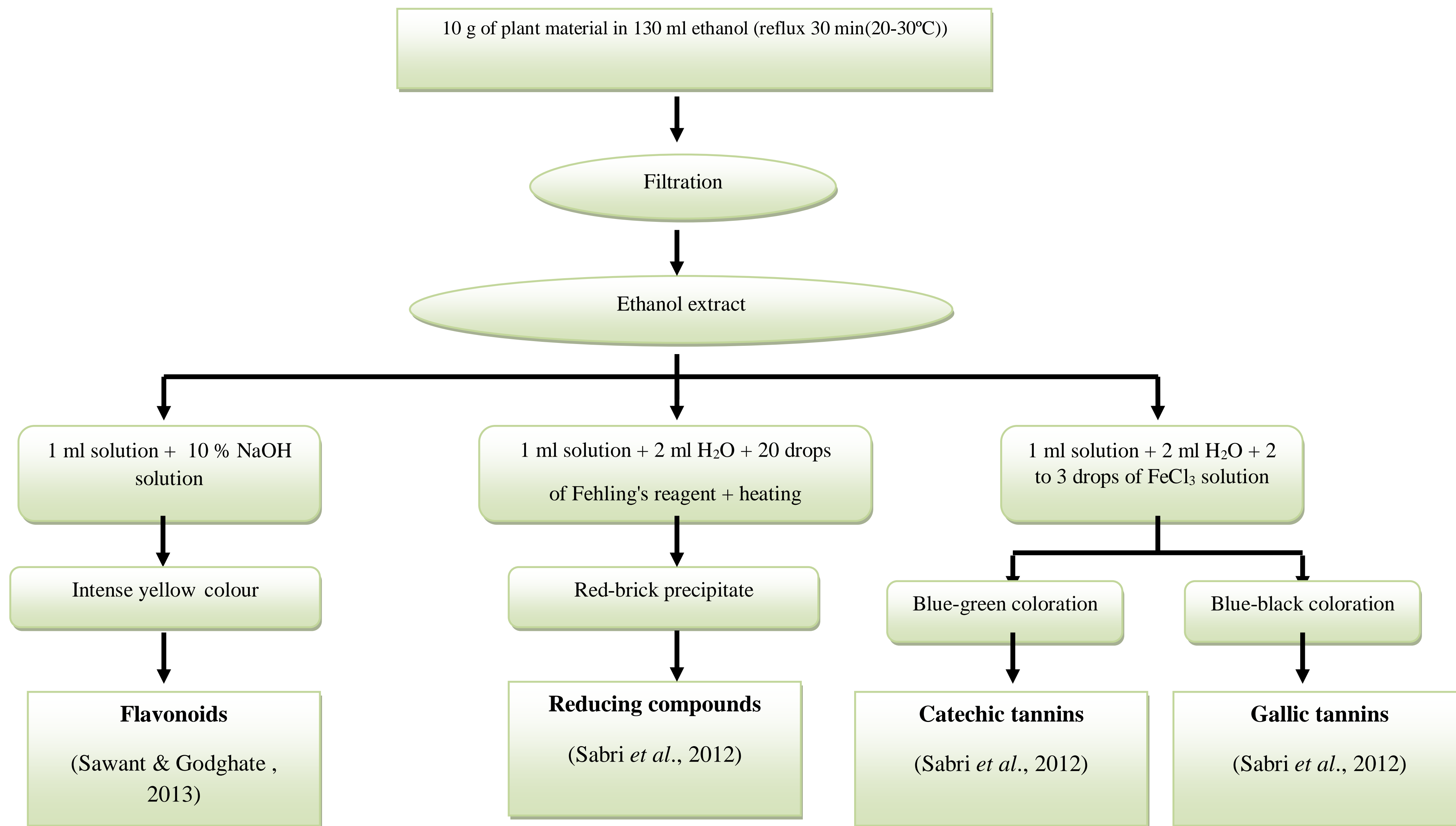


Figure 6. Phytochemical tests 2

2.3. Other secondary metabolites

2.3.1. Sterols and triterpenes

The ether extract obtained by maceration 5% ether at room temperature, filtration after 24h and solvent evaporation was dissolved in chloroform and few drops of acetic anhydride was added to it followed by concentrated sulphuric acid from side of test tube. Developments of reddish brown or purple ring at the junction indicated the presence of Sterols and triterpenes (Surana & Wagh, 2015).

2.3.2. Alkaloids

10 g of vegetable powder was macerated in 10 ml of H_2SO_4 (10%) then filtrated after 24 h on a paper washed with distilled water till obtain 10 ml of filtrate, 1 ml of the latter was introduced into two test tubes and 5drops of Mayer's reagen were added to the first tube and 5 drops Wagner's reagent was added in the second. The presence of a precipitate indicated the presence of alkaloids (Akpagu *et al.*, 2015).

2.3.3. Anthocyanins

2 ml of aqueous infusion was added to 2 ml of 2N HCl & NH_3, the appearance of pink red turns blue violet indicated presence of Anthocyanins (Sawant & Godghate , 2013).

