

Genetic Variation, Biochemical  
Contents and Wound Healing Activity  
of *Plantago major*

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Cover: RAPD banding patterns on agarose gel, structure of plantamajoside, cells in a scratch assay, *Plantago major* plant, cells showing NF- $\kappa$ B presence in nuclear and cytoplasm, pig ear wound biopsy.

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## Genetic Variation, Biochemical Contents and Wound Healing Activity of *Plantago major*

### Abstract

*Plantago major* L. (greater plantain, common plantain) has been used as a wound healing remedy in different parts of the world for centuries. Different bioactive compounds have been proposed to contribute to the wound healing properties of this plant. The present study was undertaken to investigate the impact of some genetic and environmental factors on the wound healing activity of common plantain.

Seeds of *P. major* were collected from five populations in different parts of Sweden, and were germinated and grown in a greenhouse. As expected for an inbreeding species, RAPD analyses demonstrated considerable between-population variation but very sparse within-population and within-subpopulation variation. Six major phenolic compounds were encountered in samples of *P. major*, four of which were identified for the first time in this thesis; PLMA 1–PLMA 4. Between-population and sub-population differences in the contents of these chemical compounds showed no correlation with RAPD-based estimates of genetic relatedness. The contents of these compounds differed greatly between different plant organs of *P. major*. The highest concentration of plantamajoside and PLMA 2 was found in leaves whereas the highest concentration of verbascoside was found in flower stalks and seeds. Contents were significantly higher in freeze-dried leaf samples compared to samples dried at higher temperatures. Both water and ethanol-based extracts of *P. major* leaves stimulated the cell proliferation and migration in an in vitro scratch assay, and also showed anti-inflammatory activity in an in vitro NF- $\kappa$ B assay with oral epithelial cell cultures. Similarly, these extracts stimulated wound healing activities in ex vivo tests using detached pig ears. Further breeding efforts aimed at developing *P. major* as a crop plant, and medicinal research aimed at elucidating and optimizing extracts with wound healing properties, are thus warranted.

*Keywords:* polyphenol, plantain, RAPD, scratch assay, wound healing.

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## Dedication

To my parents, my brothers, my sister and my sister in law for your love, care and motivation throughout my life.

*For every malady ALLAH created, He also created its cure. Whoever acquires such knowledge shall benefit from it, and one who ignores it will forego such benefit.*

Muhammad (PBUH)

# Contents

<b>List of Publications</b>	<b>7</b>
<b>1 Introduction</b>	<b>8</b>
1.1 Taxonomy	9
1.2 Origin and distribution	10
1.3 Biology	11
1.4 Breeding system and genetic variation	14
1.5 Bioactive chemical compounds	14
1.6 Medicinal uses of <i>P. major</i>	16
1.6.1 Anti-ulcerogenic activities	17
1.6.2 Anti-inflammatory and immunomodulatory activities	17
1.6.3 Antioxidant activities	17
1.6.4 Antiviral and antimicrobial activities	18
1.6.5 Anticarcinogenic activities	18
1.6.6 Wound healing activities	19
<b>2 Objective</b>	<b>20</b>
<b>3 Material and Methods</b>	<b>21</b>
3.1 Plant material	21
3.2 DNA analysis	22
3.2.1 DNA extraction and amplification	22
3.2.1 Data evaluation and statistical analysis	22
3.3 HPLC analysis	23
3.3.1 Sample preparation	23
3.3.2 HPLC analysis	24
3.3.3 Statistical analysis	24
3.3.4 Identification of novel polyphenolic compounds	24
3.4 Scratch assay	25
3.4.1 Experimental procedures	25
3.4.2 Data evaluation and statistical analysis	25
3.5 Porcine wound healing model	26
3.5.1 Experimental procedures	26
3.5.2 Data evaluation and statistical analysis	26
3.6 NF- $\kappa$ B assay	27
3.6.1 Experimental procedures	27

<b>4</b>	<b>Summary of Results and Discussion</b>	<b>28</b>
4.1	Genetic variation in <i>P. major</i>	28
4.2	Phytochemical variation in <i>P. major</i>	30
4.3	Effects of environmental factors on the contents of polyphenols	31
	4.3.1 Effects of drying temperature	31
	4.3.2 Effects of selection of different aerial plant organs	32
4.4	Effects of <i>P. major</i> extracts on cell proliferation and migration	33
4.5	Ex vivo wound healing properties of <i>P. major</i>	34
4.6	In vitro anti-inflammatory activities of <i>P. major</i>	35
<b>5</b>	<b>Conclusions</b>	<b>37</b>
<b>6</b>	<b>Recommendations and future prospects</b>	<b>39</b>
	<b>References</b>	<b>40</b>
	<b>Acknowledgements</b>	<b>46</b>

## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Zubair, M., Nybom, H., Lindholm, C. & Rumpunen, K. 2011. Major polyphenols in aerial organs of greater plantain (*Plantago major* L.), and effects of drying temperature on polyphenol contents in the leaves. *Scientia Horticulturae* 128, 523–529.
- II Zubair, M., Nybom, H., Ahnlund, M. & Rumpunen, K. 2012. Detection of genetic and phytochemical differences between and within populations of *Plantago major* L. (plantain). *Scientia Horticulturae* 136, 9–16.
- III Zubair, M., Ekholm, A., Nybom, H., Renvert, S., Widén, C. & Rumpunen, K. (In press). Effects of *Plantago major* L. leaf extracts on oral epithelial cells in a scratch assay. *Journal of Ethnopharmacology*.
- IV Zubair, M., Nybom, H., Lindholm, C., Brandner, J.M. & Rumpunen, K. (manuscript submitted). Promotion of wound healing by *Plantago major* L. leaf extracts – ex vivo experiments confirm experiences from traditional medicine.
- V Zubair, M., Widén, C., Renvert, S., & Rumpunen, K. (manuscript). Anti-inflammatory activity of *Plantago major* L. leaf extracts on oral epithelial cells.

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# 1 Introduction

Skin disorders, especially wounds, have been recognized as the third most common reason for medical visits in developing countries (Ryan & Cherry, 1996). Chronic wounds such as leg ulcers, diabetic foot ulcers and pressure ulcers are common both in acute care and in primary care (Lindholm *et al.*, 2005). Treatment of such wounds is very costly and time-consuming. These wounds can also reduce life quality for the patients (Lindholm *et al.*, 1993). Stimulation of the wound healing process and treatment of non-healing wounds such as diabetic foot ulcers has always been a challenge. High costs for medical wound care have often been reported; annual costs for the care and treatment of patients with chronic ulcers amount to 5–9 billion dollars in the US, while pressure ulcers constitute the third most costly condition to society after cardio-vascular and cancer diseases in the Netherlands. The development of improved wound dressings has received considerable attention lately, and more than 200 different types are presently marketed in Sweden (Grauers & Lindholm, 2011). Some of these contain bioactive compounds in order to improve the wound healing effect.

Many plants species contain bioactive compounds and have a long tradition of being used for wound healing purposes in folk medicine. According to the World Health Organization (WHO), more than 70% of the world population thus relies on herbal medicines for primary health care (Blumenthal, 1998). The most commonly used of these plant species are *Aloe vera* (true aloe), *Azardica indica* (neem), *Lantana camara* (coronitas), *Tridax procumbens* (coat buttons), *Chromolaena odorata* (Siam weed), *Hydnocarpus wightiana* (chaulmugra), *Helianthus annus* (sunflower), *Jasminum auriculatum* (jasmine), *Ginkgo biloba* (ginkgo), *Curcuma longa* (turmeric), *Centella asiatica* (gotu kola), *Cedrus deodara* (deodar), and *Plantago major* (common plantain) (Samuelsen, 2000; Raina *et al.*, 2008).



Leaves from *Plantago major* have been used in wound management for centuries in several cultures around the world. The greek physician Dioscorides thus described the use of *P. major* leaves for wound healing properties in ‘De materia medica’ already in the first century (Samuelsen, 2000). He also prescribed the leaves of *P. major* for treatment of dog bites (Roca-Garcia, 1972). *Plantago major* was mentioned for its wound healing capacity by the Islamic author Ibn El Beithar in the 12–13<sup>th</sup> century (Fleurentin *et al.*, 1983). In the Northern countries, already the vikings used *P. major* leaves for wound healing purposes (Nielsen, 1969), and the Norwegian and Swedish people call this plant ‘groblad’, which means ‘healing leaves’ (Samuelsen, 2000). Leaves of *P. major* were commonly used in the time of Shakespeare and he has mentioned it in his play ‘Romeo and Juliet’, Act I, Scene II from the period 1592–1609:

Romeo: Your plantain leaf is excellent for that.

Benvoleo: For what, I pray thee?

