Wayne Garza
Editor

Fabaceae

Classification, Nutrient Composition and Health Benefits

Plant Science Research and Practices

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FABACEAE

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WAYNE GARZA
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PREFACE

The leguminous plant Fabaceae, contains fibers, vitamins and mineral constituents. In traditional medicine, several parts of plants of the family of Fabaceae are responsible and useful for the treatment of various ailments. The authors explore the medicinal and chemical properties of Fabaceae and its different uses. The Oxytropic campestris complex is explored as well. The authors also review the detection of plant based adulterants in plant food products via the use of DNA based methods and markers. Numerous methods based on DNA analysis have contributed immensely in the food industry to monitor adulterations of food products of plant or animal origin.

Chapter 1 – In the world, many plants have a great economic and cultural importance, providing food, medicine, fuel, clothing and shelter for human beings. These functional or medicinal foods and phytonutrients or phytomedicines play positive roles in maintaining wellbeing, enhancing health and modulating immune function to prevent specific diseases. Recently, dietary supplements, particularly derived from plants have gained an increasing popularity as an alternative treatment for their low toxicity and therapeutic effects.

Leguminous plant (Fabaceae) whose seed is traditionally used for condiments, artificial flavoring and hormone production are one of the many families of vegetable. Most of them are classified as dietary supplements and not as drugs, but many report and research have proved their medicinal use. Fabaceae is classified under the Kingdom of Plantae, Division of Magnoliophyta, Class of Magnoliopsida and Order of Fabales. The Fabaceae are second to cereal crops in agricultural importance based on area harvested and total production (or yield). With some 20000 species, Fabaceae are the third largest family of higher plants.

According to the species, Fabaceae contains Fibers, Vitamins (A, C, E, …), Organics constituents (Protein, Oligosaccharides Carbohydrates, Fats, Oxalate and Phytic acid), and minerals constituents (Calcium Magnesium, Potassium, Iron, Zinc, and Nitrogen). Phytochemical investigations have revealed the presence of isoflavones, prenylated flavonoids, flavanones, flavanols, saponins, glucosides, rotenoids, chalcones, alkaloids and trypsin inhibitors. Flavonoids are the most main constituents of the Fabaceae family.

In traditional medicine, several parts of plants of the family of Fabaceae are responsible and useful for treatment of various ailments. In vitro and in vivo researches have elucidated many properties of these plants. Medicinal effect of Fabaceae can be observed in different systems of the body. This family of plants has oestrogenic, antibacterial, anti-oxidant, anti-fungal, anti-feedant and insecticidal activities. Fabaceae are used to treat polymenorrhea, anemia, ulcers and menorrhagia (during pregnancy). They help in the treatment of diarrhoea, in overcoming the protein deficiency Kwashiorkor and can also impact hypocholesterolaemic conditions, and thyroxine-induced hyperglycaemia. It is reported that Fabaceae are used to remove normal bronchitis; cure illnesses in upper breathing system, rheumatic pain and gland inflammation; prevent insomnia, stress and heart beat which is caused by nervousness; inhibit melanogenesis; regulate energy expenditure and metabolism. Other medicinal properties of Fabaceae are: anti-osteoporotic, anti-diabetic, anti-cancer, anti-nociceptive, anti-atherogenic, anti-inflammatory, anti-nephritic, laxative, sedative, diuretique, digestive, chemo-preventive and neuroprotective.

Chapter 2 – The Caatinga is a biome unique to Brazil that occupies around 11% (844,453 km^2) of the national territory and is the principal ecosystem of the Northeast region of the country. The diversity of the Caatinga flora, including the Fabaceae family, which is known for its rich variety of roughly 600 accepted species, about 150 of which are exclusive to this biome. Studies have already been conducted of the phenolic composition, cytotoxic activity and antimicrobial, antioxidant and anti-inflammatory properties of Caatinga Fabaceae, demonstrating the potential of this family from a chemical and biological point of view. The aim of this chapter is to provide further understanding of the chemical composition (especially the phenolic content) and antioxidant and antimicrobial properties of species of this family used for medicinal purposes by the local population, such as *Amburana cearensis* (Allemão) A.C. Sm., *Anadenanthera colubrina* (Vell.) Brenan, *Libidibia ferrea* (Mart.) L.P. Queiroz, *Chloroleucon extortum* Barneby; J.W. Grimes, *Erythrina velutina* Willd. and *Mimosa tenuiflora* (Willd.) Poir. The authors conducted a literature review concerning the botanical and ethnobotanical features, phytochemical composition and biological properties of this species with a special emphasis on the more abundant compounds and those with greater pharmacological potential. Spectrophotometric methods were used to determine the phytochemical content and antioxidant activity. Antimicrobial activity was evaluated by agar diffusion methods (disk) and broth microdilution (CIM). The species that had the highest phenol content was *A. colubrina*. *M. tenuiflora* had the highest tannin content. The flavonoid and coumarin content was highest in *E. velutina* and *A. cearensis*, respectively. As for the capacity to capture the free radical DPPH, the extract of *A. colubrina* provided the best result with an IC_{50} of 10.79 ± 1.16 µg/mL. *A. colubrina* produced halo of 23.3 ± 0.6 mm (*M. luteus*), *L. ferrea* of 20.3 ± 1.5 mm against *M. smegmatis*, and *M. tenuiflora* halo of 19.3 ± 1.2 mm against *S. aureus*. The extracts which stood out by having good antimicrobial activity on the MIC test were *L. ferrea* (31.25 µg/mL) and *M. tenuiflora* (62.5 µg/mL) against *M. luteus*. In the species studied, the phenolic compounds content (total phenols, tannins, flavonoids, and coumarins) is high and antioxidant and antimicrobial activity is directly related to the levels of these metabolites.

Chapter 3 – The *Oxytropis campestris* complex has a circumpolar distribution and its speciation is still not completely elucidated. There are three members of this group which occur in the Pirin Mts. Two of them, *Oxytropis urumovii* Jav. and *O. kozhuharovii* Pavlova, Dimitrov & Nikolova are alpine endemics to the North Bulgarian Pirin marble. The third one is the widespread *O. campestris* (L.) DC. They are more or less closely related to other members of the *O. campestris* complex on the Balkans. Using morphological characters and molecular techniques, including RAPDs, ITS and trnL sequences, the authors compared the two endemics to the North Bulgarian Pirin marble (*O. urumovii* and *O. kozhuharovii*), with neighbouring populations of the widespread *O. campestris* (L.) DC, as well as with other widespread relatives (*O. halleri* Bunge ex Koch) or Balkan endemics (*O. dinarica* (Murb.) Wettst).

Oxytropis urumovii is a very distinct diploid species which might be ancestral to this group and could be regarded as a palaeoendemic. The tetraploid *O. kozhuharovii* is most closely related to *O. prenja* from the Dinaric Alps, Bosnia-Herzegovina, but is a larger plant with a different facies and indumentum. It is possible that it has evolved as an allotetraploid derivative of *O. urumovii* and *O. halleri*. It is also possible that the circumpolar hexaploid *O. campestris* has evolved as an allohexaploid derivative of the diploid *O. urumovii* and a tetraploid from the Balkans, such as *O. kozhuharovii*. Additionally, *O. kozhuharovii* was found to contain

Quercetin-3-O-β-D–(3IImethoxy) -glucopyranoside. It is interesting that the authors have detected a compound in *O. kozhuharovii* not found in the other two species, which suggests that it might have come from an unknown parent that resulted in the purple pigment, e.g. *O. halleri*, *O. dinarica* (Murb.) Wettst. or *O. prenja* (Beck) Beck.

The authors observed a number of microhabitat specifics of the three *Oxytropis* species in the Pirin Mts. Here they present details about the environment - slope, exposure, bed rock, soils, vegetation and phenology. The breeding systems of the three species were studied both with direct (insect excluders in the field) and indirect (P/O ratio methods). Results are discussed. Pollinators were active only in the flowers of *O. campestris* which formed a big patch while the two endemic species were seldom visited by pollinators. Details are discussed. The authors also tested the seed germination process, development of the seedlings and *ex-situ* seedling behaviour of *Oxytropis urumovii* and *O. kozhuharovii*. Both plant species reproduce by seed and have poor vegetative propagation. Most mature test seeds would germinate within a couple of days if the seed coat was scarified. The seedlings were rather sensitive and many did not survive. The seedlings of the tetraploid *O. kozhuharovii* developed slightly better than those of the diploid *O. urumovii* and during the second year after germination several seedlings bloomed.

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Chapter 1

MEDICINAL PLANTS OF THE FAMILY OF FABACEAE USED TO TREAT VARIOUS AILMENTS

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ABSTRACT

In the world, many plants have a great economic and cultural importance, providing food, medicine, fuel, clothing and shelter for human beings. These functional or medicinal foods and phytonutrients or phytomedicines play positive roles in maintaining wellbeing, enhancing health and modulating immune function to prevent specific diseases. Recently, dietary supplements, particularly derived from plants have gained an increasing popularity as an alternative treatment for their low toxicity and therapeutic effects.

Leguminous plant (Fabaceae) whose seed is traditionally used for condiments, artificial flavoring and hormone production are one of the many families of vegetable. Most of them are classified as dietary

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supplements and not as drugs, but many report and research have proved their medicinal use. Fabaceae is classified under the Kingdom of Plantae, Division of Magnoliophyta, Class of Magnoliopsida and Order of Fabales.. The Fabaceae are second to cereal crops in agricultural importance based on area harvested and total production (or yield). With some 20000 species, Fabaceae are the third largest family of higher plants.

According to the species, Fabaceae contains Fibers, Vitamins (A, C, E, …), Organics constituents (Protein, Oligosaccharides Carbohydrates, Fats, Oxalate and Phytic acid), and minerals constituents (Calcium Magnesium, Potassium, Iron, Zinc, and Nitrogen). Phytochemical investigations have revealed the presence of isoflavones, prenylated flavonoids, flavanones, flavanols, saponins, glucosides, rotenoids, chalcones, alkaloids and trypsin inhibitors. Flavonoids are the most main constituents of the Fabaceae family.

In traditional medicine, several parts of plants of the family of Fabaceae are responsible and useful for treatment of various ailments. In vitro and in vivo researches have elucidated many properties of these plants. Medicinal effect of Fabaceae can be observed in different systems of the body. This family of plants has oestrogenic, antibacterial, anti-oxidant, anti-fungal, anti-feedant and insecticidal activities. Fabaceae are used to treat polymenorrhea, anemia, ulcers and menorrhagia (during pregnancy). They help in the treatment of diarrhoea, in overcoming the protein deficiency Kwashiorkor and can also impact hypocholesterolaemic conditions, and thyroxine-induced hyperglycaemia. It is reported that Fabaceae are used to remove normal bronchitis; cure illnesses in upper breathing system, rheumatic pain and gland inflammation; prevent insomnia, stress and heart beat which is caused by nervousness; inhibit melanogenesis; regulate energy expenditure and metabolism. Other medicinal properties of Fabaceae are: anti-osteoporotic, anti-diabetic, anti-cancer, anti-nociceptive, anti-atherogenic, anti-inflammatory, anti-nephritic, laxative, sedative, diuretique, digestive, chemo-preventive and neuroprotective.

Keywords: Fabaceae, medicinal plants, medicinal foods, phytonutrients, phytomedicines

INTRODUCTION

With recent advances in medical and nutrition sciences, natural products and health-promoting foods have attracted interest of both health professionals and the common population. New concepts have appeared with this trend, such as nutraceuticals, nutritional therapy, phytonutrients and phytotherapy (Bland, 1996; Berger, 2006; Bagchi, 2006). These functional or medicinal foods and phytonutrients or phytomedicines play positive roles in maintaining wellbeing, enhancing health and modulating immune function to prevent specific diseases. They also hold great promise in clinical therapy due to their potential to reduce side effects or low toxicity associated with chemotherapy or radiotherapy and significant advantages in reducing the health care cost (Ramaa et al., 2006). The history of plants being used for medicinal purpose is probably as old as the history of mankind. It is estimated that about 67 % of the current drugs have a natural origin (Newmann and Cragg, 2007).

Fabaceae is one of the most important plant family in the production of food for humans and livestock, as well as in the production of industrial products in agriculture (Louarn et al., 2010). Leguminous plant (Fabaceae) seeds are traditionally used for condiments, artificial flavoring and hormone production. Most of the species chemically investigated were reported to contain alkaloids, flavonoids, isoflavonoid, pterocarpan, tannins, and the free amino acid canavanine (the latter found only in legumes). The function of those that are physiologically active (i.e., often poisonous) in animals seems usually to be that of predator defense. The medical potential (especially of the alkaloids) of some of these substances, or of their synthetic derivatives, has been extensively studied.

Fabaceae are second cereal crops in agricultural importance based on area harvested and total production (or yield). With some 20000 species, Fabaceae are the third largest family of higher plants (Gepts et al., 2005; wojciechowski et al., 2003). The ecological and economical function of Fabaceae is of great importance. The unique ecological role of Fabaceae is nitrogen fixation. Legume nitrogen fixation is of prime importance in agriculture (Louarn et al., 2010). Nitrogen, element present in all proteins is an essential component of both plant and animal metabolism.

The vegetation of the forests, prairies, and deserts of most of the world is primarily dependent on the legume component of their vegetation and could not exist without it. Before the use of synthetic fertilizers in the industrial countries, the cultivation of crop plants, with the exception of rice, was dependent on legumes and plant and animal wastes (as manure) for nitrogen fertilization. As cover crops, legumes prevent or reduce soil erosion and may be plowed under as “green manure”. Legume seeds constitute a part of the diet of nearly all humans. Their most vital role is that of supplying most of the protein in regions of high population density and in balancing the deficiencies of cereal protein (Poaceae). Much of species in this family are also collected for their oil, their fiber, like fuel, for their wood, their use in medicine or in chemistry (Wojciechowski et al., 2004).

1. Clasification (Taxonomy) and Botanical Characteristics of Fabaceae

Fabaceae is classified under the kingdom of Plantae, division of Magnoliophyta, class of Magnoliopsida and order of Fabales (Petit-Aldana et al., 2014). Fabaceae or Leguminoseae commonly known as the legume, pea, or bean family, are a large and economically important family of flowering plants. It includes trees, shrubs and herbaceous plants perennials or annuals, which are easily recognized by their fruits (legume) and their compound, stipulated leaves. The group is widely distributed and is the third-largest land plant family among the angiosperms, after Orchidaceae (orchid family) and Asteraceae (aster family), with 730 genera and over 19,400 species (Judd et al., 2002) trees, shrubs, vines, and herbs.

The largest genera are *Astragalus* (over 2,400 species), *Acacia* (over 950 species), *Indigofera* (around 700 species), *Crotalaria* (around 700 species), and *Mimosa* (around 500 species), which contain around 9.4% of all flowering plant species (Magalion and Sanderson, 2001). Fabaceae occur in all terrestrial habitats occupied by plants, although the greatest number of species is in the tropics, where the group probably originated. Therefore, the Fabaceae is the most common family found in tropical rainforests and in dry forests in America and Africa (Burham and Johnson, 2004). There are also many legumes in the temperate plains, woodlands, and deserts.

The most important commercial species of fabaceae include *Glycine max* (soybean), *Pisum sativum* (garden pea), *Arachis hypogaea* (peanut [groundnut]), and *Medicago sativa* (alfalfa [lucerne]). Most woody species are tropical; herbaceous (i.e., nonwoody) species occur mainly in temperate regions.

Morphologically, Fabaceae are characterized by leaves simple to compound (pinnate, rarely palmate, or bipinnate), unifoliate, trifoliate (*Medicago, Trifolium*), sometimes phyllodic (many species of *Acacia*), or reduced to a tendril (as in *Lathyrus*), spirally arranged, with stipules present that are sometimes large and leaf-like (*Pisum*) or developed into spines (*Prosopis, Robinia*). The leaves of a few species are simple or reduced to scales.

Species of Fabaceae demonstrate a wide variety in the type of fruits (commonly referred to as "pods") they produce. Technically, the type of fruit produced by members of Fabaceae is known as a legume, defined as a dry, more or less elongate fruit derived from a single carpel that opens or "dehisces" along one or both longitudinal sutures (Judd et al. 2002). Legumes often have a single chamber (unilocular), or they may have two chambers (bilocular, e.g., in species of *Astragalus*) in the mature fruit, separated by a septum.

The family of Fabaceae has traditionally been divided into three subfamilies: Caesalpinioideae, Mimosoideae, and Faboideae (or Papilionoideae). According to APG III (2009), Bauhinioïdes is another subfamily of Fabaceae.

The subfamily Caesalpinioideae also classified as a family, Caesalpiniaceae, by some authorities (botanists) is a heterogeneous group of plants with about 161 genera and some 3000 species (wojciechowski et al., 2003). Caesalpinioideae legumes are found throughout the world but are primarily woody plants in the tropics. Their moderate secondary invasion of temperate regions is mostly by herbaceous (nonwoody) evolutionary derivatives.

The subfamily Mimosoideae (classified as a family, Mimosaseae, by some authorities) includes 76 genera and more than 3000 species (wojciechowski et al., 2003). Like Caesalpinioideae, Mimosoideae legumes are primarily woody plants of the tropics, and the few species native to temperate parts of the world are mostly herbaceous.

The subfamily Faboideae, also called Papilionoideae (classified as a family, Fabaceae or Papilionaceae, by some authorities), the largest group of legumes, includes about 483 genera and nearly 14000 species grouped into 28 tribes (wojciechowski et al., 2003; Lewis et al., 2005). Like the other subfamilies, members of Papilionoideae have their origins in the tropics, but their occupation of the arid and temperate parts of the world, mostly as herbaceous plants, is far more extensive. In the forests, prairies, and deserts, they are among the most common plants. The name of the group probably originated because of the flower’s resemblance to a butterfly (Latin: *papilio*).

2. Nutrient Composition of Fabaceae

Food legumes and seeds of some plant species of Fabaceae constitute a major source of edible nutrients such as proteins, lipids, carbohydrates, mineral elements, fatty acids and amino acids, and other important substances such as fiber and vitamins (Deshpande et al., 2001) which have some importance for health human and animal. The legume seeds generally are highest in carbohydrate compounds, followed by protein and fat.