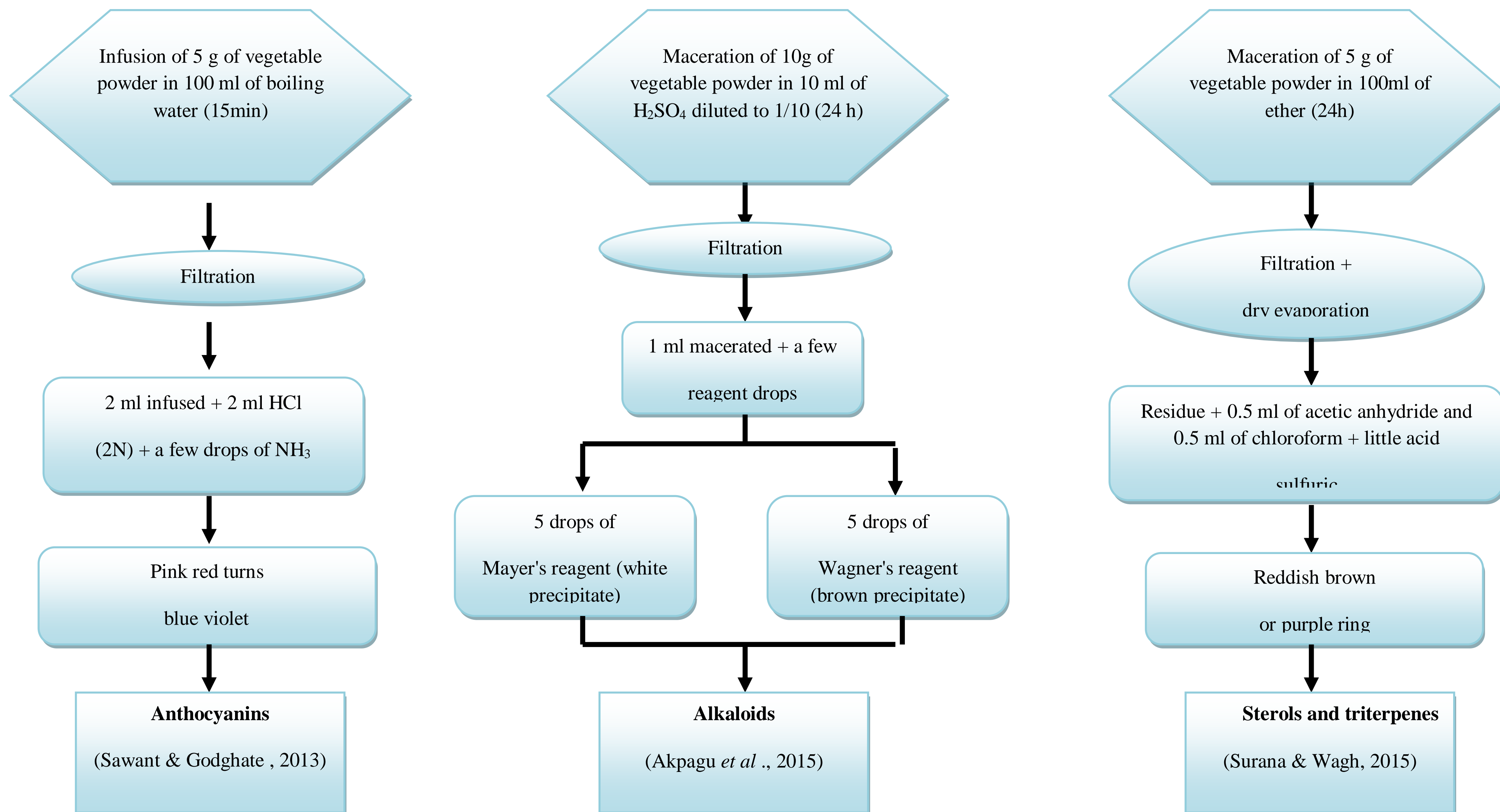


Figure 7. Phytochemical tests 3

3. Extraction of phenolic compounds

3.1. Preparation of crude methanolic extract

The powder (35 g) of *H.naudinianum* aerial parts was placed in an Erlenemeyer flask in 230 ml of methanol (70; 30) for 24 h. After filtration, the methanol was evaporated under reduced pressure in a rotary evaporator (Heidolphe) at 50 °C, and the dried residue was dissolved in 2ml methanol (Benhammou *et al.*, 2007).

Powder was delipidated with 120 ml petroleum ether for 4 to 5h in order to eliminate the lipids prior to any extraction (Figure 8).

3.2. Extract yield percentage

The extraction yield was presented in percentage (%) by the formula given by Falleh *et al* . (2008).

Yield (%) = 100 DWextr/DWsamp

where in:

DWextr: is the weight of extract after evaporation of solvent.

DWsamp: is the dry weight of organ sample.

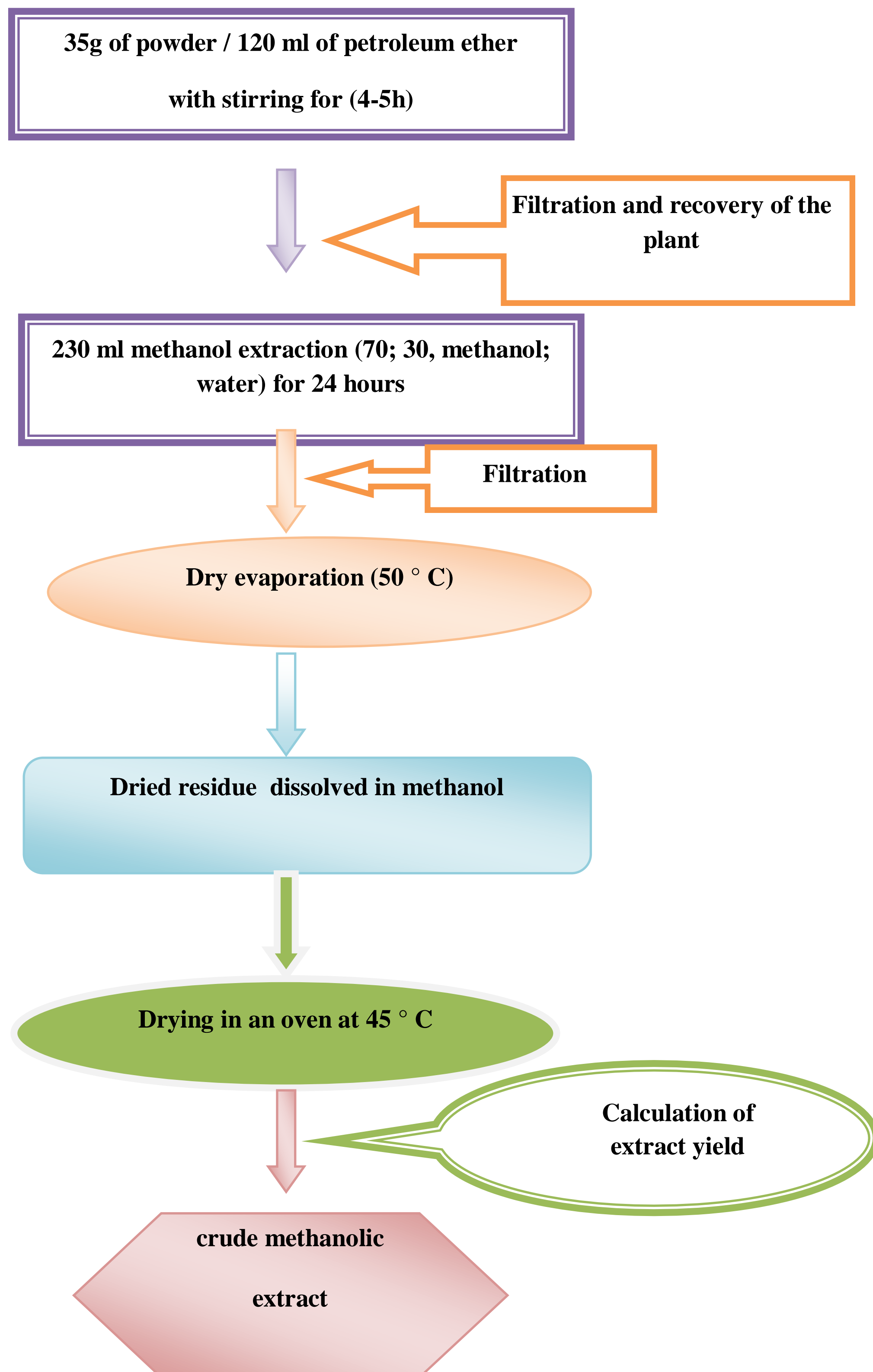


Figure 8. Preparation of crude methanolic extract

3.3. Extraction of flavonoids (ethyl acetate and butanolic fractions)

The solvents used for the liquid-liquid partition were: ethyl acetate and 1-butanol.

Before extraction, Powder was delipidated with 200 ml petroleum ether for (4-5h) in order to eliminate the lipids.

Eighty gram of plant material was macerated in 300ml ethanol for 24 h. After filtration, the ethanol was evaporated under reduced pressure in a rotary evaporator (Heidolphe) at 50 °C. The dried residue obtained was treated with 10 ml of boiling water to dissolve the flavonoids, and then put into a separating funnel and firstly extracted with 10 ml of ethyl acetate (twice), then with 10 ml of 1-butanol (twice). The two extracts were evaporated at 50 ° C, dissolved in 3 ml of methanol and then stored at 45 ° C (Benhammou *et al* ., 2009).