Romeo: For your broken shin

*Plantago major* is thus a well-known medicinal plant in many parts of the world, and it has been claimed that superficial wounds can be healed by application of leaf juice (Brondegaard, 1987). Surprisingly few scientific studies have, however, been performed with the aim to clarify the reason for these wound healing properties.

## 1.1 Taxonomy

*Plantago major* belongs to the family Plantaginaceae. The name of the genus comes from the Latin word ‘planta’ which means ‘sole of foot’ referring to the basal rosette of the broad leaves touching the ground in most of the species (Pilger, 1937). There are about 483 species in the genus *Plantago* distributed throughout the world (Tutel *et al.*, 2005).

Several species of the genus *Plantago* have been used for different purposes in traditional medicine. It is therefore very important to be able to identify the different species properly. Taxonomic classification is mainly based on morphological and anatomical traits, and e.g. leaf size and shape are often associated with the commercial use and value of the plants. However, biochemical traits and molecular markers are frequently associated with the biological activities of the species and thus species grouped together based on molecular and biochemical markers most likely possess similar biological activities.

Species of the genus *Plantago* have been used as model species for different taxonomic, medicinal and genetic studies (Morgan-Richards & Wolff, 1999).

Different taxonomic treatments have been proposed to classify the genus *Plantago*. Rahn (1996) proposed the presently most used classification of the genus *Plantago* based on 90 morphological and anatomical characters. In this classification system, *Plantago* is divided into 6 subgenera: subgenus *Plantago*, *Coronopus* Rahn, *Albicans* Rahn (includes different parts of subgenus *Plantago* sensu Pilger 1937), *Psyllium* Juss. (sensu Pilger 1937, not Rahn 1978), *Littorella* Bergius (genus *Littorella* Bergius) and *Bougueria* Rahn. *Plantago major* belongs to subgenus *Plantago*.

*Plantago major* has mostly been reported to be diploid ( $2n=2x=12$ ) (Sharma *et al.*, 1992; Mohsenzadeh *et al.*, 2008; Ozturk *et al.*, 2009; Bala & Gupta, 2011) but there is at least one report about a tetraploid *P. major* ( $2n=4x=24$ ) accession (Bala & Gupta, 2011). Two subspecies, *P. major* subsp. *major* and *P. major* subsp. *intermedia*, are often acknowledged. Although morphologically similar, they are still distinct entities with different habitat requirements (Zhukova *et al.*, 1996). A third subspecies, *P. major* subsp. *winteri*, has also been recognized but there is not much research on this subspecies. *Plantago major* subsp. *major* and *P. major* subsp. *intermedia* have distinct cytotypes. Most of the karyotypes of *P. major* subsp. *major* are more symmetrical than karyotypes of *P. major* subsp. *intermedia*, indicating that *P. major* subsp. *intermedia* is the derived type (El-Bakatoushi and Richards, 2005). Based on a molecular marker-based study on genetic structure, Morgan-Richards and Wolff (1999) proposed that the two taxa should be treated as different species, *P. major* and *P. intermedia*. *Plantago major* subsp. *major* is reported to possess wound healing activities, and we have therefore used this taxon for all our studies.

## 1.2 Origin and distribution

*Plantago major* occurs naturally in Northern Europe and Central Asia but is now distributed and naturalized almost throughout the world. It is mainly a temperate-zone plant but it extends its distribution into more extreme areas to the north and south, almost from pole to pole. The species grows from sea level to an altitude of 3500 m (Sagar & Harper, 1964; Gomez-Flores *et al.*, 2000; Velasco-Lezama *et al.*, 2006), and is especially abundant in moist areas such as river beds, coastal areas, seepage areas on hillsides, and areas subjected to water runoff from buildings and along road sides (Webb *et al.*, 1988). Pollen studies show that this species arrived to the Nordic countries 4000 years ago (Jonsson, 1983). Literature reports of its presence in England date back to 1672 while a presence in Canada was documented in 1821. The American Indians named this species ‘white man’s footprint’ because it occurred everywhere

where the Europeans had been (Samuelsen, 2000). The large distribution of this species makes it easy to access for traditional uses, and suggests that it can be grown in different climatic regions and in greenhouses.

### 1.3 Biology

Species belonging to genus *Plantago* have different growth habits varying from spring annuals to summer annuals, biennials, and perennials. Evolutionary shifts have occurred repeatedly between the annual and the perennial habit, in both directions (Primack, 1976; Samuelsen, 2000; Stanisavljevic *et al.*, 2008). *Plantago major* has both perennial and annual growth habit (Samuelsen, 2000; Velasco-Lezama *et al.*, 2006). The perennial plants produce more biomass by allocating less energy to seed production and thus can be of importance for commercial purposes.

*Plantago major* has a short, stout and erect herbaceous stem on which leaves form a basal rosette, these leaves can reach a length of up to 30 cm (Sagar & Harper, 1964; Samuelsen, 2000). The shape of the leaves varies from ovate to elliptic with parallel venation (5–9 veins). The leaf blade is entirely or irregularly toothed, and narrows towards the leaf petiole, which is of almost equal length to the leaf blade. Leaves are normally green in color, sometimes with purple shading, and are glabrous or hairy on the surface (Samuelsen, 2000). The amount of biomass produced and total number of leaves is affected by growth form of the plant and growing conditions (Warwick, 1980; Rosenhauer, 2007). Application of nitrogen fertilizers and removal of flower stalk increase the biomass production of the plant (Rosenhauer, 2007). Prostrate individuals of *P. major* produce a significantly lower number of leaves compared to the erect individuals (Warwick, 1980). Studies have not yet been made to compare the contents of important bioactive compounds between the two growth forms. Biomass production is, however, very important for commercial purposes and therefore erect individuals are preferred.

*Plantago major* produces a large number of adventitious roots, which are of whitish colour. The roots may grow up to 1 m in length (Sagar & Harper, 1964). The growth form of the plant also affects the number of the roots. Prostrate individuals of *P. major* produce a significantly lower number of roots than do the erect individuals (Warwick, 1980).

Flowering time for *P. major* generally starts from May and ends in September in the temperate zone but it can vary depending on where the plants grow (Long, 1938). Normally plants start to flower at the approximate age of 13 weeks, but plants may flower and start setting seeds just 6 weeks after germination (Sagar & Harper, 1964; Warwick & Briggs, 1980). Early

flowering reduces the biomass production by allocating energy to seed production and therefore is not preferable for commercial production (Sharma *et al.*, 1992). Flowers of *P. major* are yellowish white to brownish green in colour and have a diameter of 2–4 mm. Flowers are protogynous (stigmas are exerted 1–3 days before anthesis) (Sharma *et al.*, 1992; Samuelsen, 2000; Mao-ye & Li-guo, 2011). Inflorescence of *P. major* is a spike, which is usually simple but very rarely branched; spikes grow up to 30 cm in length (Sagar & Harper, 1964).

The fruit of *P. major* is a capsule. Large numbers of 5 mm long capsules are produced on a spike. The number of capsules per cm of spike varies between 23 and 26. Number of seeds per capsule ranges from 4 to 15 (Samuelsen, 2000; Warwick & Briggs, 1980; Sagar & Harper, 1964). Significantly less seeds are produced by the prostrate individuals of *P. major* compared to the erect individuals (Warwick, 1980).

Seeds set within three weeks after flowering. *Plantago major* plants produce a large number of seeds (up to 20,000 per plant). The seeds are very small in size ( $0.4\text{--}0.8 \times 0.8\text{--}1.5$  mm) and have an ovate to elliptic shape. The shape of the seeds varies according to the number of seeds in the capsule. The endosperm forms the major part of the seed and surrounds the embryo completely. Due to the presence of polysaccharides in the seed coat, seeds swell when moistened and can become attached to animals and humans and thus be spread over large distances (Samuelsen, 2000; Kuiper & Bos, 1992; Sagar & Harper, 1964). Seed production reduces the biomass production and therefore is not preferred for commercial purposes.



Figure 1. *Plantago major* plant with inflorescences in a greenhouse at SLU Balsgård, Sweden.



Figure 2. *Plantago major* plants in a greenhouse at SLU Balsgård, Sweden.

## 1.4 Breeding system and genetic variation

Species belonging to the genus *Plantago* have a wide range of mating systems, from inbreeders to obligate outcrossers. *Plantago major* is wind-pollinated, self-compatible and highly inbreeding (Kuiper & Bos, 1992; Samuelsen, 2000). Outcrossing rate in *P. major* subsp. *major* (10–14%) is slightly higher than in *P. major* subsp. *intermedia* (3–6%). Both species exhibit low variation within populations and high variation among populations (Wolff, 1991; Squirrell & Wolff, 2001).