Table 1. Nutrition profile of soybeans expressed per 100 g DM. (Mateos-Aparicio et al., 2008)

Composition	Soybean beans
Complex carbohydrates (g)	21
Simple carbohydrate (g)	9
Stachyose (mg)	3.300
Raffinose (mg)	1.600
Protein (g)	36
Total fat (g)	19
Saturated fat (g)	2.8
Monounsaturated fat (g)	4.4
Polyunsaturated fat (g)	11.2
Insoluble fibre (g)	10
Soluble fibre (g)	7
Calcium (mg)	276
Magnesium (mg)	280
Potassium (mg)	1.797
Iron (mg)	16
Zinc (mg)	4.8

DM: dry matter.

Along with Poaceae (the grass family), Fabaceae is the most important plant family in the production of food for humans and livestock, as well as in the production of industrial products. All the Fabaceae family, mature and imature seeds are good sources of dietary fiber and isoflavonoids- e.g. bean (*Phaseolus*), pea (*Pisum sativum*), soybean (*Glycine max*) and chickpea (*Cicer arietinum*) (Silva Dias, 2012). The Fabaceae produce other kinds of chemical substances, e.g., alkaloids, flavonoids, tannins, and the free amino acid canavanine (the latter found only in legumes). Phytochemical investigations of genus Tephrosia have revealed the presence of glucosides, rotenoids, isoflavones, chalcones, flavanones, flavanols, and prenylated flavonoids (Yinning et al., 2014). The function of those that are physiologically active in animals seems usually to be that of predator defense. The medical potential (especially of the alkaloids) of some of these substances, or of their synthetic derivatives, has been extensively studied.

In 2008, Mateos-Aparicio et al. have shown the nutrition profile of soybeans expressed per 100 g of dry matter. The legume seeds generally are highest in carbohydrate compounds, followed by protein and fat

Galactia longifolia Benth. (Fabaceae), a legume known as Kaattukollu in Tamil language, contains biochemical components with nutritional potential. The work of Thangadurai et al. (2001) has revealed that *Galactia longifolia* seeds are a good source of protein, fat, energy, some essential amino acids, fatty acids, and some minerals. Chemical study of the seed oil of *Adenanthera pavonina* L. (Fabaceae) has revealed the presence of some components with nutritive value. Senga Kitumbe et al. (2013) have confirmed that the seed oil of *Adenanthera pavonina* is rich in lipids, crude proteins, carbohydrates, amino acids, ash, fatty acids and some mineral elements compared to some other seed, nut and legume oils.

3. HEALTH BENEFITS OF FABACEAE

In the traditional medicinal system, plant extracts in various forms, concoctions or infusions and maceration are used to treat a wide range of diseases. Several parts of plants of the family of Fabaceae are then useful for the treatment of various ailments. Even in western countries, the search for alternative medication based on plant extracts is increasing. Then many Fabaceae species have shown beneficial effects for health. Medicinal effect of Fabaceae can be observed in different systems of the body. For example, the possible use of soybean in functional food design is very interesting, since the consumption of soybean protein and dietary fibers seems to reduce the risk of cardiovascular diseases and to improve glycemic control (Mateos-Aparicio et al., 2008).

Pharmacological studies on many plants of the fabaceae family have revealed theirs antibacterial, antifungal, antihypertensive, anti-oxidant, antiviral, anti-feedant, insecticidal, diuretic and hypoglycemic properties, and hepatoprotective, nephroprotective and cytotoxic effects (activities). Others plants have long been used as an abortifacient, a purgative and a vermifuge in the traditional medicine systems in some countries (Maghrani et al., 2005; Eddouks et al., 2007; Hayet et al., 2007, 2008; Koriem et al., 2009; Algandaby et al., 2010; Edrizi et al., 2010).

Some Fabaceae species are also used to treat polymenorrhea, anemia, ulcers and menorrhagia (during pregnancy). They help in the treatment of diarrhoea, in overcoming the protein deficiency Kwashiorkor and can also impact hypocholesterolaemic conditions, and thyroxine-induced hyperglycaemia. It is reported that species of Fabaceae are used to remove normal bronchitis; cure illnesses in upper breathing system, rheumatic pain and gland inflammation; prevent insomnia, stress and heart beat which is caused by nervousness; inhibit melanogenesis; regulate energy expenditure and Metabolism. Other medicinal properties of Fabaceae are: anti-osteoporotic, anti-diabetic, anti-cancer, anti-nociceptive, anti-atherogenic, anti-inflammatory, anti-nephritic, laxative, sedative, diuretique, digestive, chemo-preventive and neuroprotective.

Various plants of the Fabaceae family are used in connection with female reproductive health. Many studies have then reported the estrogenic properties of a number of Central African medicinal plants, namely, *Eryhtrina lysistemon* (Fabaceae) (Tanee et al., 2007; Njamen et al., 2007); *Millettia conraui* (Leguminosae), *Millettia drastic* (Leguminosae), *Bridelia ferruginea* (Leguminosae) (Njamen et al., 2008), and *Erythrina poeppigiana* (Fabaceae) (Djiogue et al., 2009; Djiogue et al., 2010). All the above-listed plant extracts exhibited direct estrogenic effects probably because of the presence of metabolites acting through either or both of the two estrogen receptors, receptor alpha and beta. These plants are used as contraceptives (to prevent ovulation or fertilization), abortifacients (to prevent implantation or to push out unwanted conceptus), emmenagogues (to stimulate uterine flow), or oxytocics (to stimulate uterine contractions, particularly to promote labor).

Natural products derived from some fabaceae, used as remedies could exert various activities. In vitro and in vivo researches are used to elucidate many properties of these plants. Ateba et al. (2013) have shown that *Eriosema laurentii* De Wild (Leguminosae) methanol extract has estrogenic properties and prevents menopausal symptoms in ovariectomized Wistar rats. This result justified the use of this plant for the treatment of various gynecological and menopausal problems in ethnomedicine in Cameroon.

Other studies carried out by Njamen et al. (2013) have revealed that the extracts from *Erythrina poeppigiana* (Fabaceae) have estrogen-like effects in vivo and provide evidence to support its folk empirical traditional use for the treatment of some problems related to menopause such as hot flushes and vaginal dryness.

According to Zingue et al. (2013), in vitro, the extract of *Millettia macrophylla* (Fabaceae) showed an estrogenic activity as well as an anti estrogenic activity depending on the type of estrogen receptors involved. The estrogenicity and/or antiestrogenicity of the extract may be due to the presence of isoflavonoids that are well known phytoestrogens. These results provide scientific evidence to support the folk use of *M. macropylla* as traditional medicine to alleviate menopausal symptoms among others in Cameroon.

Glabridin, a prenylated isoflavonoid of *Glycyrrhiza glabra* L. roots (European licorice, Fabaceae), has been associated with a wide range of biological properties such as antioxidant, anti-inflammatory, anti-atherogenic, regulation of energy metabolism, estrogenic, neuroprotective, anti-osteoporotic, anti-cancer and skin-whitening (Simmler et al., 2013). The bioactivity of genus Tephrosia has been studied extensively and it indicated that chemical constituents and extracts of the genus Tephrosia exhibited diverse bioactivities, such as insecticidal, antiviral, antiprotozoal, antiplasmodial and cytotoxic activities (Yinning et al., 2014). Results from researches suggest that soybean dietary fiber plays a role in the reduction of cholesterol levels in some hyperlipidemic individuals and has a major protective effect on cardiovascular disease. Moreover, it improves the glucose tolerance in some diabetic patients; it increases the wet faecal weight and reduces the caloric density in some foods. Dietary fiber seems also to have a positive effect on diarrhea and constipation and as a therapy of irritable bowel syndrome; it has anti- inflammatory and anti-carcinogenic effects on digestive system (Mateos-Aparicio et al., 2008). Erythrina (Fabaceae) is one of the worldwide represented genus which species are rich in isoflavonoids and widely used in many folkloric medicines (Bisby et al., 1994;). In sub-Saharan Africa, Erythrina species are used to treat frequent parasitic and microbial diseases, inflammation, cancer, wounds. Many fabaceae species are reported to be used traditionally to treat diabetis. For example, isoflavones from *Erythrina indica* exhibit Cytotoxic effects (activities) (Nkengfack et al., 2001); Hypoglycaemic and anti-diabetic activity of stem bark extracts of *Erythrina indica* in normal and alloxan-induced diabetic rats was also reported by Yashwant Kumar et al. (2011). It is also reported to inhibit α-amylase.

The study of Njamen et al. (2007) indicates that oral administration of the extract of *Erythrina Lysistemon* (Fabaceae) before prevented bone loss in ovariectomized rats. It had a transient effect on glucose intolerance. However *Erythrina Lysistemon* significantly increased HDL-cholesterol and decreased triglycerids level but, unlike estrogens, increased total-cholesterol and LDL cholesterol levels but with an interesting ratio total-cholesterol/HDLcholesterol.

Table 2. List of selected plants and their folkloric uses (Adjanohoun et al., 1996; Borgi Wahida et al., 2011; Carretero Accame et al., 2011; Cheryl Lans and Karla Geoges, 2011)

Botanical name (species)	Local (or vernacular) name	uses or comments about activity or indication	Part used
Accia raddiana Savi	Talh (Tunisia)	Respiratory diseases, skin ailments, toothache	Flower, fruits
Afzelia bella Harms.	Caohi (Cameroon)	Mumps	seeds
Amphimas ptericarpoides Harms.	Edii (Cameroon)	Napkin rash	Bark
Astragalus mareoticus Del.	Hachicht agrab (Tunisia)	Scorpion bite	Whole plant
Brownea latifolia	Cooper hoop (Trindad and Tobago)	Female complaints	Flower, Leaves
Calycotme villosa L.	Gandoul (Tunisia)	Cicatrizing	Leaves
Cassia obovata Colladon	Sen/Senna (Spain/England)	Laxative	Leaves
Chamaecrista mimosoides (Linn.)	Bagarouatan (Cameroon)	Dysentery	Whole plant
Cicer arietinum L.	Homs (Tunisia)	Antiseptic, kidney diseases	Leaves
Desmodium adscendens (Sw.)	Owondo bekone, ton-ton (Cameroon)	Abdominal pains, urinary tract infection, cough, dysentery.	Whole plant, leaves
Detarium microcarpum Guill. & Perr.	Nkwazi (Cameroon)	Vulvovaginitis	Stem bark
Entada polystachya	Mayoc chapelle (Trindad and Tobago)	Menstrual pain	Twigs
Erythina senengalensis DC.	Pounche bienou (Cameroon)	Fracture	Whole plant
Erytrophleum guineense G. Don.	Elon (Cameroon)	Elephantiasis (non filarial)	Stem bark
Glycyrrhiza glabra L.	Erqus (Tunisia) Regaliz/Licorice (Spain/England)	Inflammatory diseases, constipation, respiratory diseases, digestive diseases, ulcer, cancer, kidney diseases, heartburn	Roots, undergro und, stem
Guibourtia tessmennii (Harms.)	Nsim-gang, Essingang (Cameroon)	Gastroenteritis, malaria, pneumonia, used as panacea for wichcraft	Bark, roots
Milletia sanagana Hams.	Djopo (Cameroon)	Malaria	roots
Mimosa pudica	Mese marie (Trindad and Tobago)	Childbirth	

Botanical name (species)	Local (or vernacular) name	uses or comments about activity or indication	Part used
Ononis natrix L.	Mellita (Tunisia)	Toothache	Leaves
Piptadeniastrum africanum (hook. F.)	Atui, mpie, tombou (Cameroon)	Pelvic inflammatory disease, rheumatism, strangulated hernia	Bark, root bark
Pterocarpus soyauxii Taub	Nsonbankugi (Cameroon)	Gastralgia	Outer bark
Scorodophleus zenkeri	Chirum, nwinke (Cameroon)	Used for loss of appetite, abdominal pain	Stem bark
Senna alata (linn.) Roxb.	Ekon-chuwe, Ngom, To'o (Cameroon)	Constipation, jaundice, gastroenteritis, intestinal helmentiasis	Leaves, stems
Tamarindus indica Linn.	Djakpe (Cameroon)	Jaundice	Bulb
Tetrapleura tetaptera (Schum et Thonn.)	Telele, essissa (Cameroon)	Treatment of post-partum haemorrhage, abdominal pains, epilepsy, placenta retention	Fruit, root tuber
Tephrosia vogelii Hook.	Koro'o (Cameroon)	splenomegaly	Stem bark
Trigonella foenum-graecum L.	Helba (Tunisia)	Cancer, fever, digestive diseases, respiratory problems, eye diseases	Seeds
Vicia faba L.	Foul (Tunisia)	Digestive diseases, skin diseases	Fruits
Zornia latifolia	Geldandae (Cameroon)	Pharyngitis	Whole plant

The effects of crude leave extract of *Erythrina velutina* on the rodent central nervous system was investigated by Dantas et al. (2004). Their results have shown that the extract increases sleeping time induced by sodium pentobarbital in a dose-dependent manner. According to them, these results are in agreement with what is known in Brazilian traditional herbal medicine. The two most common *Erythrina* species in Brazil, *Erythrina velutina* and *Erythrina mulungu* are used for the treatment of nervous system excitation, sleepiness, convulsions and nervous coughs. These data have shown that the crude extract from the leaves of *Erythrina velutina*, causes peripheral and central effects that are dependent on the dose used. At lower doses it interferes with mnemonic process for different tasks, while at higher doses, the sedative and neuromuscular blocking actions are the main effects. Evaluation of alcoholic extract of stem bark of *Erythrina variegate* have shown anxiolytic and anticonvulsant activity in mice (Pitchaiah et al., 2008). *Erythrina crista galli* L. (Leguminosae) is a tree that grows in South America and is used in folk medicine for wound healing, as astringent, narcotic and analgesic (Toursarkissian, 1980).

In Table 2, we summarized some of the medicinal plants used deaseease treatment purposes. In this table, plants are classified according to their traditional use.

In Table 3, are summarized selected plants of Fabacea family and their pharmacological research.

Table 3. List of selected plants and their pharmacological research

Plants names	Pharmacological research	Authors
Abrus pulchellus	Studies on *In vitro* Antioxidant, Antibacterial and Insecticidal Activity of Methanolic Extract of *Abrus pulchellus* Wall (Fabaceae)	Vinayaka et al., 2009
Eriosema laurentii De Wild	*Eriosema laurentii* De Wild (Leguminosae) methanol extract has estrogenic properties and prevents menopausal symptoms in ovariectomized Wistar rats	Ateba et al., 2013
Erythrina lysistemon Hutch	Estrogenic effects of the ethyl-acetate extract of the stem bark of *Erythrina lysistemon* Hutch (Fabaceae)	Tanee et al., 2007
	Preventive Effects of an Extract of Erythrina Lysistemon (Fabaceae) on Some Menopausal Problems: Studies on the Rat	Njamen et al., 2007
	A postmenopause-like model of ovariectomized Wistar rats to identify active principles of Erythrina lysistemon (Fabaceae)	Mvondo et al., 2011a
	Effects of Alpinumisoflavone and A byssinone V-4′ -Methyl Ether Derived from Erythrina lysistemon (Fabaceae) on the Genital Tract of Ovariectomized Female Wistar Rat	Mvondo et al., 2011b
	In vitro estrogenic activity of two major compounds from the stem bark of Erythrina lysistemon (Fabaceae)	Magne Nde et al., 2012
Erythrina mildbraedii	Prenylated Flavonoids with PTP1B Inhibitory Activity from the Root Bark of *Erythrina mildbraedii*	Jang et al., 2008
	Anti-inflammatory activity of erycristagallin, a terocarpene from *Erythrina mildbraedii*	Njamen et al., 2003
Erythrina poeppigiana	Isoflavonoids from *Erythrina poeppigiana*: Evaluation of Their Binding Affinity for the Estrogen Receptor	Djiogue et al., 2009
	In vivo and in vitro estrogenic activity of extracts from *Erythrina poeppigiana* (Fabaceae)	Njamen et al., 2013

Plants names	Pharmacological research	Authors
Erythrina sigmoidea	Anti-inflammatory activities of two flavanones, sigmoidin A and sigmoidin B, from *Erythrina sigmoidea*. Planta Med. 70, 104–107.	Njamen, et al., 2004
Erythrina variegate	Pharmacological evaluation of alcoholic extract of stem bark of *Erythrina variegata* for anxiolytic and anticonvulsant activity in mice	Pitchaiah et al., 2008
Erythrina velutina	Central nervous system effects of the crude extract of *Erythrina velutina* on rodents	Dantas et al., 2004
Millettia griffoniana	Estrogenic properties of isoflavones derived from *Millettia griffoniana* Estrogenic Activity of Griffonianone C, an Isoflavone from the Root Bark of *Millettia griffoniana*: Regulation of the Expression of Estrogen Responsive Genes in Uterus and Liver of Ovariectomized Rats. Modulation of some estrogen-responsive genes in the vena cava of ovariectomised Wistar rats by griffonianone C, an isoflavone derived from Millettia griffoniana Baill. (Fabaceae), Regulation of CD1, Ki-67, PCNA mRNA expression and AKT activation in estrogen-responsive human breast adenocarcinoma cell line, MCF-7 cells by Griffonianone C, an isoflavone derived from Millettia griffoniana.	Ketcha Wanda et al., 2006 Ketcha Wanda et al., 2007 Ketcha Wanda et al., 2010 Ketcha Wanda et al., 2011
Millettia macrophylla	Effects of *Millettia macrophylla* (Fabaceae) Extracts on strogen Target Organs of Female Wistar Rat Effects of *Millettia macrophylla* (Fabaceae) Extracts on Estrogen Target Organs of Female Wistar Rat	Zingue et al., 2013a Zingue et al., 2013b
R*etama raetam* (forssk.) webb & berthel	Effects of R*etama raetam* (forssk.) webb & berthel. (fabaceae) on the central nervous system in experimental animals	Al-Tubuly et al., 2011

CONCLUSION

Many terrestrial habitats continue to provide raw materials for the discovery of newmedicinal products in view of the large diversity of its flora. Functional or medicinal foods and phytonutrients or phytomedicines play positive roles in maintaining wellbeing, enhancing health and modulating immune function to prevent specific diseases. Therefore food legumes and seeds of some plant species constitute a major source of edible nutrients such as proteins, lipids, carbohydrates, mineral elements, fatty acids and amino acids, and other important substances such as fiber and vitamins. Legume seeds are produced and consumed worldwide. They are sources of the energy, protein and other important nutrients consumed in developed and underdeveloped countries where legumes constitute a significant part of the diet. In this paper, we have reviewed that many species of the Fabaceae family can contribute to the overall health benefit. some plants have already been characterized scientifically, although to a varying degree, and several of their properties are attributed to estrogenic, oxytocic, antibacterial, antifungal, antihypertensive, anti-oxidant, antiviral, anti-feedant, insecticidal diuretic and hypoglycemic properties, and hepatoprotective, nephroprotective and cytotoxic properties (effects (activities). Others plants have long been used as an abortifacient, a purgative and a vermifuge in the traditional medicine systems. Taking into account the diversity of the world pharmacopoeia, there is still a lot to do for the phytochemical and pharmacological characterization of these medicinal plants.