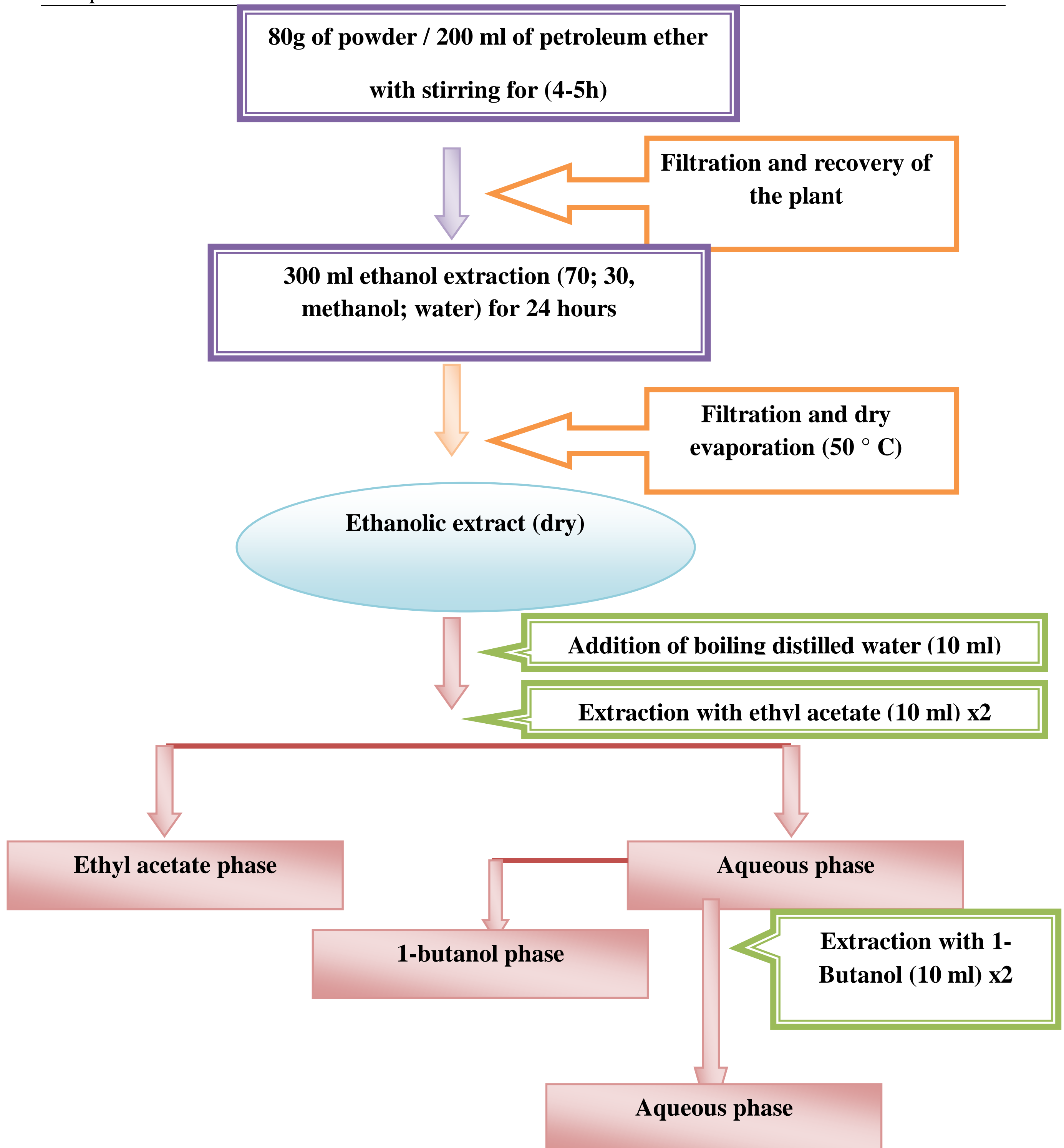


Figure 9. Extraction of flavonoids

4. Determination of phenolic compounds

4.1. Total phenolics content

Total phenolics were assayed using the Folin–Ciocalteu reagent. The application of this reagent constituted a polyphenol quantification method with enhanced sensitivity and reproducibility. The F–C reagent is a mix ture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) that reacts with phenols and non-phenolic reducing substances to form chromogens. The latter can be detected spectrophotometrically, since in alkaline conditions the oxotungstate and oxomolybdate formed in this redox reaction display a blue coloration proportional to the concentration of polyphenols (Lamuela-Raventós, 2018).

A volume of 200 µl of the crude methanolic extract of the plant was mixed with 1 ml of Folin-Ciocalteu reagent diluted 10 times with water and 0.8 ml of a 7.5 % sodium carbonate solution in a test tube. After stirring and 30 min later, the absorbance was measured at 765 nm against a blank. Gallic acid was used as a standard for the calibration curve. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry plant material. (mg GAE / g DM) (Benhammou *et al.*, 2009).

4.2. Total flavonoids content

The flavonoids content was estimated by Zhishen method with aluminum trichloride and sodium hydroxide. Aluminum trichloride as a yellow complex with flavonoids and sodium hydroxide formed a complex pink that is absorbed in a visible 510 nm (Rahmani *et al* ., 2018).

500 µl of each extract (methanol, ethyl acetate, 1-butanol and aqueous) suitably diluted, were mixed separately with 1500 µl of distilled water, followed by 150 µl of 5% sodium nitrite ($NaNO_2$). After 5 min, 150 µl of 10% (m / v) aluminum trichloride ($AlCL_3$) was added to the mixture. After 6 min of incubation at room temperature, 500 µl of sodium hydroxide (NaOH) at 4 % was added. Immediately, the mixture was completely stirred to homogenize the contents. The absorbance of the pinkish-colored solution was determined at 510 nm against a blank.

A calibration curve was performed in parallel under the same operating conditions using quercetin as a positive control.

The total flavonoid content of the plant extracts studied was expressed as milligrams of quercetin equivalents per gram of dry plant material (mg QE / g DM) (Zheishan *et al.*, 1999).

5. Statistical analysis

Experimental assay data were expressed as an average, and plus or minus the standard deviation (A±SD), using the Microsoft office Excel 2007 program.

6. Antimicrobial activity

6.1. Bacterial and fungal strains tested and media used

A collection of 8 test organisms, including bacterial and fungal strains, five of them included American Type Culture Collection (ATCC) and three were clinical obtained from the Laboratory of Microbiology, Faculty of Biology, Biskra, Algeria (Table 1). They were all stored at 4 ° C on nutrient agar, subcultured every two weeks to ensure their purity (in order to obtain a young culture, and isolated colonies). Lysogeny broth (LB) agar was used as a bacterial culture media, and LB broth was applied for cultivation of fungal strains.

Table 1. The different microbial strains tested

Gram-positive bacteria	*Staphylococcus aureus* (Clinical isolate)
	Bacillus subtilis ATCC 11774
Gram-negative bacteria	*Escherichia coli* (Clinical isolate)
	Klebsiella pneumoniae ATCC *BAA*- 1705
	Proteus mirabilis ATCC 12453
Fungi	*Candida Kefyr* ATCC 8553
	Candida albicans ATCC 10231
	Penicillium chrysogenum ATCC 10106

Chapter 04
Results and discussion

1. Phytochemical study

In this study, a chemical screening was carried out on different extracts prepared from the aerial part of *H.naudinianum* Coss using solvents of different polarity and specific revealing reagents. The aim of these tests was the demonstration of main phyto-constituents (secondary metabolites) present in the plant tissues of *H.naudinianum* Coss.