From the comparative studies on inbreeding and outcrossing species, it has become clear that in general, outcrossing species allocate much genetic variability within populations but show little differentiation between populations, whereas inbreeding species show the opposite pattern (Nybom & Bartish, 2000; Nybom, 2004). Populations of inbreeding species sometimes possess negligible genetic variability, and are then considered to be pure lines (Jain, 1976). The fact that *P. major* is self-compatible without signs of inbreeding depression is very important and can help to save time when developing inbred lines for research and/or commercial plant production.

Both the inbreeding *P. major* and the outbreeding *P. lanceolata* have shown a high degree of morphological differentiation between populations. It appears that besides the influence of the mating system, strong directional selection can decrease morphological variability in these two species (Wolff, 1991).

Genetic variation is a prerequisite for plant breeding. Variation for genes that are responsible for biomass production and for the production of bioactive chemical compounds involved in wound healing activities would be of interest for a breeding programme aimed at developing cultivars with superior characteristics.

## 1.5 Bioactive chemical compounds

Because of their natural origin and their potential use in functional food products and medicines, an increasing interest in phytochemicals has been noted in the last few decades. A large number of phytochemicals has also been reported to be present in the leaves, seeds and roots of *Plantago major*. These phytochemicals apparently have medicinal properties and can also be used as taxonomic markers (Samuelsen, 2000).

Both polysaccharides and polyphenols have been proposed to be bioactive compounds in *P. major* (Hetland *et al.*, 2000; Samuelsen, 2000). The antiviral activities of *P. major* have been reported to derive mainly from the phenolic compounds (Chiang *et al.*, 2002). Viral infections can be one of the reasons for

non-healing wounds. Phenols constitute a group of structurally related compounds containing a hydroxyl group (-OH) bound directly to an aromatic hydrocarbon group, and are present in many natural products. The phenols in natural products range from simple molecules such as the phenolic acids to highly polymerized, large polyphenolic compounds such as tannins (Jurisic Grubescic *et al.*, 2005).

Polyphenols potentially have a positive effect in protecting against certain diseases. The main source of these polyphenols is plants. The polyphenols have free radical scavenging ability and can thus act as antioxidants. This has been shown in several in vitro test systems, and is, in all likelihood, also relevant in nature. Polyphenols are considered to contribute to wound healing, and have antimicrobial and anti-inflammatory activity (Brantner & Grein, 1994).

Phenylethanoid glucosides are a group of phenolic compounds that are widely distributed in plants. Attempts have been made to use caffeoyl phenylethanoid glucosides also as taxonomic markers in the genus *Plantago* (Ronsted *et al.*, 2000; Li *et al.*, 2008).

Plantamajoside is the major known phenolic compound of phenylethanoid glucosides group in all the aerial parts of *P. major* (Zubair *et al.*, 2011, Paper I). Biological activities associated with plantamajoside include anti-inflammatory (Nishibe, 1994; Murai *et al.*, 1995; Samuelson, 2000; Turel *et al.*, 2009), free radical scavenging (Skari *et al.*, 1999) and antibacterial activities (Ravn & Brimer, 1988).

Verbascoside is another major phenolic compound of the phenylethanoid glucosides group that is present in *P. major*. The concentration of verbascoside is higher in seeds and flowering stalks of *P. major*, whereas the concentration of plantamajoside is higher in leaves (Zubair *et al.*, 2011, Paper I). Biological activities reported to be associated with verbascoside are antihepatotoxic (Xiong *et al.*, 1998), anticarcinogenic (Pettit *et al.*, 1990; Saracoglu *et al.*, 1997) and antiviral (Bermejo *et al.*, 2002). These compounds also act as protectants and repellents against herbivores in plants (Ravn & Brimer, 1988).

Flavonoids are another group of phenols found in *P. major*. The most common flavonoids in *P. major* are flavones (Kawashty *et al.*, 1994; Nishibe *et al.*, 1995). Attempts have been made to use flavonoids also as taxonomic markers in *Plantago* (Kawashty *et al.*, 1994).

Iridoid glucosides have also been used as valuable taxonomic markers in the subgenus *Plantago* and for the sections within this subgenus (Andrzejewska-Golec & Swiatek, 1984). Bartsioside and plantarenalloside are iridoid glucosides that are associated with subgenus *Psyllium* (Andrzejewska-Golec, 1997). Distribution pattern of the iridoids in *Plantago* have shown good correlation with the classification made by Rahn (1996) (Ronsted *et al.*, 2000).

The other iridoid glucosides that have been reported to be bioactive in *Plantago* are plantarenalioside, ixoroside, melampyroside (Afifi *et al.*, 1990), majoroside, aucubin, melittoside, 10-acetylaucubin (Andrzejewska-Golec & Swiatek, 1984; Ronsted *et al.*, 2003), asperuloside (Bianco *et al.*, 1984), 10-hydroxymajoroside, 10-acetoxymajoroside (Taskova *et al.*, 1999) and gardoside (Murai *et al.*, 1996).

Ronsted *et al.* (2003) performed a study on chemotaxonomy and evolution of *Plantago* and concluded that the common presence of 5-hydroxylated iridoids and caffeoyl phenylethanoid glucoside ( $\beta$ -hydroxyacteoside) in the two subgenera *Coronopus* and *Plantago* support a close relation between these taxa. They also concluded that compounds of majoroside type could be of taxonomic importance within subgenus *Plantago*. There are taxonomic differences in chemical composition between different subgenera, species and subspecies of genus *Plantago* and these differences are useful for taxonomic identification and selection of species that can be valuable for wound healing activities.

Triterpenic acid (specifically ursolic acid) from *Plantago* has been reported to be a bioactive compound possessing anti-inflammatory activities (Zacchigna *et al.*, 2009).

We have recently found four major novel polyphenolic compounds: PLMA 1 ( $C_{34}H_{44}O_{20}$  mw = 772.2 g/mol,  $\beta$ -D-Glucopyranoside, 2-(3,4-dihydroxyphenyl)ethyl 3-O- $\beta$ -D-glycopyranosyl-, 4-[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoate], 6-O-2,3,4,5-tetrahydroxycyclopentyl); PLMA 2 ( $C_{35}H_{46}O_{20}$  mw = 786.2 g/mol,  $\beta$ -D-Glucopyranoside, 2-(3,4-dihydroxyphenyl)ethyl 3-O- $\beta$ -D-glycopyranosyl-, 4-[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate], 6-O-2,3,4,5-tetrahydroxycyclopentyl); PLMA 3 ( $C_{23}H_{26}O_{11}$  mw = 478.1 g/mol,  $\beta$ -D-Glucopyranoside, 2-(3,4-dihydroxyphenyl) ethyl 4-[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoate] and PLMA 4 ( $C_{22}H_{20}O_{12}$  mw = 476.1 g/mol,  $\beta$ -D-Glucopyranosiduronic acid, 4-(3,5-trihydroxy-7-methoxy-4-oxo-4H-1-benzopyran-2-yl)phenyl). The biological activities of these compounds are hitherto unidentified (Zubair *et al.*, 2012, Paper II).

## 1.6 Medicinal uses of *Plantago major*

In response to disappointment with modern medicine, many botanical, especially herbal products have gained popularity for the treatment of a wide range of ailments and diseases such as common cold, skin disorders, wounds, hypertension, inflammation, tumours, insomnia, and even cancer (Blumenthal *et al.*, 2006). *Plantago major* has been used in traditional medicine for many centuries in several cultures around the world mainly for wound healing and



other associated activities (Samuelsen, 2000). The biological activities of *P. major* that have been reported so far are as follows:

#### 1.6.1 Anti-ulcerogenic activities

Ulcer is a kind of wound that can be defined as a break in the skin or the mucous membrane with loss of surface tissue, disintegration and necrosis of epithelial tissue. *Plantago major* leaf extracts have shown an anti-ulcerogenic effect against alcohol- and aspirin-induced gastric ulcer (Atta *et al.*, 2005; Than *et al.*, 1996). Yesilada *et al.* (1993) reported after a series of experiments in Turkey, that a combined methanol and water extract of *P. major* increased the inhibition of ulcer formation by 40% compared to the control group, while a water extract produced a 37% increase and a methanol extract a 29% increase compared to the control group.

Phipps and Mahmood (2006) also reported that *P. major* leaf extracts possess beneficial compounds that control ulcer formation induced by ethanol.

#### 1.6.2 Anti-inflammatory and immunomodulatory activities

Phenolic compounds from *Plantago* have immunomodulatory activities (Chiang *et al.*, 2003). Methanol extracts as well as isolated compounds from the aerial parts of *P. major* have shown anti-inflammatory activities using COX-1 and 12-LOX enzymes (Beara *et al.*, 2010). Extracts of *P. major* have also shown antinociceptive (reducing painful stimuli) effects in mice, which can be one of the factors responsible for the anti-inflammatory activities (Atta & El-Sooud, 2004). Protection against inflammatory mediators can thus be one of the ways in which extracts of *P. major* contribute in wound healing (Mahmood & Phipps, 2006).