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Chapter 2

CAATINGA FABACEAE: BOTANICAL AND ETHNOBOTANICAL FEATURES, PHYTOCHEMICAL COMPOSITION AND BIOLOGICAL PROPERTIES

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ABSTRACT

The Caatinga is a biome unique to Brazil that occupies around 11% (844,453 km^2) of the national territory and is the principal ecosystem of the Northeast region of the country. The diversity of the Caatinga flora, including the Fabaceae family, which is known for its rich variety of roughly 600 accepted species, about 150 of which are exclusive to this biome. Studies have already been conducted of the phenolic composition, cytotoxic activity and antimicrobial, antioxidant and anti-inflammatory properties of Caatinga Fabaceae, demonstrating the potential of this family from a chemical and biological point of view. The aim of this chapter is to provide further understanding of the chemical composition (especially the phenolic content) and antioxidant and antimicrobial properties of species of this family used for medicinal purposes by the local population, such as *Amburana cearensis* (Allemão) A.C. Sm., *Anadenanthera colubrina* (Vell.) Brenan, *Libidibia ferrea* (Mart.) L.P. Queiroz, *Chloroleucon extortum* Barneby; J.W. Grimes, *Erythrina velutina* Willd. and *Mimosa tenuiflora* (Willd.) Poir. We conducted a literature review concerning the botanical and ethnobotanical features, phytochemical composition and biological properties of this species with a special emphasis on the more abundant compounds and those with greater pharmacological potential. Spectrophotometric methods were used to determine the phytochemical content and antioxidant activity. Antimicrobial activity was evaluated by agar diffusion methods (disk) and broth microdilution (CIM). The species that had the highest phenol content was *A. colubrina*. *M. tenuiflora* had the highest tannin content. The flavonoid and coumarin content was highest in *E. velutina* and *A. cearensis*, respectively. As for the capacity to capture the free radical DPPH, the extract of *A. colubrina* provided the best result with an IC_{50} of 10.79 ± 1.16 µg/mL. *A. colubrina* produced halo of 23.3 ± 0.6 mm (*M. luteus*), *L. ferrea* of 20.3 ± 1.5 mm against *M. smegmatis*, and *M. tenuiflora* halo of 19.3 ± 1.2 mm against *S. aureus*. The extracts which stood out by having good antimicrobial activity on the MIC test were *L. ferrea* (31.25 µg/mL) and *M. tenuiflora* (62.5 µg/mL) against *M. luteus*. In the species studied, the phenolic compounds content (total phenols, tannins, flavonoids, and coumarins) is high and antioxidant and antimicrobial activity is directly related to the levels of these metabolites.

1. INTRODUCTION

The Caatinga is a biome unique to Brazil that occupies around 11% (844,453 km^2) of the national territory and is the principal ecosystem of the

Northeast region of the country and home to approximately 27 million people. This region has a semi-arid climate with irregular rains and a mean annual precipitation of less than 620 mm. The vegetation, typically xerophilic, is highly diverse, including a large number of endemic species, comprising deciduous, thorny and succulent plants.

This is considered to be of fundamental importance for the local population, owing to the variety of plants and animals used for a range of purposes, including food, fuel (wood and charcoal), building materials (fences) and medicine (Almeida et al., 2010).

In this region, various ethnobotanical studies have been conducted and these have confirmed the immense potential of the plants used in local folk medicine, which have antimicrobial, antidiabetic and antidiarrheal properties. These studies suggest that these properties are related to the presence of secondary metabolites, such as flavonoids and tannins (Siqueira et al., 2012).

This broad body of traditional knowledge can be explained in part by the diversity of the Caatinga flora, with around 170 botanical taxa, including the Fabaceae family, which is known for its rich variety of roughly 600 accepted species, about 150 of which are exclusive to this biome, making it of even greater interest from a scientific point of view (Monteiro et al., 2011; Reflora, 2015).

This growing interest in studying the Caatinga has enabled greater investigation of the species of the Fabaceae family and various properties attributed to them by folk medicine have now been confirmed (Giulietti et al., 2004). Studies have already been conducted of the phenolic composition, cytotoxic activity and antimicrobial, antioxidant and anti-inflammatory properties of Caatinga Fabaceae, demonstrating the potential of this family from a chemical and biological point of view (Desmarchelier et al., 1999; David et al., 2007; Trentin et al., 2011; Araújo et al., 2014).

The aim of this chapter is to provide further understanding of the chemical composition (especially the phenol content) and antioxidant and antimicrobial properties of species of this family used for medicinal purposes by the local population, such as *Amburana cearensis* (Allemão) A.C. Sm., *Anadenanthera colubrina* (Vell.) Brenan, *Libidibia ferrea* (Mart.) L.P. Queiroz, *Chloroleucon extortum* Barneby; J.W. Grimes, *Erythrina velutina* Willd. and *Mimosa tenuiflora* (Willd.) Poir.

2. THE SPECIES STUDIES

This chapter begins by examining the botanical and ethnobotanical features, phytochemical composition and biological properties of the species under study, as reported in the literature, with a special emphasis on the more abundant compounds and those with greater pharmacological potential.

We go on to present experimental results regarding the phenol content and antioxidant and antimicrobial properties of these species.

2.1. *Amburana cearensis* (Allemão) A. C. Sm.

2.1.1. Botanical Features

Amburana cearensis A.C. Smith is a species widely found in South America, in countries such as Bolivia, Paraguay, Peru and Brazil (Tropicos, 2015). *A. cearensis* is synonymous with *Torresea cearensis* Allemão and, along with *Amburana acreana* (Ducke) A.C. Sm., is one of only two representatives of the genus (Tropicos, 2015). In Brazil, it can be found in the Northeast, Southeast and Midwest regions, growing in Caatinga, Cerrado and Atlantic Forest areas (Lima, 2015). *A. cearensis* is commonly known in the Northeast of Brazil as *cumaru*, *amburana*, or *imburana-de-cheiro* (Albuquerque et al., 2007a).

The popular nomenclature of *A. cearensis* may be misleading in terms of its identification in regions where it is known as *cumaru*, as this may be confused with another homonymous leguminous plant called *Dipteryx odorata* (Aubl.) Willd. Both species are rich in coumarin. *Commiphora leptophloeos* (Mart.) J.B. Gillett, commonly known as *imburana-de-espinho* may also be misidentified as *A. cearensis* (Maia, 2004).

The species is a tree-sized plant that may grow to a height of up to 10 meters in the Caatinga regions and up to 20 meters in the Zona da Mata. Its leaves are compound, imparipinnate (occasionally paripinnate), with 5 to 9 leaflets, alternate, long and petiolate. The trunk is covered by a dark brown bark whose outer layer peels off in thin layers to form red and grayish-brown patches. The plant produces white blossom in September and the fruit takes the form short bean-pods. Each fruit has a single seed, which is flat, winged and black in appearance and has a strong smell similar to that of other parts of the plant (Pio-Correa, 1984; Almeida et al., 2010).

2.1.2. Ethnobotanical Features

An inventory of medicinal plants used by 32 members of a traditional community in the Northeast region of Brazil included 121 species and *A. cearensis* was the one with the greatest use value (Baptistel et al., 2014). In another ethnobotanical study, *A. cearensis* was again the most frequently cited species, familiar to 77% of those interviewed, recommended as a syrup for treating fatigue (Paulino et al., 2012).

The stem bark is widely used to treat respiratory disorders in the form of a *cumaru* syrup and this is produced on an industrial scale by the Federal University of Ceará Pharmacy School's *Farmácias Vivas* Program and by some private companies (Canuto; Silveira, 2006).

The wood is widely used to make furniture, doors and windows, and seeds, owing to their characteristic agreeable smell are used to perfume clothes (Lorenzi, 2002) and to repel insects. The seeds may also be used to produce a fine powder, called *rapé-de-imburana*, used to induce sneezing when treating nasal congestion (Maia, 2004). The seeds are also employed in folk medicine as an anti-spasmodic and emmenagogic agentand to treat rheumatic disorders (Tigre, 1968; Braga, 1976).

2.1.3. Phytochemical Composition

Negri et al. (2004) identified a predominant presence of coumarin derivatives in the stem bark of *A. cearensis,* including 1,2-benzopyrone, dihydrocoumarin and scopoletin. The species was also found to contain an anthraquinone (chrysophanol); phenylpropanoids (*trans*-methyl 3,4-dimethoxycinnamate; *cis*-methyl 3,4-dimethoxy-cinnamate; methyl 3-methoxy-4-hydroxy-cinnamate; methyl 4-hydroxy benzoate; methyl 3,4-dihydroxybenzoate; methyl 3-hydroxy-4-methoxybenzoate; catechol; guaiacol; α-ethoxy-*p*-cresol; 4-hydroxy-benzenemethanol; 4-(methoxymethyl)phenol; 2,3-dihydrobenzofurane); triterpenoids (lupeol; α,β-amyrins; scalene); steroids (γ-sitosterol; ergost-5-en-3β-ol; 24,26-dimethylcholesta-5,22-dein-3β-ol); and aliphatic compounds (methyl palmitate; ethyl palmitate; *n*-undecene; methyl eicosanoate; 9(Z)-methyl octadecenoate; 9,17(Z)-octadecadienal).

Canuto and Silveira (2006) note that, despite the proven properties and wide use of *cumaru* syrup, only four constituents of the stem bark of *A. cearensis* have been reported: coumarin (Figure 1a), iso-kaempferide (Figure 1b), amburoside A (Figure 1c) and amburoside B (Figure 1d). These authors succeeded in isolating and identifying eleven chemical constituents of the ethanolic extract of trunk bark: coumarin, sucrose, two phenol acids (vanillic acid and protocatechuic acid), five flavonoids (afrormosin, isokaempferide, kaempferol, quercetin and 4'-methoxy-fisetin), a phenol glucoside (amburoside A) and a mixture of glucosilated β-sitosterol and stigmasterol.

Phytochemical investigation of the resin of *A. cearensis* allowed the isolation of a new compound: 3',4'-dimethoxy-1'-(7-methoxy-4-oxo-4Hcromen-3-yl) benzo-2',5'-quinone, together with six known compounds identified as: 4,2',4'-trihydroxychalcone, 7,8,3',4'-tetra methoxyisoflavone, 4,2',4'-trihydroxy-3-methoxychalcone, 3,4,5-trimethoxy cinnamaldehyde, 3',4'-dimethoxy-7-hydroxy isoflavone and 6,7,4'-trimethoxy-3'-hydroxy isoflavone (Bandeira et al., 2011).

2.1.4. Biological Properties

The bronchodilatory activity of *A. cearensis* has been scientifically validated for treatment of respiratory disorders, owing to the presence of coumarin and other compounds acting synergistically. Some phenol glucosides exhibit anti-malarial, anti-protozoal, anti-fungal and antibacterial properties *in vitro* (Bravo et al., 1999). Furthermore, flavonoids isolated from the bark of *A. cearensis* may contribute to the spasmolytic properties cited in the popular use of the plant (Canuto; Silveira, 2003).

There have also been reports of immunomodulatory properties of the hydroalcoholic extract and its coumarins and reduction of rat's paw edemainduced by the antigen, an effect that may be linked to the production of immunoglobins.

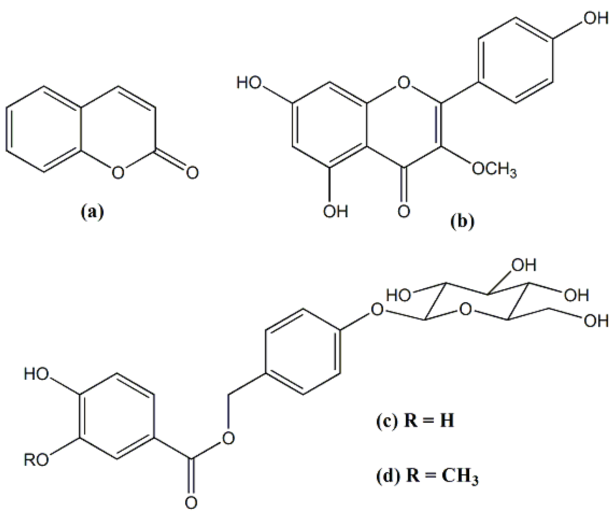


Figure 1. Isolated compounds of the stem bark of *A. cearensis*: (a) coumarin, (b) isokaempferide, (c) amburoside A and (d) amburoside B.

It was also found that the increase in vascular permeability induced by acetic acid was inhibited by the extract, suggesting a possible effect on the mediators produced during acute inflammation (Marinho et al., 2004).

In a study conducted by Trevisan and Macedo (2003) to select plants with anticholinesterase properties for treatment of Alzheimer's disease, *A. cearensis* was the species that produced the best result, with the extract inhibiting 100% of enzymatic activity at a concentration of 2.3 mg/mL.

With regard to neuroprotective properties, amburoside A (Figure 1c) acted as in antioxidant, with a neuroprotective effect in the test of neurotoxicity induced by 6-hydroxydopamine, suggesting beneficial effects in the treatment of neurodegenerative diseases similar to Parkinson's (Leal et al., 2005). The same substance also exhibited hepatoprotective properties, an effect that is related to the presence of hydroxyl groups in the structure of amburoside A, as observed with other natural phenols (Leal et al., 2008a). Dicoumarol, another coumarin found in this species, has hypoprothrombinemic properties and competes with vitamin K, through antagonistic action with the liver enzyme that participates in the synthesis of prothrombin (Diniz et al., 1998).

Evaluation of the clinical toxicity of the syrup of *A. cearensis* in patients voluntarily treated with a daily dose of 20 mL for 30 consecutive days did not reveal any alteration in the clinical-laboratory parameters of the patients (Leal et al., 2006).

2.2. *Anadenanthera colubrina* (Vell.) Brenan

2.2.1. Botanical Features

Anadenanthera colubrina (Vell.) Brenan is a species widely distributed in South America, from the southernmost part of the continent to the Northeast region of Brazil and is characteristic of dry forests. The genus contains two species *A. colubrina* and *A. peregrina* (L.) Speg. (Tropicos, 2012; Morim, 2012) and it is difficult to distinguish the two (Schultes; Hofmann, 2000). *A. colubrina* has two varieties: *A. colubrina* var. cebil (Griseb.) Altschul and *A. colubrina* (Vell.) Brenan var. colubrina (Morim, 2012).

The popular names of *A. colubrina* include *cobi*, *angico*, *angico branco*, *angico de caroço* and *angico-monjolo* (Oliveira, 2002). The species grows to a height of 3-19 m and its stem bark is often covered in cone-shaped aculeus, which is a striking characteristic of the species (Schultes; Hofmann, 2000). The density and the size of the aculeus are used to differentiate such trees in one region of the Caatinga, where the local people classify them as *caroço* or *angico liso*, the latter having a straighter trunk and few and small or no aculeus (Soldati; Albuquerque, 2010).

Some phenological studies suggest that this species sheds its leaves in the dry season and is more abundant in the rainy season and that individual plants can go for more than three years without flowering and producing fruit (Lima; Rodal, 2010).

2.2.2. Ethnobotanical Features

The deciduous nature of this species means that the prime resources for the people of the Caatinga region are the bark and the trunk. Local people use the bark to dye hammocks (Oliveira, 2002).

The trunk can be used to build houses and fences, to cure leather, for veterinary purposes (dairy production), for animal fodder, technology and handicrafts (simple pipes and ornaments) (Albuquerque et al., 2005; Ferraz et al., 2006; Monteiro et al., 2006b, Lucena et al., 2007, Soldati; Albuquerque, 2010; Lucena et al., 2012). Studies show that *A. colubrina* is a preferred source of fuel, as wood or for charcoal production, in rural parts of Caatinga, since it has a high calorific value, is slow burning, easy to gather and produces little ash (Ramos et al., 2008a; Ramos et al., 2008b).

In the Northeast of Brazil, this species is widely used to treat respiratory disorders and the stem bark can be macerated in wine to a produce a remedy for coughs and bronchitis (Agra et al., 2008). The bark is used as an anti-inflammatory and for magical-religious rituals (Albuquerque et al., 2007a).

In the Caatinga, the leaves, flowers and fruit may be used to treat anemia, asthma, bronchitis, whooping cough, inflammation of the lungs, influenza, constipation, stomach pain, cancer, heart murmurs, lesions, diphtheria, chapped feet and gastritis, and as an expectorant, wound healing agent, and blood-thinner (Albuquerque et al., 2007b).

It has also been found to be recommended for snake bites, rheumatic and/or back pain, digestive and urinary disorders, trauma, childbirth disorders, cosmetics, and blood conditions (Ferraz et al., 2006).

2.2.3. Phytochemical Composition

The bark of *A. colubrina* contains various secondary metabolites, including tannins, flavonoids, quinones, triterpenes and cyanogenic glucosides (Tokarnia, 1999; Almeida et al., 2005, Araújo et al., 2008; Alencar et al., 2010). Its leaves have been found to contain 12 different compounds: alnusenol, lupenone, lupeol, betulinic acid, α-amyrin, β-amyrin, β-sitosterol, stigmasterol, apigenin, 4-hydroxybenzoic acid, cinnamic acid and anadantoflavone (Figure 2) (Gutierrez-Lugo et al., 2004).

The seeds have characterized as containing tryptamines, especially bufotenin (5-hydroxy-N,N-dimethyltryptamine), which has a hallucinogenic effect, 5-methoxy-N-methyltryptamine, N,N-dimethyltryptamine, dimethyltryptamine-N-oxide and 5-hydroxy-dimethyltryptamine-N-oxide (Schultes; Hoffmann, 2000; Moretti et al., 2006).