The detection of these chemical compounds was based on tests for the solubility of the constituents, precipitation and turbidity reactions and a specific color change.

The experimental results of the phytochemical tests carried out on the ground plant material of the aerial part of *H.naudinianum* Coss .showed the presence of flavonoids, tannins (catechic tannins) and reducing compounds.

The demonstration of flavonoids in the ethanolic extract was confirmed by the appearance of an intense yellow color.

Tannins were present with significant intensity in both aqueous and ethanolic extracts. Its presence was confirmed by a positive reaction with the ferric chloride solution, giving a greenish blue color, so it was catechic tannins.

The positive test for reducing compounds showed their presence in with an appearance of a red-brick precipitate (Figure 10).

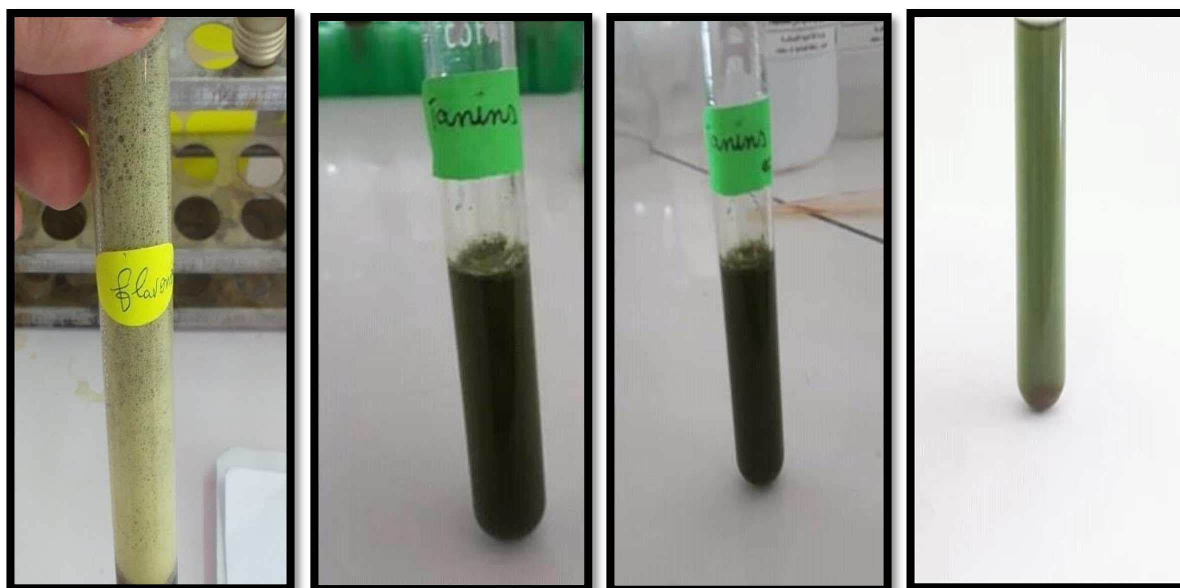


Figure10. Metabolites present in *H.naudinianum* Coss. aerial parts.

Starch, saponosides, gallic tannins, sterols and triterpenes, alkaloids and anthocyanins were completely absent in the present study (Table 2).

Table 2. Negative results of the phytochemical tests

Starch

Saponosides

Gallic tannins

Alkaloids

Anthocyanins

Sterols and triterpenes

All the results of the phytochemical tests carried out were summarized in the table below (Table3).

Table 3. Experimental results of phytochemical tests carried out on *H. naudinianum* Coss. aerial parts.

Research of			***H. naudinianum* Coss.** Aerial part
Aqueous extract	**Starch**		-
	Saponosides		-
	Tannins		++
Ethanolic extract	**Flavonoids**		+++
	Tannins	**Gallic tannins**	-
		Catechic tannins	++
	Reducing compounds		+
Alkaloids			-
Sterols and triterpenes			-
Anthocyanins			-

The results were classified according to:

Strong positive reaction: +++ Moderately positive reaction: ++

Weakly positive reaction: + Negative reaction: -

Previous works on phytochemical study have demonstrated the strong presence of flavonoids in the genus *Hedysarum* and showed that both of flavonoids and catechic tannins are abundant and potent phenolic compounds in this genus (Tibe *et al.*, 2011 ; Dong *et al.*, 2013; Ben salah *et al.*, 2015; Bourezzane, 2018; Liu *et al.*, 2019 ; Rufino-moya *et al.*, 2019) which is comparable to the present results, excluding the reducing compounds already found and reported in the seeds of the Turkish endemic *H. pogonocarpum* Boiss .(Tonguç *et al.*, 2019) and also for the first time in aerial parts of *H. naudinianum* Coss.

Unlike previous studies on *Hedysarum* species (Nechepurenko *et al.*, 2008; Dang *et al.*, 2013; Dong *et al.*, 2013), *H. naudinianum* aerial parts had registred a deffernt chemical composition represented by the absence of : saponosides, sterols, triterpenes, alkaloids and anthocyanins .

2. Extract yields

The extraction of the most abundant phenolic compounds in *H.naudinianum* allowed to calculate the yield of each extract (methanol, ethyl acetate, n-butanol, and water). The yield which has been determined relative to 100 g of dry and ground plant material was expressed as a percentage. The results obtained were presented in Table 4:

Table 4. Extract yields of *H.naudinianum*

Extracts	Yields %
Methanol/ water (70; 30)	12.19
Ethyl acetate extract	0.85
N-butanol extract	5.16
Aqueous extract	3.88

The results obtained showed that the highest yield was the methanolic extract (70%) (12.19%) followed by the butanolic fraction (5.16%) then the aqueous extract (3.88%) and finally the ethyl acetate fraction which was lowest yield (0.85%). Present results were in accordance with a previous studies reporting that solvents with very high solvent polarity, such as water, did not give good extraction results (Liu *et al.,* 2000*;* Chirinos *et al.*, 2007 ; Sayari *et*

al., 2016).

Moreover, Falleh *et al*. (2008) reported that the solubility of phenolic compounds was actually governed by the type of solvent used and the degree of polymerization of phenolics, that's why methanol was recommended and frequently used for the extraction of phenolics.

These results agreed with those obtained by Dong *et al*. (2013)who reported that flavonoids are the most abundant phenolic compounds in *Hedysarum* species.

3. Determination of phenolic compounds

3.1. Calibration curve for total phenols assay

This curve was established using gallic acid as a reference and the results were expressed as mg gallic acid equivalent per gram of dry matter (mg GAE/ g DM).The calibration curve was established with a correlation coefficient R^2=0,991(Figure 11).

