Preliminary investigation of *Herniaria incana* Lam.

Determination of the total flavonoid content, antioxidant properties and free radical scavenging capacity

Master Thesis in Pharmacy

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Summary

In traditional medicine, herbs with antioxidant properties have been used to treat various conditions. Promising results of antioxidant capacity, may suggest that further investigations might lead to development of new prophylactic and therapeutic drugs for a numerous of oxidative diseases.

The aim of this master thesis is to evaluate some of the antioxidant properties of *Herniaria incana* Lam.. This is the first time the plant is being investigated with respect to potential antioxidant effect, but several of its close relatives have been used in traditional medicine in treatment of various diseases.

The total flavonoid content and the antioxidant capacity of the methanolic extract of *H. incana*, were determined using UV-VIS spectroscopy by the aluminum nitrate method, the Folin-Ciocalteu reducing capacity assay, the phosphomolybdenum assay and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, respectively. The results are presented as the mean and the standard deviation of the parellels performed.

The total flavonoid content was estimated to be 20.46 ± 0.07 microgram quercetin equivalents per milligram of dry weight plant extract. The Folin-Ciocalteu reducing capacity was estimated to be 35.0 ± 1.6 microgram gallic acid equivalents per milligram of the dry weight plant extract. The phosphomolybdenum reducing capacity was estimated to be 0.84 ± 0.25 millimol α-tocopherol equivalents per gram of the dry weight plant extract, or 362 ± 106 microgram α-tocopherol equivalents per milligram of the dry weight plant extract. The DPPH free radical scavenging capacity was presented as the concentration of reductant necessary to decrease the initial DPPH concentration by fifty percent. (EC_{50}). The EC_{50} of *H. incana* was determined to 71.1 ± 8.6 μg/mL, compared to that of 6.6 ± 1.0 μg/mL for ascorbic acid.

These preliminary investigations, showed that *H. incana* has a moderate content of flavonoids and a moderate antioxidant capacity. Since these are the first results of this kind for *H. incana*, they represent a contribution to better understanding of its chemical composition, pointing the direction for future studies.

Abbreviations

AC	Antioxidant capacity
ATE	α-tocopherol equivalents
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
DPPH-A	The DPPH free scavenging assay
Dw	Dry weight
FCA	The Folin-Ciocalteu reagent assay
FCRC	The Folin-Ciocalteu reducing capacity
FDA	Food and Drug Administration
GAE	Gallic acid equivalents
HAT	Hydrogen atom transfer
HIV	Human immunodeficiency virus
Mo	Molybdenum
PMA	The phosphomolybdenum assay
PMRC	Phosphomolybdenum reducing capacity
QUE	Quercetin equivalents
R^2	The coefficient of determination
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Standard deviation
SET	Single electron transfer
TFC	Total flavonoid content
TPC	Total phenolic content
UV-VIS	Ultraviolet–visible
W%	Weight percentage

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1 Introduction

1.1 The aim of the master thesis

In the last years, there have been great focus on the possible health benefits of natural substances with antioxidant properties. This has resulted in an enormous increase of research on the antioxidant capacity of different plant extracts.

In traditional medicine, herbs with antioxidant properties have been used to treat various conditions. Promising results of antioxidant capacity may suggest that further investigations might lead to development of new prophylactic and therapeutic drugs for a numerous of oxidative diseases, such as cancer and cardiovascular diseases [1, 2].

Species of *Herniaria* from the plant family Caryophyllaceae, have been used in traditional medicine in treatment of various diseases such as hypertension and kidney stones [3, 4]. The work in this master thesis is a preliminary investigation of *Herniaria incana* Lam., as a part of a larger project aimed at the study of its chemical composition. The aim of this master thesis was to evaluate some of the antioxidant properties of this plant species. To the best of our knowledge, this is the first time the plant has been investigated with respect to potential antioxidant effect.

There are no published data on the chemical composition of *H. incana*. However, other *Herniaria* species have been found to contain flavonoids and saponins [5, 6, 7]. Flavonoids are a class of secondary metabolites known for their ability to scavenge free radicals and their strong antioxidant activity [8]. Hence, an assay determining the flavonoid content of *H. incana* has been performed.

Antioxidants can act by many mechanisms. The reducing capacity is an important property of antioxidants and it is easy to test it *in vitro*. Three different assays investigating the reducing capacity of the plant extract have been performed; the Folin-Ciocalteu assay, the phosphomolybdenum assay and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay. These assays are regularly used in studies investigating antioxidant properties of plant extracts [9, 10, 11].

Saponins are a group of secondary metabolites that have shown various biological activity such as antimicrobial activity and anticancer activity [12]. Isolation and structure elucidation of saponins in *H. incana* will also be subject to the investigation of the plant in the larger project.

1.2 Natural substances as a source of development of new drugs

Drugs of natural origin have been classified as ”original natural products, products derived semisynthetically from natural products, or synthetic products based on natural product models [13]”.

For thousands of years natural products have been used in traditional medicine and as natural poisons [14]. Chemical investigations of such natural products have lead to the discovery of important drugs such as aspirin, digitoxin, morphine, quinine and pilocarpine [14]. The discovery of penicillin revolutionized the drug discovery research [14]. Pharmaceutical companies started culturing microorganisms in the search for new antibiotics, resulting in drugs such as streptomycin, chloramphenicol, chlortetracycline, cephalosporin C, erythromycin and vancomycin [14].

From the early 1980s, it was believed that the future of drug development was in combinatorial chemistry technology, causing the formation of libraries containing hundreds of thousands to millions of new compounds. This resulted in a decreased interest in the research of natural products in the pharmaceutical industry [15].

The idea was that screening of these compounds would give new lead drugs simply by the weight of number [14]. However, it was noticed that these synthetic compounds lacked “the complexity normally associated with bioactive natural products, items such as multiple chiral centers, heterocyclic substituents, and polycyclic structures“ [15]. Although combinatorial chemistry has offered a great advantage in the drug development, the technology has been more useful in making moderations to lead compounds, than making derivatives from scratch [15]. In fact, only one *de novo* combinatorial compound has been approved as drug between 1981 an 2007, the kinase inhibitor Sorafenib, approved by the Food and Drug Administration (FDA) in 2005 for renal carcinoma [15].

Cragg *et. al.* mentioned in 1997 that “only 5-15 % of the approximately 250 000 species of higher plants have been systematically investigated for the presence of bioactive compounds, while the potential of the marine environment has barely been tapped” [13]. Fifteen years later the number is certainly higher, but still there is an unknown potential of compounds waiting to be discovered. Newman and Cragg have gone through all new approved agents from 1981 to 2006. Of 155 drugs used in cancer therapy from 1940s to 2007, 73 % are drugs of natural origin and 47 % being natural products or directly derived from a natural product. [16]. This demonstrates that natural compounds, or compounds derived from natural products, still play an important part in discovery and development of new drugs[14].

1.3 Antioxidants and free radicals

An antioxidant is defined as "a molecule capable of slowing or preventing the oxidation of other molecules" [17], or as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [17].

A simple search for “antioxidant” or “antioxidants” on ISI Web of Science (09.05.2012), gives 33 683 hits for the timespan 1945-2000 and 111 392 hits for the timespan 2000-2012. This demonstrates the enormous increase in antioxidant research over the last decade.

A free radical is a higly reactive molecule or chemical species that contains one or more unpaired electron [18]. These unpaired electrons make the radical very reactive and cause oxidative stress, defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage” [19].

Oxidation can harm various biological systems by damaging lipids, proteins, enzymes, carbohydrates and deoxyribonucleic acid (DNA). This can result in membrane damage, fragmentation or random cross linking of molecules and even lead to cell death induced by DNA fragmentation and lipid peroxidation [19]. A relationship is reported between oxidative damages and various diseases, such as cardiovascular diseases, cancer, liver diseases, neurodegenerative disorders, aging, autoimmune diseases, diabetes and atherosclerosis [1, 2].

Antioxidants are believed to be efficient prophylactic and therapeutic agents in many oxidative diseases [19]. Several secondary metabolites from plants are proven to be good antioxidants, e.g. phenolic acids, flavonoids and carotenoids [1] (see section 1.4).

Antioxidants have several mechanisms of action [20]:

- inhibition by generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS)
- scavenging effect
- reducing capacity
- metal chelating capacity
- activity as antioxidative enzymes
- inhibition of oxidative enzymes

The mechanism the antioxidant act by may be dependent on the reaction system, and the radicals or oxidant sources involved [17].

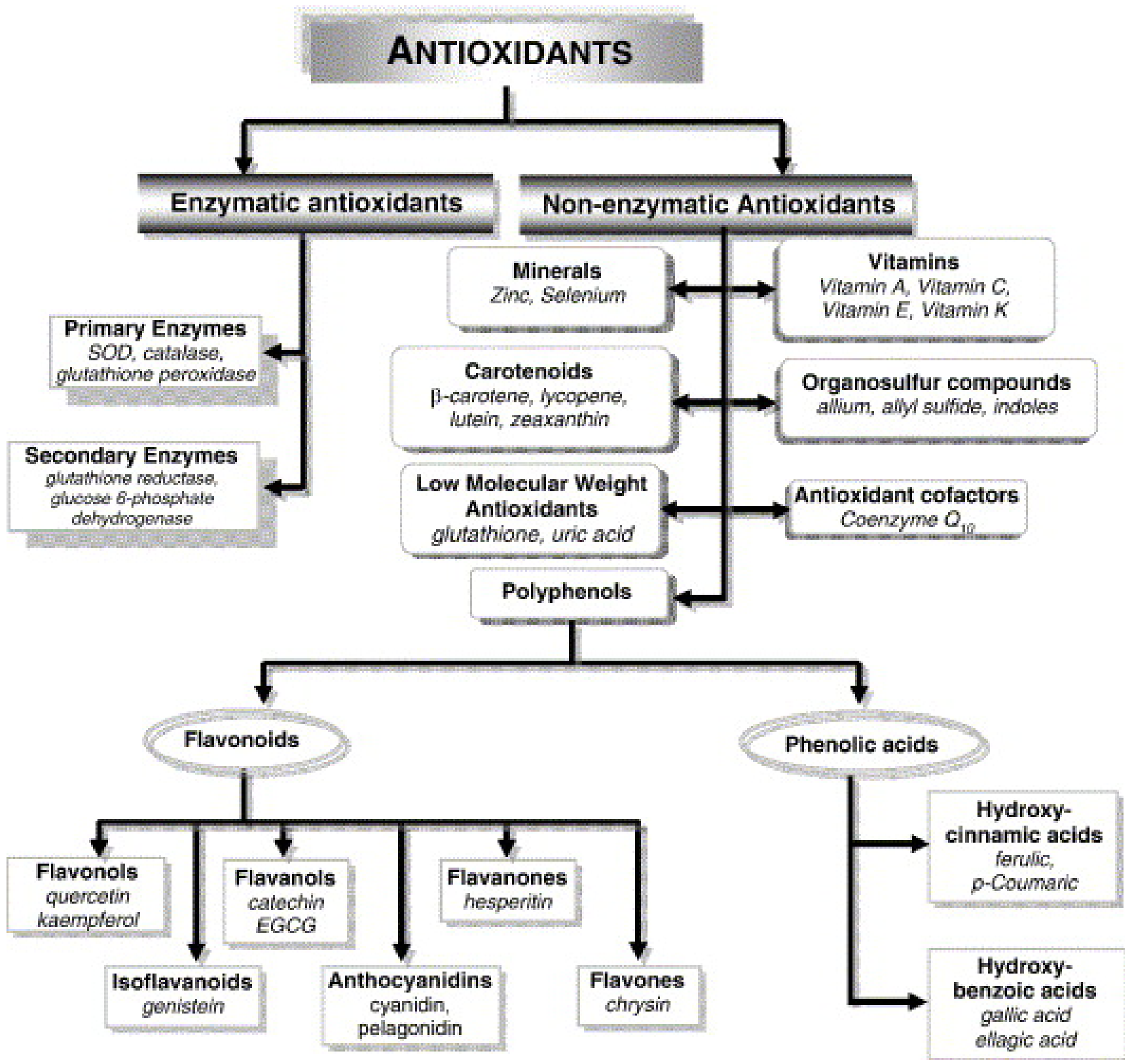


Figure 1: *Classification of antioxidants as enzymatic and non-enzymatic [19].*

There are two main classes of antioxidants, enzymatic and non-enzymatic, as shown in figure 1 [19]. Examples of enzymatic antioxidants are superoxide dismutase, gluthathione peroxidase and catalase [19, 17]. Examples of non-enzymatic are minerals, vitamins, carotenoids, organosulphur compounds, low molecular weight antioxidants, antioxidant cofactors and polyphenols. Vitamins, carotenoids, flavonoids and phenolic acids are examples of types of dietary antioxidants [19].

The humane antioxidant defense is equipped with enzymatic, hydrophilic and lipophilic radical scavengers. Some of these agents are produced by the body itself, but most of them derives from dietary sources. In medical conditions caused by oxidative stress, the balance between oxidants and antioxidants are off, and the humane antioxidant defense

against ROS are weakened [19].

1.4 Secondary metabolites with antioxidant properties

Most of the plant constituents used medicinally are secondary metabolites, meaning that they are "substances formed in plants, but not participants of the metabolic processes which are necessary for the life and development of the plant" [21]. The composition of secondary metabolites in plants is dependent on various factors , among others on the soil properties, time for harvesting, and climate [22]. Typical classes of compounds known for their antioxidant potential are isoprenoids and polyphenols [23].

Isoprenoids is a term used for groups of compounds derived from isoprene (2-metyl-1,3-butadien) [24]. Examples of different types of isoprenoids are the terpenes (mono, sesqui, di, and triterpenes), carotenoids and steroids. The most powerful antioxidants of the isoprenoids are the carotenoids and the abietane diterpenes. Isoprenoids are generally more lipophilic than polypehnols. Carotenoids, like β-carotene and lycopene, are known for their chemopreventive activities, probably contributed by an antioxidant cascade [23].

Polyphenols in plants are a structural group of secondary metabolites, containing aromatic functional groups. They are derived from either the shikimic acid pathway, or from the polymalonate pathway, or by a combination of these. Phenolic compounds have been found to exhibit a variety of bioactivities, such as anti-allergic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects [25]. Known groups of polyphenols are xanthones, stilbenes, anthraquinones, flavonoids, lignans, neolignans, lignins and condensed tannins [25]. The flavonoids are an important type of polyphenols that are known as great antioxidants [24]. The flavonoids are more closely discussed in section 1.5.

Other compounds that are known for their antioxidant properties are tocopherols and vitamin C. Tocopherols are powerful free radical scavengers. Vitamin C is essential and needs to be in the diet of humans [19].

1.5 Flavonoids

Flavonoids are a large group of polyphenolic secondary metabolites found in ferns and higher plants. They occur both in free state and as glycosides. Most flavonoids are yellow

and they are present as dissolved glycosides in the cell sap of flowers, contributing to the colours. A group of flavonoids called anthocyanidines contribute to red, blue or purple colours in flowers, depending on the pH of the cell sap. The colours are important in pollination for attracting seed dispersers, and in feeding on plants. Some flavonoids have a very bitter taste, which repels caterpillars from feeding on the plant [24, 26] .