#### 1.6.3 Antioxidant activities

Oxidative stress is one of the major factors causing chronic and degenerative diseases, including atherosclerosis, cancer and Parkinson's disease, and is also involved in aging (Halliwell, 2000; Young & Woodside, 2001). Antioxidant substances possess the ability to protect the body from damages caused by free radical-induced oxidative stress (Souri *et al.*, 2008). Antioxidants can be effective in prevention of the free radical formation by scavenging and suppression of disorders mentioned above (Halliwell, 2000; Young & Woodside, 2001). A number of medicinal plants are promising sources of potential antioxidants (Souri *et al.*, 2008), including green leaves and leaf-based tea of *P. major* (Campos & Lissi, 1995) as well as ethanol extracts of *P. major* leaves (Mohamed *et al.*, 2011). Phenols have been reported to be the main

bioactive compounds responsible for antioxidant activities of *P. major* (Souri *et al.*, 2008; Stanisavljevic *et al.*, 2008; Beara *et al.*, 2009). Environmental factors such as altitude affect the antioxidant activities differently in roots and leaves of *P. major*; antioxidant activities of roots increase with an increase in altitude whereas antioxidant activities of leaves instead decrease (Argueta *et al.*, 1994; Ren *et al.*, 1999). Oxidative stress can slow down the healing process for internal wounds such as stomach ulcer and can also cause problems in controlling inflammation. However, a biochemical energy supply is a basic requirement for the wound healing process, and oxygen is essential for the production of biological energy equivalents (e.g. ATP). Therefore, a balanced and sufficient oxygenation of tissue is a prerequisite for adequate energy levels and thus for the wound healing process (Schreml *et al.*, 2010).

#### 1.6.4 Antiviral and antimicrobial activities

Leaf extracts and certain pure compounds from the leaf extracts of *P. major* possess antiviral activities. Phenolic compounds are the major bioactive compounds found in extracts of *P. major* that exhibit potent anti-herpes and anti-adenoviral activities (Chiang *et al.*, 2002).

Extracts of *P. major* have shown antimicrobial activity against yeasts and bacteria (Stanisavljevic *et al.*, 2008). Leaves have also traditionally been used for the treatment of skin bacterial infections (Holetz *et al.*, 2002). Viral and microbial infections can increase the time needed for wound healing, therefore controlling viral and microbial infections can help in the healing process.

#### 1.6.5 Anticarcinogenic activities

Leaf extracts of *P. major* have been utilized for treatment of skin cancer (Samuelsen, 2000). Lithander (1992) reported that an aqueous extract of *P. major* showed a prophylactic effect on mammary cancer in mice. In this study, a leaf-derived extract was injected subcutaneously in mice that had developed cancer. After 60 weeks, only 18.2% of the treated mice had tumours as compared to 93.3% of the untreated. Crude extracts of *P. major* have also shown inhibitory effects on Ehrlich ascites carcinoma (EAC). *Plantago major* is registered and recommended by the National Cancer Institute (NCI, USA). *Plantago* extracts have cytotoxic effects on breast adenocarcinoma and melanoma cell lines (Galvez *et al.*, 2003). Recently it has also been reported that hot as well as cold extracts from *P. major* leaves and seeds possess anticarcinogenic activities (Mohamed *et al.*, 2011).

### 1.6.6 Wound healing activities

Clinical experiences with *P. major* leaf extracts indicate that there is a promotion of the wound healing process. However, the mechanisms explaining the potential healing properties have hitherto been obscure (Mahmood and Phipps, 2006; Velasco-Lezama *et al.* 2002; Amini *et al.*, 2010; Zubair *et al.*, in press, Paper IV). An aqueous extract of *P. major* leaves stimulated wound healing in vivo using a rat model (Mahmood and Phipps, 2006). In another study, methanol and aqueous extracts of *P. major* leaves showed stimulating effects on the healing of burn wounds in rats (Amini *et al.*, 2010). Leaf extracts have also shown enhancement of cell proliferation and migration in vitro (Velasco-Lezama *et al.* 2002; Zubair *et al.*, in press, Paper III). The cell proliferation and migration are crucial parts of the wound healing process. The German Commission E has approved *P. major* for internal use to ease coughs and mucous membrane irritation associated with upper respiratory tract infections, and in topical use for skin inflammation and superficial wounds (Blumenthal, 1998).

## 2 Objectives

The objectives of this study were

1. To determine what aerial plant organs are most useful for production of a specific polyphenolic compound, and to determine the effects of different drying temperatures on the content of major polyphenolic compounds in *P. major* leaves.
2. To use DNA markers for investigating genetic variation, among and within populations, as well as level of homogeneity in offspring after open pollination and after selfing.
3. To investigate variation in the contents of polyphenolic compounds of *P. major* originating from different populations and subpopulations.
4. To assess the effect of different extracts of *P. major* leaves on cell proliferation/migration in vitro.
5. To assess the wound healing properties of *P. major* leaf extracts using an ex vivo wound healing porcine model.
6. To assess the anti-inflammatory activities of different extracts of *P. major* in vitro.

## 3 Material and methods

### 3.1 Plant material

Seeds of *Plantago major* were collected from 5 wild plants at 5 localities (each locality was considered as one population) in Sweden in 2005: from Helmershus and Dalby (both in Skåne, southernmost Sweden), Blekinge (southeastern Sweden), Västergötland (western Sweden) and Stockholm (eastern Sweden) (Table 1). Seeds from each plant were kept in a separate seed bag, and sown in separate seed trays.

**Table 1.** Collection locations of *P. major* seeds with latitude and longitude.

Population	Area of collection	Latitude	Longitude
2	Helmershus, Skåne	56°1'N	14°2'E
4	Dalby, Skåne	55°6'N	13°3'E
5	Verkö, Blekinge	56°2'N	15°2'E
10	Värmdö, Stockholm	59°2'N	18°3'E
12	Gråbo, Lerum, Västergötland	57°7'N	12°0'E

In paper I, seeds from one plant (P2-5) at Helmershus and from two plants (P4-4 and P4-5) at Dalby were sown in a greenhouse and leaves of the emerging plants were sampled and screened for content of total phenols (Rosenhauer, 2007). Selected plants from these two populations, with comparatively high contents of total phenols, were then isolated by use of perforated bags to produce selfed seeds. Presumably very homogenous

offspring families P2-5a4, P2-5a10, P4-4a3, P4-5b7 and P4-5b10, obtained from those seeds, were chosen for the study.

All the five populations were sown in a greenhouse and were used in paper II. Germinating seeds were obtained from all the plants except from one plant in population 5 (Blekinge) and three plants in population 10 (Stockholm). Plants germinated from one mother plant were treated as one subpopulation. From each subpopulation 10 plants were selected for DNA analysis and 7 plants for HPLC analysis.

In paper III, seeds from population 4 (Dalby) were collected after two generations of selfing. These seeds were then sown in the greenhouse and leaves of the emerging plants were used.

In paper IV, leaves of the plants emerging from population 12 (Gråbo, Lerum, Västergötland), were used for the first and second experiments, while leaves of one-generation selfed plants of this population were used for the third experiment.

In paper V, leaves of the plants one-generation selfed plants of population 12 (Gråbo, Lerum, Västergötland), were used.

## 3.2 DNA analysis

### 3.2.1 DNA extraction and amplification

In paper I and II, DNA was extracted from young leaf tissue (10 µg) using the E.Z.N.A.<sup>TM</sup> SP Plant DNA Mini Kit (VWR, Stockholm) following the manufacturer's protocol. The concentration and size of DNA was visually estimated after staining with 3 µL of ethidium bromide using the DNA low mass ladder<sup>TM</sup> (Invitrogen, Life Technologies Carlsbad, CA, USA) by electrophoresis in 2% agarose gels.

Genomic DNA was amplified using a standard polymerase chain reaction (PCR) protocol, briefly as follow: 45 cycles of 94 °C for 15 s, 36 °C for 45 s, 72 °C for 1.5 min. Amplification was done in a final volume of 25 µL, which consisted of approximately 30 ng of genomic DNA, 2.5 µL of 10X reaction buffer, 0.5 µL of DNTPs with 10 mM of each DNTP, 1.6 µL of 25 mM MgCl<sub>2</sub>, 0.2 µL of 5 units/µL *Taq* DNA polymerase (Thermo Fisher Scientific, Surrey) and 1.0 µL of primer (5 µM) (Operon Biotechnologies, Cologne, Germany).

### 3.2.2 Data evaluation and statistical analysis

Based on readability, reproducibility and polymorphism of the obtained bands, primers were selected for RAPD analysis in paper I and paper II. Scoring was

done to produce binary data matrices with presence of band as “1” and absence of band as “0”.