The trunk also produces an exudate rich in arabinogalactane, which consists of a heteropolysaccharide complex containing arabinose, mannose, galactose, rhamnose and hexuronic acid at a molar ratio of 63:1:20:6:10 (called ARAGAL) (Delgogo et al., 1998; Moretão et al., 2003, Moretão et al., 2004).

2.2.4. Biological Properties

ARAGAL affects the immune system, increasing the number of activated macrophages both *in vitro* and *in vivo* and has exhibited phagocytic activity in mice. It also produces a 26-fold increase in tumor necrosis factor alpha (TNFα), demonstrating antitumor activity against Sarcoma-180, inhibiting ascites (63%) and solid tumors (38%) in mice (Delgogo et al., 1998; Moretão et al., 2003, Moretão et al., 2004). Apart from ARAGAL, anadantoflavone has been shown to be effective against platelet lipoxygenases (12-hLO) and human reticulocytes (15-hLO), apigenin against 15-hLO and lupeol, lupenone, α-amyrin inhibit soya lipoxygenase (15-sLO), suggesting action against cancer and inflammations (Moody et al., 1998; Gutierrez-Lugo et al., 2004; Horrillo et al., 2007).

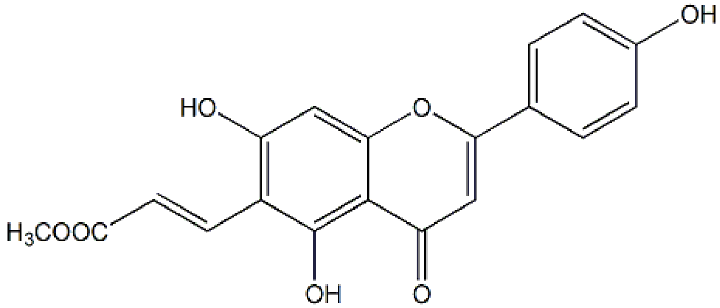


Figure 2. Structure of anadantoflavone, a flavonoid found in the leaves of *Anadenanthera colubrina* (Vell.) Brenan.

Melo et al. (2010) found a high antioxidant capacity, although methanolic extracts of leaves had a proliferative effect on lung carcinoma cells and little antiproliferative effect on larynx tumor cells, showing that they are not effective in combating this kind of tumor.

The 70% hydroethanolic extract of *A. colubrine* was not found to be active against the following microorganisms: *Escherichia coli*, *Enterobacter aerogenes*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Providencia* spp., *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Staphylococcus aureus* and *Staphylococcus* spp. *coagulase* (Gonçalves et al., 2005). However, other studies have shown that this extract is effective against strains of *Staphylococcus aureus* and Salmonella sp. (Palmeira et al., 2010; Kluczynik1 et al., 2010).

2.3. *Chloroleucon extortum* Barneby; J. W. Grimes

2.3.1. Botanical Features

The genus *Chloroleucon* (Benth.) Britton; Rose is found across Brazil and seven species are known: *C. acacioides* (Ducke) Barneby; J. W. Grimes, *C. dumosum* (Benth.) G. P. Lewis, *C. extortum* Barneby; J. W. Grimes, *C. foliolosum* (Benth.) G. P. Lewis, *C. mangense* (Jacq.) Britton; Rose, *C. tenuiflorum* (Benth.) Barneby; J. W. Grimes, *C. tortum* (Mart.) Pittier. It can be found in the North, Northeast and Midwest regions, especially in Caatinga and Atlantic Forest environments (Iganci, 2015).

The genus is characterized by spiny branches, buds wrapped in pearly scales, generally bimorphic flowers and spiral-shaped pods (Fernandes, 2011). *C. extortum* is a tree of around four meters in height, with thorny axillary branches. It has bipinnate leaves with 4 to 5 pairs of leaflets and 22 to 25 pairs of linear leaflets with sickle-shaped pinnae, and moniliform spiral-shaped fruit (Giulietti et al., 2009).

C. extortum is a deciduous heliophile and selective xerophile characteristic of the Caatinga, found in well-drained soils. It produces a large number of viable seeds per year and these are easily disseminated by the wind. It is in blossom from the end of July through September and the plant completely sheds its leaves. The fruit reaches maturity between late September and mid-October (Isam, 2015).

2.3.2. Ethnobotanical Features

The parts of *C. extortum* are used by the local people for various purposes, ranging from food to fuel, to construction, technology and medicine. The bark is used in folk medicine to cure infections, dandruff and hair-loss and to counter head lice (Silva, 2011). There is no information in the literature on the botanical and ethnopharmacological features or chemical composition of this species. Almeida et al. (2011) include it in the list of Brazilian plants for which little data is available (Almeida; Souza; Queiroz, 2011). The experimental data in this chapter are thus being presented for the first time and will provide a significant contribution to knowledge of this species.

2.4. *Erythrina velutina* Willd.

2.4.1. Botanical Features

The genus *Erythrina* contains around 120 species found in tropical regions with a hot climate and is divided into two sub-genera. Twelve of these species are found in Brazil, eight of them native to the Northeast region of the country (Silva, 2013). *Erythrina velutina* Willd. is a deciduous, heliophilic arboreal species (Santos, 2013), measuring 6 to 10 meters in height (Vasconcelos et al., 2011), with an 80-cm diameter trunk. It has alternating compound and trifoliate leaves, sustained by apetiole 6-14 cm in length. Its fruits are leguminous and it bears reddish orange or red flowers and tough flexible leaflets (Carvalho, 2008). *E. velutina* has five synonyms: *Chirocalyx velutinus* Walp., *Corallodendron velutinum* (Willd.) Kuntze, *E. aculeatissima* Desf., *E. aurantiaca* Ridl. and *E. splendida* Diels (Tropicos, 2015).

The species is commonly known as *mulungu*, *canivete*, *sanaduí*, *mulungu-da-caatinga*, *pau-de-coral* and *sananduva* (Silva et al., 2011).

It is found in some countries in South America, including Brazil, Venezuela, Ecuador and Peru and also in Central America (Tropicos, 2015). In Brazil, it is mostly found in the Northeast region and in the broad-leaved semi-deciduous forests of Minas Gerais, in the Caatinga and Cerrado regions (Lima; Martins, 2015).

2.4.2. Ethnobotanical Features

E. velutina is used for ornaments, woodwork, handicrafts and industrial purposes (Santos et al., 2013) to dye clothes and in folk medicine, although its safety and effectiveness remain to be proved (Carvalho, 2008).

The infusion or decoction of the stem bark is used to treat insomnia, coughs and worms, and as a tranquilizer and sedative; (Agra et al., 2008), it is also used to help bring gum abscesses to maturity (Centenaro et al., 2009), and to treat bronchitis and hemorrhoids (Craveiro et al., 2008), and has sudorific and pectoral emollient properties (Silva et al., 2011). The bark of *E. velutina* also produces a yellow tincture with tannic properties. The dried fruit is rolled into a cigar and used as a local anesthetic and to treat toothache (Agra et al., 2008; Carvalho, 2008).

2.4.3. Phytochemical Composition

As it contains a wide variety of species, there are many studies isolating and characterizing the secondary metabolites of *Erythrina*, especially erythrin-type tetracyclic alkaloids. However, other kinds of alkaloids and metabolites have also been isolated from a very wide variety of species of the genus, including orientalin (Flausino-Júnior et al., 2007), flavones and prenylated isoflavones (Waffo et al., 2006).

A study by Carvalho et al. (2009) using aqueous extract of the leaves of *E. velutina* found a variety of secondary metabolites, such as flavonoids, triterpenes, steroids, phenols, xanthones, tannins, alkaloids and saponins. A study by Ozawa et al. (2008) isolated an indole alkaloid, hypaphorine, from the methanolic extract of the seeds of the species (Figure 3). In 2011, the same research group succeeded in isolating four new alkaloids from the seeds (Ozawa et al., 2011). Chromatogram analysis of a hexane fraction the ethanolic extract of the bark of *E. velutina* found cinnamic acid, phenic acid, β-sitosterol and lupeol (Virtuoso et al., 2005).

The stem bark has been used to isolate an oleanane-type triterpene, an erythrinian alkaloid and three different types of flavonoid, one described for the first time in the genus *Erythrina* (Cabral, 2009).

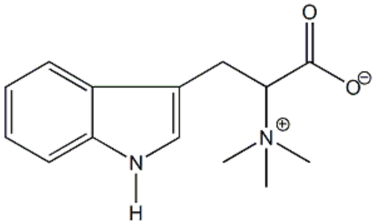


Figure 3. Chemical structure of the indole alkaloid hypaphorine isolated from *Erythrina velutina* Willd.

2.4.4. Biological Properties

E. velutina has been studied from a pharmacological point of view by various authors, although mostly in connection with activity relating to the central nervous system, owing to the presence of alkaloids in its composition. Most of these studies have used the raw extract with a low percentage of isolated metabolites obtained from the species.

Some studies have demonstrated anxiolytic/sedative, antinociceptive, anti-inflammatory and antibacterial properties of extracts from this species (Marchioro et al., 2005; Virtuoso et al., 2005; Vasconcelos et al., 2011).

The indole alkaloid hypaphorine showed a significant increase of 33% in the duration of sleep compared with the control group in mice (Ozawa et al., 2008). Ribeiro et al. (2006) have shown the anxiolytic effect of a 30% hydroethanolic extract of this species when used in rats submitted to models of anxiety and depression, providing support for the use of the species as a tranquillizer in folk medicine.

This study suggested that this effect results from a specific subset of defensive behaviors, which are, in clinical practice, called general anxiety. An antinociceptive effect and depressive action on the central nervous system were observed by Vasconcelos et al. (2004) in treatment of laboratory animals with the hydroalcoholic extract of the bark of *E. velutina*.

Another study by Vasconcelos et al. (2011) showed significant anti-inflammatory properties for *E. velutina*, since there was a significant reduction in dextran-induced edema in rats pre-treated with a 70% hydroethanolic extract of the bark of the species compared to the control group.

E. velutina was also used by Virtuoso et al. (2005) to demonstrate antibacterial properties. The raw hydroethanolic extract of the bark exhibited a minimal inhibitory concentration (MIC) of 9.77 mg/mL for *Staphylococcus aureus* and *Streptococcus pyogenes*.

The extract of the part used also exhibited intermediary activity for other microorganisms, such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella tiphymurium*.

2.5. *Libidibia ferrea* (Mart.) L. P. Queiroz

2.5.1. Botanical Features

The genus *Libidibia* (DC.) Schltdl contains 10 species, which are found in dry neotropical forests, principally in South America (Tropicos, 2015). *Libidibia ferrea* (Mart.) L.P. Queiroz, commonly known as *pau-ferro* or *jucá*, is a species native to Brazil, primarily occurring in the Amazonian and Caatinga biomes (Crepaldi et al., 1998).

As a result of an updated taxonomy produced by Queiroz (2009), the species *Caesalpinia ferrea* Mart. and its varieties have been included in *L. ferrea.* This species of tree can grow to 15 meters in height and has a smooth dark trunk with irregular patches of white. The leaves of this species are bi- and imparipinnate, its flowers dychlamideousin the form of yellow-colored terminal panicles and its fruit (pods) are fleshy, indehiscent and reddish in color (Di Stasi; Hiruma-Lima, 2002).

2.5.2. Ethnobotanical Features

Ethnobotanical studies have shown that *L. ferrea* is an economically important species with multiple uses, such as fodder, carpentry, construction and fuel production. *L. ferrea* is also used in landscape gardening for parks and public squares (Rizzini; Mors, 1995) and to reforest degraded areas (Lorenzi, 2002).

A survey conducted by Albuquerque et al. (2007a) of medicinal plants from the semi-arid region of the Northeast of Brazil noted that all parts of the plant are used to treat a variety of diseases, including heart murmurs, throat afflictions, bronchitis, anemia, swelling, back pain, injuries, labyrinthitis, kidney problems, inflammations in general, stress and fatigue. Agra et al. (2008) found that the stem bark of *L. ferrea* is used to treat anemia, diarrhea and dysentery.

Ferreira Júnior et al. (2011) evaluated the model of utilitarian redundancy to investigate the preference for plants native to the Caatinga used as anti-inflammatories by those interviewed and found that use of the extract of *L. ferrea* to treat contusions, pain in the uterus and kidneys, cuts and skin wounds and throat inflammation is scientifically recognized. Gazzaneo et al. (2005) assessed knowledge and use of medicinal plants by specialists from a Mata Atlântica community in the State of Pernambuco and found that the fruit and stem bark are used to treat skeletal, muscle, and connective tissue disorders.

2.5.3. Phytochemical Composition

Phyochemical investigation of the hydroalcoholic extract of the stem bark and leaves of *L. ferrea* has revealed the presence of saponins, steroids, coumarins, flavonoids, tannins and other phenolic compounds (Gonzalez et al., 2004). Almeida et al. (2005) also conducted preliminary phytochemical tests and identified the presence of phenolic and quinonic compounds.

Tannins are the main class of compounds isolated from *L. ferrea*, including gallic acid, ellagic acid, methyl gallate, catechin and epicatechin (Souza et al., 2006, Vasconcelos et al., 2011). Nozaki et al. (2007) isolated an amorphous yellow powder from the stem bark of *L. ferrea* and identified it as a chalcone derivative called pauferrol-A. This is a chalcone trimer with a cyclobutane ring (Figure 4).

2.5.4. Biological Properties

Studies have shown that ellagic acid extracted from the bark of *L. ferrea* inhibits aldose-reductase, an enzyme involved in the complications of diabetes (Ueda et al., 2001).

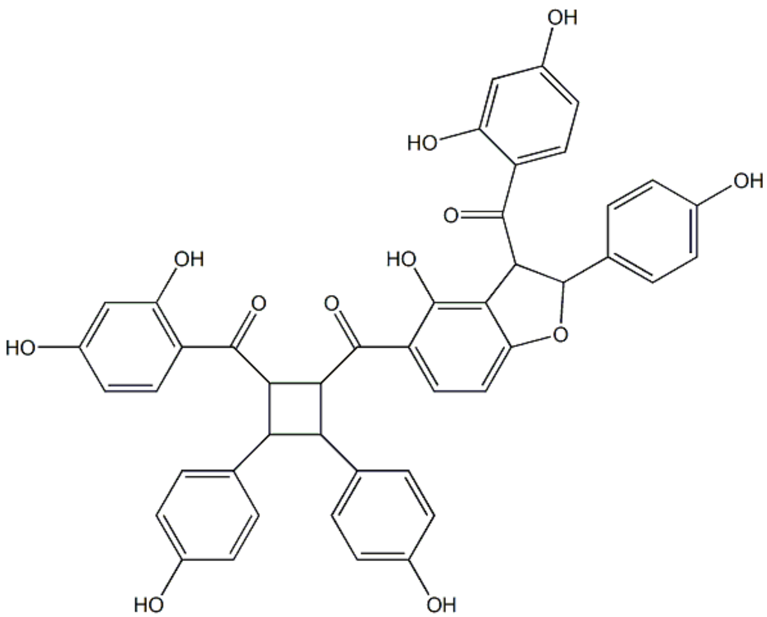


Figure 4. Pauferrol-A, a chalcone trimer with cyclobutane ring isolated from the bark of *Libidibia ferrea* (Mart.) L.P. Queiroz.

Giazzi et al. (1991) showed that the aqueous extracts of the bark of this species partially inhibit the larvae of ancylostomids, while the hydroalcoholic extracts inhibit them completely. Gallic acid and methyl gallate isolated from the fruits of *L. ferrea* significantly reduced the number of papillomas induced by 12-O-tetradecanoylforbol-13-acetate (TPA) in mice, indicating anti-cancer properties (Nakamura et al., 2002). The dry extract of the fruit of *L. ferrea*

(300mg/kg, v.o.) has been shown to exhibit anti-inflammatory, anti-ulcer and peripheral analgesic properties (Bacchi; Sertié, 1991). Menezes et al. (2007) showed *in vitro* that the dry extract of the bark of *L. ferrea* causes vasodilation of the mesenteric artery in rats and hypotension associated with tachycardia.

Studies of raw extracts of *L. ferrea* have revealed anti-ulcer properties (Bacchi; Sertié, 1994; Bacchi et al., 1995) and restriction of coronary circulation, possibly through action on the smooth musculature of vessels, with secondary electrocardiographic alterations. Other studies of *L. ferrea* have found it to be toxic to the liver. These authors point out that this species, which is widely used by the local people, should be controlled and used with care, in view of its toxic effects (Di Stasi, 2002).

2.6. *Mimosa tenuiflora* (Willd.) Poir.

2.6.1. Botanical Features

Mimosa tenuiflora (Willd.) Poir., commonly known in the semi-arid region as *jurema preta*, is used by indigenous tribes and the local people of these regions for medicinal purposes (Dourado; Conceição; Santos-Silva, 2013). Its synonyms are *Acacia hostilis* Mart., *Acacia tenuiflora* Willd., *Mimosa cabrera* H. Karst., *Mimosa hostilis* Benth., *Mimosa limana* Rizzini and *Mimosa nigra* Huber (Tropicos, 2015). It is a bushy plant ranging from 1-8 meters in height, with thorns arranged irregular on the internodes. Its bitter-tasting astringent roots are long and have a dark brown outer cuticle. The thorny stem is dark brown on the outside, light brown on the inside, and wine red in between. The brown bark is fibrous with lengthwise fissures a resinous astringent taste and sweetish smell. It has small white flowers arranged in isolated ears 4-8 cm in length. The fruit is takes the form of adehiscent pod, 2.5-5 cm in length containing 4-6 seeds. The oval-shaped seeds are smooth and dark brown. It is axerophilic, deciduous species that flowers and bears fruit in the driest part of the year, when it has no foliage (Mello, 1955; Pio-Correa, 1984; Sangirardi Junior, 1989; Camargo-Ricalde, 2000).

2.6.2. Ethnobotanical Features

In the Northeast of Brazil, some indigenous tribes use *M. tenuiflora* wine, a "miraculous drink" made, generally, of the stem bark of the species, for communication with the spirit world, diagnosis of disease, and to receive oracular advice and visions, as it is considered an “entheogenic” plant (Albuquerque, 1997).