Fruits and vegetables are a giant source for human dietary flavonoids. Flavonoids are known for their good antioxidant capacity, and they are belived to be of great health benefits to humans. At least four thousands different compounds of flavonoids have been isolated, and it is the largest group of naturally occuring phenols [24]. There are several subclasses of flavonoids; chalcones, flavones, isoflavonoids, aurones, anthocyanidines, flavanones and flavonols [24, 26].

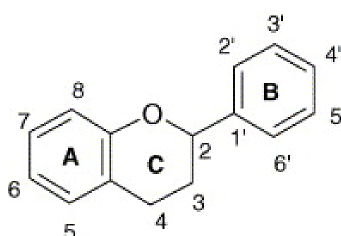


Figure 2: *Generic structure of a flavonoid [25]*

1.5.1 Biosynthesis and structure

The basic structure of flavonoids are shown in figure 2, and constitutes of two aromatic rings connected by a 3-carbon chain. In plants, aromatic rings can be formed by two pathways; the shikimic acid pathway and the acylpoymalonate pathway. Flavonoids are biosynthesized in a combination of these two pathways. A simple overview of the biosynthesis is shown in figure 3. First the amino acid phenylalanine derived from shikimic acid is used as a precursor to yield p-coumaric acid via cinnamic acid. P-coumaric acid acts as a starting compound in a polyketide synthesis, and is the source for the first aromatic ring. In the next steps three malonyl-CoA molecules are used to get three acetate residues, which are incorporated into the structure. This is followed by ring closure, giving the second aromatic ring. Further ring closure involving the 3-carbon chain and this hydroxyl group can form a 6-membered or a 5-membered ring. Subsequent hydroxylation, reductions and methylations leads to the different classes of flavonoids shown in figure 4. The addition of the sugar residues is probably one of the last reactions in the biosynthesis. The different compounds are separated by their substituents and the position of the sugar residues [27, 24].

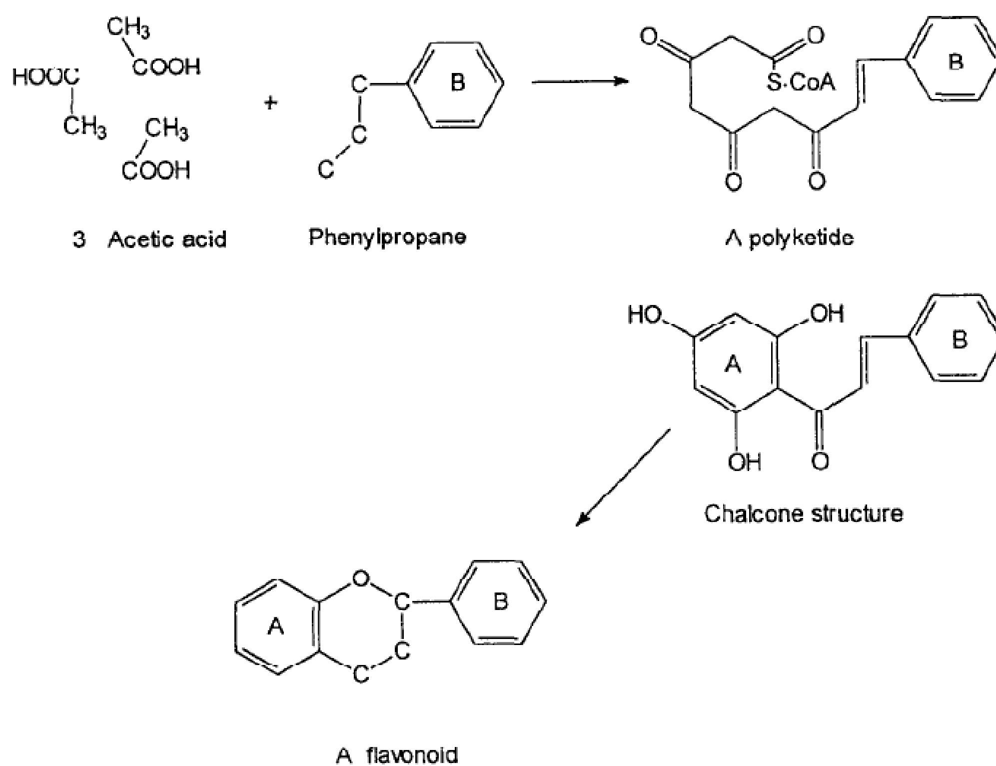


Figure 3: *The biosynthesis of flavonoids. Three acetate residues are incorporated into the polyketide starting compound, giving rise to the second aromatic ring [27].*

1.5.2 Biological and pharmacological activity

The pharmacological activity of flavonoids were first described by Szent-Gyorgyi in 1938, as an ability to prevent capillary bleeding and fragility associated with scurvy. The effect became referred to as the vitamin P activity, however the effect is not regarded as entirely proven[24].

Later flavonoids have been reported having many pharmacological effects among others anti-inflammatory, antihepatotoxic, antitumor, antimicrobial, antiviral, enzyme inhibiting, antioxidant activity, and central vascular system effect [24]. They have also been investigated for their inhibitory activity against human immunodeficiency virus protease ((HIV)-1 protease). Flavonols, and especially quercetin, was found to be the most potent inhibitors of HIV-1 protease [5]. However, most of the investigations are performed *in vitro*, and several of the activities are yet to be proven in *in vivo* assays [24].

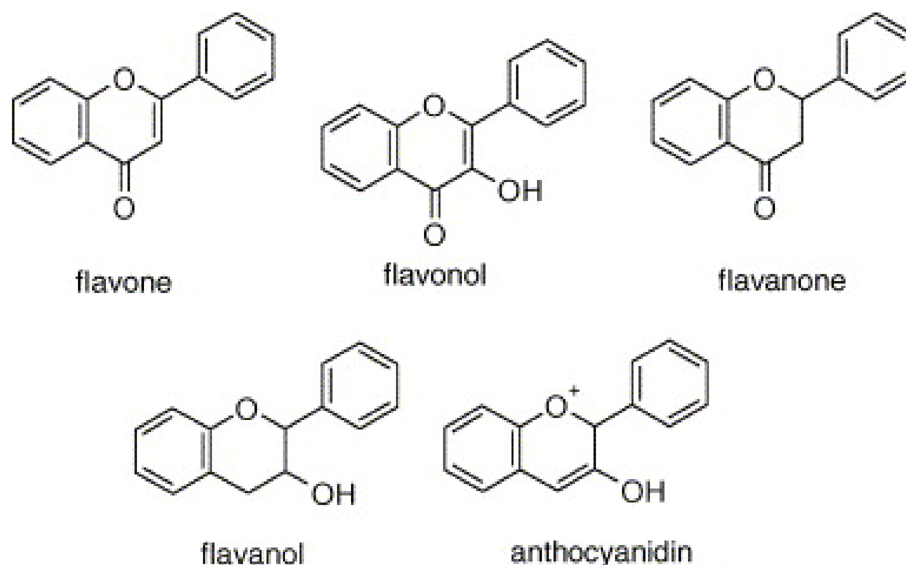


Figure 4: *Generic structure of the major classes of flavonoids [25]*

Epidemiological studies show that flavonoids have beneficial effect in protection against cardiovascular diseases. However, there is no correlation found between intake of flavonoids and a reduced risk of cancer [28, 24]. "The French paradox", the fact that despite of similarity in cholesterol levels and fat intake, the French have much lower incidence of coronary heart disease than the Americans, is believed to be related to the French's high intake of red wine. Red wine is a good source of flavonoids, like quercetin and myricetin [24].

There are several difficulties involved in the study of dietary flavonoids. They undergo structural changes in the gastrointestinal tract, which makes it difficult to extrapolate results from *in vitro* studies to *in vivo*. This makes it very important to distinguish between experiments concerned with intake of flavonoids through the orally route and experiments concerned with other routes. The food itself may contain compounds interacting with flavonoids. The effect of flavonoids seems to be mainly protective, thus it is harder to study than effective treatment. Flavonoids have several effects, but it seems that there is no effect for which they are solely responsible. The metabolism and pharmacokinetics of flavonoids in humans are not very well understood, and depletion studies are difficult to perform, since it is hard to compose a diet free of flavonoids [24].

1.6 *Herniaria incana* Lam.

Herniaria incana Lam. (figure 5(a)) is a flowering seed plant belonging to the *Herniaria* genus in the Caryophyllaceae family, also known as the pink family [29]. It is commonly

known as gray rupturewort. The duration of gray rupturewort is perennial, which means that it will grow for more than two years [29]. An overview of the classification of *H. incana* is shown in table 1.

Species of *Herniaria* are found all over the world. Some of the more known species are *Herniaria glabra* and *Herniaria hirsuta*, commonly known as smooth and hairy rupturewort, respectively [30]. Traditionally, extracts from *Herniaria* species were believed to be an efficient remedy for hernia, hence the genus name [31].

"Substances, herbs or preparations are classified as drugs in Norway if they have a property that allows them to actually prevent, treat or relieve disease, disease symptoms or pain or affect physiological functions in humans or animals" [32]. One of *H. incana*'s close relatives, *Herniaria glabra* (figure 5(b)), is classified as a drug in the Norwegian list of herbs under the Norwegian name "brokkurt" [32].

Table 1: *Classification of Herniaria incana Lam. [29].*

Category	**Scientific name**	**Common name**
Kingdom	*Plantae*	Plants
Subkingdom	*Tracheobionta*	Vascular plants
Superdivision	*Spermatophyta*	Seed plants
Division	*Magnoliophyta*	Flowering plants
Class	*Magnoliopsida*	Dicotyledons
Subclass	*Caryophyllidae*	-
Order	*Caryophyllales*	-
Family	*Caryophyllaceae*	Pink family
Genus	*Herniaria* L.	Rupturewort
Species	*Herniaria incana* Lam.	Gray rupturewort

1.6.1 Traditional use

Although, there is no published data of the therapeutic use of *H. incana*, several other closely related species, have been used in traditional medicine for treatment of various conditions. Aerial parts of *Herniaria fontanesii* have been used in Moroccan traditional medicine in the treatment of lithiasis and as a diuretic [6]. *H. hirsuta* has traditionally been used in the treatment of kidney stones [3]. *H. glabra* has been used as an astringent, diuretic and expectorant [33, 7], and aerial parts have been used in the treatment for hypertension [4].

(a) *Herniaria incana Photo: Enzo De Santis [34]*

(b) *Herniaria glabra. Photo: Biopix.dk: JC Schou [35]*

Figure 5: *The plants (a) Herniaria incana, commonly known as gray rupturewort, and its close relative (b) Herniaria glabra, commonly known as smooth rupturewort*

1.6.2 Chemical composition

The chemical composition of *H. incana* is not known, although other species of *Herniaria* have been found to contain flavonoids and saponins. Species from the *Herniaria* genus are reported to contain polyphenolics. Stefova *et. al.* investigated *Herniariae* herba (the aerial parts of the plant [22]) and found the flavonoid quercetin and an unidentified flavonol [5]. Several papers have investigated the saponin content in *Herniaria* species, and many new saponins have been isolated and identified [6, 7].

1.6.3 Bioactivity

In vivo experiments have shown a diuretic and antihypertensive effect of *H. glabra* [33]. A study on the effect of aqueous extract from *H. hirsuta* L. on experimentally nephrolithiasic rats show beneficial effect, and confirms the benefit of using the plant as a preventive agent against the formation of calcium oxalate kidney stones [3]. A variety of other medicinal uses of *H. glabra* has also been reported, as the treatment of dropsy, catarrh of the bladder, cystitis, kidney stones, gout, hernias, jaundice, nerve inflammation, respiratory disorders, removing excess of mucus in the stomach, to increase the flow of urine, and in treatment of digestive and renal cancer [33].

1.7 Determination of the antioxidant capacity

In order to evaluate a plant extract‘s potential, it’s common to determine the antioxidant capacity. The term antioxidant capacity expresses the ability of an antioxidant to scavenge free radicals [36]. Several terms that describe the antioxidant capacity are being used, e.g. activity, efficiency, power, parameter, potential and potency. Some assays report the results as the total antioxidant capacity. However, the antioxidant capacity measured in a single assay can only reflect those specific chemical conditions in that assay, and it would be erroneous to generalize the data as indicators of total antioxidant capacity[2].

No single assay available provides all necessary information. To get the best possible impression of the overall antioxidant capacity, several assays should be applied investigating different aspects of the antioxidants properties [17]. It must be emphasized that the results from the *in vitro* assays based on chemical reactions, in no way can be understood to express the *in vivo* activity. These assays do not measure bioactivity, *in vivo* stability, retention of antioxidants by tissues and reactivity *in situ* [2].

Antioxidants deactivate free radicals mainly by two mechanisms; hydrogen atom transfer (HAT) or single electron transfer (SET) [17]

1.7.1 HAT-based assays

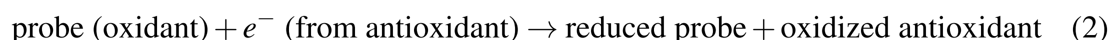
HAT-based assays measure the ability of an antioxidant to quench free radicals by hydrogen donation [17]. Equation (1) show how the the antioxidant (AH) terminates the free radical ($X\cdot$) by the transfer of a hydrogen atom [17].

$$X \cdot + AH \rightarrow XH + A\cdot \qquad (1)$$

HAT reactions are usually quite rapid (gone to completion within seconds or minutes), and they are dependent on solvent and pH. The presence of reducing agents (including metals) influences the reaction and can lead to erroneously high apparent reactivity [17]. The quantification is usually based on kinetic curves [2].

1.7.2 SET-based assays

SET-based assays measure the ability of an antioxidant to "transfer one electron to reduce any compound, including metals, carbonyls and radicals" [17]. Equation (2) shows that the antioxidant losses a electron to the probe (oxidant), with the result that the antioxidant is oxidized and the probe is reduced [2].



$$\text{probe (oxidant)} + e^{-} \text{ (from antioxidant)} \rightarrow \text{reduced probe} + \text{oxidized antioxidant} \qquad (2)$$

The reactions are usually slow and can require a long time to complete [17]. Trace compounds and contaminants, like metal ions, may interfere with the results. The indicator of the endpoint of the reaction is the redox reaction with the oxidant.

1.7.3 Comparison of HAT- and SET-based assays

In both HAT- and SET-based assays, the radical (or oxidant) scavenging capacity is measured, instead of the preventative antioxidant capacity [2].

The hydrogen transfer in HAT-based assay is an important step in the free radical chain reaction, suggesting that the HAT-assays are more relevant for measuring chain-breaking antioxidant capacity. In SET-based assays the reducing capacity of the antioxidant is measured, which is not directly related to its scavenging effect. However, the reducing capacity is also an important property of an antioxidant [2].

The antioxidant capacity assays in this thesis are mainly SET-based.

2 Materials

2.1 Plant material

The plant material used in this investigation origins from the location Gabrovačko Brdo (Niš, Serbia, see figure 6), and was harvested and identified in June 2009 and May 2010 by Bojan Zlatković (University of Niš, Department of Biology and Ecology, Niš, Serbia). A voucher specimen was deposited in the Herbarium collection at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš under the acquisition number 6581.