For paper I, inter-sample similarities within and between offspring families were obtained with Nei's unbiased measure of genetic distances with the software package Popgene, version 1.31. Only polymorphic bands were included in the analysis.

For paper II, a similarity matrix was created with Jaccard's similarity index (Jaccard, 1908) and a dendrogram was constructed based on this similarity matrix data by applying a UPGMA cluster analysis, with the software package NTSYS-pc, version 1.80 (Rohlf, 1993). Analysis of molecular variance (AMOVA) was performed to partition the total molecular variance between populations, between subpopulations and within subpopulations with Arlequin version 3.01 software programme (Excoffier *et al.*, 2005). A population genetic analysis was performed to estimate the genetic variation between populations ( $G_{st-pop}$ ), between subpopulations within populations ( $G_{st-subpop}$ ) and within subpopulations  $H_s$ , with the Popgene software package version 1.32 (Yeh *et al.*, 1997).

### 3.3 HPLC analysis

#### 3.3.1 Sample preparation

For paper I, aerial plant parts (leaves, flower stalks and seeds) of *P. major* were harvested from each offspring family separately. Samples were then pooled inside each family and three technical repeats were used from each family for further analysis. The samples were freeze-dried and the dried samples were then ground (IKA Analytical mill A10, IKA Labortechnik, Janke & Kunkel GmbH & Co. KG, Staufen, Germany) to form a fine powder to compare the polyphenol contents in different plant organs. Leaves were also harvested to study the effect of different leaf drying temperatures on the contents of polyphenols. For this study leaves were freeze-dried as well as dried by convection at 30 °C, 40 °C and 50 °C.

For paper II, leaves were harvested separately from 7 of the exactly same plants as in the molecular marker analyses. The samples were freeze-dried and ground as in paper I. The samples were analysed individually.

In paper I and II, 25 mg of the ground sample was extracted in 1.2 mL of 50% ethanol and 50% water solution in an ultrasonic bath for 15 min. The solution was centrifuged at 13 000 rpm for 10 minutes, and 1 mL of the supernatant was used for analysis without any further sample clean up.

For paper III, the leaves were freeze-dried and ground as in paper I. For making the extracts, 10 g of the ground sample were extracted in 100 mL of

50% ethanol while another 10 g of the powder were extracted in 100 mL of distilled water. The solvents were removed by a vacuum evaporator at 30 °C and dissolved in sterilized PBS. These samples were then used for HPLC analysis and for a scratch assay.

### 3.3.2 HPLC analysis

For all the papers (I, II and III), the HPLC analyses were performed using diode array detection. A Synergi hydro-RP 80A (250 x 4.60 mm, 4 $\mu$ ) column (temperature was set to 24 °C and the injection volume was always 10  $\mu$ L) was used for the separation of polyphenols. A gradient at a flow rate of 1 mL/min was used to elute the compounds. The mobile phases in HPLC consisted of a solution of acetic acid (1%) and acetonitrile (5%) in water (solution A), and a solution of methanol (5%) in acetonitrile (solution B). Solution A was filtered through a 3  $\mu$ m isopore membrane before use. The gradient was as follows: 82% A, 18% B for 0–10 min; 79% A, 21% B for 10–13 min; 20% A, 80% B for 13–14 min; 82% A, 18% B for 14–16.1 min and then 82% A, 18% B to the end of the run (18 min). Quantification of all the polyphenolic compounds was performed at 328 nm following optimization of the conditions.

### 3.3.3 Statistical analysis

For all the studies in papers I, II and III, the contents of polyphenolic compounds were calculated as milligram per gram of dried plant tissue (mg/g dw) using a standard curve. Microsoft Excel software was used to make the calculations and prepare the figures. Minitab 16.0 (Minitab, State College, PA, USA) software was used for the analysis of variance and Tukey's test.

### 3.3.4 Identification of novel polyphenolic compounds

The high mass accuracy MS and tandem mass spectrometry (MS/MS) analysis was performed to elucidate the structure of the novel polyphenolic compounds as described in paper II. The samples were injected (eight  $\mu$ L) onto a 4.60 x 250 mm 4.0  $\mu$ m Synergi C18 column (Phenomenex) connected to a Thermo Accela LC system. For the chromatographic separation solutions A, which consisted of 1% formic acid in 5% acetonitrile / 94% water, and B, which consisted of 5% methanol acid in acetonitrile. The flow-rate of 500  $\mu$ L min<sup>-1</sup> was used to elute the compounds, using a mobile phase composed of 18–21% B (0–11 min), 21–80% B (11–15.5 min), and finally 80% B for 3.5 min. The mobile phase was then changed to 18% B after 21 min and the column was equilibrated for 3 min before the injection of next sample.



## 3.4 Scratch assay

### 3.4.1 Experimental procedures

For paper III, the oral epithelial cells were maintained and cultured for assay in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and L-glutamine (Sigma; final concentration 2mM), at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> (Heraeus Instruments GmbH, Germany).

The cells were seeded into 12-well cell culture plates at a concentration of  $2 \times 10^5$  cells/mL, to produce a nearly confluent cell monolayer. A linear wound was generated in the cell monolayer using a sterile 200  $\mu$ L plastic pipette tip (approximately 1 mm wide scratch). All the cellular debris was removed by washing the wells with sterile PBS. DMEM medium (1 mL) supplemented with 10% FBS and 10  $\mu$ L of PBS was used as a negative control and DMEM medium (1 mL) supplemented with 10% FBS and platelet derived growth factor (PDGF) (5 ng/mL) was used as positive control. Three different extracts (water and ethanol-based extracts, respectively, of dried leaves, and a water-based extract of fresh leaves) as well as one combination (equal volumes of the two extracts of dried leaves) were tested, each in three different concentrations: 10.0, 1.0 and 0.1 mg/mL. These plant extracts were added to a set of 3 wells per dose.

### 3.4.2 Data evaluation and statistical analysis

To evaluate the results, digital pictures (1712 x 1368 pixels) were taken at different time intervals (0 hour, 4 hours, 8 hours, 18 hours and 24 hours) after creation of the scratches. The pictures were then evaluated using Image J 1.44o (Wayne Rasband, National Institute of Health, USA) software for Mac. The width of the scratch was measured (expressed in pixels) at each time interval (4 hours, 8 hours, 18 hours and 24 hours) and compared to the width of the scratch immediately after the creation of the scratch (0 hour). Observations were taken from 100 measurements for each experimental unit for each time interval.

Statistical analyses of data from the scratch assay were carried out using Minitab 16 software (Minitab, State College, PA, USA) and a graph was made using Microsoft Office Excel 2008 for Mac.

## 3.5 Porcine wound healing model (patent no 10317400)

### 3.5.1 Experimental procedures

For paper IV, three sets of experiments were carried out at the Department of Dermatology and Venereology, University Hospital Hamburg-Eppendorf, Germany. Pig ears were obtained for the experiments from a local slaughterhouse in Hamburg, Germany. All the pigs were of the same type (crossbred Yorkshire/Deutsches Edelschwein) and 6 months of age. The ears were properly washed after arrival in the laboratory and disinfected. Punch biopsies were taken (6 mm of diameter) from the plicae of the ear. The parts of dermis, subcutis and fats were removed from the punch biopsies. Wounds were created in biopsies by removing the epidermis and dermis from the centre of each biopsy (a circle of 3 mm diameter). These biopsies were then placed on gauze in a 12-well culture plate filled with Dulbecco's modified Eagle's medium (DMEM) supplemented with hydrocortisone, fetal calf serum, penicillin, and streptomycin in a way that epidermis was exposed to the air and only the dermis was in contact with the medium. The biopsies were treated with PBS (negative control) and three different concentrations (1.0, 0.1 and 0.01 mg/mL of dry weight) of both water and ethanol-based *P. major* leaf extracts directly to the wounds. The culture dishes containing biopsies were incubated at 37 °C with 10% CO<sub>2</sub>. After 24 hours of incubation, biopsies were again treated with a second dose of the same plant extracts and incubated for another 24 hours. The biopsies were frozen in isopentane pre-cooled with liquid nitrogen and then stored at -80 °C until evaluation.

### 3.5.2 Data evaluation and statistical analysis

Reepithelisation at both wound margins was evaluated under light microscope (Leica, Wetzlar, Germany). For evaluation, a score was used in which no progress in wound healing was indicated by 0, sparse progress in wound healing by 1, large progress in wound healing by 2, regeneration of a monolayer of epidermis by 3, and regeneration of a multi-layered epidermis was indicated by 4, and all values inbetween were also applicable (e.g. 1.1, 1.2, 1.3, ...). Data were normalized to the standard (PBS) before statistical analysis.