Studies have shown the potential of *M. tenuiflora* for the treatment of infections (Heinrich et al., 1992), treating minor burns, lesions, and eczemas (Tellez; Dupoy de Guitard, 1990), and its antimicrobial properties (Lozoya et al., 1989; Meckes-Lozoya et al., 1990a). However, several other reported uses have not been examined: effectiveness against hair loss; headaches, and toothaches; internal problems such as stomach acidity, gastritis, peptic and duodenal ulcers, colitis, and hemorrhoids; and herpes, acne, and parasite-related diseases (Camargo-Ricalde, 2000).

Studies have also shown the capacity of the species to cure leather with good penetration and dispersion in the skins, dispensing with the need for dispersants. The physical-mechanical characteristics of skins cured with the tannins of *M. tenuiflora* were superior to those of other species, showing that this plant also has the potential to be used in the tanning of hides (Lima et al., 2014).

2.6.3. Phytochemical Composition

Various substances with biological properties have been isolated from the stem bark of *M. tenuiflora* (Souza et al., 2008). These substances include three steroids: campesterol-3-O-beta-D-glucopiranosil, estigmasterol-3-O-beta-D-glucopiranosil and beta-sitosterol-3-O-beta-D-glucopiranosil (Anton et al., 1993); three terpenoids: mimonoside A, mimonoside B and mimonoside C, the first two triterpenoid glucosides and the third a triterpenoid saponin (Anton et al., 1993; Jiang et al., 1991; Jiang et al., 1992); and two indole alkaloids: 5-hidroxy-tryptamine and N,N-dimethyltryptamine (Figure 5) (Meckes-Lozoya et al., 1990b), the latter also being found in the root bark (Melo; Bandeira, 1961).

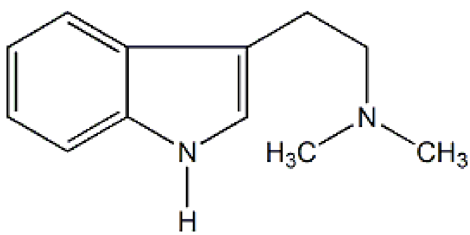


Figure 5. The indole alkaloid, N,N-dimethyltryptamine, isolated from the stem bark and roots of *Mimosa tenuiflora* (Willd.) Poir.

In addition to N,N-dimethyltryptamine, Meckes-Lozoya et al. also reported finding compounds of a phenolic character (including hydroxinamic acids, flavonoids and tannins), terpenic compounds (phytosterols and triterpenic acids), other indole alkaloids and triterpenic saponins (Meckes-Lozoya et al., 1990a). Other studies have found chalcones: kukulkane A or 2',4'-dihydroxy-3',4'-dimethoxichalcone; and kukulkane B or 2',4',4-trihydroxy-3'-methoxichalcone (Camargo-Ricalde, 2000).

Anton et al. (1993) also found tannins linked to the sugars xylose, rhamnose and arabinose and the triterpenoid lupeol.

2.6.4. Biological Properties

The substance responsible for the psychoactive properties of *M. tenuiflora* is an indole alkaloid (N,N-dimethyltryptamine), which is inactive when taken orally (Meckes-Lozoya et al., 1990b). For the hallucinatory effects to occur, it is also necessary to ingest the substances containing MAO inhibitors (such as β-carbolines), which will then permit the action of N,N-dimethyltryptamine (Schultes; Hofmann, 1980).

According to Ott (2002), there may be MAO inhibitors in the *M. tenuiflora* extract, which would explain the observed effects. The hallucinogenic effect that accompanies the use of *M. tenuiflora* is similar to that of LSD-25, although apparently of faster effect and shorter duration, as mydriasis and arterial hypertension are significantly intense (Corbett, 1977).

Pereira et al. (2009) tested the antifungal properties of *M. tenuiflora* against strains of *Candida* spp. Isolated from cases of sub-clinical mastitis in cows in the State of Pernambuco. The ethanolic extract of the species exhibited fairly satisfactory antifungal activity against *C. albicans*, with a result better than those obtained with the standard drug fluconazol (Pereira et al., 2009).

The antimicrobial action of ethanolic extract of *M. tenuiflora* against strains of *Staphylococcus aureus* from clinical isolates had higher minimum inhibitory concentrations (MIC) of 0.18 mg/mL for 16 isolates and 0.36 mg/mL for the other 14 isolates and for the standard strain (Padilha et al., 2010). One study has shown the effectiveness of the extract of *M. tenuiflora* at different concentrations against 25 strains of *S. aureus*, the main cause of mastitis, with inhibition haloes of between 6 and 25 mm (Bezerra et al., 2009).

However, it should also be noted that congenital malformations have been found to be caused by ingestion of *M. tenuiflora* in ruminants in the semi-arid region of the Brazilian Northeast. The percentage of malformations varies according to species of animal, 4.3% for sheep, 1.84% for cattle and 1.81% for goats (Dantas et al., 2010).

Genotoxic effects of *M. tenuiflora* have been investigated using both the micronucleus test and bacterial reverse mutation assay on *Salmonella typhimurium*. The results show that the extract is not mutagenic in the absence of an exogenous metabolizing system and does not produce an increase in the frequency of the micronucleus and is characterized as a non-mutagenic agent in these conditions (Silva et al., 2013).

3. EXPERIMENTAL SECTION

This section briefly reports on the methods used for all experimental procedures.

3.1. Determination of the Total Phenolic Content (TPC) and Total Tannin Content (TTC)

The TPC of the extracts was determined by the Folin-Ciocalteu method and the residual phenolic content was determined by precipitation of casein followed by Folin-Ciocalteu, where the TTC is the difference between the levels of total and residual phenols (Amorim et al., 2008). The TPC was calculated from 0.2 mL of diluted extract (1 mg/mL, w/v), 0.5 mL of aqueous solution of Folin-Ciocalteu (10%, v/v), 1 mL of aqueous solution of sodium carbonate (7.5%, w/v) and 8.3 mL of distilled water. The solution was allowed to stand in the dark for 30 minutes and the absorbance was measured at 760 nm. To calculate the residual phenolic content, 6 mL of diluted extract (1 mg/mL, w/v) and 12 mL of distilled water was agitated for 3 hours with 1 g of casein, filtered and adjusted to a final volume of 25 mL with distilled water.

The residual phenolic content was determined with 1 mL of the filtrate by the Folin-Ciocalteu method. The TTC was calculated as the difference between the content of total phenols and residual phenols.

TPC and TTC were expressed as one milligram of tannic acid per gram of sample (mg TAE/g). The samples were evaluated in triplicate. The calibration equation for tannic acid was $y = 0.047x + 0.127$ ($R^2 = 0.985$).

3.2. Determination of Total Flavonoid Content (TFC)

The TFC of the extracts was estimated using a colorimetric method based on the formation of a flavonoid-aluminum complex (Peixoto Sobrinho et al., 2008). The TFC was calculated using 0.2 mL of diluted extract (1 mg/mL, w/v), 0.12 mL of glacial acetic acid, 2 mL of pyridine in methanol (20%, v/v), 0.5 mL of aluminum chloride in methanol (5%, w/v) and 7.18 mL of distilled water. The solution was allowed to stand in the dark for 30 minutes and the absorbance was measured at 420 nm.

The results were expressed as one milligram of rutin per each gram of sample (mg RE/g). The samples were evaluated in triplicate. The rutin calibration equation was y = 0.026x + 0.020 (R^2 = 0.997).

3.3. Determination of the Coumarin Content (CC)

The CC was determined using the colorimetric assay described by Osório and Martins (2004) with some adjustments. The coumarin content was calculated using 0.5 mL of diluted extract (1 mg/mL, w/v), 500 µL of lead acetate solution (5%, w/v) and 9 mL of distilled water.

In this solution, 2 mL were transferred to new test tubes and added to 8 ml of hydrochloric acid solution (0.1M, v/v). The solution was allowed to stand in the dark for 30 minutes and the absorbance was measured at 320 nm. The results were expressed as one milligram of coumarin (1,2-benzopyrone) per gram of sample (mg CE/g). The samples were evaluated in triplicate. The coumarin calibration equation was y = 0.022x + 0.005 (R^2 = 0.994).

3.4. Evaluation of Antioxidant Activity (AOA)

The free-radical scavenging activity (DPPH) assay was performed in triplicate, based on the method described by Peixoto Sobrinho et al. (2011). Different concentrations (100-1000 µg/mL) of the diluted extract (0.5 mg/mL, w/v) were added to 3 mL of DPPH in methanol (40 µg/mL, w/v) in a test-tube. The solution was allowed to stand for 30 minutes in the dark and the absorbance was then measured at 517 nm. Measurements were compared with a negative control solution of DPPH (40 µg/mL, w/v) and were then used as blank concentrations of 0.5 mL of the diluted extract with 3 mL of methanol. The final result of the DPPH assay was calculated from a calibration curve obtained for the percentage of radical scavenging activity (Equation RSA) versus concentrations of diluted extracts and expressed as the IC_{50}, i.e., the inhibitory concentration of the sample required to reduce the absorbance of the negative control by 50%.

$$RSA(\%) = \frac{\text{ABS}_{\text{negtive control}} - (\text{ABS}_{\text{sample}} - \text{ABS}_{\text{blank}})}{\text{ABS}_{\text{negative control}}} \times 100$$

3.5. Antibacterial Test Using the Agar Diffusion Method

The antimicrobial activity of the extracts was evaluated by the agar diffusion method, using the paper disk technique (Bauer et al., 1966). The extracts were tested on the following standard strains of Gram-positive bacteria: *Staphylococcus aureus* (DAUFPE 01), *Bacillus subtilis* (DAUFPE 16), *Enterococcus faecalis* (DAUFPE 138) and *Micrococcus luteus* (DAUFPE 06); Gram-negative bacteria: *Escherichia coli* (DAUFPE 224), *Pseudomonas aeruginosa* (DAUFPE 39) and *Serratia marcescens* (DAUFPE 398); Acid-fast bacilli: *Mycobacterium smegmatis* (DAUFPE 71); and yeast *Candida albicans* (DAUFPE 1007).

Disks of 6 mm in diameter were impregnated with 10 µL of each diluted extract (200.000 µg/mL, w/v). The inoculates at 0.5 on the McFarland scale (10^8 CFU/mL) were plated with 20 mL of Mueller-Hinton agar. The positive control used gentamicin at 10 µg/disk and ketoconazole at 30 µg/disk. The plates were pre-incubated for 3 hours at room temperature, allowing for the full diffusion of the extracts. These diffused extracts were then incubated aerobically at 37 ± 1 °C for 24 hours and the antibacterial activity was assessed by measuring the inhibition zones, in accordance with the parameters suggested by Alves et al. (2000): Inhibition zones <9 mm were considered inactive; 9-12 mm, less active; 13-18 mm, active; and >18 mm, very active.

3.6. Determination of Minimum Inhibitory Concentration (MIC)

The MIC evaluation was adapted from the microdilution methodology proposed by the Clinical and Laboratory Standards Institute (CLSI, 2010) with bacteria that exhibited an inhibition zone ≥ 15 mm. Each well of sterile 96-well microplates received 180 µL of Mueller-Hinton broth, 20 µL solutions of different concentrations of the extracts (3.93 to 2000 µg/mL) and 10 µL of inoculum (*S. aureus*, *B. subtilis* e *M. luteus*) corresponding to 0.5 on the McFarland scale (10^8 CFU/mL). The controls used were as follows: 1) 100 µL of Muller-Hinton broth (control of sterility); 2) 100 µL of Muller-Hinton broth and 20 µL of inoculum to 0.5 McFarland scale (control of microbial growth). The microplates were incubated at 37 ± 1 °C for 24 hours and thereafter, 50 µL of aqueous resazurin (2.5 mg/mL, w/v) was added as a developer. This test was performed in triplicate.

3.7. Statistical Analysis

The Shapiro-Wilk test confirmed the normality of the data obtained. Data were expressed as means ± standard deviation and were analyzed using Kruskal-Wallis followed by the Dunn test.

A Spearman correlation test was used to compare the phenolic content with the antioxidant and agar diffusion (inhibition zone) tests of the samples. A p value < 0.05 was considered significant. BioEstat 5.3 software was used to perform statistical analysis and GraphPad Prism 5 to plot graphs.

4. Results and Discussion

The results will be presented for doses of total phenols, tannins, flavonoids and coumarins along with the antioxidant and antimicrobial properties (disk test and minimum inhibitory concentration) of the species studied.

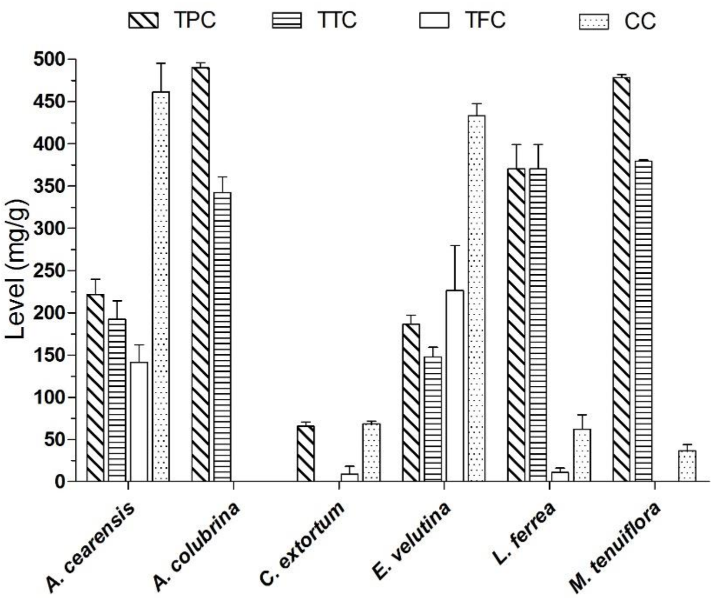
4.1. Phytochemical Composition and Antioxidant Properties

The total phenolic content (TPC), total tannin content (TTC), total flavonoid content (TFC), coumarin content (CC) and inhibitory concentration for reduced absorbance in 50% (IC_{50}) for antioxidant activity can be found in Table 1 and are presented as a graph in Figure 6. The species that had the highest phenol content was *A. colubrina* with 490.36 ± 5.80 mg/g. However the content of this species was not significantly different from that of *L. ferrea* and *M. tenuiflora*. *M. tenuiflora* had the highest tannin content (379.50 ± 1.51 mg/g), but was not significantly different from *L. ferrea* and *A. colubrina*.

The flavonoid content was highest in *E. velutina* with 226.39 ± 53.35 mg/g and coumarin content was highest in *A. cearensis* with 461.40 ± 33.84 mg/g. As for the capacity to capture the free radical DPPH, the extract of *A. colubrina* provided the best result with an IC_{50} of 10.79 ± 1.16 µg/mL.

This species proved to be more effective than the positive control, ascorbic acid, with an IC_{50} of 14.90 µg/mL, indicating that the antioxidant power of this species is comparable to that of ascorbic acid.

However, despite having the strongest antioxidant properties of all the plants tested, the species was not significantly different from *L. ferrea*, *M. tenuiflora* and *A. cearensis*. The phytochemical analysis of the six species studied in this chapter showed a positive correlation between TPC and TTC (rs = 0.8286, p = 0.0415) and there was no correlation between the other secondary metabolites present. There was a negative correlation between TPC and TFC and antioxidant activity in the capture of free radicals (rs = -0.9429, p = 0.0048 and rs = 0.8117, p = 0.0498, respectively), but there was no correlation between this activity and TTC or CC.



TPC = Total phenolic content, TTC = Total tannin content, TFC = Total flavonoid content, CC = Coumarin content.

Figure 6. Total phenol, tannin, flavonoid and coumarin content obtained for six medicinal species of the Fabaceae family found in the Caatinga.

Table 1. Total phenol, tannin, flavonoid and coumarin content expressed as mean ± standard deviation for Caatinga Fabaceae species

Scientific Name	Common Name	TPC (mg EAT/g)	TTC (mg EAT/g)	TFC (mg ER/g)	CC (mg EC/g)	IC_{50} (μg/mL)
Amburana cearensis	Imburana açú	221.56 ± 18.12^{acd}	192.20 ± 22.17^{acd}	141.45 ± 20.73^{ac}	461.40 ± 33.84^{b}	332.89 ± 21.32^{ab}
Anadenanthera colubrina	Angico	490.36 ± 5.80^{b}	342.08 ± 18.78^{ab}	ND	ND	10.79 ± 1.16^{b}
Chloroleucon extortum	Jurema Branca	65.45 ± 4.97^{c}	ND	8.55 ± 9.94^{bc}	68.52 ± 3.21^{ab}	584.33 ± 62.49^{ac}
Erythrina velutina	Mulungu	186.28 ± 10.86^{ac}	147.89 ± 11.33^{ac}	226.39 ± 53.35^{a}	433.33 ± 14.29^{ab}	838.05 ± 78.77^{a}
Libidibia ferrea	Jucá	370.33 ± 28.82^{abd}	370.33 ± 25.87^{bd}	10.68 ± 5.45^{bc}	62.12 ± 17.21^{ab}	21.92 ± 1.32^{ab}
Mimosa tenuiflora	Jurema Preta	478.46 ± 3.61^{bd}	379.50 ± 1.51^{b}	ND	36.51 ± 7.27^{ab}	19.13 ± 0.38^{bc}
Control (ascorbic acid)						14.90

TPC = Total phenolic content, TTC = Total tannin content, TFC = Total flavonoid content, CC = Coumarin content, IC_{50} = Inhibitory concentration for reduced absorbance of 50%, ND = Not detected. Values followed by the same letter in column are not statistically different according to Kruskall Wallis followed by the Dunn test ($p < 0.05$).

The correlation between TPC and TTC was predicted, since the same methodology was used for the doses of these metabolites and the samples used to produce the doses of these compounds were bark, corroborating the results found by Araújo et al. (2008) who observed that, in medicinal plants from the Caatinga, tannins are found in greater percentages in samples of bark. No correlation was found for the other metabolites. Some authors have shown a correlation between total phenolic compounds and antioxidant activity (Wojdylo et al., 2007).

A study by Li et al. (2009) showed a good correlation between phenolic compounds and antioxidant capacity, suggesting a significant contribution on the part of this group of metabolites to this activity.

In the case of flavonoids, Orcić et al. (2011) showed a good correlation between antioxidant activity and fractions of vegetal extracts rich in glycosidic flavonoids, suggesting that this kind of flavonoid contributes to antioxidant activity.