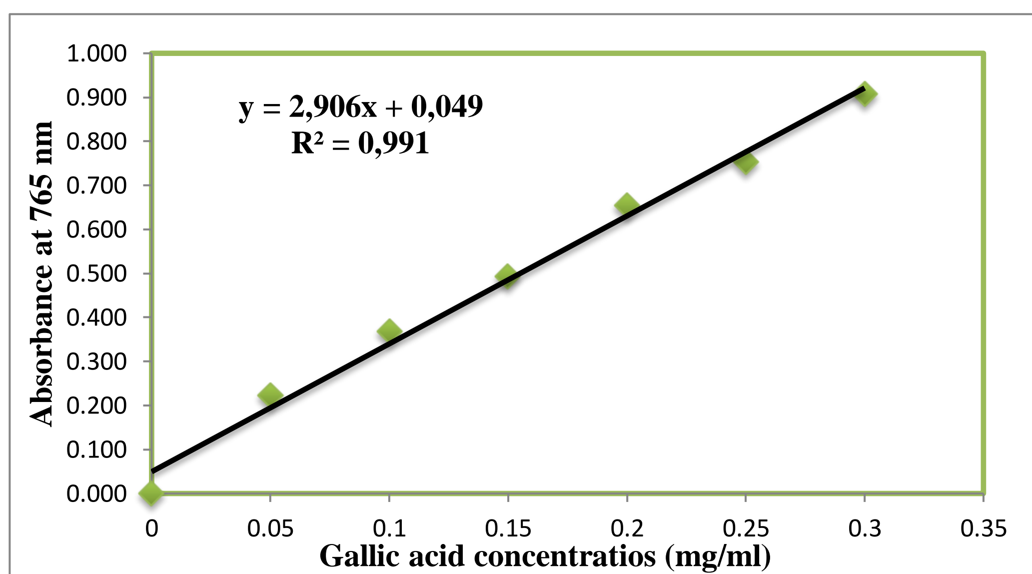


Figure11. Gallic acid calibration curve.

The polyphenol content of the aerial parts methanolic extract of *H. naudinianum* Coss. was 506.67 ± 5.56 mgGAE / g DM , this value showed that the methanolic extract was very rich in phenolic compounds which was a comparable to prervious studies on a *Ononis* L. (Fabaceae), Tawaha *et al*.(2007) and Mhamdi *et al*. (2014) revealed that the total phenol content of methanolic extract was 21.1 ± 0.7 mgGAE/g DW and 51 mgGAE/g DW, respectively.

3.2. Calibration curve for total flavonoids assay

This curve was established using quercetin as a reference and the results were expressed as mg quercetin equivalent per gram of dry matter (mg (QE) / g DM). The calibration curve was

established with a correlation coefficient R^2=0,980 (Figure 12).

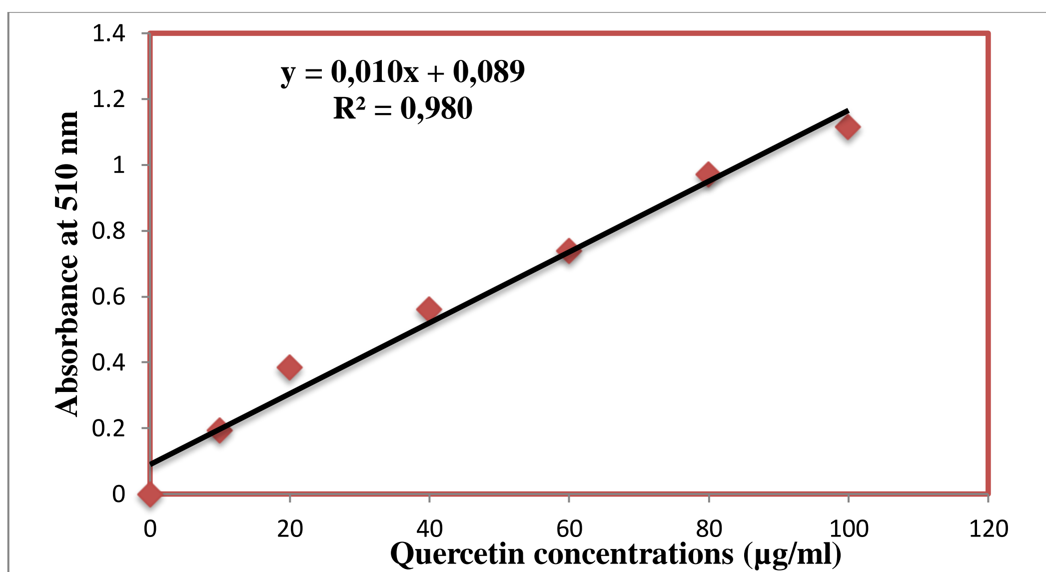


Figure 12. Quercetin calibration curve for the determination of flavonoids.

As shown in Table 5, the methanolic extract had the highest flavonoids content (255±13 mgQE/g DM), followed by the butanol extract (173 ±11 mg QE/g DM). Variations in total flavonoids content of the four extracts may be attributed to the polarities of compounds present (Sayari *et al*., 2016).

According to Uyar *et al*. (2017), butanol extract of the Turkish *H.aucheri* was found to be the richest in flavonoid (66.3 ±1.3 mg QE/g DM) followed by methanol extract (42.6±1.4 mg QE/g DM).

Table 5. Flavonoids content of the extracts of the aerial part of *H.naudinianum* Coss.

Extract	Flavonid content (mgQE/g DM)
Methanolic extract	255±13
N-butanol extract	173 ±11
Ethyl acetat extract	91± 11
Aqueous extract	53.5 ±5.5

The phenolic contents of a plant depend on a number of intrinsic (genetic) and extrinsic (environmental, handling and storage) factors (Falleh *et al* ., 2008), the main reason why content

of flavonoids must be determined lies in the fact that they constitute the class most important phenolic with more than 5,000 compounds already described (G´omez-Caravaca *et al*., 2006).

Chapter 05

Synthesis of scientific articles

Because the world health situation, The experimental part could not be continued and the evaluation of biological activities (antioxidant and antimicrobial) could not be released, for this reason , a review chapter was added as supplemented.

1. Introduction

Hedysarum, a genus of the Fabaceae (leguminosae) family, has around 300 annual and perennial species, these species are distinguishable by different morphology, mating systems, biological cycles and geographical origin, they are also widely used as medicinal plants (Baatout *et al*., 1990; Uyar *et al*., 2017). The phytochemistry and pharmacology of *Hedysarum* species have attracted increasing worldwide attention among those involved in the research and development of new drugs (Dong *et al*., 2013).

There are various species among the genus *Hedysarum*, which have been shown to have different pharmacological actions. This study was intended to summarize the biological activities that have been investigated in plants of genus *Hedysarum*.

2. Results and Discussion

Biological activities studied in some species of genus *Hedysarum* were summarized in table 6.

Table 6. Biological activities of the plants in genus *Hedysarum*.

Plant species	Geographical origin	Parts used	Biological activities	Source reference
Hedysarum polybotrys Hand.-Mazz	China	The roots	Anti-tumor	(Li *et al*., 2008)
Hedysarum Carnosum Desf.	Tunisia (Sousse)	The flowers	Antioxidant	(Ben salah *et al*., 2015)
	Tunisia (Kalbia Sebkha)	The seeds		(Hafsi *et al*., 2016; Hafsi *et al* ,2017)
Hedysarum alpinum	Mongolia	The whole herb	Antimicrobial	(Gonchig *et al*., 2008)
Hedysarum inundatum				
Hedysarum aucheri Boiss	Turkey	The whole herb	Antioxidant, anti microbial, cytotoxic	(Uyar *et al*., 2017)
Hedysarum caucasicum Bieb.	Russia	The grass (aerial parts)	Antimicrobial	(Serebryana *et al*., 2020)

2.1. Biological activities

2.1.1.Antioxidant activity

Antioxidant activities from different solvent extracts (Water, polyphenol ,methanol and n-butanol) of both *H.carnosum* and *H.aucheri* were determined by different coloremetric methods; scavenging 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals, Cupric-Ion-Reducing Antioxidant Capacity(CUPRAC), ferric reducing power (FRAP) and L'acide 2,2'-azino-bis(3-éthylbenzothiazoline-6-sulphonique) (ABTS) radical Scavenging assay, results are presented in table 7.