The plant was dried for ten days at a shadowed place and then stored in a dark place protected from light. The soil and roots were removed and the aerial parts [1] were used for further extractions.

Figure 6: *A map of Niš, Serbia: The origin of the plant material [38]*

[1]The parts of the plant above the ground [37].

2.2 List of chemicals

Substance	**Formula**	**Purity**	**Supplier**
α-tocopherol	$C_{29}H_{50}O_2$	$\geq$ 96% (HPLC)	Sigma
Aluminum nitrate nonahydrate	$Al(NO_3)_3 \cdot 9H_2O$	$\geq$ 98%	Sigma-Aldrich
Ammonium molybdate	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	USP	Sigma-Aldrich
Ascorbic acid	$C_6H_8O_6$	$>$ 99.5%	Fluka AG
1,1-diphenyl-2-picryl-hydrazyl	$C_{18}H_{12}N_5O_6$	90%	Aldrich
Ethanol	CH_3CH_2OH	$>$ 99.8%	Sigma-Aldrich
Folin-Ciocalteu reagent	-	-	Sigma-Aldrich
Gallic acid	$C_7H_6O_5$	98,00%	Acros Organics
n-Hexane	C_6H_{14}	HPLC	VWR Prolabo
Methanol	CH_3OH	HPLC	VWR Prolabo
Potassium acetate	CH_3COOK	90%	Chemi-Teknik
Quercetin	$C_{15}H_{10}O_7$	$\geq$ 98% (HPLC)	Sigma
Sodium phosphate	Na_3PO_4	96%	Aldrich
Sulfuric acid	H_2SO_4	95-97%	Merck KgaA

2.3 List of laboratory equipment

Equipment	**Specification**	**Supplier**
Automatic pipettes	50-200 μL, 200-1000 μL, 1-10 mL,	Finnpipettes
Disposible UV-cells	2.5 mL makro	Brand
Disposible UV-cells	1.5 mL semi-micro	Brand
Heating bath	B-490	BUCHI
Heath plate / Magnetic stirrer	MR 3001 K	Heidolph
Rotary evaporator	Rotavapor RII	BUCHI
Rotary evaporator	Rotavapor R-200	BUCHI
Thermometer	EKT 3001	Heidolph
Ultrasonic cleaner	3 L	VWR International
UV-VIS spectrophotometer	double-beam	Cecil (CE9500) 9000 Series
UV-VIS spectrophotometer	single-beam	Shimadzu UV Mini-1240
Vacuum controller	CVC2	Vaccubrand
Vacuum controller	V-850	BUCHI
Vacuum pump	V-700	BUCHI
Weight, analytical	BL210 S	Sartorius
Weight	BL610	Sartorius

3 Methods

3.1 Ultraviolet–visible spectroscopy

The different assays performed in this thesis are all based on Ultraviolet–visible (UV-VIS) spectrophotometry.

UV-VIS spectrophotometry is based on the principle that when monochromatic electromagnetic radiation is sendt trough a solution of an analyte, parts of the radiation will be absorbed by the analyte, while the rest passes through. By detecting the intensity of the radiation before and after it has passed through the sample, the absorbance of the analyte can be measured [39]:

$$A = log\frac{I_0}{I} \tag{3}$$

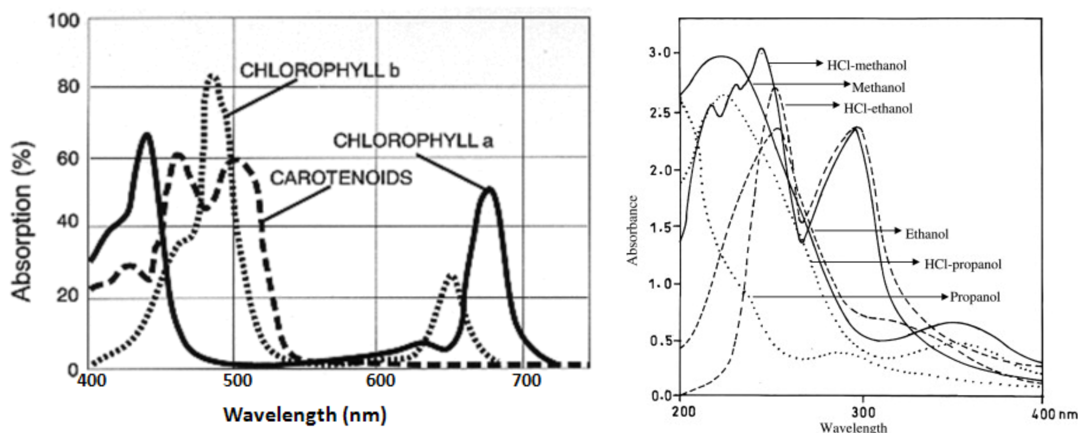
Where A is the absorbance, I_0 is the intensity of the radiation before absorption and I is the intensity of the radiation after absorption. The absorbance depends on the wavelength of the radiation, the solvent used, the pH of the sample and the temperature [39].

The Beer-Lambert law describes the relationship between the absorbance and the concentration of the sample [40]:

$$A = \varepsilon lc \tag{4}$$

According to Beer-Lambert's law, the absorbance (A) is proportional to the pathlenght of the sample cell (l), and the concentration of the sample (c) [39]. The molar absorptivity (ε) is the characteristic of a substance that describes how much light that is absorbed at a particular wavelength [40]. The Beer-Lambert law is applicable to dilute solutions with a concentration below 0.01 M [40]. A higher concentration may affect the charge distribution of the molecules and their ability to absorb radiation. This could result in a deviation from Beer-Lamberts law [39].

Substances that absorb radiation in the same wavelength region as the test chemical, may interfere with the absorbance [1]. The solvent must not absorb at the wavelength of measurement [39]. The wavelengths of measurement in this thesis are 415, 517, 695 and 760 nm. The solvents used (distilled water, methanol and ethanol), do not absorb radiation above 210 nm [39]. Most polyphenols exhibit two major absorption bands in



(a) *Absorption spectrum of chlorophyll and carotenoids [41]* (b) *Absorption spectrum of polyphenols in finger mellet [42]*

Figure 7: *The asbortion spectrums for (a) chlorophyll a,b and carotenoids, and (b) polyphenols from finger mellet.*

the UV-VIS region, one band in the 320-385 nm range and the other band in the 250-285 nm range [43]. An example of the absorption spectrum of polyphenols can be seen in figure 7(b). None of the wavelengths used in the assays coincides with the solvents or the polyphenolic absorption bands. Chlorophyll and carotenoids strongly absorbs light in both the 400 nm and the 600 nm region [44], and may influence the absorbance of samples with plant extract. An example of the absorption spectrum of chlorophyl and carotenoids can be seen in figure 7(a). Removal of chlorophyll from the plant extract has therefore been considered as necessary.

It is important that the standards, the samples and the references are prepared in the same way, so that the solvent, pH and possible presence of other substances, are the same for the standard and the sample [39, 45]. "The standard curve should be set up in the absorbance range 0.2 - 0.8 because this range offers the greatest precision" [39].

3.2 Statistical analysis

All graphs are drawn in Gnuplot version 4.6, using the stats function for making the linear regression lines and estimating the coefficient of determination (R^2). The linear regression equation and the value of the coefficient of determination were found to be the same in Gnuplot and in Open Office version 3.2.0.

Open Office version 3.2.0 was used to find the probit and the standard deviation, using the functions NORMSINV and STDEV, respectively.

3.3 Extraction of *Herniaria incana*

Extraction with methanol is a commonly known method for extraction of natural substances [46, 47, 10, 48, 49, 50]. “The application of ultrasound to methanolic extraction gave a signicant reduction in extraction time and an increase in maximum yield” [46].

Since chlorophyll absorbs light at UV regions similar to the applied wavelengths in some of the assays (see section 3.1), removal was necessary. Chlorophyll, in addition to non-polar compounds as carotenoids, may be removed by dissolving the dry extract in distilled water and washing with n-hexane, diethyl ether or chloroform [12]. Attention must be payd, that the substances responsible for the antioxidant activity are not removed from the extract. Shui and Peng [51] found only a small loss of antioxidant activity when further extraction with hexane were performed, thus hexane was chosen to remove the chlorophyll from the plant extract.

Some secondary metabolites may be sensitive to light, hence the plant extract should be protected from light exposure.

3.3.1 Chemicals and equipment: ultrasound-assisted methanolic extraction

- n-Hexane
- Methanol
- *Herniaria incana*
- Aluminum foil
- Büchner funnel
- Filter paper (Schleicher and Schuell no. 589)
- Preweighed round-bottom flasks.
- Rotary evaporator
- Ultrasonic bath
- Vacuum pump

3.3.2 Procedure: ultrasound-assisted methanolic extraction

1. Accurately measure out 10.0 g of *Herniaria incana*, carefully broken into smaller pieces, and put it into a round-bottom flask.
2. Add 500 mL of methanol.
3. Sonificate the plant material on ultrasonic bath for 20 minutes.
4. Let stay for 12 hours covered with aluminum foil.
5. Have the plant material on ultrasonic bath for 20 minutes.
6. Pour out the methanolic plant extract and add new 500 mL of methanol to the plant residue.
7. Repeat the procedure five times.
8. Filter the methanolic plant extract twice under vacuum using an Büchner funnel and filterpaper no. 589 (Schleicher and Schuell).
9. Evaporate the extract til dryness under vacuum at 40 °C using a rotary evaporator.
10. Measure the weight and calculate the yield.
11. Keep the dry plant extract in refrigerator at 2.2 °C covered with aluminum foil until further use.

3.3.3 Chemicals and equipment: extraction with n-hexane

- Distilled water
- Methanolic extract of *Herniaria incana*
- n-Hexane
- Aluminum foil
- Beakers to collect the different phases in.
- Preweighed round-bottom flasks.
- Rotary evaporator
- Separating funnel, 250 mL

- Preweighed round-bottom flasks.

3.3.4 Procedure: extraction with n-hexane

1. Dissolve 0.25 or 1.4 g of the dry methanolic extract in 25 or 50 mL of distilled water, respectively, and transfer it into a separating funnel.
2. Add an equal amount of n-hexane (25 or 50 mL) and shake.
3. Let the mixture rest until the layers have separated (20 minutes).
4. Pour the hexane phase into a clean round-bottom flask and keep in the refrigerator covered by aluminum foil.
5. Repeat the procedure three times.
6. Evaporate the aqueous phase under vacuum at 40 °C using a rotary evaporator.
7. Add a few drops of hexane and evaporate until dryness.
8. Measure the weight and calculate the yield.
9. Keep the dry plant extract in refrigerator at 2.2 °C covered with aluminum foil until further use.

3.4 Estimation of the total flavonoid content

Flavonoids are known to scavenge free radicals and have strong antioxidant activity [8], hence it is interesting to investigate the total flavonoid content of the plant extract.

The total flavonoid content of the methanolic extract of *Herniaria incana* will be determined using a UV-VIS spectrophotometer, according to the method of Hsu [52].

When the plant extract is incubated with aluminum nitrate, aluminum nitrate forms an acid stable complex with the keto group and either the hydroxyl group in ring A or ring C of flavonoids (see figure 2 in section 1.5.1). It also forms acid unstable complexes with orthodihydroxyl groups in ring A or ring B. The complexes absorbs light strongly at 415 nm and can be detected on UV-VIS spectroscopy [53]. The absorbance of the complexes formed between the plant extract and aluminum nitrate are compared with that of a standard, often quercetin [52].

3.4.1 Chemicals and equipment

- Aluminum nitrate
- Ethanol, 80 %
- Extract of *Herniaria incana*
- Potassium acetate
- Quercetin
- 2 automatic pipette
- Disposable UV cells, 2.5 mL
- 2 graduated pipette, 5 mL
- 10 test tubes (5 mL) with cap
- UV-VIS spectrophotometer: Cecil (CE9500) 9000 Series
- 1 volumetric flask with cap, 500 mL
- 1 volumetric flask with cap, 100 mL
- 5 volumetric flasks with cap, 50 mL
- 5 volumetric flasks with cap, 10 mL

3.4.2 Procedure: The aluminum-nitrate method

1. Make the 80 % ethanol solution (450 mL 99.8 % ethanol + 50 mL distilled water).
2. Prepare the 10 % (10 g/ 100 mL) aluminum nitrate solution. Accurately measure out 1 g of aluminum nitrate and quantitatively transfer it to a 10 mL volumetric flask. Adjust the volume to 10 mL with 80 % ethanol. Shake.
3. Prepare the 1 M potassium acetate solution. Accurately measure out 981.4 mg of potassium acetate and quantitatively transfer it to a 10 mL volumetric flask. Adjust the volume to 10 mL with 80 % ethanol. Shake.
4. Prepare the standard solutions using quercetin dissolved in 80% ethanol as standard (2.5, 5, 10, 15, 20 μg/mL): accurately measure out 25 mg of quercetin. Quanti-

tatively transfer it to a 100 mL volumetric flask. Adjust the volume with 80% ethanol. Shake well.

5. Transfer 0.5, 1, 2, 3 and 4 mL, respectively, of this solution into five 50 mL volumetric flasks. Adjust the volume with 80% ethanol. Shake well.
6. Prepare the sample solutions. Measure out 5.0 mg, 7,5 mg and 10.0 mg of the extract and dissolve it in 80% ethanol to a total volume made up to 10.0 mL. Use the ultrasonic bath to dissolve.
7. Into 10 test tubes put 0.1 mL of 10 % aluminum nitrate, 0.1 mL of 1 M potassium acetate and 4.3 mL of 80 % ethanol.
8. Add an aliquot of 0.5 mL of the sample/standard/solvent (control) to the test tubes. Mix. Note the time.
9. Incubate at room temperature for 40 minutes.
10. Measure the absorbance of the supernatant at 415 nm. 80 % ethanol as blank.
11. Make a standard curve of quercetin by plotting the concentration of quercetin against the absorbance. Make a linear curve (Y = ax + b) by linear regression.
12. Calculate the total flavonoid content of the plant extract as μg quercetin equivalents per milligram dry weight (dw) plant extract, using the equation obtained from the standard curve.
13. Calculate the mean and standard deviation of the parallels.

3.5 The Folin-Ciocalteu reagent reducing capacity assay

The method was introduced by Singleton and Rossi in 1965 as a simple way to determine the total phenolic content of wine [54], and has been used to determine the total phenolics in natural products. However, the Folin-Ciocalteu reagent can be reduced by many non-phenolic compounds, e.g. vitamin C and Cu(I). Thus the term "total phenolic content" is not suitable, unless interfering species are considered or removed [20]. Several correlations between the Folin-Ciocalteu assay and other electron-transfer assays confirm the value of the Folin-Ciocalteu reagent for the assessment of antioxidant capacity [20].

The exact chemical nature of the Folin-Ciocalteu reagent is not known, but it is believed to contain complex polymeric ions formed from phosphomolybdic and phosphotungstic

heteropoly acids [54, 2]. The basic mechanism of the assay is a single electron transfer reaction between the reductants and molybdenum (Mo). As showed in equation (5), the electron from the antioxidant reduces molybdenum from Mo(VI) to Mo(V) [2]. This results in the formation of a blue complex absorbing light at 750-765 nm [20]. A linear correlation between the absorbance of the complex and concentration of the reducing species has been established [54].

$$Mo(VI) + e^- \rightarrow Mo(V) \tag{5}$$

The Folin-Ciocalteu reducing capacity will be estimated according to the procedure of Singleton *et. al.* [55] and gallic acid is used as standard. The results are expressed as microgram gallic acid equivalents (GAE) per milligram of dry plant extract.