4.2. Antimicrobial Activity

Following the parameters described by Alves et al. (2000), the extracts of *A. colubrina*, *L. ferrea* and *M. tenuiflora* were found to be very active against strains of Gram-positive bacteria and alcohol acid resistant bacilli (Table 2). *A. colubrina* produced haloes of 18.0 ± 1.0 mm (*S. aureus*), 23.3 ± 0.6 mm (*M. luteus*) and 23.3 ± 0.6 mm (*M. smegmatis*), *L. ferrea* of 19.3 ± 1.5 mm and 20.3 ± 1.5 mm against *S. aureus* and *M. smegmatis*, respectively, and *M. tenuiflora* haloes of 19.3 ± 1.2 mm and 17.7 ± 0.6 mm against *S. aureus* and *E. faecalis*, respectively. These results are significant in that the extracts produced haloes similar to those of gentamicin, suggesting potential antimicrobial properties for these species.

Plant extracts are considered to have good antimicrobial activity with an MIC < 100 µg/mL, moderate activity with an MIC of 100 to 500 µg/mL, weak activity with an MIC of 500 to 1000 µg/mL and to be inactive with an MIC > 1000 µg/mL (Tanaka et al., 2005). According to these parameters, the extracts that stand out are those that were moderately active on the MIC test: *A. colubrina*, *L. ferrea* and *M. tenuiflora* against *S. aureus* with MICs of 500, 250 and 500 µg/mL, respectively; *A. colubrina* against *M. luteus* with an MIC of 250 µg/mL. The MICs of *L. ferrea* (31.25 µg/mL) and *M. tenuiflora* (62.5 µg/mL) against *M. luteus* (Table 3) are also worthy of note.

Table 2. Results for antimicrobial activity (disk test) of hydroalcoholic extracts of Caatinga species of the Fabaceae family

Microorganisms	Hydroalcoholic Extracts (2,000µg/disk) (haloes in mm)						Standard	
	1	2	3	4	5	6	Gentamicin (10 µg/disk)	Cetoconazol (30 µg/disk)
S. aureus	14.3	18.0	-	-	19.3	19.3	23.0	-
B. subtilis	11.3	16.7	-	-	12.3	14.7	28.0	-
E. faecalis	14.7	15.0	-	-	17.7	14.7	15.0	-
M. luteus	12.3	23.3	-	12.3	14.0	16.3	31.0	-
E. coli	-	-	-	-	-	-	23.0	-
P. aeruginosa	-	-	-	-	-	-	21.0	-
S. marcescens	-	-	-	-	-	-	15.0	-
M. smegmatis	12.0	23.3	-	-	15.0	20.3	33.0	-
C. albicans	-	-	-	-	-	-	-	40.0

Extracts: 1 – *Amburana cearensis*; 2 – *Anadenanthera colubrina*; 3 – *Chloroleucon extortum*; 4 – *Erythrina velutina*; 5 – *Libidibia ferrea*; 6 – *Mimosa tenuiflora*; (–): No activity.

Table 3. Minimum inhibitory concentration (MIC) in µg/mL for hydroalcoholic extracts of Caatinga Fabaceae

Microorganisms	Hydroalcoholic extracts (µg/mL)					
	2		5		6	
	MIC	CMB	MIC	CMB	MIC	CMB
S. aureus	500	>2000	250	>2000	500	>2000
B. subtilis	1000	>2000	2000	>2000	-	-
M. luteus	250	>2000	62.5	1000	62.5	500
E. faecalis	-	-	2000	>2000	-	-

Extracts: 2 – *Anadenanthera colubrina*; 5– *Libidibia ferrea*; 6 – *Mimosa tenuiflora*; (–): No activity.

Like Trentin et al. (2011), who commented in their studies on the potential of Caatinga plants to combat microorganisms, and Rosado-Vallado et al. (2000), who found antimicrobial activity against Gram-positive rather than Gram-negative bacteria in Fabaceae, the Caatinga species of Fabaceae selected for the present study also proved to be promising on microbiological investigation, showing good activity against Gram-positive bacteria. In the species studied, the total phenolic content correlated positively with antimicrobial activity (disk test) against *B. subtilis*, *E. fecalis*, *M. luteus* and *M. smegmatis* (rs = 0.9276; rs = 0.9122; rs = 0.9276; rs = 0.9276, respectively).

The tannin content was positively correlated with activity against two microorganisms: *S. aureus* and *E. fecalis* (rs = 0.9710; rs = 0.8827, respectively), with no correlation for the other metabolites and inhibition haloes for the other microorganisms tested. None of the extracts tested showed an inhibition halo for *E. coli*, *P. aeruginosa*, *S. marcescens* or *C. albicans*. The correlation results obtained corroborate the findings of Shan et al. (2007), who found a group of species to present a correlation between total phenolic content and antimicrobial activity agains five microorganisms, with R^2 of between 0.93 and 0.73.

CONCLUSION

This chapter has demonstrated the therapeutic uses of Caatinga species belonging to the Fabaceae family. Further investigation of these revealed that they are present in the life of the local population, in the construction industry (carpentry), religious rituals and the treatment of various diseases.

We also found rich chemical compositions with a wide diversity of compounds isolated and scientifically tested pharmacological activity.

In the species studied, the phenolic compounds content (total phenols, tannins, flavonoids, and coumarins) is high and antioxidant and antimicrobial activity is directly related to the levels of these metabolites.

L. ferrea is a species especially worthy of note in view of its potential as a new antimicrobial medication against *M. luteus*.

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Chapter 3

ANOTHER BRICK IN THE WALL OF THE *OXYTROPIS CAMPESTRIS* COMPLEX WITH AN EMPHASIS OF THREE MEMBERS OF THIS GROUP FROM PIRIN MTS, THE BALKANS

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ABSTRACT

The *Oxytropis campestris* complex has a circumpolar distribution and its speciation is still not completely elucidated. There are three members of this group which occur in the Pirin Mts. Two of them, *Oxytropis urumovii* Jav. and *O. kozhuharovii* Pavlova, Dimitrov & Nikolova are alpine endemics to the North Bulgarian Pirin marble. The third one is the widespread *O. campestris* (L.) DC. They are more or less closely related to other members of the *O. campestris* complex on the Balkans. Using morphological characters and molecular techniques, including RAPDs, ITS and trnL sequences, we compared the two endemics to the North Bulgarian Pirin marble (*O. urumovii* and *O. kozhuharovii*), with neighbouring populations of the widespread *O. campestris* (L.) DC, as well as with other widespread relatives (*O. halleri* Bunge ex Koch) or Balkan endemics (*O. dinarica* (Murb.) Wettst).

Oxytropis urumovii is a very distinct diploid species which might be ancestral to this group and could be regarded as a palaeoendemic. The tetraploid *O. kozhuharovii* is most closely related to *O. prenja* from the Dinaric Alps, Bosnia-Herzegovina, but is a larger plant with a different facies and indumentum. It is possible that it has evolved as an allotetraploid derivative of *O. urumovii* and *O. halleri*. It is also possible that the circumpolar hexaploid *O. campestris* has evolved as an allohexaploid derivative of the diploid *O. urumovii* and a tetraploid from the Balkans, such as *O. kozhuharovii*. Additionally, *O. kozhuharovii* was found to contain Quercetin-3-O-β-D–(3IImethoxy) -glucopyranoside. It is interesting that we have detected a compound in *O. kozhuharovii* not found in the other two species, which suggests that it might have come from an unknown parent that resulted in the purple pigment, e.g. *O. halleri*, *O. dinarica* (Murb.) Wettst. or *O. prenja* (Beck) Beck.

We observed a number of microhabitat specifics of the three *Oxytropis* species in the Pirin Mts. Here we present details about the environment - slope, exposure, bed rock, soils, vegetation and phenology. The breeding systems of the three species were studied both with direct (insect excluders in the field) and indirect (P/O ratio methods). Results are discussed. Pollinators were active only in the flowers of *O. campestris* which formed a big patch while the two endemic species were seldom visited by pollinators. Details are discussed. We also tested the seed germination process, development of the seedlings and *ex-situ* seedling behaviour of *Oxytropis urumovii* and *O. kozhuharovii*. Both plant species reproduce by seed and have poor vegetative propagation. Most mature test seeds would germinate within a couple of days if the seed coat was scarified. The seedlings were rather sensitive and many did not survive. The seedlings of the tetraploid *O. kozhuharovii* developed slightly better than those of the diploid *O. urumovii* and during the second year after germination several seedlings bloomed.

Keywords: ITS sequence, phylogeny, RAPD, *Oxytropis kozhuharovii, O. urumovii, O. campestris* breeding systems, pollination ecology, fruit set

INTRODUCTION

The genus *Oxytropis* has about 300 species distributed through temperate, montane and boreal regions of the northern hemisphere. It is closely related to an even larger and more widespread genus, *Astragalus* (2500 species), from which it is distinguished only by a mucronate tooth on the abaxial side of the keel. It seems possible that *Oxytropis* is an artificial grouping however, Wojciechowski et al. (1993) and Sanderson & Wojciechowski (1996) show that three species of *Oxytropis*, including *O. campestris*, are sisters to a much larger sample of *Astragalus* which forms a single clade, but studies involving a larger number of *Oxytropis* species are awaited.

Some species in both the genera *Oxytropis* and the closely related and larger *Astragalus* are 'locoweeds', selenium accumulators, or contain the alkaloid swainsonine and thus are harmful to stock (Demeuov et al., 1998, Ralphs & James, 1999, Ralphs et al., 2000, Torell et al., 2000, Gardner et al., 2001, Stegelmeier et al., 2001, Pfister et al., 2001). Toxic alkaloid quinolizidine compounds were detected with thin layer chromatography in three *Oxytropis* species from China, namely *O. kansuensis*, *O. ochrocephala and O. glabra* (Xiezhong & Ruming**,** 1995). Some *Oxytropis* species are found to have high to very high alkaloid contents (more than 1%, e. g. *Oxytropis ambigua*, Lugmanova et al., 2006). Four species of *Oxytropis,* in North America i.e. *O. besseyi* Rydberg, *O. campestris* (L.) DC., *O. lambertii* Pursh, and O. *sericea* Nuttall ex Torrey & Gray, are cited as toxic or potentially so due to the presence of swainsonine (Ralphs et al., 2002). However further investigations reveal a rather interesting reason for the presence of swainsonine - a fungal endophyte, *Embellisia* spp., and *Undifilum oxytropis* has been implicated in the synthesis of swainsonine in *Oxytropis* and *Astragalus* species (Ralphs et al., 2008). A survey of the major locoweeds was conducted to verify the presence of the endophyte and to relate endophyte infection with swainsonine concentrations. Species found to contain the fungal endophyte and produce substantial amounts of swainsonine were *A. wootoni*, *A. pubentissimus*, *A. mollissimus*, *A. lentiginosus*, and *O. sericea*. *Astragalus* species generally had higher concentrations of swainsonine than *Oxytropis.* Swainsonine was not detected in *A. alpinus*, *A. cibarius*, *A. coltonii*, *A. filipes*, or *O. campestris*. The endophyte could not be cultured from *A. mollissimus* var. *thompsonii* or *A. amphioxys*, but endophyte DNA was detected by polymerase chain reaction, and only 30% of these samples contained trace levels of swainsonine. Further research is necessary to determine if the endophyte is able to colonize these and other species of *Astragalus* and *Oxytropis* and determine environmental influences on its growth and synthesis of swainsonine (Gardner et al., 2001, Ralphs et al., 2008, Cook et al., 2009).

More than 127 chemical constituents have been isolated from the genus *Oxytropis*, including flavonoids, flavonones, chalcones, isoflavones, isoflavanones, dihydroflavones, alkaloids, saponins, lignans and others compounds (Elisens & Denford, 1982, Sakanyan & Blinova, 1986, Sun & Jia, 1990, 1997; Sun et al., 1989, 1990, 1991, 1992, Pkhonasa, 1991, Yamaguchi et al., 2002, Li et al., 2012).

Section *Orobia*, one of the richest in species within the subgenus *Oxytropis*, includes 27 species and subspecies within the Arctic. It is one of the most difficult sections with respect to taxonomy, and it probably has given rise to the sections *Baicalia* and *Glaeocephala*. It is extremely polymorphic with several very close aggregates of species forming parallel series, which recall the series of homologous variation sensu N.Vavilov. In section *Orobia* is included the *Oxytropis campestris* complex. In the *O. campestris* complex the lowest ploidy level in Eurasian species and races is 2n = 48, whereas in western North America and far-east Asia there are taxa with both 2n = 16 and 32, which are not always morphologically distinct: e.g. *O. gorodkovii* in the easternmost Chukotka Peninsula, (Yurtsev, 1986, 1988, 1994, 1997, 1999; Yurtsev et al., 1978, 1994). At the same time, morphometric analyses reveal significant differences between some North American taxa - the tetraploid *O. campestris* var. *chartacea*, and the hexaploid *O. campestris* var. *johannensis* (Chung et al., 2004). In other cases, polyploidy has accompanied and consolidated evolutionary adaptation to contrasting cold treeless environments (as in *O. sverdrupii* 2n = 48, *O. wrangelii* 2n = 64; or *O. viscida* 2n = 16 and 32, *O. middendorffii* 2n = 48) (Yurtsev, 1986, 1988, 1994, 1997, 1999; Yurtsev et al., 1978, 1994).

The Pirin horst SW Bulgaria is a neotectonic block structure that has inherited elements of Precambrian, Palaeozoic, late Mesozoic and Palaeogene structures. It has a Neogene - Quaternary origin. The rocks involved are extremely complex, dating from a wide variety of geological periods. They have mostly been metamorphically modified and folded in several deformation events. Much of the northern Pirin is marble - Razlozki and Sinanitsa anticlines (Zagorchev, 1994, 1995a, b, 1998). Velčev & Kenderova (1994) separated three glaciations, Mindell, Riss and Wurm, on the basis of thermoluminiscence analyses of correlative deposits from the Western foot of Pirin. There are three acaulescent alpine *Oxytropis* species in the Pirin Mts (Kožuharov, 1976, Pavlova et al., 1999) and they all belong to the *O. campestris* complex, namely *Oxytropis campestris* (L.) DC.**,** *O. kozhuharovii* Pavlova, Dimitrov & Nikolova and *O. urumovii.* Jav. (the last two local endemics for the marbles of this mountain). Here we present a compilation of several research papers of ours (Kožuharova, 2000, Kožuharova et al., 2007, 2012 a, 2012b, Kožuharova and Richards, 2009) as well as some additional unpublished data on breeding systems, pollination ecology and free pollination fruit set.

MATERIAL AND METHODS

Study Sites and Habitat Observations

The field observations were conducted in the marbleised karst region of North Pirin Mts., namely in its impressive alpine area at elevations between 2170 and 2640 m a. s. l. (Figure 1). The terrain includes the main watershed with Vihren peak and the next highest peaks around it, their slopes built of marble. The period of investigations of wild populations was during the summers of 1995, 1996, 2001, 2002 and 2005. The *ex situ* observations were conducted during the period 2006-2010. The exact geographic location of all sites was determined using a global positioning receiver Garmin GPS 12, Datum WGS 1984, UTM projection. Elevation was double checked with an altimeter. Slope and exposure were recorded and described both in the field and using the global positioning system (GPS) methods.

The geomorphology of the Northern Pirin marble ridges was analysed a from soil genesis point of view. Soil samples (two samples at each study site) were taken from the rooting zone of study plants. Each sample was taken from an area of 20-30 cm^2 and 4 cm depth. The soil characters were measured after a standard methodology at Newcastle University in January 2002. Particular attention was paid to the comparison between the soils collected from the sites of the endemic *O. urumovii* and *O. kozhuharovii* to those of the widespread *O. campestris*.

Figure 1. The Northern Pirin marble ridges. Inhabited mostly by *Oxitropis urumovii* found in small patches. Here are the peaks Kutelo 1 and Kutelo 2, Banski Suhodol, Bayuvi dupki, Razložki Suhodol, Kamenititza and behind it is Okadenski cirque.

Associated vegetation was recorded in the close vicinity in order to check microhabitat specifics. The plants were identified after Jordanov (1963-1995) and Kožuharov (1992). Approximate abundance evaluation of the plant species was done after Drude scale descendingly as follows: Soc. (sociales), Cop.$_3$ (copiosae$_3$), Cop.$_2$ (copiosae$_2$), Cop.$_1$ (copiosae$_1$), Sp. (sparsae), Sol. (solitariae) (Jaroshenko, 1961). We chose to use the scale of Drude as this approach has particular importance for evaluation of those plant species that grow in close vicinity to the three *Oxytropis* species and blooming simultaneously with them. It is all with respect to the analyses of pollination ecology (competition for pollinators).

Samples for Morphological and DNA Analyses

In late July 2001, the authors visited 10 sites with *Oxytropis* in the northern Pirin Mts: six with *O. campestris*, four with *O. urumovii* and the only known site for *O. kozhuharovii*. At each site, samples of leaves, flowers and fruits were taken from a minimum of five individuals for morphological examination, and leaf material was dried with silica gel for later DNA extraction. In August 2002, material of *O. halleri* subsp. *halleri* was collected from five individuals at each of the two sites on the northern coast of Scotland, and in July 2003, material of *O. dinarica* subsp. *weberi* was collected from northwest Macedonia.

Morphological Analysis Protocol

A minimum number of two flowers per plant and a minimum of five plants per site were rehydrated in warm dilute alcohol, dissected into components, dehydrated and mounted on a sheet. The sheets were digitalized (images scanned at 1:1). A total of 14 characters were measured on flowers and a further three measurements were made of leaves. The flower characters were measured digitally using Adobe Photoshop 5.0. Measurements of the leaves were made using a pocket micrometer. Descriptive statistics were calculated for individuals within populations, and using this data, for populations for each species. To analyse morphological differences between taxa, average readings per population were used for *O. campestris*, but measurements were only taken for single populations of *O. urumovii* and *O. kozhuharovii*, and for these species mean readings per individual were computed.

Inspection showed that six floral characters were differentiated between at least two of the species examined, and these were used in a principal components analysis (PCA), using a covariance matrix. Two of the three vegetative characters examined differentiated at least two of the taxa.