Table 7. Antioxidant activity of plants in genus *Hedysarum*

<table>
<tr><th>Species</th><th>extracts</th><th>DPPH(IC_{50}) mg /ml</th><th>CUPRAC
mg/ml</th><th>ABTS
µg/ml</th><th>FRAP
mg/ml</th><th>Source reference</th></tr>
<tr><td rowspan="3">H.carnosum Desf.</td><td>Water</td><td>2</td><td>NT</td><td>NT</td><td>NT</td><td>(Hafsi et al., 2016)</td></tr>
<tr><td>Polyphenol</td><td>2</td><td>NT</td><td>NT</td><td>NT</td><td>(Hafsi et al., 2017)</td></tr>
<tr><td rowspan="2">Butanol</td><td>20.35</td><td>NT</td><td>19.03</td><td>70.60</td><td>(Ben salah et al., 2016)</td></tr>
<tr><td rowspan="2">H. aucheri</td><td>NT</td><td>0.823</td><td>52</td><td rowspan="2">NT</td><td rowspan="2">(Uyar et al., 2017)</td></tr>
<tr><td>Water</td><td>NT</td><td>0.325</td><td>22</td></tr>
</table>

NT: non tested

As shown in table 7, n-butanol extract from Tunisian (Sousse) *H.carnosum* exhibited a significant activity for DPPH test compared to water and polyphenolic extracts (Kalbia Sebkha, Tunisia) with IC_{50} value 2 and 20 mg/ml , respectively. This variation may be due to the different area. (Ben salah *et al.*, 2016; Hafsi *et al.*, 2016; Hafsi *et al* ., 2017).

Turkich *H.aucheri* butanolic extract showed higher activity with 0.823 mg/ml for CUPRAC assay than water extract 0.325 mg/ml. Variation may be due to the solvents polarities (Djeridane *et al.,*2006).

For ABTS assay, Tunisian *H.carnosum* n-butanol fraction showed a lower antioxidant activity than Turkish *H.aucheri* with 19.03 and 52 µg/ml**,** respectively (Ben salah *et al.*, 2016;Uyar *et al.*, 2017). This assay was especially related to the phenolic composition and significant correlation could be found between their total phenolic-flavonoid contents and antioxidant activities. It has been reported in literature that the results of total phenol and

flavonoid contents of some plant species had a correlation with other in vitro antioxidant activity method(DPPH). Fitriansyah & Fidrianny (2017) revealed that total phenolic content in *Sesbania sesban* leaves extract had significant and negative correlation with DPPH method (r = -0.943, p< 0.01). In addition, a high correlation was found between different antioxidant assays and total phenol content of *Onobrychis* species extracts (R2 > 0.55 to 0.98; 6,25±0,28 to 10,38±0,33 mgGAE/g DM, respectively) (Karamian & Asadbegy,2016).

For FRAP assay, Tunisian *H.carnosum* n-butanol extract showed a remarkable antioxidant activity with 70.60 mg/ml (Ben salah *et al*., 2016).

2.1.2. Antimicrobial Activity

The antimicrobial activities of plant extracts (Ethanol, methanol, n-butanol, hexane, DCM and water) were determined by means of the disc diffusion method (the diameters of inhibition zones were measured in mm, against microbial strains , The results are given in Table 8

Almost all of these plant extracts were active against gram positive bactertia *Staphylococcus aureus.* The inhibition zones were varied from (8 to 15 mm.). Moreover, antibacterial activity was observed approximately in order of *H. caucasicum* extact > *H. alpinum* extract > *H. aucheri* extracts.

H.aucheri and *H.caucasicum* displayed an important antibacterial activity against gram negative bacteria *E.coli* compared to previous study (Gonchig *et al*., 2008; Uyar *et al*., 2017; Serebryanaya *et al*., 2020). Besides, the Turkish *H.aucheri* exhibited an antibacterial activity (8 to10 mm) superior to the Russian *H.caucasicum* (8mm) (Uyar *et al*., 2017; Serebryanaya *et al* ., 2020).

H. caucasicum , *H.alpinum* and *H.aucheri* were active against both gram negative and positive bacteria but they have shown most stronger antibacterial activities against gram positive (Gonchig *et al*., 2008; Uyar *et al* ., 2017; Serebryanaya *et al*., 2020) .

Gonchig *et al*. (2008) asserted that *H.inundatum* exhibited an antibacterial activity only against gram negative bacteria compared to *H.alpinum* , although, both of species are from to the same region.

Regarding the antifungal activity, *H.aucheri* showed more active against a yeast *C.albicans* (Uyar *et al*., 2017).

Table 8. Antimicrobial activity of plants in genus *Hedysarum*

Method				Disc diffusion method (Inhibition zone in mm)									
Species				***Hedysarum caucasicum* Bieb.**	***Hedysarum inundatum***	***Hedysarum alpinum***	***Hedysarum aucheri* Boiss**						
Extracts				Ethanol (80%)			MeOH	BuOH	Hexan	H2O	DCM	CF	NYS
Microorganisms	**Gram Negaive**	*Ec*	ATCC	–	na	na	8	10	9	8	8	14	–
			675	8	–	–	–	–	–	–	–	–	–
			0157H7	–	–	–	8	10	9	8	8	13	–
		Sal.t		12	–	–	10	10	10	8	8	14	–
		Sh.s 3d		18	–	–	–	–	–	–	–	–	–
		P.a		–	8,4	10,5	–	–	–	–	–	–	–
	Gram Positive	*E.f*		–	na	na	9	8	10	9	8	17	–
		S.e	ATCC	–	–	–	8	8	10	8	8	12	–
			Wood46	16	–	–	–	–	–	–	–	–	–
		S.a		15	na	13,3	10	10	10	8	8	20	–
		E.fm		–	–	–	8	8	10	8	8	15	_
		M.l		–	na	na	–	–	–	–	–	–	–
		B.s		10	–	–	–	–	–	–	–	–	–
		B.a		10	–	–	–	–	–	–	–	–	–
		S.c		14	–	–	–	–	–	–	–	–	–
	yeast	*C.a*		–	–	–	10	11	10	10	8	_	18
Source reference				(Serebryanayan *et al.*, 2020)	(Gonchig *et al.*, 2008)		(Uyar *et al.*, 2017)						

Microorganisms: ***E .c***, *Escherichia coli* ; ***S.a***, *Staphylococcus aureus* ; ***E.f*** ,*Enterococcus faecalis;* ***S.e***, *Staphylococcus epidermidis* ; ***Sal.t***, *Salmonella thyphimurium* ; ***E.fm***, *Enterococcus faecium* ; ***P.a***, *Pseudomonas aeruginosa* ; ***M.l***, *Micrococcus luteus;* ***B.s***, *Bacillus subtilis* ; ***B.a***, *Bacillus anthracoides*; ***S.c***, *Staphylococcus ceureus* ; ***Sh.s 3d***, *Shigella sonnei 3d;* ***C.a***, *Candida albicans.*

References: CF, Ceftazidime (30 µg/disc); **NYS,** Nystatin (20 µg/disc).