3.5.1 Chemicals and equipment

- Distilled water
- Ethanol
- Extract of *Herniaria incana*
- Folin-Ciocalteu reagent
- Gallic acid
- Sodium carbonate anhydrous
- Aluminum foil
- 1 automatic pipette, 0.500 mL
- Disposable UV cells, 2.5 mL
- Measuring ship
- 2 pipettes, 5 mL
- Test tubes with caps.
- UV-VIS spectrophotometer: Cecil (CE9500) 9000 Series
- 3 volumetric flasks with cap, 10.0 mL

- 8 volumetric flasks with cap, 100 mL
- 1 volumetric flasks with cap, 50 mL

3.5.2 Procedure: the Folin-Ciocalteu reagent reducing capacity assay

1. Turn on the UV-apparatus.
2. Cover all volumetric flasks and test tubes with aluminum foil.
3. Prepare the sample solutions. Accurately measure up 0.1000 g of plant extract and quantitatively transfer it into a 10 mL volumetric flask. Adjust the volume to 10 mL using distilled water.
4. Prepare the 7.5% (7.5 g/100 mL) sodium carbonate solution. Accurately measure out 7.5 g of sodium carbonate anhydrous and quantitatively transfer it to a 100 mL volumetric flask. Adjust the volume with distilled water.
5. Make the Gallic Acid stock solution. Accurately measure out 0.050 g of dry gallic acid and dissolve it in 10 mL ethanol in a 100 mL volumetric flask. Adjust the volume to 100 mL with distilled water.
6. Prepare the standard solutions using gallic acid dissolved in distilled water (5, 10, 15, 25 and 50 (μg/mL)): quantitatively transfer 1, 2, 3, 5 and 10 mL of the gallic acid stock solution into five 100 mL volumetric flasks, respectively. Adjust the volume with distilled water. Shake well.
7. Dilute the Folin-Ciocalteu reagent. Using a graduated pipette measure up 10 mL of the Folin-Ciocalteu reagent and transfer it into a 100 mL volumetric flask. Adjust the volume to 100 mL with distilled water.
8. Using an automatic pipette transfer 0.5 mL of the samples into test tubes. One sample of each standard and three parallels of the plant extract.
9. Add 2.5 mL of diluted Folin-Ciocalteu reagent to the sample in the test tubes. Use a graduated pipette. Shake. Note the time. (Prepare a new one every 4 minute.)
10. After 2 minutes add 2 mL 7.5% sodium carbonate. Shake. Note the time.
11. Let stand for 2 hours in room temperature.
12. Measure the absorbance at 760 nm against the control (distilled water and reagent).

13. Make a graph by plotting absorbance against the concentrations of the standard curve. Find the equation. Determine the Folin-Ciocalteu reducing capacity as μg gallic acid equivalents of mg dw plant extract by using the standard curve.

14. Calculate the mean and standard deviation of the parallels.

3.6 The phosphomolybdenum reducing capacity assay

The antioxidant capacity of *Herniaria incana* is determined using the phosphomolybdenum assay as proposed by Prieto *et. al.* [56]. The method was first used to evaluate the antioxidant capacity of corn and soybean seeds, and have later been extended to evaluate the antioxidant capacity of plant extracts [49, 57, 58].

It is a spectrophotometric method based on the reduction of Mo(VI) to Mo(V) (SET-based) by the sample analyte followed by the formation of a green phosphate/Mo(V) complex at acidic pH. The absorbance maximum of the complex is measured at 695 nm [56]. The results are presented as mmol α-tocopherol equivalents per gram of dried plant extract [57].

3.6.1 Chemicals and equipment

- α-tocopherol
- Ammonium molybdate
- Concentrated sulfuric acid
- Extract of *Herniaria incana*
- Methanol
- Sodium phosphate
- 1 automatic pipette, 0.3 mL
- 1 automatic pipette, 3.0 mL
- Disposable UV cells: 2.5 mL
- Heath plate
- 9 vials with cap

- 9 test tubes with caps
- Thermometer
- UV-VIS spectrophotometer: Shimadzu UV Mini-1240
- 1 volumetric flasks with caps, 50 mL

3.6.2 Procedure: The phosphomolybdenum method

1. Make the reagent solution: 0.6 M sulfuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate tetrahydrate. Accurately measure up 1.6 mL of sulfuric acid, 0.2295 g of sodium phosphate and 0.2472 g of ammonium molybdate and quantitatively transfer it to a 50 mL volumetric flask. Adjust the volume with methanol. First measure up the sulfuric acid into a small cleaner beaker containing ca 12 mL of methanol. Use this to transfer the sodium phosphate and ammonium molybdate into the volumetric flask. Be careful and do not allow contact of the reagent or any other solutions with aluminum foil.
2. Prepare the sample solutions. Measure up 0.0500 g in a vial and dissolve it in 1 mL of methanol.
3. Prepare the standard solutions using α-tocopherol dissolved in methanol (150 mg/mL). Preweigh a vial with a cap containing 5 mL of methanol. Add an appropriate amount (0.7500 g) of α-tocopherol using a plastic spoon. Weigh the sample glass with cap again. Calculate the concentration. If necessary dilute the stock solution.
4. Transfer 100, 200, 400, 600, 800, 1000 and 1300 μL respectively, of this solution into vials. Make up the volume with methanol, so that the total volume is 5 mL. Shake well.
5. Put 3.0 mL of reagent solution in each of the 9 test tubes.
6. To the test tubes add 0.3 mL of sample (three parallels) / standard (one parallel) / methanol(control). Prepare a new one every 5 minutes.
7. Cap the tubes and incubate in a boiling water bath [9] at 95 °C for 90 minutes.
8. Cool the samples to room temperature (wait for fifty minutes). Measure the absorbance of the aqueous solution at 695 nm against the control.
9. Make a standard curve. y = absorbance and x = concentrations of α-tocopherol

in mmol/mL. Find the equation. Express the total antioxidant capacity as mmol equivalents of α-tocopherol per gram of dried plant extract.

10. Make a standard curve. y = absorbance and x = concentrations of α-tocopherol in μg/mL. Find the equation. Express the total antioxidant capacity as μg equivalents of α-tocopherol per milligram of dried plant extract.

11. Calculate the mean and standard deviation of the parallels.

3.7 The DPPH free radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay is one of the most popular antioxidant assays used by almost 90 % of antioxidant studies [1]. DPPH is a stable and commercially available organic nitric radical giving a purple solution in methanol [1]. The assay is based on an electron-transfer (SET) reaction, where DPPH is reduced by the sample to 2,2-diphenyl-1-picrylhydrazine, which gives a yellow solution [20]. The SET-based reaction is shown in figure 8.

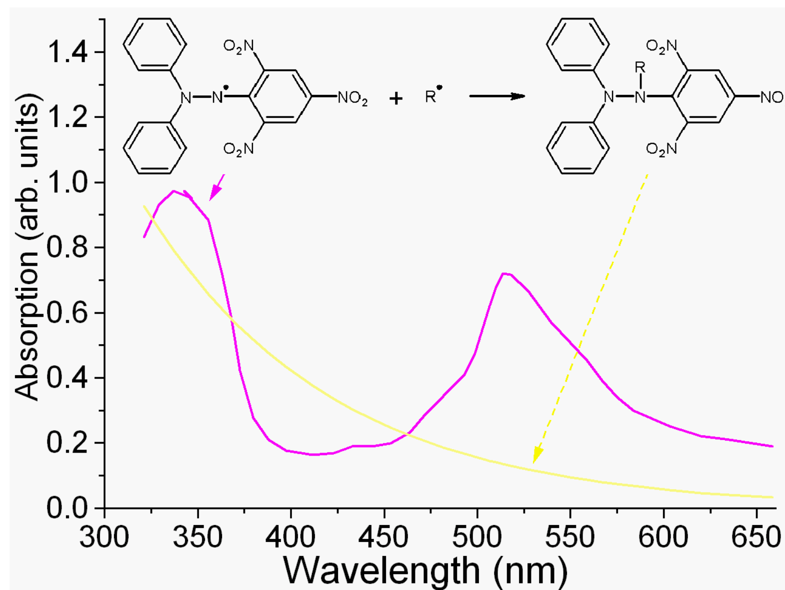


Figure 8: *The reduction of 2,2-diphenyl-1-picrylhydrazyl to 2,2-diphenyl-1-picrylhydrazine, and their respective absorption spectrum [59]*

The DPPH free radical scavenging capacity is determined using UV-VIS spectroscopy according to the method of Molyneux [60].

The purple DPPH solution can be detected using UV-VIS spectroscopy at 515-528 nm. By letting the DPPH react with the plant extract at different concentrations, the scav-

enging activity can be evaluated by measuring the decrease in the absorbance [20]. The scavenging activity is often represented as inhibition percentage (I%) [61]:

$$I\% = \frac{Abs_0 - Abs_1}{Abs_0} \tag{6}$$

Abs_0 = The absorbance of the negative control

Abs_1 = The absorbance of the sample/standard

Several factors may influence the results, such as solvent, pH, concentration of the reagent, concentration of the samples, reaction time and processing data [60, 61].

The antioxidant capacity is proportional to the decrease in absorbance. This relationship is not linear [1, 61]. The results are often presented as EC_{50}, the amount of antioxidant necessary to decrease the initial DPPH concentration by 50 % [62]. The EC_{50} value of the plant extract will be compared with a standard, in this case ascorbic acid. A lower value of EC_{50} indicates greater antioxidant activity [63].

When plotting the scavenging activity of the sample versus the concentration, an s-shaped curve is given, as showed in figure 9. In literature many ways for obtaining the EC_{50} are reported. Some articles report that the EC_{50} values are obtained from regression lines, without mentioning the regression model used [11]. One way to obtain the EC_{50} is to apply linear regression to the linear parts of the curve. Nikolova *et. al* calculated the EC_{50} by sigmoid non-linear regression model [64]. In an article by Locatelli *et. al.*, it is argued that the accuracy of the obtained EC_{50} value, achieved by linear regression from the linear parts of the plot, would be significantly affected [61]. A linear relation between the percentage of inhibition and sample concentration is confirmed only for a limited range of concentrations, and it is indicated that it would be erroneous to employ the linear regression for the calculation of EC_{50} over 70% [61]. Locatelli *et. al.* suggested to calculate the EC_{50} value by the Probit regression model, as it's was found to fit well to the antiradical activity curves. The Probit regression model has also been used in other studies evaluating the DPPH free radical scavenging assay [61].

Some of the main limits of the DPPH assay is the non-existence of DPPH or similar compounds in biological systems, and the steric accessibility of DPPH radicals. Smaller molecules have better access to the radical site of the DPPH molecule and therefore may show higher antioxidant activity than larger molecules [20].

As mentioned earlier, chlorophyll and other substances, as carotenoids, may absorb light at the wavelength of measurement and interfere with the results. This may be avoided to

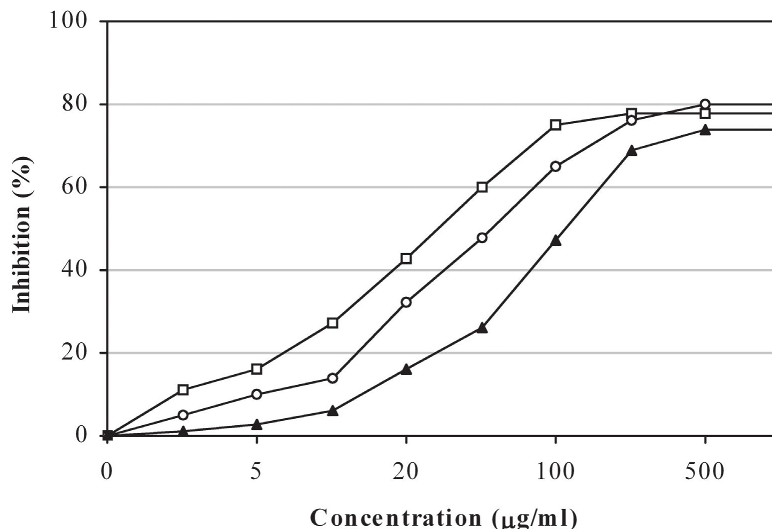


Figure 9: *An example of the s-shaped curve given when the DPPH scavenging activity is plotted against the concentration of the samples [65]*

a certain degree by using the plant sample without reagent as a blank, which is normal procedure in the DPPH assay.

The DPPH solution decomposes with time and should be used within two hours after preparation. The effect of temperature is more dramatic than the effect of time, so it is better to keep for a short period of time at room temperature, than refrigerating the solution [62]. DPPH is light sensitive, and the samples should be protected from strong light [66].

3.7.1 Chemicals and equipment

- Ascorbic Acid
- DPPH
- Extract of *Herniaria incana*
- Methanol
- Aluminum foil
- Appropriate automatic pipettes with tips
- Card board box
- Disposable UV cuvettes 1.5 mL

- Magnet
- Magnetic stirrer
- 9 vials with cap
- 18 test tubes with caps
- UV-VIS spectrophotometer: Shimadzu UV Mini-1240
- 2 volumetric flasks with caps, 100 mL

3.7.2 Procedure: the DPPH free radical scavenging assay

1. Turn on the single beam UV apparatus one hour before use.
2. Mark a clean test tube with Ca. This will be the blank to the negative control.
3. Mark a clean vial with Cb. This will be the negative control.
4. Mark eight clean test tubes with 1a, 2a, 3a, 4a, 5a, 6a, 7a and 8a. These will be containing the blanks to the samples.
5. Mark eight clean vials with 1b, 2b, 3b, 4b, 5b, 6b, 7b and 8a. These will be containing the samples.
6. Make the stock solution of a) the standard (ascorbic acid) (0.25 mg/mL) or b) Plant sample (*Herniaria incana* (0.80 mg/mL).

   a) Accurately measure out 0.0100 g of ascorbic acid and quantitatively transfer it into a 100 mL volumetric flask. Make up the volume with Methanol.

   b) Accurately measure out 0.090 g of dry methanolic extract of *Herniaria incana* and dissolve it in 6 mL of methanol.
7. Make standard solutions of a) ascorbic acid or b) plant extract. Eight different concentrations of the stock solution. Pre-tests with various concentrations were performed in order to find the concentration range which corresponds to the linear dependence of absorption versus concentration.

   a) Ascorbic acid (2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0 (μg/mL)): Transfer 0.100, 0.200, 0.300, 0.400, 0.500, 0.600, 0.700 and 0.800 mL of the stock solution into eight test tubes using an automatic pipette. Make the total volume up to 5 mL by

adding 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3 and 4.2 mL of methanol, respectively, using an automatic pipette.

b) Plant extract (60, 87, 113, 141, 164, 188, 211, 233 (μg/mL)): Transfer 0.200, 0.300, 0.400, 0.500, 0.600, 0.700, 0.800 and 0.900 mL of the stock solution and add 5 mL of methanol using an automatic pipette.