The experiment was repeated three times at different time intervals between 2008 and 2011, and each time the experiment was performed in triplicate (one pig was used as one replication). Statistical analyses were performed using Minitab software version 16 (Minitab, State College, PA, USA) and Microsoft Office Excel 2008 for Mac was used to make a graph. Significance of the differences between the extracts and the control was revealed using paired Student's t-test.

## 3.6 NF- $\kappa$ B assay

### 3.6.1 Experimental procedures

For paper V, the oral epithelial cells were maintained and cultured for assay as in paper III. The cells were seeded into 96-well cell culture plates at a concentration of  $5 \times 10^4$  cells/mL, to produce a nearly confluent cell monolayer. DMEM medium (147  $\mu$ L) with 3  $\mu$ L of PBS was used as a negative control for the study. Water and ethanol-based extracts as well as one combination (equal volumes of the two extracts derived from dried leaves) were tested for anti-inflammatory activities, each in three different concentrations: 1.0, 0.1 and 0.01 mg/mL. These plant extracts were added to a set of 6 wells per dose and incubated for 47 h at 37 °C with 5% CO<sub>2</sub>. After 47 hour of treatment with plant extracts and negative control, three wells of each concentration of both plant extracts and negative control were treated with 0.02 ng/mL of LPS and incubated for 1 hour. The cells were then washed twice with PBS for 2 minutes and fixed with 1% formaldehyde, and finally sent to Imagen Biotech Ltd Manchester, UK, for evaluation.

## 4 Summary of Results and Discussion

### 4.1 Genetic variation in *P. major*

For paper II, the main objective was to estimate amount and partitioning of genetic variation in five different populations of *P. major* using RAPD markers, and to investigate a possible association between genetic and biochemical variability. RAPD markers were used due to the technical simplicity and cost-effectiveness, and the previously proven ability to estimate genetic diversity in *P. major* (Wolff, 1991; Morgan-Richards & Wolff, 1999). Other molecular markers that have been used in *P. major* are allozyme and microsatellite markers (Wolff, 1991; Morgan-Richards & Wolff, 1999; Squirrell & Wolff, 2001; Hale & Wolff, 2003).

The RAPD analyses showed that levels of differentiation among populations appear to be associated with the geographic distance between populations in our study. A cluster analysis produced two major clusters, one with the two populations sampled in Skåne and the other with the remaining three populations, sampled in Blekinge, Stockholm and Västergötland (Fig. 3). These two major clusters were then divided into 2 and 3 subclusters, respectively, that corresponded perfectly with the origination of the samples from the different populations.

Genetic variation between the five populations was calculated as  $G_{st} = 0.801$ . Variation among subpopulations within populations was also estimated, and reached 0.747 in population 2, 0.844 in population 4, 0.600 in population 5, 0.374 in population 10, and 0.836 in population 12. The mean within-subpopulation variation calculated across all 19 subpopulations was  $H_s = 0.022 \pm 0.0055$ .

As expected from previously published studies in inbreeding species, the DNA marker data obtained in our study show that a high proportion of genetic variation in *P. major* occurs between populations rather than within

populations. Similar results were obtained also in the previous studies on *P. major*, based on morphological, biochemical and molecular markers (Wolff, 1991; Morgan-Richards & Wolff, 1999; Squirrell & Wolff, 2001). This shows that it will be quite easy to develop a genetically homogeneous plant material for commercial production of selected lines (cultivars) of *P. major*.

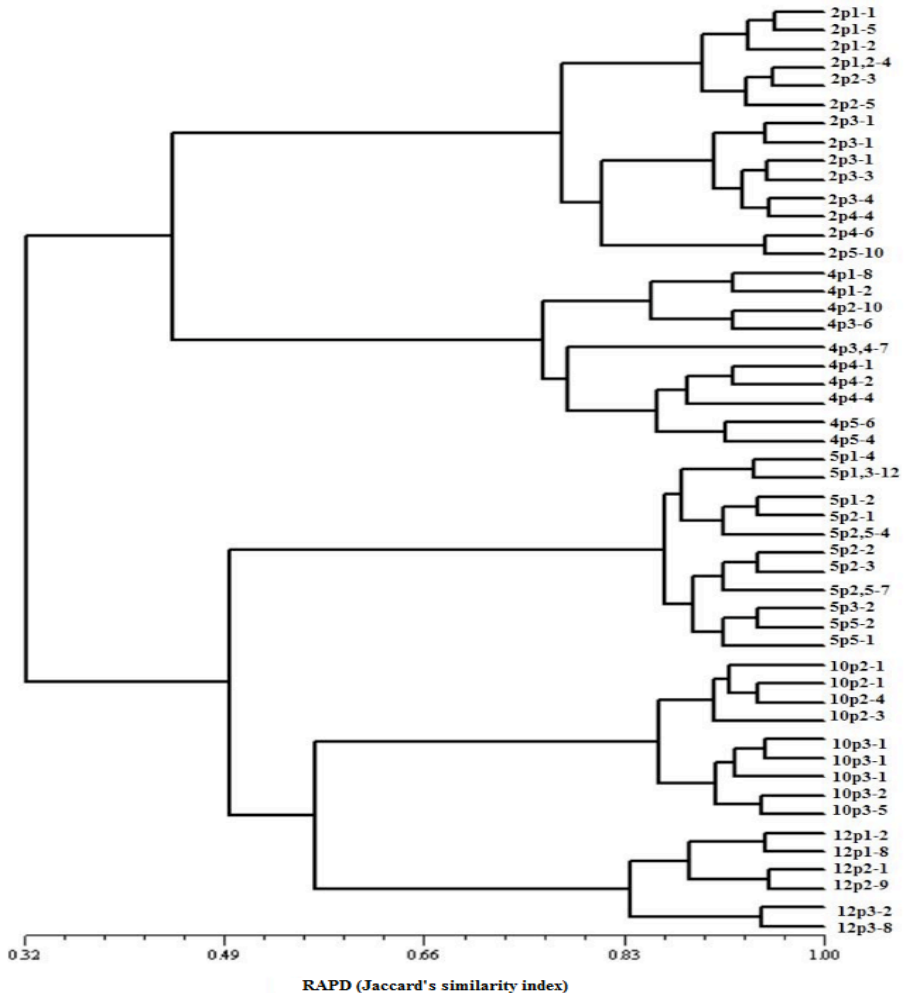


Figure 3. UPGMA dendrogram of *Plantago major* based on Jaccard's similarity index using RAPD data, showing two major clusters corresponding to geographic origination. The first number on the label represents population, the second number represents subpopulation and the third number represents the number of individuals sharing the same RAPD profile.

## 4.2 Phytochemical variation in *P. major*

For paper II, the objective was to investigate variation, among and within populations, in the contents of polyphenolic compounds of *P. major* and to investigate a possible association with genetic variability. The dendrogram produced from a cluster analysis based on polyphenol contents, showed very little similarity with the dendrogram obtained with molecular markers (Fig. 4). The difference in dendrograms based on contents of phenolic compounds and DNA markers, respectively, suggests that the expression of the studied polyphenolic compounds is affected by, e.g., epigenetic inheritance or a very early environmental influence on the newly germinated seedlings, before randomization. The dendrogram produced only a few subclusters where subpopulations from the same population occurred closely together, like 2-1 and 2-4, 5-1 and 5-3, 4-2 and 4-5, and 5-2 and 5-5, with the latter being inseparable. These results suggests that the selection of proper cultivation conditions along with proper cultivar is very important for commercial production of *P. major*.

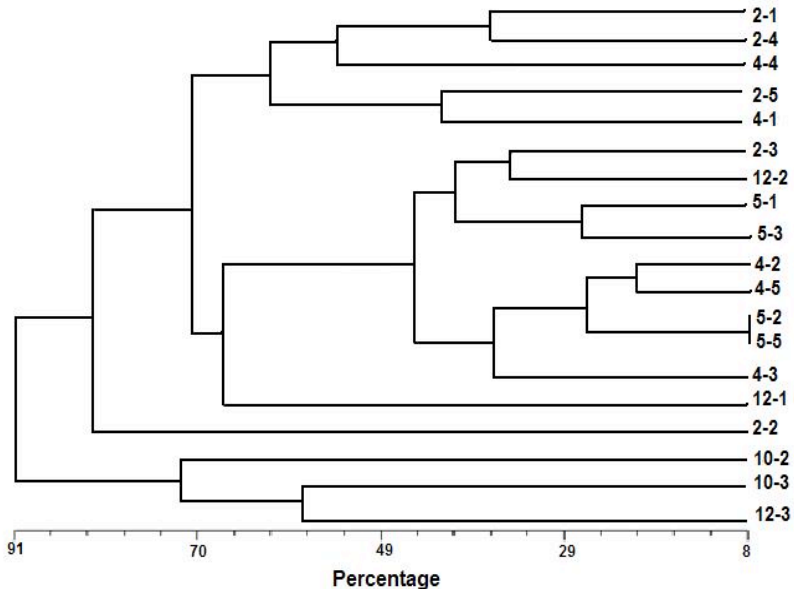


Figure 4. UPGMA dendrogram of subpopulations from five different populations of *Plantago major* based on Jaccard's similarity index, screened for the content of five polyphenolic compounds. The first number on the label represents the population and the second number represents the subpopulation.