BuOH, Butanol extract; **MeOH,** Methanol extract; **H2O,** Aqueous extract; **DCM,** dichloromethane extract.

na: not active (inhibition zone was less than 0.1 mm)/ _ : not tested / **nd:** not detected .

2.1.3. Anti-tumor Activity

According to Li *et al.*(2008), the anti-tumor activities of a water-soluble polysaccharide HPS-1isolated from the roots of *H.polybotrys* Hand.-Mazz against two kinds of human solid cancer cell lines; human gastric cancer MGC-803 and human hepatocellular carcinoma HEP-G2 in vitro were determined by 3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) assay. The results showed that the HPS-1 presented significant anti-tumor activities in vitro especially in human HEP-G2 cells (Figure13).

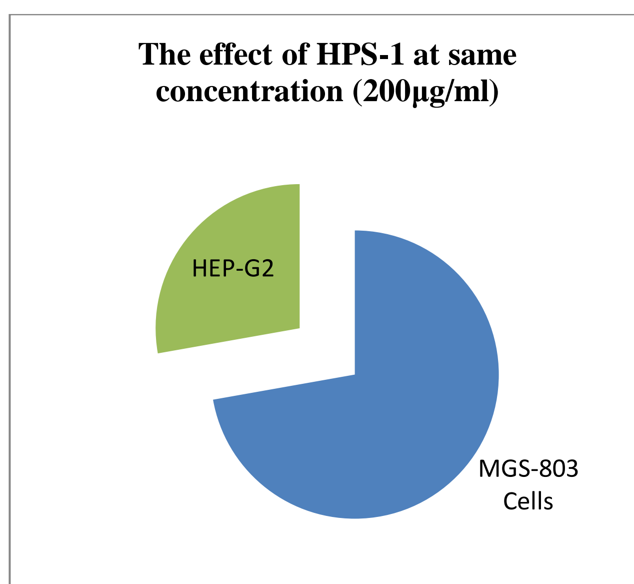


Figure 13. Comparaison between the effects of HPS-1 against two kinds of human cells

2.1.4. Cytotoxic Activity

Cytotoxic activity of Turkish *H.aucheri* extracts (MeOH, BuOH, Hexane ,H2O and DCM) were determined by MTT assay against a panel of human cancer ;Cancer coli-2 (CaCo-2) , Michigan Cancer Foundation-7 (MCF-7) , Uppsala 87 Malignant Glioma (U-87MG) , Human Lung Carcinoma Epithelial Cells (A549) , Henrietta's cancer cells (HeLa) , prostate cancer cell 3 (PC3) and normal cell lines; Human embryonic kidney 293 (HEK293) (Uyar *et al.*, 2017), results are presented in Figure 14.

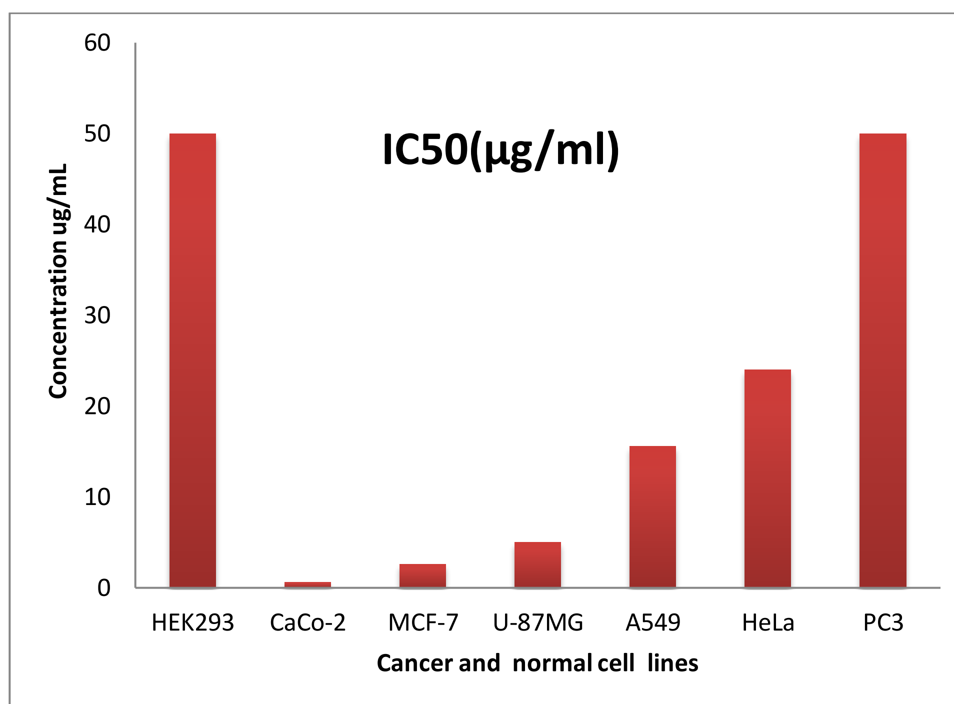


Figure 8. IC_{50} values for DCM extract in cancer and normal cell lines

DCM extract showed remarkable cytotoxic activity against all the tested cancer cells. The morphological changes of cancer cells were observed all over the post-treatment with DCM extract for 48 h exposure. DCM extract showed exceptional cell growth inhibition on cancer cells and had better IC_{50} values, followed by A549 and HeLa cells with IC_{50} values 15.6 and 24 mg/ml, respectively. Both PC3 and HEK293 cell lines exhibited an IC_{50} values above 50 mg/ml. (Uyar *et al*., 2017)

The percentage of cell viability of the tested cell lines following the exposure to DCM extract at 0.5µg/ml concentrations are given in Figure15.

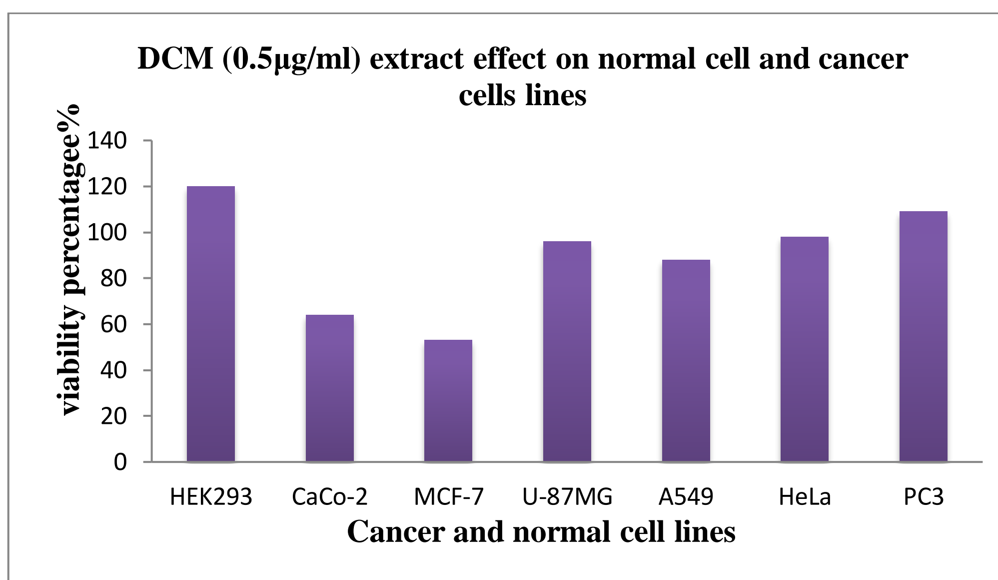


Figure 9. DCM (0,5µg/Ml) extracts effect on normal cell and cancer Cell lines

Based on these results, DCM extract exhibited a cytotoxicity against all tested cancer cells in a dose-dependent manner. CaCo-2, MCF-7, and U87MG were the most sensitive cells against the DCM extract, respectively while A549 and HeLa cells had relatively higher viability. Even though viability of U87MG, A549, and HeLa cells was high at low concentrations, their viability dropped substantially at higher concentrations. On the other hand, healthy cells HEK293 were quite resistant to the DCM extract exposure and no considerable cell death (Uyar *et al* ., 2017).