8. Using an automatic pipette transfer 2 mL of methanol into the vials marked with a and Cb and at least 4 mL methanol into Ca.

9. Using an automatic pipette transfer 2 mL of standard solution into the corresponding vials. Example: 2 mL of standard solution S1 into 1a and 1b. 2 mL of standard solution S2 into 2a and 2b.osv...

10. Make the DPPH solution (0.1 mM). Accurately measure out 0.0039 g of DPPH and quantitatively transfer it into a 100 mL volumetric flask. Shake it well. Dissolve using a magnetic stirrer. Cover it with aluminum foil for protection against light.

11. With 4 minutes interval add 2 mL of DPPH solution to all sample glass marked with b. Start with Cb. Shake well (20 shakes) and put it in the card board box.

12. Let stand for 30 minutes in the dark (card board box), before measuring the absorbance at 517 nm against it's own blank. Example: Autozero the spectrophotometer with Ca, 1a, 2a before measuring the absorbance of Cb, 1b, 2b...

13. Calculate the scavenging activity [62]:

$$I = \frac{Abs_0 - Abs_1}{Abs_0} \tag{7}$$

14. Calculate the Probit of the scavenging activity. Make a graph by plotting log of the concentrations against the Probit of the scavenging activity.

15. Make a linear regression line and generate an equation Y = aX + b. From this equation calculate EC_{50}. The Probit of 50 % scavenging is zero, thus the EC_{50} is calculated as:

$$EC_{50} = 10^{\frac{-b}{a}} \tag{8}$$

16. Calculate the mean and standard deviation of the parallels.

4 Results

4.1 Extraction of *Herniaria incana*

Figure 10 shows the extract after extraction with methanol, before it is evaporated to dryness. The methanolic extraction of *H. incana* had a deep green colour. The first extract looks like a paste and is difficult to get completely dry. After extraction with hexane and removal of oily compounds, the extract looks more like a powder.

Figure 10: *The methanolic extract of Herniaria incana had a dark green colour. Photo: S.M. Parmer*

Table 2 shows yield of the extractions. Extract A and B origins from 2009, while extract C origins from 2010. Extract A was used to determine the total flavonoid content (TFC), extract B was used in the Folin-Ciocalteu reducing capacity assay (FCA), while extract C was used in the phosphomolybdenum assay (PMA) and in the DPPH free radical scavenging assay (DPPH-A).

Table 2: *Weight percent yield from the extraction of Herniaria incana*

Extraction	**Harvested**	**Yield 1 (w%)**	**Yield 2 (w%)**	**Total yield (w%)**	**Used in:**
A	08.06.2009	14.85	82.87	12.31	TFC
B	08.06.2009	14.85	93.87	13.94	FCA
C	18.05.2010	14.47	83.81	12.13	DPPH-A, PMA

Yield 1 is the yield of extract from the crude drug after methanolic extraction, yield 2 is the yield of extract from the first extract after extraction with hexane, while the total yield represents the yield after extraction with hexane in relation to the crude drug. The total yield of extractions A, B and C is 12.31, 13.94 and 12.13 w% respectively.

See appendix D.1 for more detailed data.

4.2 Estimation of the total flavonoid content

The total flavonoid content was determined by the aluminum nitrate method with UV-VIS spectrophotometry according to the method of [52]. Figure 11 shows the standard curve of quercetin, with absorbance of the aluminum-nitrate complex plotted on the y-axis and the concentration of quercetin in microgram per milliliter on the X-axis. The equation f(x) = 0.0604x-0.0027 (R^2 = 0.9973) was obtained by linear regression. From this equation the total flavonoid content (TFC) of the plant extract was calculated as micrograms of quercetin equivalents (QUE) per milligram of the dry plant extract.

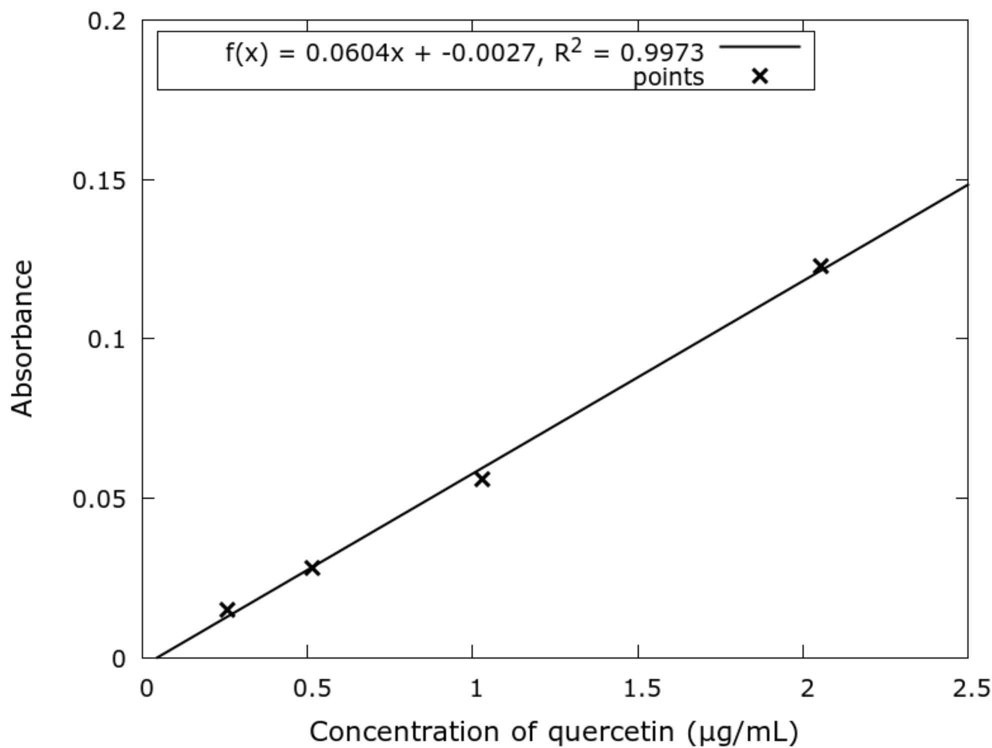


Figure 11: *The standard curve of quercetin used to determine the total flavonoid content of Herniaria incana*

Table 3 show the concentration, the absorbance, the QUE calculated from the equation and the TFC of the plant samples. The results are presented as mean ± standard deviation (SD) of three parallels. The total flavonoid content of *H. incana* was estimated to be 20.46 ± 0.07 microgram quercetin equivalents per milligram of the dry weight extract. For example of calculation see appendix E.1.

See appendix D.2 for more detailed data.

Table 3: *The estimation of the total flavonoid content of Herniaria incana*

Sample	Concentration (mg/mL)	Absorbance	QUE (μg/mL)	TFC (μg QUE/mg)
1	0.024	0.027	0.49172	20.48841
2	0.055	0.065	1.12086	20.37929
3	0.070	0.084	1.43543	20.50615
Mean	-	-	-	20.46
SD	-	-	-	0.07

4.3 The Folin-Ciocalteu reagent reducing capacity assay

The Folin-Ciocalteu reagent reducing capacity (FCRC) was determined with UV-VIS spectrophotometry using the method of Singleton *et. al* [55]. Figure 12 shows the standard curve of gallic acid, with absorbance of the reaction complex plotted on the Y-axis and the concentration of gallic acid in microgram per milliliter on the X-axis. The equation f(x) = 0.1171+0.0500 (R^2 = 0.9970) was obtained by linear regression. From this equation the Folin-Ciocalteu reagent reducing capacity of the plant extract was calculated as micrograms of gallic acid equivalents (GAE) per milligram of the dry plant extract.

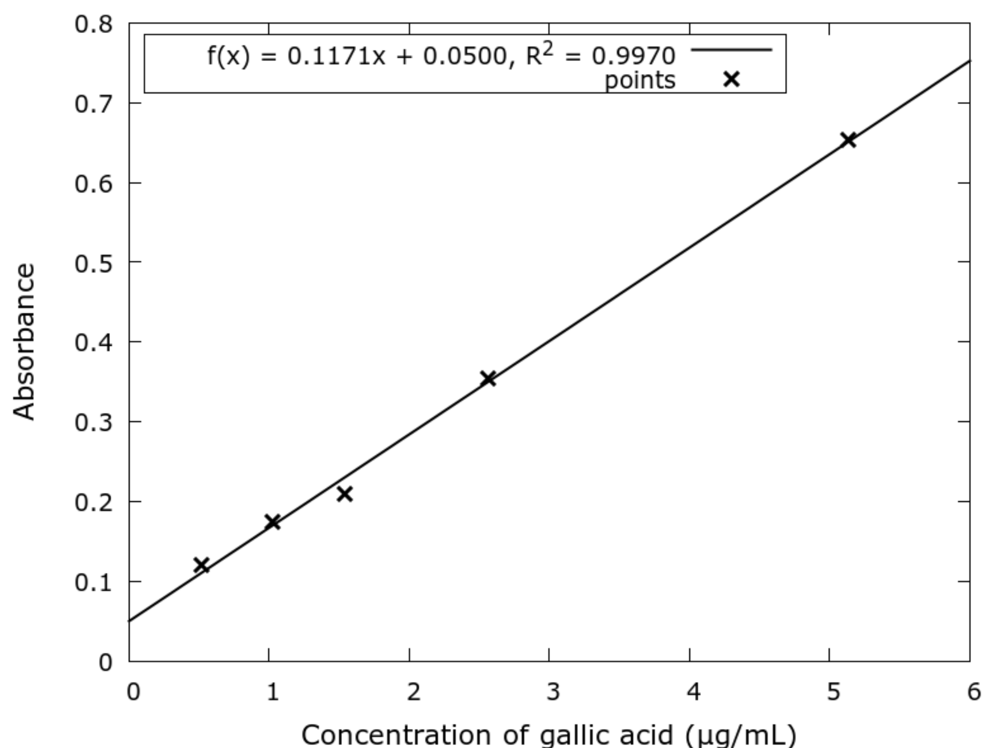


Figure 12: *The standard curve of gallic acid used to determine the Folin-Ciocalteu reducing capacity of Herniaria incana*

Table 4 show the concentration, the absorbance, the GAE calculated from the equation

and the FCRC of the plant samples. The results are presented as mean ± standard deviation of three parallels. The Folin-Ciocalteu reagent reducing capacity of *H. incana* was estimated to be 35.0 ± 1.6 microgram gallic acid equivalents per milligram of the dry weight extract. For example of calculation see appendix E.2.

Table 4: *The estimation of the Folin-Ciocalteu reducing capacity of Herniaria incana*

Sample	**Concentration (mg/mL)**	**Absorbance**	**GAE (μg/mL)**	**FCRC (μg GAE/mg)**
1	0.1018	0.446	3.38173	33.21930
2	0.1018	0.478	3.65500	35.90369
3	0.1018	0.478	3.65500	35.90369
Mean	-	-	-	35.0
SD	-	-	-	1.6

See appendix D.3 for more detailed data.

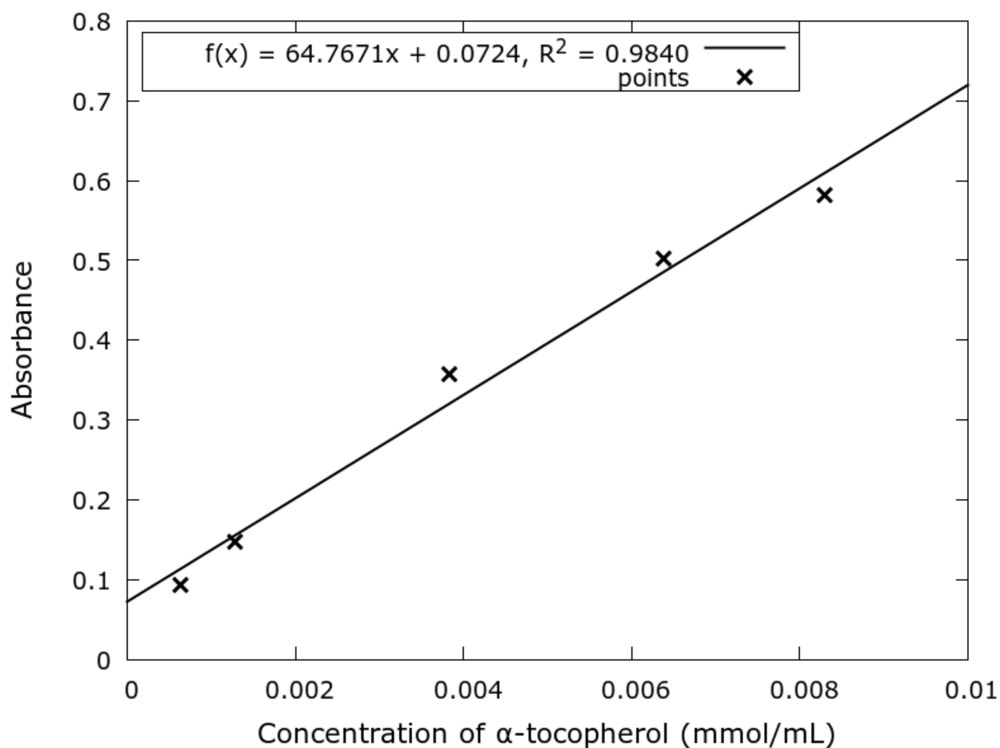
4.4 The phosphomolybdenum reducing capacity assay

The antioxidant activity was determined by the phosphomolybdenum assay using UV-VIS spectrophotometry as described by Prieto *et. al.* [56]. Due to different manners for presenting the results in the literature, the results are presented in two different ways. This will make it easier to compare the results of the phosphomolybdenum reducing capacity of *H. incana* with that of other plant species.