## 4.3 Effects of environmental factors on the contents of polyphenols

### 4.3.1 Effects of drying temperature

For paper I, one of the objectives was to evaluate the effects of different drying temperatures on the contents of polyphenolic compounds in *P. major* leaf extracts.

There was a clear tendency that higher drying temperatures reduced the contents of each polyphenolic compound investigated in our study (Table 2). The content of P1 (PLMA 2) was 58% higher in freeze-dried samples compared to samples dried at 50 °C, content of plantamajoside was 68% higher, and content of P3 (PLMA 4) was 52% higher in average. There were no significant differences between different drying treatments for the contents of P1 and P3, whereas content of plantamajoside was significantly reduced at 50 °C.

Many of the phytochemicals, including some polyphenols, are heat sensitive. The findings of our study are in agreement with results from a previous study on bioactive compounds in *P. lanceolata* L. (narrow leaf plantain) being sensitive to different drying treatments (Tamura & Nishibe, 2002). As compared to fresh biomass, iridoideglycosides catapol and aucubin decreased by 50% and 25%, respectively, when leaves of *P. lanceolata* were dried for 8 h at 60 °C, and verbascoside (acetoside) decreased by 29%. Our study showed that the contents of polyphenolic compounds in *P. major* leaves decreased with an increase in drying temperatures, and that the highest contents were obtained in the freeze-dried samples. Since the freeze-drying process is very expensive for large-scale production, drying may have to be carried out in a regular convection dryer instead, preferably set at no higher than 30 °C.

**Table 2.** Tukey's *post hoc* test ( $p = 0.05$ ) for differences in content of polyphenols (mg/g of dry weight) effected by different drying temperatures in *Plantago major*. (For the contents of each polyphenol, significance among treatments is indicated by different letters).

Polyphenol	Freezedrying	30 °C	40 °C	50 °C
PLMA 2*	1.21 a	1.16 a	1.07 a	0.76 a
Plantamajoside	14.46 a	13.48 a	12.08 ab	8.56 b
PLMA 4*	0.41 a	0.26 a	0.21 a	0.27 a

\*PLMA 2 and PLMA 4 as in paper II.

#### 4.3.2 Effects of selection of different aerial plant organs

For paper I, one of the objectives was to determine differences among aerial plant organs in the contents of a specific polyphenolic compound.

The results obtained from the HPLC chromatogram of extracts from different aerial plant organs revealed nine polyphenolic compounds. Six of these compounds occurred in minute quantities (less than 100  $\mu\text{g/g dw}$ ) and were therefore difficult to quantify properly with our analytical procedures. The three major phenolic compounds consisted of plantamajoside ( $\text{C}_{29}\text{H}_{36}\text{O}_{16}$  mw = 640.6 g/mol), verbascoside ( $\text{C}_{29}\text{H}_{36}\text{O}_{15}$  mw = 624.6 g/mol) and PLMA 2. Of these compounds, plantamajoside was the most prominent in all aerial plant organs except in the seeds. Verbascoside was the most prominent phenolic compound in seeds, and the second most prominent in flower stalks.

There was considerable variation between different aerial plant organs regarding the content of these three compounds (Table 3). Our study is in agreement with Gray *et al.* (2003), who stated that polyphenols are usually not evenly distributed within the plant, and that contents are also affected by age of the plant used for the analysis.

Our study showed that selection of the appropriate plant organ is crucial for obtaining optimum amounts of a specific polyphenolic compound. These results may be helpful for future selection of plant material for commercial purposes. Annual plants, which start seed setting within 6–8 months after germination, are quick to produce flower stalks and seeds and will yield the highest concentration of verbascoside, whereas perennial plants are more suitable for leaf production in order to obtain high contents of PLMA2 and plantamajoside.

**Table 3.** Tukey's *post hoc* test ( $p = 0.05$ ) for differences among different plant organs of *Plantago major* in content of major polyphenols (mg/g of dry weight). (For each polyphenol, significance among plant organs is indicated by different letters).

Polyphenol	Young leaves	Old leaves	Flower stalks	Seeds
PLMA2	0.31 b	0.74 a	0.16 c	0.02 d
Plantamajoside	10.87 a	3.71 b	13.03 a	0.17 b
Verbascoside	0.02 b	0.01 b	2.30 a	2.28 a

\*PLMA 2 as in paper I.



#### 4.4 Effects of *P. major* extracts on cell proliferation and migration

For paper III, the objective was to assess the effect of different *P. major* leaf extracts on cell proliferation/migration in vitro using a cell scratch assay and oral epithelial cells (OEC).

Numerous in vitro, in vivo and ex vivo assays have been proposed, and are now available and used for evaluation of the wound healing process in mammalian skin. Among these, the scratch assay is one of the most valuable and inexpensive method to study and quantify processes that belong to the second phase of wound healing, namely the proliferation and migration of cells. This method is very helpful for investigation and comparison of the various factors that influence the wound healing process including the formation of new tissue (Liang *et al.*, 2007).

The effects of three different concentrations (10.0, 1.0 and 0.1 mg/mL) of water extracts (both fresh and dried leaves) and ethanol-based extracts (dried leaves) of *P. major* as well as a combination of the two dried leaf-containing extracts on cell proliferation/migration, was studied under the established optimum conditions. The study revealed significant differences among the different extracts (Fig. 5). Most of the extracts tested in our study increased the proliferation/migration of the OEC compared to the negative control. A concentration of 1.0 mg/mL (on dry weight basis) appears to be optimal regardless of type of extract, and among the other concentrations, 0.1 mg/mL was always better than 10 mg/mL.

The results are in agreement with Velasco-Lezama *et al.* (2005), who reported that the aqueous and methanol-based extracts of *P. major* possess in vitro cell-proliferation activities. From our study it can be concluded that the extracts of *P. major* leaves stimulate the cell proliferation and migration and thus stimulate the wound healing process in vitro.

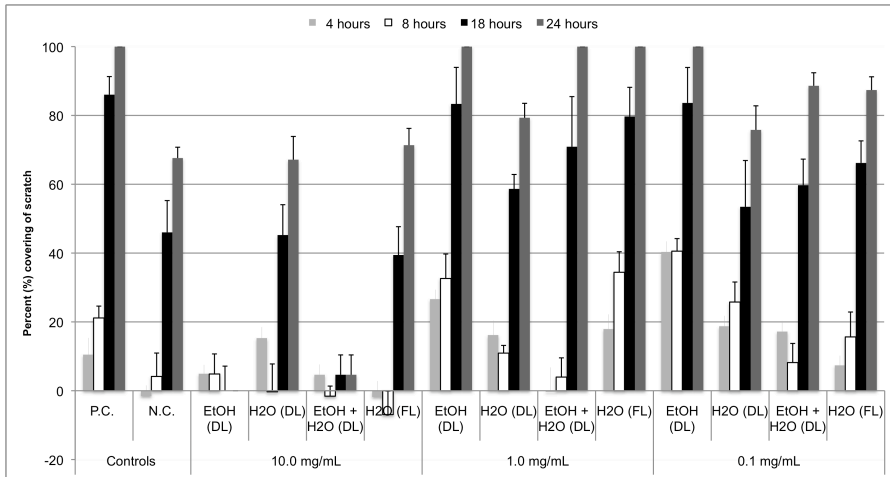


Figure 5. Effects of *P. major* leaf extracts of both fresh leaves (FL) and dried leaves (DL) on cell proliferation/migration in vitro using a cell scratch assay. Phosphate buffer saline (PBS) was used as negative control (N.C.) and platelet derived growth factor (PDGF) as positive control (P.C.). Bars represent the mean  $\pm$  standard error of mean SEM.

#### 4.5 Ex vivo wound healing properties of *P. major*

In paper IV, the objective was to assess the wound healing properties of *P. major* leaf extracts using an ex vivo wound healing porcine model.

The bioactive compounds from plant origin that stimulate the wound healing process need to be tested in an appropriate manner. Immediate clinical studies on the effects of novel substances on the wound healing process in humans are not acceptable due to ethical restrictions. Several different in vitro, in vivo and ex vivo models have therefore been proposed and are currently being used for initial investigations of the effects of different bioactive compounds on wound healing. The most ideal wound healing model should be as similar to the human skin as possible. An ex vivo porcine wound healing model is so far considered to be one of the best models for initial investigation of the effects of bioactive compounds (Brandner *et al.*, 2006; Brandner *et al.*, 2008; Pollok *et al.* 2011).