As results, it is notable to say that *H.aucheri* is probably one of the most promising members of genus *Hedysarum* (Uyar *et al* ., 2017).

3. Conclusion

The importance of *Hedysarum* biological activities that has been investigated directed toward the improvement of new and competent production of *Hedysarum*'s compounds .This review reflected the various uses potential of *Hedysarum* species.

Conclusion

The present study aimed to prepare four extracts (methanolic, ethyl acetate, *1*-butanol and aqueous) from *Hedysarum naudinianum* Coss. areal parts, to determinate phytochemical composition and to evaluate its biological activities.

Due to the current conditions of the new virus covid 19 in the world, it was not possible to complete the practical side of this work. To remedy this healthy situation , an additional chapter was included and the biological activities were continued as review paper.

The results of phytochemical screening confirmed the richness of *H. naudinianum* Coss. in phenolic compounds (catechic tannins and flavonoids). The total phenols and total flavonoids contents of *H.naudiniaun* extracts have been determined using the Folin–Ciocalteu colorimetric method and aluminum chloride colorimetric assay, respectively. Methanolic extract (70%) was found to be the richest in flavonoid and phenolic compounds.

Based on the synthesis of research that has already done, the genus *Hedysarum* has shown that it expresses many biological activities (antioxidant, antimicrobial, anti-tumor and cytotoxic).

There are still many perspectives on this subject:

- To evaluate the antioxidant activities (DPPH, FRAP, ABTS, CUPRAC and β-carotene bleaching methods),
- To determine the antimicrobial potential (CMI and CMB) of *H.naudinianum* Coss.
- Other alternative uses characterize Sulla. It is a special resource for bees and allows high quality honey production.

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Appendices

Appendix 01: Characterization reagents

Starch

Starch is characterized by a specific reagent known as starch. The latter was prepared as follows:

- Dissolve 1.2 g of iodine in 50 ml of distilled water containing 2.5 g of potassium iodide;
- Heat for 5 minutes;
- Dilute to 500 ml.

Starch detection is carried out as follows:

- Heat 5 ml of the test solution with 10 ml of saturated NaCl solution in a water bath until it boils;
- Add the starch reagent.

A positive test is revealed by the appearance of a blue-purplish color

Alkaloids

The characterization of alkaloids is done by:

• **Mayer's reagent:** the preparation of this reagent is carried out as follows:

- Dissolve 1.358 g of HgCl2 in 60 ml of water;
- Dissolve 5 g of KI in 10 ml of water;
- Mix the two solutions then adjust the total volume to 100 ml of water.

The alkaloids with this reagent give a white precipitate

• **Wagner's reagent:** This reagent was prepared as follows:

- Dissolve 2 g of KI and 1.27 of I2 in 75 ml of water;
- Adjust the total volume to 100 ml of water.

The alkaloids with this reagent give a brown precipitate.

Appendix 02

Articles's references

Chapter 04: Results and discussion

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Abstracts

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Abstracts

The present study was designed to determinate the photochemical composition and evaluate antimicrobial and antioxidant activities of *Hedysarum naudinianum* extracts. Photochemical screening revealed the presence of flavonoid, catechic tannins and reducing compounds in the extracts of aerial parts of *H. naudinianum.* The dry aerial parts of *H. naudinianum* was extracted with methanol and ethanol, and then the latter was fractioned with ethyl acetate, n-butanol, and water. Four fractions (methanol, ethyl acetate, n-butanol, and water) were examined for the total phenolic and flavonoid contents. Methanolic extract was found to be the richest in flavonoids (255±13 mg QE/g DM) and phenolic (506.67 ± 5.56 mg EAG/ g DM) compounds. Furthermore, biological activities (antioxidant, anti-microbial,anti-tumor and cytotoxic) of Hedysarum species were summarized as a short chapter synthesis.

Keywords: *Hedysarum naudinianum Coss*. Photochemical screening, anti-microbial, antioxidant, flavonoids, total phenols, biological activities synthesis.

Resumé

La présente étude a été conçue pour déterminer la composition phytochimique et évaluer les activités antimicrobiennes et antioxydantes des extraits de *Hedysarum naudinianum Coss.* Le phytochimique a révélé la présence de flavonoïdes, de tanins catéchiques et de composés réducteurs dans les extraits des parties aériennes de *H. naudinianum.* Les parties aériennes sèches de *H. naudinianum* ont été extraites avec du methanol et de l'éthanol, puis ce dernier a été fractionné avec de l'acétate d'éthyle, n-butanol et de l'eau. Quatre fractions (méthanol, acétate d'éthyle, n-butanol et eau) ont été examinées pour la teneur totale en phénol et en flavonoïdes. L'extrait méthanolique s'est avéré d'être le plus riche en flavonoïdes (255 ± 13 mgEQ / g MS) et en composés phénoliques (506,67 ± 5,56 mgEGA / g MS). De plus, les activités biologiques (antioxydantes, antimicrobiennes, antitumorales et cytotoxiques) des espèces Hedysarum ont été résumées dans un court chapitre de synthèse.

Mots clés: *Hedysarum naudinianum*, screening phytochimique, antimicrobien, antioxydant, flavonoïdes, phénols totaux, synthèse d'activités biologiques

ملخص

صممت الدراسة الحالية لتحديد التركيب الكيميائي النباتي وتقييم الأنشطة المضادة للميكروبات ومضادات الأكسدة لمستخلصات *Hedysarum naudinianum Coss*. كشفت الفحص الكيميائي النباتي عن وجود مركبات الفلافونويد ، والمركبات العفصية ، والمركبات المختزلة في مستخلصات الأجزاء الهوائية من *H. naudinianum*. تم استخلاص الأجزاء الهوائية الجافة من *H. naudinianum* بالميثانول والإيثانول ، ثم تم تجزئة الأخير باستخدام أسيتات الإيثيل ، بيوتانول والماء تم فحص أربعة أجزاء (ميثانول ، أسيتات إيثيل ، ن- بيوتانول وماء) لمعرفة المحتوى الكلي للفينول والفلافونويد. تم العثور على أن الجزء الميثانولي هو الأغنى بمركبات الفلافونويد (255 ± 13 مجم مكافئ / غرام DM) والمركبات الفينولية (506.67 ± 5.56 مجم / جم DM). بالإضافة ، تم تلخيص الأنشطة البيولوجية (مضادات الأكسدة ، مضادات الميكروبات ، مضادات الأورام والسمية الخلوية) لأنواع Hedysarum في فصل تجميعي قصير

الكلمات المفتاحية: *Hedysarum naudinianum Coss*. الفحص الكيميائي النباتي ، مضادات الميكروبات ، مضادات الأكسدة ، مركبات