Molecular Analysis Protocol

DNA was extracted using a CTAB with chloroform method (Weising et al., 1995) from five individuals per sampling location. We amplified a total of 46 polymorphic RAPD fragments using four primers (Operon Technologies): OPA8 (12 polymorphic bands), OPA12 (15 polymorphic bands), OPA6 (11 polymorphic bands), and OPA4 (8 polymorphic bands) in 25µl reactions. Each reaction consisted of 1 x Taq buffer (16 mM (NH4)2SO4, 67 mM Tris-HCl, 0.01% Tween-20), 2.0 mM $MgCl_2$, 0.08 mM each dNTP, 0.2 µM primer, 1.0U Taq (Bioline) and 0.5 µl template DNA. The reaction cycle was 94ºC for 3 min, then 45 cycles of 94ºC for 30 sec, annealing temperature for 30 sec, 72ºC for 1.5 min, with a final extension of 72ºC for 4 min. Annealing temperatures were 40ºC for OPA8 and OPA12, 36ºC for OPA6 and OPA4. All reactions were performed in a PTC-100TM thermocycler (MJ research). PCR products were visualised on 1.4% agarose gels stained with ethidium bromide. We regarded the presence/absence state of RAPD bands, comparable between species and individuals, as a data matrix of bi-state characters varying between each individual. We viewed the relationships between these as a single linkage dendrogram, based on cluster analysis available within the Principle Components Analysis program in the MINITAB 14 statistical package.

Nei's genetic distance between sampling locations was calculated from the RAPD presence/absence data using RAPDDIST 1.0 (Black, 1995), with 1000 bootstrap replications. A consensus neighbour-joining tree was calculated from the bootstrap replicate Nei's distance matrices using PHYLIP 3.57c (Felsenstein, 1993), and was visualised using TREEVIEW 1.6.1 (Page, 1996).

The chloroplast TrnL intron was amplified for 24 individuals (9 *O. campestris*, 8 *O. urumovii*, and 7 *O. kozhuharovii*) using primers TrnL-c and TrnL-d (Taberlet et al., 1991) in 25 μl reactions containing 1x Taq buffer, 2.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 1.0U Taq (Bioline), and 0.5 μl template DNA. The reaction cycle was 35 cycles of 93°C for 1 min, 50 °C for 1 min, 72°C for 2 min. The internal transcribed spacer region (ITS) was also amplified for each of these 24 individuals using the primers ITS4 and ITS5 (White et al., 1990) and the same PCR component concentrations as for the TrnL intron amplification. The reaction cycle for ITS was 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min, with a final extension of 72°C for 4 min.

PCR products were sequenced directly. All PCR products were purified using QIAquick® PCR Purification Kits (Qiagen). Purified PCR products were then sequenced using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems) following manufacturer's recommended conditions, and sequences detected on an ABI 310 Prism® automated sequencer (Applied Biosystems). Sequences were aligned and edited using ProSequence (Filatov 2002).

Most species in this study had been characterised cytologically previously, but the chromosome number of *O. dinarica* was unknown. Material was collected by A. Novotna from the meadows of Popova Šapka, Šar Planina, NW Macedonia in July 1996 (voucher kept in personal collection in the Faculty of Pharmacy, Sofia). The haploid chromosome number in the pollen grains was studied by the acetocarmine method suggested by Heywood (1967) and Taschetto & Pagliarini (2004) on seven buds at different stage and two flowers. As a control we used 10 flowers and buds of *O. campestris* from the Pirin and Rila Mts, with a known chromosome number (n = 24).

Secondary Compounds Analysis

The plant material for this survey was collected in 2001, 2002 and 2005 from the Pirin Mts. Only a leaf or two per plant (from a few plants) were collected in order to avoid damage to the plants. As we had a restricted quantity of plant material, it was necessary to decide what tests should be performed. We chose to take the flavonoids test screening path. Dried powdered herb of each plant species were extracted with 70% aqueous EtOH. at 70°C for 2 hours. After removing under reduced pressure the ethanol the water fraction was treated with $CHCl_3$. The residue was treated with EtOAc. The fractions of the supernatant were reduced to ½ the volume and were concentrated to dryness under reduced pressure to give a crude EtOAc mixture. 10 mg of the EtOAc mixture were hydrolyzed with 5% H_2SO_4 for 1 hour and the solid residue was washed to neutral with water and dissolved in methanol. The crude EtOAc extract was identified by TLC comparison with authentic samples in three chromatographic systems.

Flowering Phenology

The flowering phenology of *O. campestris, O. urumovii* and *O. kozhuharovii* was investigated by estimating buds, flowers and wilted flowers ratios periodically.

Breeding Systems and Pollination Ecology

The possibility for spontaneous self-pollination was tested, by covering of inflorescences and flowers at the bud stage with fine mesh insect excluders. Control open-pollinated flowers were also covered subsequently to prove the lack of influence on the normal seed set by the fine mesh insect excluders. Indirectly the breeding system was estimated using the method of pollen-ovule ratio. This was calculated by dividing the number of pollen grains in a flower by the number of ovules in the same flower. Pollen-ovule ratio values for a plant species are mostly average values of several aggregations from different individuals and/or populations. The counts were done after a slightly modified standard protocol (Dafni, 1992) and a Beurker chamber was used. All pollen grains were counted on the grid (in a volume of 3mm x 3mm x 0,9mm) and recalculation was done accordingly to the dilution.

Figure 2. View from Vihren peak towards the northeast slope. Under the view point is vertical cliff of about 1000 m, which runs down into the cirque named Kazan (marble bed rock). Inhabited mostly by *Oxitropis urumovii* found in small patches.

The insect visitors (in the sense of Faegri and van der Pijl, 1971) were investigated after a transect method combined with observation of a plant group, according to the specifics of the plants' population structure (Dafni 1992). The transect method was chosen in order to avoid higher recording of social insects near their nest (Dlusskii, personal communication). Observations presented here refer to three main study sites (Figures 2-4). Some more details are presented in our previous publications (Kožuharova, 2000). In total, 21 days and 140 hours of observations (in 1995, 2001, 2002) are presented as summarized/average data to investigate the relative pollinator activity. Several periods of time (from 60 to 150 minutes) were spent on each study site at different times of the day. The observations covered different meteorological and phenological conditions. All observed visitors were recorded. The behaviour of pollinators was observed. Visitation rate is presented as number of visitors in the site per time unit (minute).

Figure 3. Vihren peak (all marble bed rock) and wide grassy saddle named Kabata (contact zone with silicate bed rock). Inhabited by *Oxitropis campestris* found in big patches.

Figure 4. Žultite skali above Okadenski cirque. Marble bed rock and next is contact zone with silicate bed rock. Inhabited by *Oxitropis kozhuharovii* found in several small and one big patches.

An insect sample was collected for detailed identification. It is deposited in personal collections. Most of the observed bumblebees were collected and narcotized with ethyl-acetate and after extraction of their pollen loads released again (Heinrich, 1979a). Pollen contamination was avoided by catching each bumblebee separately. Pollen identification and counting (at least 200 pollen grains after Louveaux et al., 1978) was conducted under light microscope “Nikon” and magnification X 320 and X 800.

The plant species flowering in close neighbourhood, simultaneously with the investigated ones were listed (Table 2). The pollinators of the actively visited plant species were recorded in order to obtain an idea of the pollinator sharing (Table 6).

Free Pollination Fruit Set and Seed Set, Seed Germination and *Ex Situ* Ontogenesis

The free pollination fruit set was tested by counting matured fruits (legumes) versus empty wilted flowers per representative sample of inflorescences at fruit stage. The seed set was tested by counting the matured seeds and non fertilized ovules per legume just before its opening to release the seeds.

Several sets of seeds (20-135 at a time) kept cool (4°C, in the fridge, but not frozen), or kept at room temperature, were processed for germination at a natural light - dark photoperiod. This took place in Sofia during the period 20th February-6th May 2006, 5th March-5th May 2007, and 12th March-20th April 2008 at temperatures between 10-25°C - first in greenhouse conditions, and later outdoor on a shaded place with south-west exposure. Scarification of the seeds was undertaken with a needle (in 2006) or with fine sand paper (in 2007 and 2008). The seeds were placed in Petri dishes on wet filter paper. As controls, seed samples previously kept cool were soaked in water before germination without scarification. Once the seeds had germinated, the seedlings were transferred individually to plastic pots filled with a mix of 30% rough marble sand, 30% sieved humus, 30% sieved good soil, 10% perlite or with a mix of 60% silty brown soil and 40% rough marble sand. Watering was from below at first.

When seedlings were at the 2-6 true leaf stage, they were transported to the experimental rock garden in the foot hills of Pirin Mts. near Dobrinishte village. They were planted singly into the flower beds with fine marble gravel top-dressing. Observations on the *ex situ* ontogenesis were performed periodically.

Statistic Analysis

Descriptive statistics were applied to analyze the data. One-wayANOVA analysis was used to examine differences in free pollination fruit set of *O. kozhuharovii* at 3 different patches and altitudes of the population, one (site wp 16) consisted of few individuals at 2170 m a. s. l.; a second (wp 37) consisted of numerous individuals at 2225 m a. s. l. and the third one consisted of few individuals at 2300 m a. s. l.

DISTRIBUTION OVERVIEW

There are several species of *Oxytropis* in the mountains of the Balkans (Table 1, Hayek, 1927; Leins & Merxmüller, 1968; Diklich, 1972; Kožuharov, 1976; Chrtek & Chrtkova, 1983; Strid, 1986). In this study we focus on the acaulescent species with semi-bilocular and almost completely bilocular legumes.

HABITAT SPECIFICS: SOILS AND SOIL FORMING PROCESSES OF THE STUDY SITES, SPACE DISTRIBUTION OF THE *OXYTROPIS* POPULATIONS MICRO-RELIEF AND VEGETATION

The high-elevation ecosystems are known by extreme temperatures and large diurnal variation in growing-season temperatures, in conjunction with high levels of ultraviolet radiation, accompany large variation in the amount of precipitation or in other words as harsh habitats (Bowman, 2001, Bowman et al., 2002).

The studied populations are localized in the criolithogenic belt. Here is found periglacial relief which is a result of crionivalic processes with periodical freezing and unfreezing of the soil and the weathering crust. The mean yearly temperature is -3°C, the rainfall is about 1200 mm and 60-80% of it is snow, which remains about 180 days. The mean snow cover is about 80 cm thick. The growth period lasts three to three and a half months, when the mean temperature is above 3°C, but temperatures above 10°C occur rarely. Due to the karst terrain there are no lakes (except a single very small one). The significant rainfall combined with low evaporation and steep slopes cause soils to have a high moisture status, although 90% of the rainfall passes rapidly through the profile; 1 kg of soil absorbs about 3 kg of water, helped in part by a present layer of dead vegetation. Regeneration processes are slow, so that the mean vegetation cover is about 50%. The soil is primitive, poorly developed and its cover is rather loose.

Table 1. Distribution of the acaulescent *Oxytropis* species in the Balkans: A compilation after Hayek, 1927; Leins & Merxmüller, 1968; Diklich, 1972; Kožuharov, 1976; Chrtek & Chrtkova, 1983; Strid, 1986

Oxytropis species with yellow flowers	*Oxytropis* species with purple flowers
O. dinarica subsp. *dinarica* (Murb.) Wettst. Mt Velez,1600-1800-2300 m;	*O. kozhuharovii* Pavlova, Dimitrov & Nikolova (2n = 32), Pirin marbles, above 2500 m;
O. dinarica (Murb.) Wettst. subsp. *velebitica* Chrtek & Chrtkova: Mt Velebit, 1400-1600 m;	*O. prenja* (Beck) Beck, 2350 m shist, Gramos and mountains of the W part of the Balkan peninsula;
O. dinarica subsp. *weberi* Chrtek & Chrtkova: Mt Korab, 2000 m, Popova Šapka, Šar Planina, NW Macedonia (2n = 16);	*O. halleri* subsp. korabensis (Kümmerle & Jav.) Chrtek & Chrtková, Mt Korab, Prizren (Kosovo);
O. urumovii Jáv. (2n = 16), Pirin marbles, above 2500 m;	*O. halleri* Bunge ex Koch: Carpathians and Slovenia (2n = 32);
O. campestris (L.) DC. subsp. *campestris* (2n = 48), Pirin and Rila marbles, at about 2500 m, Prizren (Kosovo), Carpathians.	*O. purpurea* (Bald.) Markgr. (2n = 16), limestone ridges 1900-2800, Olimbos, Albania, Macedonia

Vihren peak (2915 m) is the fourth highest peak on the Balkans. It is narrow, bare, with steep rocky slopes, and in some places with vertical crags and cliffs. It is piled with marble boulders (Figures 2 and 3). The soil is primitive, poorly developed and its cover is rather loose. The southern slope of Vihren peak is very steep, bare and soilless. Under it is situated the wide grassy saddle named Kabata (Figure 3). The eastern part of Kabata is dissected by the cirque bearing the same name, formed in the glaciations of the Quaternary and covered with grass vegetation and scattered here and there dwarf pines. It goes down to the U-shaped valley of Bunderitza river, where during the Quaternary flowed one of the most massive glaciers in Pirin Mts. The southwest slope of Vihren Peak is formed by a smoothed cliff named Stenata which runs down more than 1000 meters into the Vlachina river valley. The northeast slope is a vertical cliff of about 1000 m, which runs down into the cirque named Kazan (meaning 'cauldron').

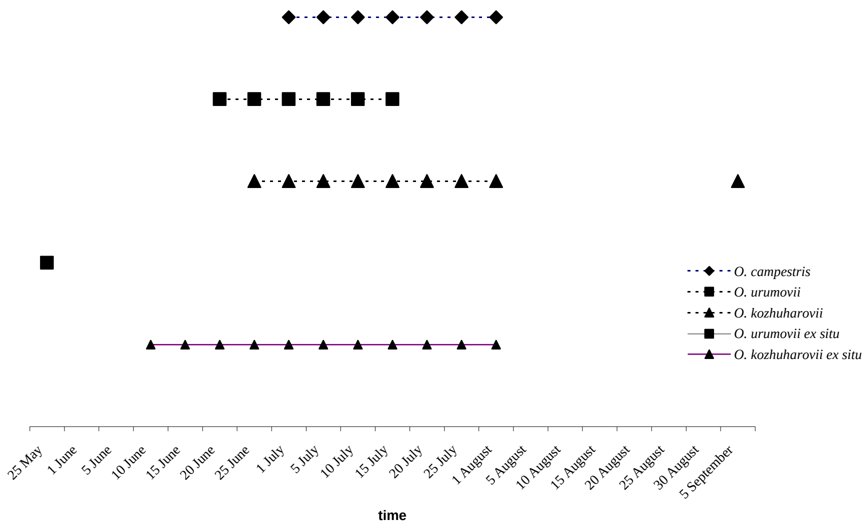


Figure 5. Flowering periods of the three species *in situ* and *ex situ*.

Towards the north-west is developed the main ridge of the marble massive. Here are the peaks Kutelo 1 and Kutelo 2, Banski Suhodol, Bayuvi dupki, Razložki Suhodol, Kamenititza (Figure 1). The ridge is dangerously narrow so that there is a part named Končeto (meaning "The horse back" and it is no wider than that indeed, Figure 1). All are marble bed rock.

The area of Žultite skali (meaning Yellow rocks) above Okadenski cirque is slightly isolated from the main ridge (Figures 1 and 4). Here dominates marble bed rock and next is contact zone with silicate bed rock.

The population structure of all *Oxytropis* species in Pirin Mts. is of a mosaic type. The patches of populations of each *Oxytropis* species are to some extent isolated in space. This is especially true for *O. kozhuharovii* which basically has no geographic overlap with the other two species (Figure 4, details in Kožuharova et al., 2012a). The populations of *O. urumovii* and *O. campestris* are partially sympatric. Thus in the area of Vihren peak the population is more or less separated in space from that of *O. urumovii*. They grow together only at some of the study sites. In the highest part of cirque Kazanazite (Figure 1) together with *O. urumovii* there are few plants *O. campestris*.

The floristic composition of plant communities of all the investigated species of *Oxytropis*, is rather similar although in each patch of their populations the individuals are surrounded by different plant species – differences refer to both qualitative and quantitative characteristics. It is worth a mention, however, that *O. campestris* was not observed growing in close vicinity with *Potentilla apennina*. The differences of the habitats are more or less expressed in the pH, and basic rock (Table 2). *O. campestris* and *O. kozhuharovii* are more associated with the contact zone with the granites while *O. urumovii* is strictly on the marbles. *O. kozhuharovii* occurs at slightly lower altitudinal range compared to the other two species. *O. campestris* shows the widest range with regard to altitude.

The high elevation with harsh climate and marble terrain causes an extremely poor soil-forming process and scanty soil hidden between the marble boulders, rock cracks, grooves, and fissures (Ninov, 1982). These are the conditions at our study sites. The granulometric composition of the soils, determined by sieving them (2 mm mesh sieve) revealed that the soils consisted of rough particles - sand and pebbles. The nature of the marble weathering is the reason for the fragmented soil cover, poor development and functioning as well as poor interaction with the vegetation. The index of the potential bio-production is 0,1 - the same as in the tundra or in the desert. Thus most of the soils here have poor morphology and quality. They have a ”cryo” temperature regime and are defined as Cryrendolls. Such soils are rare for Bulgaria. They occur only here in Pirin and rarely in Slavianca Mts. Being formed on hard rock they are defined in details, at a lower taxonomic level as Lithic Cryrendolls (ST) or Lithic Leptosols – Rendzic Leptosols (F.A.O.). Usually the rendzic leptosols contain a high quantity of pebbles. Their vegetation is grassy, represented by the genera *Festuca, Sesleria, Carex* etc. and rarely shrubby so they are used as pastures. These soils often contain carbonate. They have high values of pH and a moderate supply of phosphorus, potassium, calcium, and magnesium. The analysis showed that pH varied from slightly acid (5.82 at site inhabited by *O. campestris*) to slightly alkaline (7.70 at site inhabited by *O. urumovi*). It is interesting to notice that at one and the same site with 3-15 m distances between the samples the values of pH vary markedly. Despite the humid climate the soils are not acid due to the high content of carbonate in the marbles. In the soils developed on marbles are observed vertical belts, a specific caused by the geomorphologic conditions, which is autochthon. The content of humus is comparatively high. Extractable phosphorus (P) in the soil is between 20.05 mg and 23.89 mg (details in Kožuharova et al., 2012a).