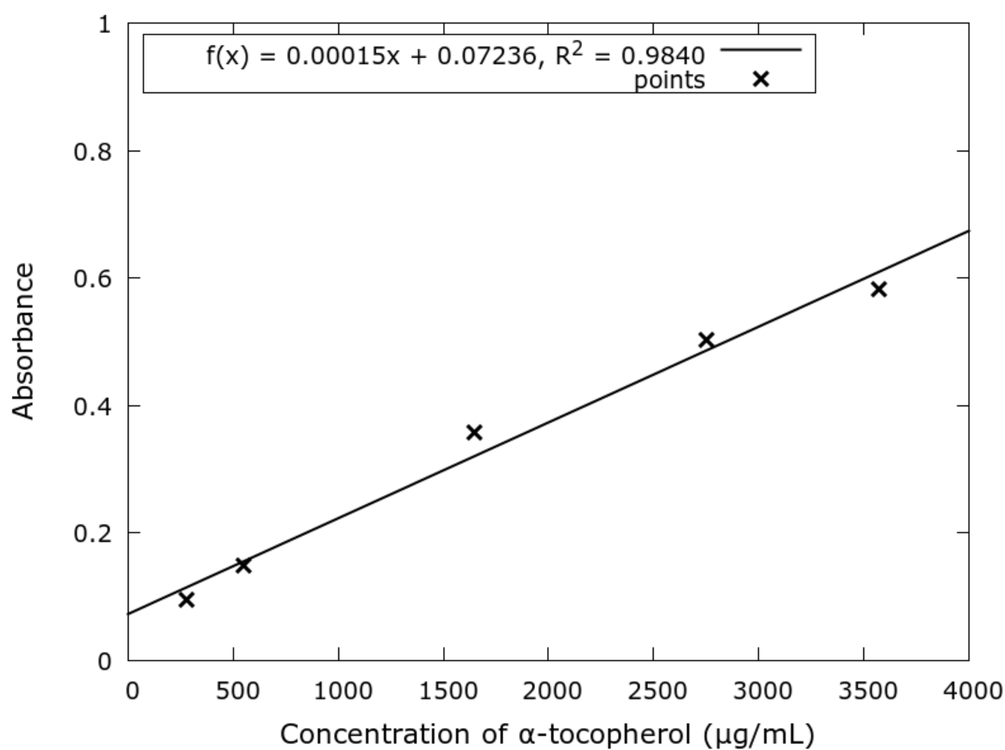
Figure 13 shows the standard curves of α-tocopherol, with absorbance of the complex formed plotted on the Y-axis and the concentration of α-tocopherol on the X-axis presented as mmol/mL and μg/mL, respectively. The equations f(x) = 64.7671x + 0.0724 (R^2 = 0.9840) and f(x) = 0.00015x + 0.07236 (R^2 = 0.9840) were obtained by linear regression. From this equations the phosphomolybdenum reducing capacity (PMRC) of the plant extract, was calculated as millimols of α-tocopherol equivalents (ATE) per gram of the dw plant extract, and micrograms of ATE per milligram of the dw plant extract.

Table 5 show the concentrations, the absorbance, the ATE calculated and the PMRC of the plant samples. The results are presented as mean ± standard deviation of three parallels. The phosphomolybdenum reducing capacity of *H. incana* was estimated to be 0.84 ± 0.25 millimol α-tocopherol equivalents per gram of the dry weight extract, or 362 ± 106 μg α-tocopherol equivalents per milligram of the dry weight extract. For example of calculation see appendix E.3.

It was observed that the reagent rapidly formed a blue colour when it came in contact with aluminum foil or air.



(a) *α-tocopherol expressed as mmol/mL*



(b) *α-tocopherol expressed as μg/mL*

Figure 13: *The standard curve of α-tocopherol used in the phosphomolybdenum reducing capacity assay presented as (a) mmol/mL and (b) μg/mL*

Table 5: *The phosphomolybdenum reducing capacity assay of Herniaria incana presented as (mmol ATE/g) and (μg ATE/g)*

Sample	**Concentration (g/mL)**	**Absorbance**	**ATE (mmol/mL)**	**PMRC (mmol ATE/g)**
1	0.004173	0.249	0.002727	0.6535
2	0.004173	0.273	0.003113	0.7460
3	0.004173	0.374	0.004657	1.1160
Mean	-	-	-	0.84
SD	-	-	-	0.25
Sample	**Concentration (mg/mL)**	**Absorbance**	**ATE (μg/mL)**	**PMRC (μg ATE/mg)**
1	4.1727	0.249	1177.60	282.22
2	4.1727	0.274	1344.27	322.16
3	4.1727	0.374	2010.93	481.93
Mean	-	-	-	362
SD	-	-	-	106

See appendix D.4 for more detailed data.

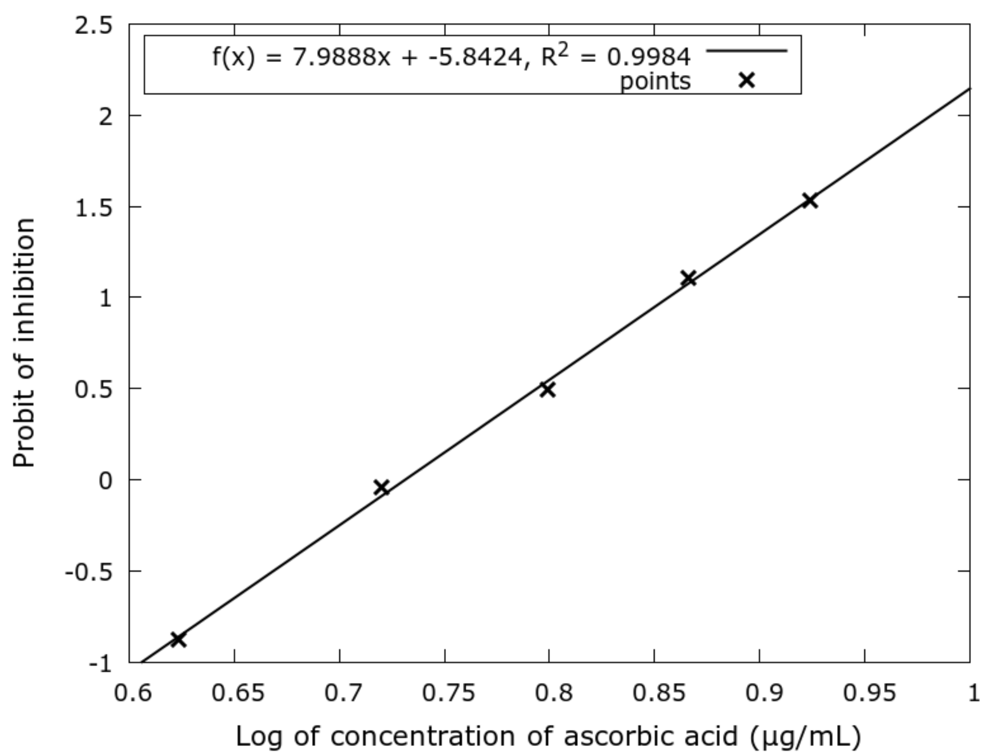
4.5 The DPPH free radical scavenging assay

The DPPH free radical scavenging capacity of ascorbic acid and *H. incana* was determined using UV-VIS spectrophotometry, and are presented as the concentration of reducing reagent necessary to decrease the initial DPPH concentration by fifty percent (EC_{50}) [60]. Figure 14(a) and 14(b) show an example of the standard curves of ascorbic acid and *Herniaria incana*, respectively. See appendix C for the standard curves of the other parallels. The Probit of the scavenging activity is plotted on the Y-axis, while the log concentration of the reductant is plotted on the X-axis. The equations, Y = aX + b, were obtained by linear regression. The a-value, b-value, R^2 and EC_{50} for all the parallels of ascorbic acid and *H. incana* are presented in table 7 and 6, respectively. For example of calculation see appendix E.4.

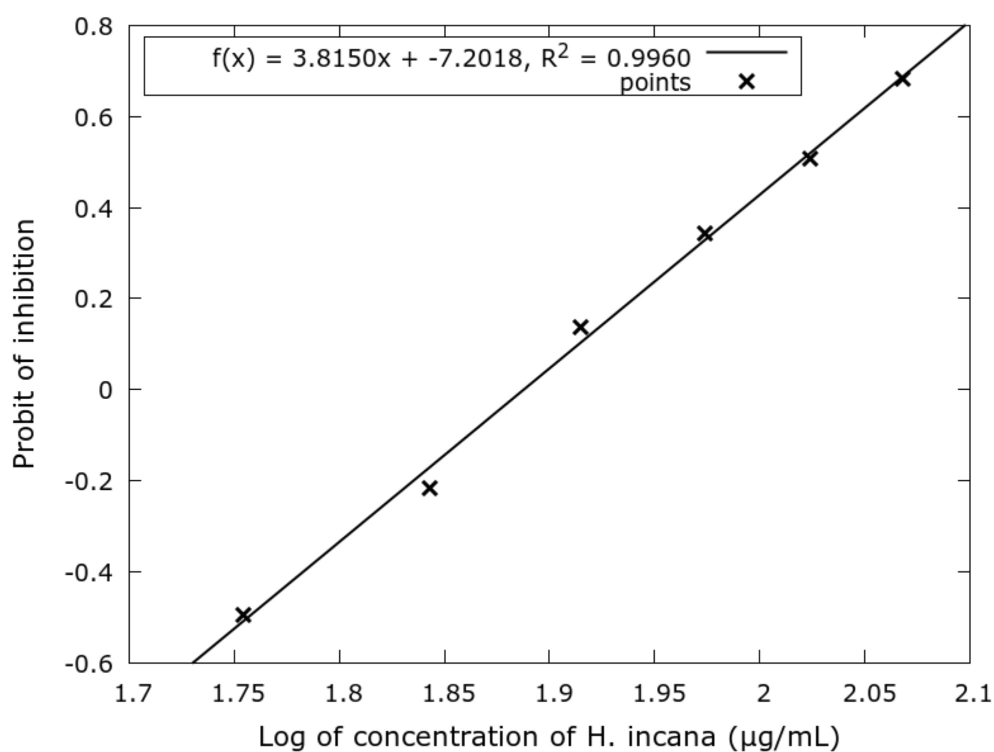
Table 6: *The results from the DPPH free radical scavenging of Herniaria incana*

Date	**a**	**b**	$\mathbf{R^2}$	$\mathbf{EC_{50}}$ **(μg/mL)**
12.04.12	3.8150	-7.2018	0.9960	77.23
13.04.12	3.2147	-5.8283	0.9990	65.02
Mean	-	-	-	71.1
SD	-	-	-	8.6

The results from the DPPH free radical assay of ascorbic acid are shown in table 7. The EC_{50} was determined to 6.6 ± 1.0 μg/mL. The results are presented as mean ± standard deviation of five parallels.



(a) *The DPPH free radical scavenging of ascorbic acid 09.04.12*



(b) *The DPPH free radical scavenging of Herniaria incana 12.04.12*

Figure 14: *Examples of standard curves used to calculate the EC_{50} value of the DPPH free radical scavenging of (a) ascorbic acid and (b) Herniaria incana*

Table 7: *The results from the DPPH free radical scavenging of ascorbic acid*

Date	**a**	**b**	**R^2**	**EC_{50} (μg/mL)**
02.04.12	7.4117	-6.2679	0.9926	7.17
04.04.12	8.5174	-6.3430	0.9895	5.56
06.04.12	10.8883	-9.2774	0.9938	7.11
09.04.12	7.9888	-5.8424	0.9984	5.39
10.04.12	10.5707	-9.3396	0.9870	7.65
Mean	-	-	-	6.6
SD	-	-	-	1.0

Table 6 shows the DPPH free scavenging of *H. incana*. The EC_{50} was determined to 71.1 $\pm$ 8.6 μg/mL. The results are presented as mean $\pm$ standard deviation of two parallels.

See appendix D.5 for more detailed data.

5 Discussion

5.1 Extraction of *Herniaria incana*

H. incana was first extracted in methanol and subsequently with hexane, in order to remove substances like carotenoids and chlorophyll, that might interfere with the UV absorbance in the following assays (see section 3.1). An emulsion of the aqueous and the hexane phase was easily formed during shaking of the separating funnel. Most of the emulsion settled and separated after twenty minutes.

There are little variation of yield of the different extracts. Extract B, used in the Folin-Ciocalteu assay, has a slightly higher second yield than the others. This may be due to less emulsion formed during the extraction, and subsequently lesser amount of loss of water-soluble substances. It may also mean that less hexane-soluble substances have been extracted from the aqueous phase, with the consequence of more chlorophyll in extract B.

Due to a lack of more crude drug from 2009, plant extract C is from another year and month than extract A and B. Although they are harvested in the same area, factors like climate, soil and month of harvest may effect the amount of secondary metabolites with antioxidant activity in the plant. This fact needs to be taken into account when the results from the different assays are compared.

5.2 Estimation of the total flavonoid content

Flavonoids are known for their antioxidant properties, and the flavonoid content of the investigated plant is of interest when evaluating its antioxidant properties. A positive correlation between the content of flavonoids and the antioxidant capacity in plant extracts have been found [9]. The methanolic extract of *H. incana* was found to have a content of 20.46 $\pm$ 0.07 microgram quercetin equivalents per milligram of the dry weight extract.

There is little data on the flavonoid content of different *Herniaria* species, so the estimated content of flavonoids in *H. incana* can not be compared to that of other *Herniaria* species.

In a study of *Wrightia tomentosa*, the flavonoid content was estimated to 16.9 and 20.3 μg quercetin equivalents per mg of dry ethanolic extract [9]. Investigations of *Polygonum*

aviculare showed a total flavonoid content of 112.7 mg quercetin equivalents per g of dry ethanolic extract, corresponding to 112.7 μg/mg [52]. The results of flavonoid content of *Wrightia tomentosa*, *Polygonum aviculare* and *H. incana* are obtained by the same method.

The flavonoid content estimated for *Wrightia tomentosa* corresponds well with the results obtained for *H. incana*, while that reported for *Polygonum aviculare* is considerable higher. This may suggest that *H. incana* have moderately content of flavonoids. More data from similar studies must be reviewed before a conclusion of the significance of the flavonoid content of *H. incana* can be drawn.

The absorbance of the standard curve are a bit lower than recommended (see section 3.1). This may have resulted in lower precision. The coefficient of determination (R^2) of the equation describing the standard curve have a value of 0.9973, suggesting that the standard curve has a good linear correlation, even though the absorbance are a bit low.

5.3 The Folin-Ciocalteu reagent reducing capacity

The Folin-Ciocalteu reagent assay is a very popular antioxidant assay for the estimation of the reducing capacity of a plant extract. The results are presented as the total phenolic content or the Folin-Ciocalteu reducing capacity (FCRC), and reported as mg GAE/g.

The majority of the Folin-Ciocalteu reducing capacity results reported for different plant extracts lies between 13 and 50 mg gallic acid equivalents per gram dry weight extract [67, 10, 48]. Higher values of 155 mg/g [10] and 677 mg/g [52] have also been reported. The reducing capacity of *H. incana* is estimated to 35.0 $\pm$ 1.6 μg GAE/mg, suggesting a moderately good ability to scavenge the Folin-Ciocalteu reagent, compared to other plant extracts.

The standard curve of gallic acid have a good linear approximation with a R^2 of 0.9970.

There is little data of the Folin-Ciocalteu reducing capacity of different *Herniaria* species, so the Folin-Ciocalteu reducing capacity of *H. incana* can not be compared to that of other *Herniaria* species.

5.4 The phosphomolybdenum reducing capacity assay

The results of the phosphomolybdenum reducing capacity assay are presented in a various different ways in the literature. In the original article the results are presented as 100-200 nmol α-tocopherol / g of seeds from corn and soybeans [56]. Other studies have presented the results as 1000-2300 mmol α-tocopherol/g of turmeric oil [68], 0.06-118 μmol α-tocopherol/g of fresh tissue from various fruit peel, seed or leaf[50], 4.2-8.3 μg/mg dry extract of bark and leaf from *Wrightia tomentosa* [9], 3.2-5.4 mM α-tocopherol acetate/g dry mass of aerial parts from *Hypericum perforatum*, *Hypericum empetrifolium* and *Hypericum triquetrifolium* [57] and 14-23 mM α-tocopherol acetate/g dry mass of leaves from *Ficus carica* [58].