Both water- and ethanol-based extracts of *P. major* leaves showed stimulating effects in the wound healing process in the ex vivo porcine wound healing model (Fig. 6). There was a clear dose response pattern in the stimulation of the wound healing process for the different concentrations of the leaf extracts. The highest wound healing activities were observed in samples

treated with ethanol-based extracts with a concentration of 1.0 mg dry weight of *P. major* leaves per mL of phosphate buffer saline (PBS).

These results are in line with the previous studies and reports in which both fresh leaves and extracts of *P. major* leaves have shown wound healing properties in different models (Gorin *et al.*, 1966; Brondegaard, 1987; Samuelson, 2000; Mahmood & Phipps, 2006; Amini *et al.*, 2010). From the results of our study, it can be concluded that the extracts of *P. major* leaves contain bioactive compounds that can stimulate the wound healing activities in an ex vivo wound healing model.

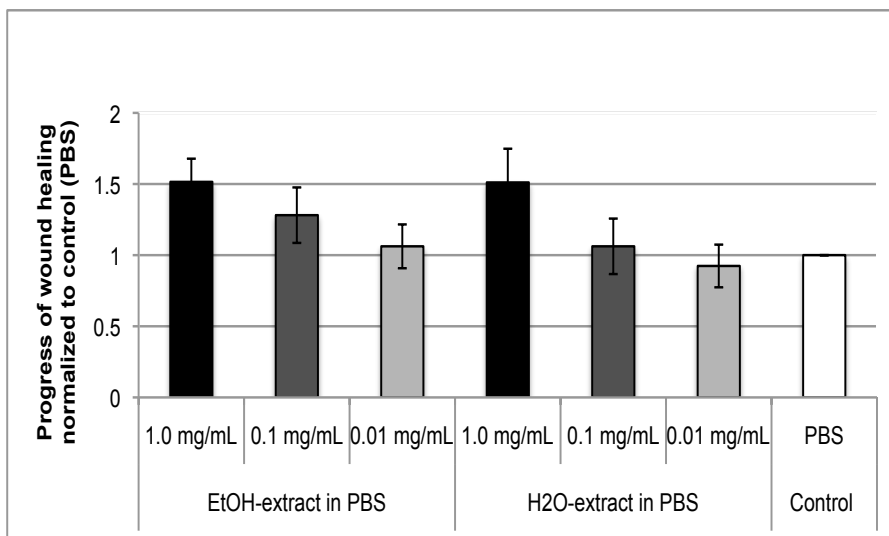


Figure 6. Effects of water and ethanol-based extracts of *Plantago major* leaf (dissolved in PBS) in three different concentrations; 1.0, 0.1 and 0.01 mg/mL, on wound healing activities in an ex vivo porcine wound healing model. PBS was used as control. For evaluation a 5-step score was used, 0: no progress in wound healing, 1: sparse progress in wound healing, 2: substantial progress in wound healing, 3: regeneration of a monolayer of epidermis, and 4: regeneration of a multi-layered epidermis. All values in between were also applicable. The results were then normalized to the PBS standard. Bars represent the mean  $\pm$  S.E.M. (the mean is taken from the triplicates of three experiments).

#### 4.6 In vitro anti-inflammatory activities of *P. major*

In paper V, the objective was to assess the anti-inflammatory activities of different extracts of *P. major* in vitro.

Numerous in vitro, in vivo and ex vivo assays have been proposed, and are now available and used for evaluation of the anti-inflammatory activities of plant extracts. Among these, the NF-kB assay is one of the most valuable assays to study and quantify the movement of nuclear factor kB. This method

is very helpful for investigation and comparison of the various factors that influence the anti-inflammatory activities of crude plant extracts and pure compounds (Milward *et al.*, 2007).

The anti-inflammatory activities of three different concentrations (1.0, 0.1 and 0.01 mg/mL) of both water- and ethanol-based extracts of *P. major* leaves as well as a combination of both extracts were studied under the established optimum conditions. The study revealed significant differences among the different concentrations of plant extracts (Fig. 7). Most of the extracts tested in our study showed increased anti-inflammatory activities compared to the negative control except for the lowest concentration (0.01 mg/mL). A concentration of 1.0 mg/mL (on dry weight basis) appears to be optimal regardless of type of extract.

The results are in accordance with Beara *et al.* (2010), who reported that the methanol extracts of *P. major* leaves as well as compounds isolated from the leaves possess *in vitro* anti-inflammatory activities. Anti-inflammation is one of the major factors involved in the wound healing process. Anti-inflammatory activities can therefore be one of the ways in which extracts of *P. major* contribute in the wound healing process (Mahmood & Phipps, 2006).

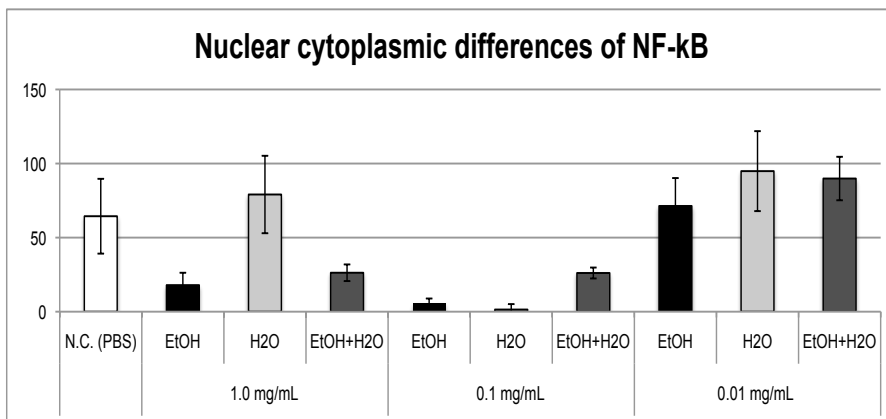


Figure 6. Anti-inflammatory activities of water and ethanol-based extracts of *Plantago major* leaf (dissolved in PBS) in three different concentrations; 1.0, 0.1 and 0.01 mg/mL, in an *in vitro* NF-kB assay with 1 hour of exposure to *E.coli* LPS 2 ng/mL. PBS was used as control.

## 5 Conclusions

Based on our studies, the following conclusions can be drawn:

- I The phenolic compounds in the leaves of *P. major* are heat sensitive. The maximum contents of polyphenolic compounds were obtained in the freeze-dried samples of *P. major* leaves, and contents decreased with increasing drying temperatures. Since freeze-drying is an expensive process for commercial purposes, it may however be necessary to use a convection dryer, set at no higher than 30 °C.
- II The concentration of the contents of polyphenolic compounds differs greatly in different plant organs of *P. major*. To obtain optimum amounts of a specific polyphenol, it is crucial to select the most appropriate plant organ, the production of which can be affected by choosing annual or perennial plant types.
- III The contents of polyphenolic compounds in *P. major* leaves do not reflect the DNA-marker based pattern of genetic relatedness. This discrepancy suggests that the expression of the studied polyphenols is affected by, e.g., epigenetic inheritance or a very early environmental influence on the newly germinated seedlings, before potting and randomization.
- IV Both ethanol-based and water extracts of fresh and dried leaves of *P. major* had stimulating effects on cell proliferation and migration in an in-vitro scratch assay on human epithelial cells. Careful optimization of dosages for the treatments is needed.
- V Both ethanol-based and water extracts of dried leaves of *P. major* had stimulating effects on wound healing activities in an ex vivo model suggesting that bioactive compounds in this plant could be useful for wound healing also in humans. Careful optimization of both water and ethanol-based extract is needed.
- VI Both ethanol-based and water extracts of *P. major* leaves showed anti-inflammatory activities in an in vitro NF-kB assay on human epithelial

cells. Careful optimization of the dosage of both water and ethanol-based extract is needed.

## 6 Recommendations and future prospects

- I We found that the highest concentration of especially the ethanol-based extract, with a high content of polyphenols, prevented cell proliferation/migration in the scratch assay as well as wound healing in the ex vivo model. Therefore dosages of both water and ethanol-based extracts need to be carefully optimised for further research on *P. major* as wound healing remedy.
- II Considerable attention should be given to determine the role of the different specific polyphenolic compounds as well as other compounds like polysaccharides in *P. major* on cell proliferation/migration and other wound healing activities.
- III When the most efficient compounds have been determined, more research is needed on genetic and environmental factors that affect the content and preservation of these compounds in a plant-based extract.
- IV Following sufficient research and development, a modern wound healing dressing with a *P. major* extract as bioactive compound is envisioned.

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