The huge difference of order, and the diverse manners of presenting the results in the distinct studies, make the interpretation of the results more difficult. It was considered best to compare the reducing capacity of the methanolic extract of *H. incana* with the results of other dried herbs, hence it is presented as both (mmol ATE/g) and (μg ATE/g). The phosphomolybdenum reducing capacity of *H. incana* was estimated to be 0.84 $\pm$ 0.25 millimol α-tocopherol equivalents per gram of the dry weight extract, or 362 $\pm$ 106 μg α-tocopherol equivalents per milligram of the dry weight extract.

The reducing capacity of *H. incana* determined by the phosphomolybdenum method is around four times lower than that reported for the three *Hypericum* species [57], and fifteen times lower than that reported for *Ficus carica* [58]. On the other side, the reducing capacity of *H. incana* is much higher (362 μg/mg) than that reported for *Wrightia tomentosa* [9].

It was observed a blue colour, when the reagent was exposed to aluminum foil or air. A possible reason for the formation of the blue colour is oxidation of the reagent. This may have affected the accuracy of the standard curve. Although precautions were taken for avoiding formation of the blue colour, two of the standard solution samples had different colours and much higher absorbance than the other samples. These two samples were excluded from the standard curve. Further investigations of stability of the reagent would be necessary in order to reveal the effect the formation of the blue colour may have on the accuracy of the standard curve.

The standard curve of α-tocopherol has a good linear approximation with an R^2 of 0.9840. The standard deviation of the plant samples show low precision. This might be improved by performing more parallels.

In the phosphomolybdenum assay, a blue colour was formed in some of the samples. The

results did not show very good precision. The precision might be improved by performing more parallels, however, due to difficulties in this assay, it should be considered if it would be good to replace this experiment in future studies.

5.5 The DPPH free radical scavenging assay

The DPPH free radical scavenging assay is one of the most used assays for evaluation of the antioxidant properties of a plant sample. Despite the huge number of data available, comparison of the results from different studies can not be directly compared to each other. Several factors may influence the results, such as solvent, pH, concentration of the reagent, concentration of the samples, reaction time and processing data [60, 61]. In many articles it is not mentioned how the EC_{50} value is calculated, which makes it difficult to compare results from different studies [62].

The EC_{50} of the plant extracts are compared to that of a standard, usually ascorbic acid [60]. Mishra *et. al.* pointed out that the EC_{50} reported for ascorbic acid varied enormously from 5.85 to 110.77 μg/mL [69], demonstrating the differences in the results due to the factors mentioned above.

The EC_{50} of *H. incana* was determined to 71.1 $\pm$ 8.6 μg/mL, compared to that of 6.6 $\pm$ 1.0 μg/mL for ascorbic acid.

Nikolova *et. al.* considered plant extract with EC_{50} below fifty μg/mL to exhibit strong DPPH radical scavenging activity, plant extracts with EC_{50} between fifty and hundred μg/mL to possess significant activity, and those with an EC_{50} above 200 μg/mL to have little activity [64].

The EC_{50} has been determined to 54 to 62 μg/mL for leaves, twigs and stem bark of *Canarium album* compared to 78.25 μg/mL for asorbic acid [70]. The EC_{50} for the leaf, stem bark and root bark of *Acacia confusa* determined to 89 to 113 μg/mL, compared to 118 μg/mL for ascorbic acid [63]. The EC_{50} for bark and leaf ethanolic extract of *Wrightia tomentosa* estimated to 75 and 135 μg/mL compared to 3.1 μg/mL for ascorbic acid [9].

According to Nikolovas classification, *H. incana* exhibit a moderately DPPH radical scavenging activity. Keeping in mind that the variations of the procedure may effect the outcome of the results, *H. incana* has similar DPPH free radical scavenging activity compared to the other plant species mentioned above. However, when the EC_{50} of the plant extracts are compared to that of ascorbic acid, *H. incana* and *Wrightia tomentosa*

have much higher value of EC_{50} than ascorbic acid, while *Canarium album* and *Acacia confusa* have lower values of EC_{50} than ascorbic acid. Reminding that a lower value of EC_{50} means a higher radical scavenging acitivty, this may indicate that *H.incana* has similar DPPH scavenging activity as the bark of *Wrightia tomentosa*, and lower than *Canarium album* and *Acacia confusa*.

There are very few studies that have evaluated the DPPH free radical scavenging of *Herniaria* species, but one article reported little antioxidant activity (EC_{50} more than 200 μg/mL) for *H. glabra*, compared to 3.15 for quercetin [64]. This may indicate that *H. incana* is a better DPPH free radical scavenger than its close relative *H. glabra*.

The standard curves of ascorbic acid shows a good linear approximation with a R^2 between 0.9870 and 0.9984. The standard curves of *H. incana* shows a good linear approximation with a R^2 of 0.9960 and 0.9990.

The standard deviation of the EC_{50} values of *H. incana* shows that the results do not have great precision. Only two experiments were performed. By performing more experiments the precision of the assay could bee improved.

It was also found that dissolving the DPPH with magnetic stirrer did not affect the accuracy of the experiment, but made it easier to perform than with manual shaking.

6 Conclusion

The rise of interest of the investigation in this thesis, is the possibility of a health benefit of antioxidants and the possibility of isolating active compounds that may be used as lead compounds in the development of new drugs.

The evaluation of the antioxidant capacity of the plant extract is complicated by the fact that it is hard to compare results from different studies. Small variations in the procedures affects the obtained results, making it difficult to compare the results.

This preliminary investigation of *H. incana* showed that the methanolic extract of the plant had a moderately content of flavonoids, which are known for their antioxidant properties. All of the three *in vitro* assays investigating the antioxidant properties of the plant, showed that it had a moderately antioxidant reducing capacity, even though the plant material originated from different years. This may indicate that the various factors affecting the plant composition, did not vary significantly in these successive years. The obtained results suggest that *H. incana* have moderately antioxidant properties. The effect showed in these *in vitro* experiments, can not be be extrapolated to *in vivo* antioxidant capacity without further investigations.

Other important antioxidant parameters, as the ability to inhibit generation of and scavenge ROS/RNS, ability to chelate metals, activity as antioxidative enzymes and inhibition of oxidative enzymes were not investigated in this thesis. Since lipophilic compounds, as chlorophyll and carotenoids, were removed by extraction with hexane, their possible impact on the antioxidant activity of *H. incana* were not measured. For a full screening of the antioxidant capacity of *H. incana* more assays, investigating other antioxidant mechanisms, should be applied, and the different extract phases should also be investigated.

Available literature indicates that no previous antioxidant properties studies have been performed on *H. incana*, and there are also very few publications on other *Herniaria* species. Since these are the first results of this kind for *H. incana*, they represent a contribution to better understanding of its chemical composition, pointing the direction for future studies.

6.1 Further studies

The obtained results suggest a considerable content of antioxidant compounds, which belong to the class of phenolic compounds and flavonoids. Therefore, future work should include chemical investigation of flavonoids, as the most important antioxidants.

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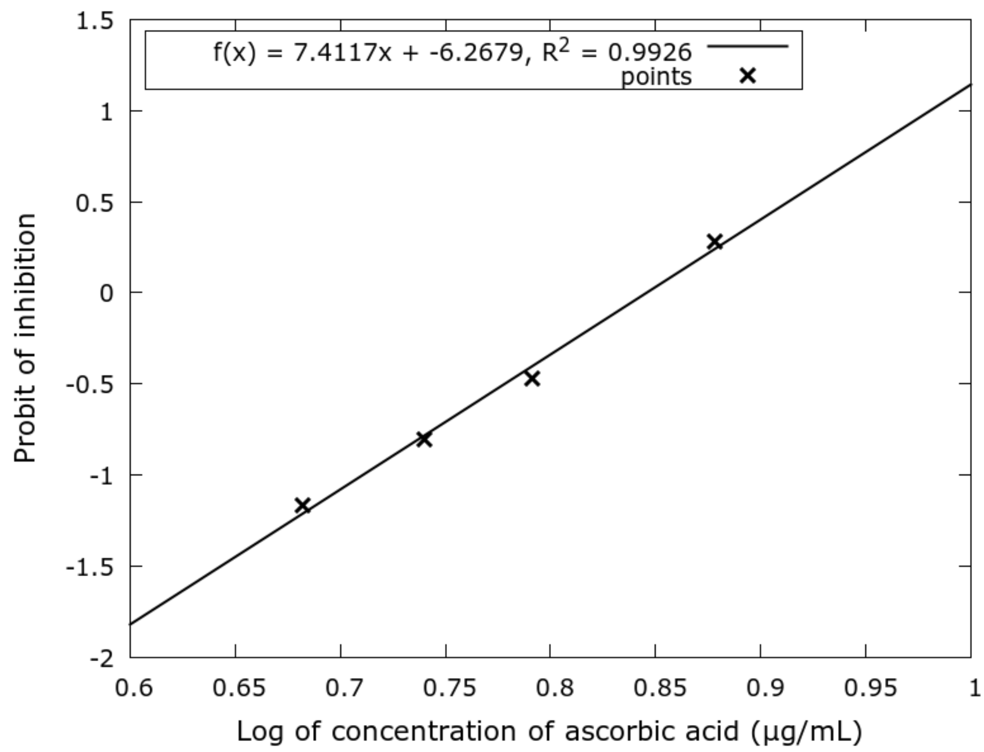

Figure 15: *DPPH free radical scavenging of ascorbic acid 02.04.12*

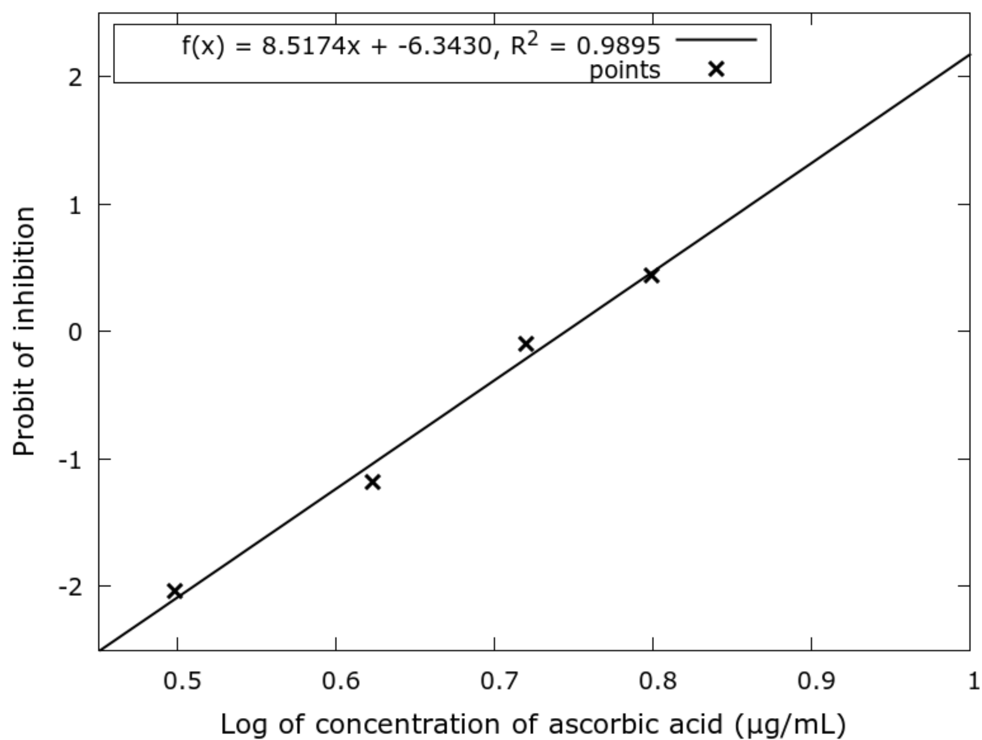

Figure 16: *DPPH free radical scavenging of ascorbic acid 04.04.12*

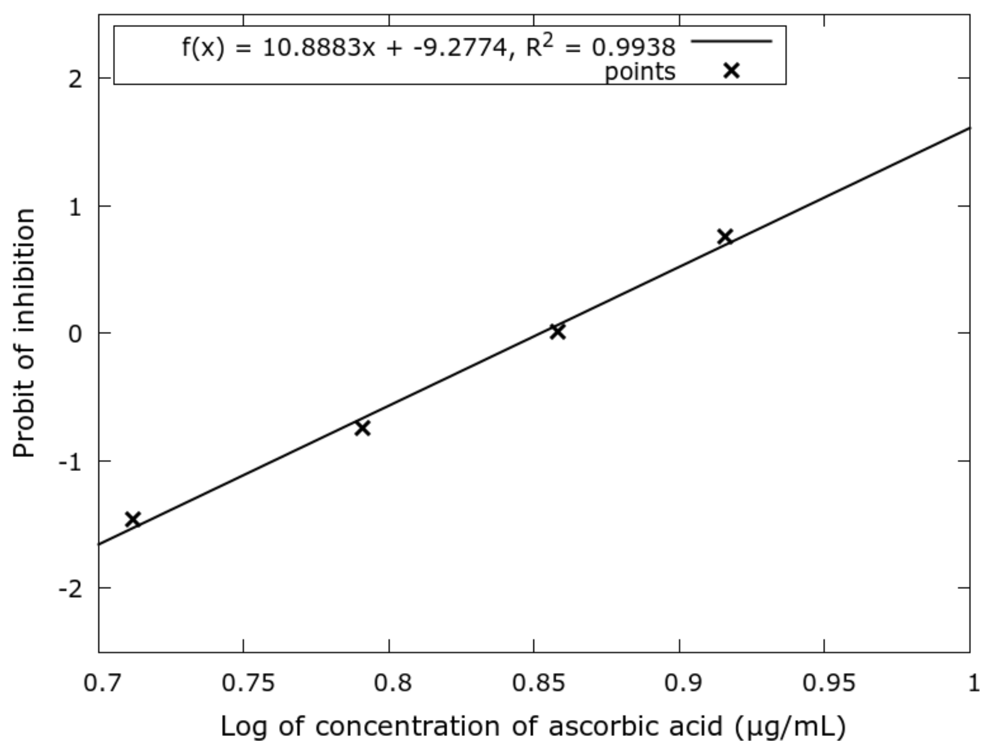

Figure 17: *DPPH free radical scavenging of ascorbic acid 06.04.12*

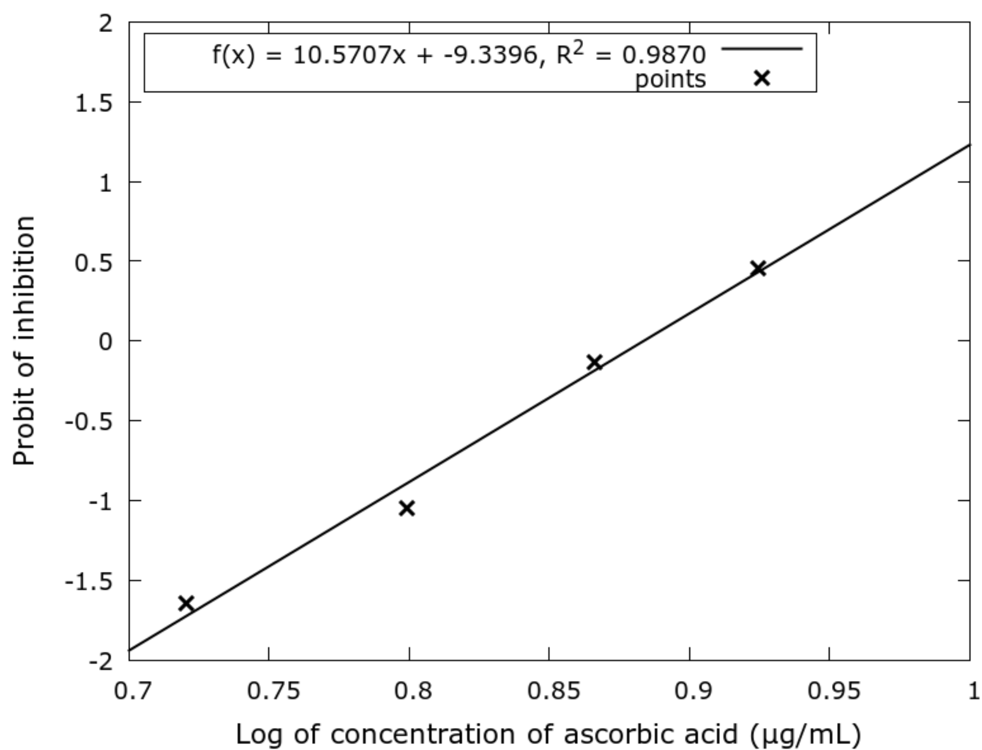

Figure 18: *DPPH free radical scavenging of ascorbic acid 10.04.12*

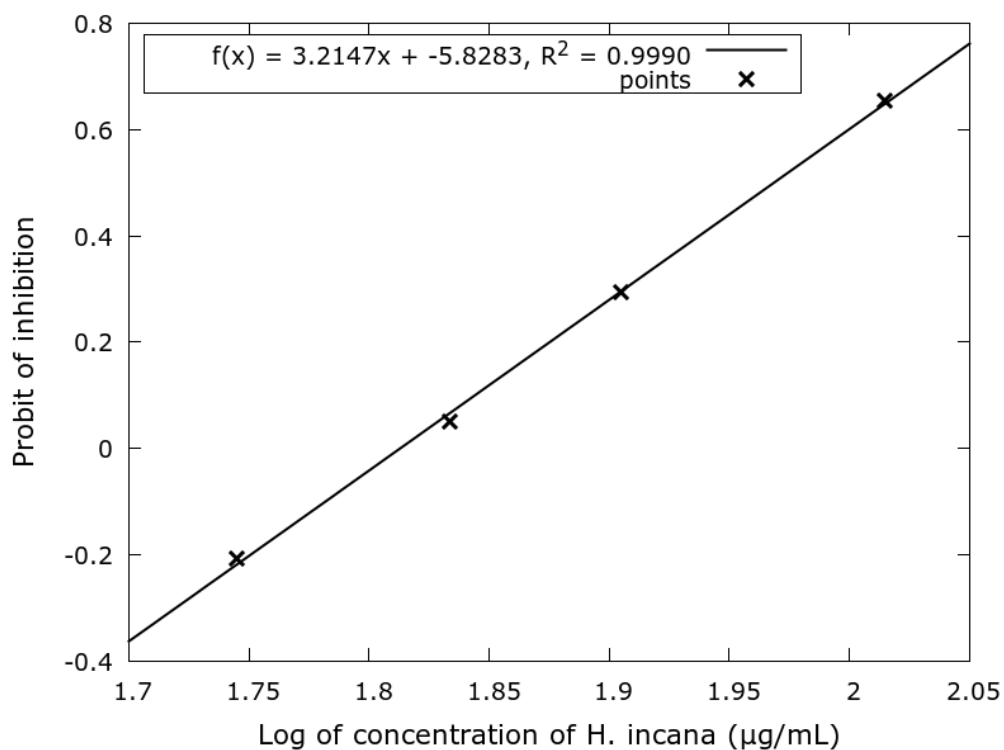

Figure 19: *DPPH free radical scavenging of Herniaria incana 13.04.12*

D Data

D.1 Extraction of *Herniaria incana*

Methanolic extraction 1 (A and B):

Dry plant (g): 10,0159
Dry MeOH extract (g): 1,4876
Yield 1 (w%): 14,8523

Extraction with hexane 09.11.11 (extract A):

MeOH extract (g): 0,2213
Dry aq. Extract (g): 0,1834
Yield 2 (w%): 82,8739
Total yield (w%): 12,3087

Extraction with hexane 16.11.11 (extract B):

MeOH extract (g): 0,3212
Dry aq. Extract (g): 0,3015
Yield 2 (w%): 93,8667
Total yield (w%): 13,9414

Methanolic extraction 2 (extract C):

Dry plant (g): 9,5373
Dry MeOH extract (g): 1,38
Yield 1 (w%): 14,4695

Extraction with hexane 03.02.12 (extract C):

MeOH extract (g): 1,38
Dry aq. Extract (g): 1,1566
Yield 2 (w%): 83,8116
Total yield (w%): 12,1271

D.2 Estimation of the total flavonoid content

Table 8: *Data of the quercetin standard curve used in the total flavonoid content assay 12.11.11*

standard	concentration (μg/mL)	Absorbance
1	0.257	0.015
2	0.514	0.028
3	1.028	0.056
4	2.056	0.123

D.3 The Folin-Ciocalteu reagent reducing capacity assay

Table 9: *Data of the gallic acid standard curve used in the Folin-Ciocalteu reducing capacity assay 26.01.12*

standard	concentration (μg/mL)	Absorbance
1	0.513	0.12
2	1.026	0.174
3	1.539	0.21
4	2.565	0.355
5	5.13	0.653

D.4 The phosphomolybdenum reducing capacity assay

Table 10: *Data of the α-tocopherol standard curve used in the phosphomolybdenum reducing capacity assay 21.03.12*

standard	concentration (μg/mL)	concentration (mmol/mL)	Absorbance
1	274.981	0.0006384384	0.094
2	549.963	0.0012768768	0.148
3	1099.92	0.0025537537	0.366
4	1649.89	0.0038306306	0.358
5	2199.85	0.0051075074	0.520
6	2749.81	0.0063843843	0.503
7	3574.76	0.0082996996	0.582

D.5 The DPPH free radical scavenging assay

Table 11: *Data from the DPPH free radical scavenging of ascorbic acid 02.04.12*

Sample	**C (μg/mL**	**Log C**	**Absorbance**	**I**	**Probit (I)**
negative control	0	-	0.487	-	-
1	4.8067	0,6818	0.457	0.06160	-1.54146
2	5.4933	0.7398	0.411	0.15605	-1.01079
3	6.1800	0.7909	0.354	0.27310	-0.60346
4	7.5533	0.8781	0.202	0.58521	0.215254

Table 12: *Data from the DPPH free radical scavenging of ascorbic acid 04.04.12*

Sample	**C (μg/mL**	**Log C**	**Absorbance**	**I**	**Probit (I)**
negative control	0	-	0.533	-	-
1	3.15	0.4983	0.522	0.02063	-2.04074
2	4.20	0.6232	0.470	0.11819	-1.18403
3	5.25	0.7201	0.287	0.46153	-0.09655
4	6.30	0.7993	0.175	0.67166	0.444528

Table 13: *Data from the DPPH free radical scavenging of ascorbic acid 06.04.12*

Sample	**C (μg/mL**	**Log C**	**Absorbance**	**I**	**Probit (I)**
negative control	0	-	0.587	-	-
1	5.15	0.7118	0.545	0.07155	-1.464342
2	6.18	0.7909	0.453	0.22827	-0.744525
3	7.21	0.8579	0.290	0.50596	0.014946
4	8.24	0.9159	0.131	0.77683	0.761535

Table 14: *Data from the DPPH free radical scavenging of ascorbic acid 09.04.12*

Sample	**C (μg/mL**	**Log C**	**Absorbance**	**I**	**Probit (I)**
negative control	0	-	0.559	-	-
1	4.20	0.6232	0.453	0.18962	-0.87928
2	5.25	0.7201	0.289	0.48300	-0.04261
3	6.30	0.7993	0.174	0.68872	0.492253
4	7.35	0.8662	0.075	0.86583	1.106901
5	8.40	0.9242	0.035	0.93738	1.533212

Table 15: *Data from the DPPH free radical scavenging of ascorbic acid 10.04.12*

Sample	**C (μg/mL**	**Log C**	**Absorbance**	**I**	**Probit (I)**
negative control	0	-	0.521	-	-
1	5.25	0.7201	0.495	0.04990	-1.645784
2	6.30	0.7993	0.444	0.14779	-1.045947
3	7.35	0.8662	0.288	0.44721	-0.132696
4	8.40	0.9242	0.169	0.67562	0.4554960

Table 16: *Data from the DPPH free radical scavenging of H.incana 12.04.12*

Sample	**C (μg/mL**	**Log C**	**Absorbance**	**I**	**Probit (I)**
negative control	0	-	0.555	-	-
1	56.7888	1.75426	0.383	0.3099	-0.496105
2	69.6954	1.84320	0.325	0.4144	-0.216203
3	82.1410	1.91456	0.247	0.5549	0.1381902
4	94.1500	1.97382	0.203	0.6342	0.3430889
5	105.7448	2.02425	0.170	0.6936	0.5063476
6	116.9466	2.06798	0.137	0.7531	0.6844458

Table 17: *Data from the DPPH free radical scavenging of H.incana 14.04.12*

Sample	**C (μg/mL**	**Log C**	**Absorbance**	**I**	**Probit (I)**
negative control	0	-	0.515	-	-
1	55.5555	1.74472	0.300	0,4174	-0.208355
2	68.1818	1.83366	0.247	0.5203	0.0511282
3	80.3571	1.90502	0.198	0.6155	0.2937721
4	103.4482	2.01472	0.132	0.7436	0.6547614

E Example of calculations

E.1 Estimation of the total flavonoid content

Example of calculation of TFC (μg QUE/mg) for sample 1:

From the standard curve of quercetin (Figure 11), the equation f(x) = 0.0604x - 0.0027 is obtained, where f(x) is the absorbance and x is the concentration of quercetin (μg/mL).

From this equation we have that:

$$\text{QUE} = \frac{(\text{Absorbance} + 0.0027)}{0.0604} \tag{9}$$

We also know that:

$$TFC(\mu\text{g QUE/mg}) = \frac{QUE}{\text{sample concentration(mg/mL)}} \tag{10}$$

Put together 9 and 10 and get the expression:

$$TFC(\mu\text{g QUE/mg}) = \frac{\frac{(\text{Absorbance}+0.0027)}{0.0604}}{\text{sample concentration(mg/mL)}} \tag{11}$$

Put in the data of sample 1 and get:

$$TFC(\mu\text{g QUE/mg}) \text{ of sample 1} = \frac{\frac{(0.027+0.0027)}{0.0604}}{0.024 \text{ (mg/mL)}} = 20.48841(\mu\text{g QUE/mg}) \tag{12}$$

E.2 The Folin-Ciocalteu reagent reducing capacity assay

Example of calculation of FCRC (μg GAE/mg) for sample 1:

From the standard curve of gallic acid (Figure 12, the equation f(x) = 0.1171x +0.0500 is obtained, where f(x) is the absorbance and x is the concentration of gallic acid (μg/mL).

From this equation we have that:

$$\text{GAE} = \frac{(\text{Absorbance} - 0.0500)}{0.1171} \tag{13}$$

We also know that:

$$FCRC(\mu\text{g GAE/mg}) = \frac{GAE}{\text{sample concentration(mg/mL)}} \tag{14}$$

Put together 13 and 14 and get the expression:

$$FCRC(\mu\text{g GAE/mg}) = \frac{\frac{(\text{Absorbance}-0.0500)}{0.1171}}{\text{sample concentration(mg/mL)}} \tag{15}$$

Put in the data of sample 1 and get:

$$FCRC(\mu\text{g GAE/mg}) = \frac{\frac{(0.446-0.0500)}{0.1171}}{0.1018(\text{mg/mL})} = 33.21930(\mu\text{g GAE/mg}) \tag{16}$$

E.3 The phosphomolybdenum reducing capacity assay

Example of calculation of FCRC (mmol ATE/g) for sample 1:

From the standard curve of α-tocopferol (figure 13(a), the equation f(x) = 64.7671x + 0.0724 is obtained, where f(x) is the absorbance and x is the concentration of $alpha$-tocopherol (mmol/mL).

From this equation we have that

$$\text{ATE} = \frac{(\text{Absorbance} - 0.0724)}{64.7671} \tag{17}$$

We also know that:

$$PMRC(\text{mmol ATE/g}) = \frac{ATE}{\text{sample concentration(g/mL)}} \tag{18}$$

Put together 17 and 18 and get the expression:

$$PMRC(\text{mmol ATE/g}) = \frac{\frac{(\text{Absorbance}-0.0724)}{64.7671}}{\text{sample concentration(g/mL)}} \tag{19}$$

Put in the data of sample 1 and get:

$$PMRC(\text{mmol ATE/g}) = \frac{\frac{(0.249-0.0724)}{64.7671}}{0.0041727(\text{mg/mL})} = 0.6535(\text{mmol ATE/g}) \tag{20}$$

Example of calculation of FCRC (μg ATE/mg) for sample 1:

From the standard curve of α-tocopferol (figure 13(b), the equation f(x) = 0.00015x + 0.07236 is obtained, where f(x) is the absorbance and x is the concentration of *alpha*-tocopherol (μg/mL).

From this equation we have that

$$\text{ATE} = \frac{(\text{Absorbance} - 0.07236)}{0.00015} \tag{21}$$

We also know that:

$$PMRC(\mu\text{g ATE/mg}) = \frac{ATE}{\text{sample concentration(mg/mL)}} \tag{22}$$

Put together 21 and 22 and get the expression:

$$PMRC(\mu\text{g ATE/mg}) = \frac{\frac{(\text{Absorbance}-0.07236}{0.00015}}{\text{sample concentration(mg/mL)}} \tag{23}$$

Put in the data of sample 1 and get:

$$PMRC(\mu\text{g ATE/mg}) = \frac{\frac{(0.249-0.07236)}{0.00015}}{4.1727(\text{mg/mL})} = 282.2154(\mu\text{g ATE/mg}) \tag{24}$$

E.4 The DPPH free radical scavenging assay

Example of calculation of EC_{50} for *H. incana* 12.04.12:

The data from the parallel 12.04.12 is shown in table 16.

Calculate the scavenging activity of the absorbances:

$$I = \frac{Abs_0 - Abs_1}{Abs_0} \tag{25}$$

sample 3:

$$I = \frac{0.555 - 0.247}{0.555} = 0.634234234234234 \quad (26)$$

Calculate the Probit of the scavenging activity using the Norminsv function in OpenOffice 3.2. Make a graph by plotting log of the concentration against the Probit of the scavenging activity. Make a linear regression line and generate an equation Y = aX + b. The equation Y = 3.8150x - 7.2018, was obtained. From this equation, claculate EC_{50}. Probit of 50 % scavenging is zero, thus the EC_{50} is calculated as:

$$EC_{50} = 10^{\frac{-b}{a}} \quad (27)$$

The EC_{50} of *H. incana* 12.04.12 is calculated:

$$\mathrm{EC}_{50} = 10^{\frac{7.2018}{3.8150}} = 77.23(\mu\mathrm{g/mL}) \quad (28)$$