The populations of all three studied *Oxytropis* species occupy habitats which belong to Montane tall-herb, grassland, fell-field and snow-bed vegetation. These are alpine and sub alpine open calcicolous herbaceous and alpine calcicolous herbaceous communities near melting snow-patches. Here is found psychrophytous and cryophytous hecistothermal vegetation in the alpine woodless belt; calciphilous cryophytous grass formations *Kobresieta myosuroides, Cariceta kitaibelianae, Seslerieta korabensis* and small shrub formations *Dryeta octopetalae, Saliceta reticulatae*, etc. It dominates a limited number of phytocoenoses occurring at approx 2500 m alt. The category of these habitats is endangered to critically endangered. The habitats are included in Annex № 1 of BDA. The localities of this habitat are within the borders of Rila and Pirin National Parks. Some of the most representative localities are in sites of the European Ecological Network NATURA 2000 in Bulgaria, (Bondev, 1991, Asenov, 2006, Tzonev et al., 2009. Roussakova, 2011) and in most cases this is habitat - 6170 Alpine and subalpine calcareous grasslands described for Pirin Mts by Roussakova (2009). On the slopes of Vihren and Sinanitsa peaks in Pirin Mts., where the slope is 30-45° and the altitude is at and above 2500 m, the phytocoenoses are dominated by *Sesleria korabensis*. In some coenoses, the rare and relic species *Carex rupestris* occurs as a co-dominant or with lower abundance. In some phytocoenoses the abundance of *Carex kitaibeliana* is high, while in others, *Sesleria coerulans* is common. In some places these species are edificators (not only in Pirin Mts.). The local endemic to Pirin Mts., *Oxytropis urumovii*, is a rare species (Roussakova 2011). It is true for *O. kozhuharovii* too and its population is even more restricted in space (Kožuharova et al., 2007, Kožuharova et al., 2012a).

FLOWERING PHENOLOGY

The divergence in flowering periods is a potential mechanism for reproductive isolation between closely related and partially sympatric species. *In situ O. campestris*, *O. urumovii* and *O. kozhuharovii* have not only spatial but also slight phenological isolation although there is some overlap in the flowering periods. *O. urumovii* blooms slightly earlier than the other two (Figure 5). The same pattern was observed in *ex situ* conditions - *O. urumovii* blooms earlier than *O. kozhuharovii* and this period takes place earlier compared to the native populations which is not surprising bearing in mind that the *ex situ* site is almost 2000 m lower than the native ones (Kožuharova et al., 2012a).

How to Distinguish the Species: Morphological Analysis

Using pairs of flower characters, the only combination which completely separated the species was calyx teeth length and keel length, although *O. urumovii* also has longer bracts and a longer calyx than the other species, and is undoubtedly the most distinct of the four species studied. Generally, the yellow-flowered *O. dinarica* falls closest to the purple-flowered *O. kozhuharovii* morphologically. *Oxytropis kozhuharovii* has narrower and less numerous leaflets than most *O. campestris*. In general, the hexaploid *O. campestris* tends to fall between the diploid *O. urumovii* and the tetraploid *O. kozhuharovii*, morphologically (Kožuharova et al., 2007).

Oxytropis urumovii is a distinctive species compared on first sight to the other Balkan *Oxytropis* acaulescent species (with a semi-bilocular or almost completely bilocular legume, Table 3, Figure 6).

The whole plant, including the fruit, is rather densely covered with long (2-3 mm) patent whitish hairs, which contrast with the scape and calyx, the green colour of which is often suffused with black (Figure 7). Amongst the European species of *Oxytropis*, *O. urumovii* is diagnosed by being acaulescent, having leaves with about eight pairs of leaflets, nearly free stipules (adnate to the petiole for no more than one quarter), calyx-teeth which are much shorter than the tube, yellowish flowers and an ovoid semi-bilocular legume (Figures 6 and 7, Leins & Merxmüller, 1968; Kožuharov, 1976). It is diploid with $2n = 16$ (Kruscheva, 1986; Pavlova, 1996). The karyotype is symmetrical, consisting of $2n$ = 4m + 12sm = 16 small and medium size chromosomes. (Pavlova, 1996). In addition, the author notes that endopolyploidy ($2n = 48$) is also observed and that is how she interprets the previous report of a hexaploid number (Andreev, 1981). It is immediately distinguished from O. *campestris* by the much longer, denser and generally patent indumentum (usually sparse and semi-appressed in *O. campestris*), flowers that turn reddish rather than blackish at the tip, nearly free stipules (in *O. campestris* stipules are adnate to the petiole for one-third to three-quarters of their length), and a smaller legume (see also Leins & Merxmüller, 1968; Kožuharov, 1976). In general, it has a dwarfer and stiffer habit than the rather lax and sprawling *O. campestris*. According to Pavlova (1996), the karyotype of the population *O. campestris* in the Pirin Mts consists of 2*n* = 24m + 20sm+4st = 48, and the chromosomes are of medium size.

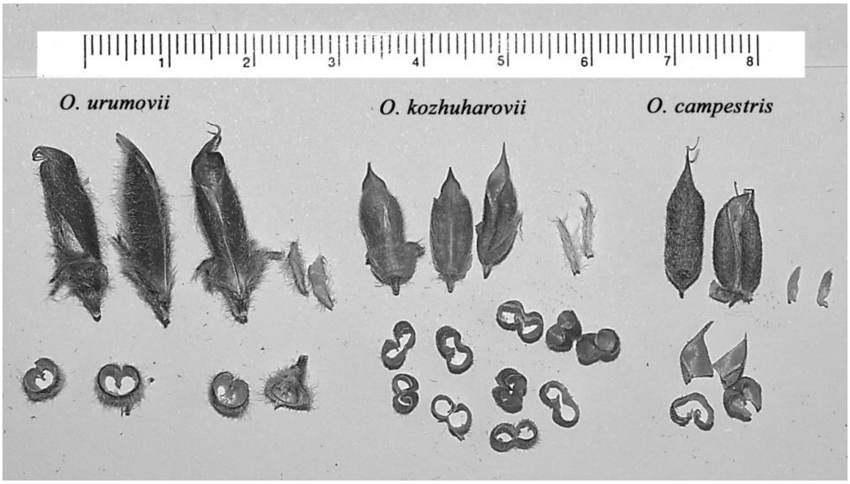


Figure 6. Legumes of *O. urumovii, O. kozhuharovii* and *O. campestris:* Views of the central vein, accretion, and (below) transverse section.

Leins & Merxmüller (1968) included *O. campestris* subsp. *dinarica* Murb. within *O. urumovii*, but Chrtek & Chrtkova (1983) reinstated the combination of Wettstein (1892) - *O. dinarica* (Murb.) Wettst., and described two new subspecies for this taxon, which occurs from Croatia (subsp. *velebitica*) to Bosnia and Montenegro (subsp. *dinarica*) and to Macedonia and Albania (subsp. *weberi*) (Table 1). In general, *O. dinarica* lies between *O. campestris* and *O. urumovii*, not least in general habit. It resembles the former species by the stipules which are adnate to the petiole for half their length, but the indumentum more resembles *O. urumovii*, except on the fruit on which the short (<1 mm) hairs are similar to those of *O. campestris*. The calyx of *O. dinarica* is diagnostic, for the calyx teeth are only about one-fifth the length of the calyx tube, but in the other two species they are more than one-quarter the length of the tube. In all three of these species the flowers are yellowish. *O. dinarica* is reported as diploid (2*n* = 16) for the first time in this paper. The haploid chromosome number in the pollen grains of *Oxytropis dinarica* was counted as n = 8. It is clearly a distinct species, so that *O. urumovii s. s.* can be regarded as being endemic to the Bulgarian Pirin Mts.

Table 2. Habitat specifics of *Oxytropis urumovii O. Campestris* and *O. kozhuharovii*

	Oxytropis urumovii.	*Oxytropis campestris.*	*Oxytropis kozhuharovii*
Bed rock	Strictly on the marble (Figures 1 and 2)	Very close to the contact zone with the granites (Figure 3)	Marble, gneisses and gneiss-schist formations near glacier deposits (boulders, pebble gravels) and granites (Figure 4)
The patches of the population	Small and not dense scattered within a comparatively larger territory	Large dense patch at the southern side of Vihren peak in the area of Kabata	forms one big patch (about three thousand individuals) and a couple of small ones consisting by 5-30 individuals. The total number of plants in the population is estimated to about 3170 individuals
Slope	Both on steep slopes and on a flat ground such as the "bottom" of Kazan II (Figures 1 and 2).	The dense patches are usually seen on the big flat surfaces. Few plants are on steep slope	Some of the plants grow on very steep slopes: In practice part of the population is on a snow-slip gully.
Microhabitat preferences	Even when the slope is steep the plants tend to grow on small flat surfaces that look like stairs. The plants are found on silty brown earth amongst marble blocks, sometimes in pockets of big rocks	When the slope is steep the plants inhabit small flat surfaces that the terrain offers. The largest part of its population grows on silty brown earth on the table land in the area of Kabata.	Plants tend to grow on small flat surfaces that look like stairs (Figure 4). Stabilized scree with fine silty brown earth amongst mixed marble and siliceous blocks
pH of the soil	7.27 (min = 6.94, max = 7.70)	6.79 (min = 5.82, max = 7.79)	7.42 (min = 7.29, max = 7.55)
Extractable phosphorus (P) in the soil	21.67 mg (min = 20.22, max = 23.89)	20.69 mg (min = 20.05, max = 21.62)	20.84 mg (min = 20.72, max = 20.95).

Table 2. (Continued)

	Oxytropis urumovii.	*Oxytropis campestris.*	*Oxytropis kozhuharovii*
Plant community dominated by:	*Festuca valida* (Uechtr.) Penz. Soc. *Sesleria korabensis* (Kumm. et Javorka) Deryl Soc. *Carex kitaibeliana* Degen ex. Bech. Soc. *Daphne velenovskyi* Halda, (this area is notable for this restricted endemic, Halda, 1981) Cop.$_3$ *Anthyllis vulneraria* L., Cop.$_3$, *Saxifraga ferdinandi-coburgii* Kellerer et Sund, Cop.$_3$, *Onobrychis pindicola* Hausskn., Cop.$_3$ *Potentilla apennina* Ten., Cop.$_3$, *Linum capitatum* Kit ex Schultes, Cop.$_3$ *Acinos alpinus* (L.) Moench., Cop.$_3$ *Centaurea achtarovii* Urum., Cop.$_2$, *Thymus thracicus* Velen, Cop.$_2$, *Helianthemum nummularium* (L.) Miller, Cop.$_2$, *Rhodax canus* (L.) Fuss, Cop.$_2$, *Cerastium alpinum* L., Cop.$_2$, *Aster alpinus* L., Cop.$_1$, *Achillea ageratifolia* (Sibth. Et Sm.) Boiss. Cop.$_1$, etc.	*Festuca valida* (Uechtr.) Penz., Soc. *Sesleria korabensis* (Kumm. et Javorka) Deryl Soc. *Onobrychis pindicola* Hausskn., Cop.$_3$ *Cerastium alpinum* L., Cop.$_3$ *Gentiana verna* L., Cop.$_3$ *Thymus thracicus* Velen., Cop.$_3$ *Genista depressa* Bieb., Cop.$_3$ *Acinos alpinus* (L.) Moench., Cop.$_3$ *Rhodax canum* (L.) Fuss, Cop.$_2$ *Armeria alpina* Wild., Cop.$_2$ *Antennaria dioica* (L.) Gaertner, Cop.$_2$ *Alyssum cuneifolium* Ten., Cop.$_1$ *Aster alpinus* L., Cop.$_1$, *Achillea ageratifolia* (Sibth. Et Sm.) Boiss., Cop.$_1$ *Jasione bulgarica* Stoj. & Stef. Cop.$_1$	*Festuca valida* (Uechtr.) Penz., Soc. *Sesleria korabensis* (Kumm. et Javorka) Deryl Soc. *Carex kitaibeliana* Degen ex. Bech. Soc. *Onobrychis pindicola* Hausskn., Cop.$_3$ *Chamaecytisus absinthioides* (Janka) Kuzmanov. Cop.$_3$, *Daphne oleoides* Schreber, Cop.$_3$, *Saxifraga ferdinandi-coburgii* Kellerer et Sund, Cop.$_3$ *Anthyllis montana* L., Cop.$_2$ *Anthyllis vulneraria* L., Cop.$_2$, *Potentilla apennina* Ten., Cop.$_2$, *Jurinea mollis* (L.) Reichenb. Cop.$_1$ Note *O. kozhuharovii* plants occupy the open soil patches between the big tufts of *Sesleria korabensis* and small shrubs of *Chamaecytisus absinthioide*

Legend: Drude scale (Jaroshenko, 1961) descendingly as follows: Soc. (sociales), Cop.$_3$ (copiosae$_3$), Cop.$_2$ (copiosae$_2$), Cop.$_1$ (copiosae$_1$), Sp. (sparsae), Sol. (solitariae) (details in Kožuharova at al. 2012a).

Figure 7. *Oxytropis urumovii* is rather densely covered with long patent whitish hairs, which contrast with the scape and calyx, the green colour of which is often suffused with black.

Unlike the species mentioned previously, the other Pirin endemic *Oxytropis*, *O. kozhurahovii* is a tetraploid with $2n = 32$ (Pavlova et al., 1999). It is only known from a single locality from the northernmost part of the Pirin, the Yavorov anticline, where it was discovered and described very recently (Pavlova et al., 1999). *Oxytropis kozhurahovii* is the only Bulgarian *Oxytropis* with blue or purple flowers. However, at least one and possibly two purple-flowered species occur in the region around the border between Macedonia and Albania, some 220 km to the west, notably Mt Korab (Table 1). Here an endemic subspecies of the widespread species *O. halleri* Bunge ex Koch, *O. h.* subsp. *korabensis* (Kummerle & Jav.) Chrtek & Chrtkova, occurs (Table 1). As discussed by Pavlova and coauthors (1999), *O. kozhurahovii* closely resembles members of the *O. halleri* complex, but differs in the structure of the legume which, like that of *O. campestris* and *O. urumovii,* lacks a septum in the dorsal valve (semi-bilocular, Figure 6). All subspecies of *O. halleri* have a septum in the dorsal valve, although that in subsp. *korabensis* is narrow and poorly developed. Stipules are another feature in which *O. kozhurahovii* resembles more the *O. campestris* complex than *O. halleri*. Most forms of *O. halleri* have stipules with several longitudinal veins, whereas those of *O. kozhurahovii* are single-veined. However, the reduced stipules of *O. halleri* subsp. *korabensis* have only 1-2 veins. We would add that the indumentum of the *O. halleri* complex tends to be velutinous, but that in *O. kozhurahovii* is longer and patent.

In our opinion, the most distinctive feature of *O. kozhurahovii* is the indumentum of the calyx, particularly the apex, which is very densely covered with long white hairs, which equal or exceed the calyx teeth (Figure 8). This contrasts strikingly with the rest of the plant in which the patent indumentum is long but sparse and inconspicuous.

Although it resembles *O. halleri* superficially, it seems likely that *O. kozhurahovii* is in fact more closely related to *O. prenja* which also has semi-bilocular legumes. From an examination of the herbarium material at *K* and *E*, it is clear that the latter is a dwarfer plant in which the scape exceeds the rather prostrate foliage. Decisively, it is scarcely hairy even on the calyx. It is also said to have fewer pairs of leaflets per leaf (usually 6-7 rather than 10-11 for *O. kozhurahovii),* but this disagrees with our findings for the latter species (Figure 4 in Kožuharova et al., 2007). Also, according to Leins & Merxmüller (1968), *O. prenja* is said to have a longer (15-19 mm) standard which is emarginate at the apex, and a shorter keel tooth (‘beak’) of only 0.5 mm. However, although Figure 4 in Pavlova and coauthors (1999) appears to show an emarginate standard in *O. prenja,* the apex of the standard in this species is said in Table 1 to be ‘convex’, and the standard illustrated does not appear to differ in length from that of *O. kozhurahovii* at about 1.3 mm. Also, although Pavlova and coauthors (1999) state that the length of the keel tooth in the latter species is ‘circa 1 mm’ (diagnosis) or ‘1-1.5 mm’ (final line), our study

(Kožuharova et al., 2007) found that it varied between 0.3 and 0.8 mm, and did not differ from that of *O. prenja* in this regard.

Figure 8. Indumentum of the calyx of *Oxytropis kozhurahovii*, particularly the apex, is very densely covered with long white hairs, which equal or exceed the calyx teeth, while the indumentum of the whole plant is long but sparse and inconspicuous.

Table 3. Key to Balkan species of *Oxytropis* with semi-bilocular or almost completely bilocular legume

1	Plant caulescent, stem > 20 cm; calyx teeth > calyx tube; flowers yellow (widespread subalpine)	*O. pilosa*
1*	Acaulescent alpine; calyx teeth < half length of tube; flowers yellow or purple	2
2	Calyx teeth < 25% length of calyx tube; hairs on fruit 2-3 mm, dense; flowers yellow (Croatia to Albania and Macedonia, three subspecies)	*O. dinarica*
2*	Calyx teeth > 25% length of calyx tube; hairs on fruit usually less than 1 mm (except occasionally *O. urumovii*); flowers yellow or purple	3
3	Fruit bilocular, with a septum in the dorsal valve; stipules with (1-) 2 or more veins; leaf indumentum velutinous (the hairs would be less than 0.5 mm long, although it is their soft dense quality that is important); flowers purple (widespread, but only in Mt Korab in the Balkans, e.g. widespread subsp. *halleri* 2*n* = 32, subsp. *velutina* - 2*n* = 16 restricted to the Alps, but in Mt Korab subsp. *korabensis*)	*O. halleri*
3*	Fruit semi-bilocular, lacking a septum in the dorsal valve; stipules one-veined; leaf indumentum usually pilose or sericeous but not velutinous; flowers yellow or purple	4
4	Stipules free; calyx <1 cm; indumentum dense; inflorescence peduncle somewhat exceed the subtending leaf; raceme 4-12-flowered, flowers cherry-red; peduncle and legume with long, patent hairs (N Greece, Albania, SW Macedonia)	*O. purpurea*
4*	Stipules adnate to petiole	5
5	Stipules adnate to petiole for 25% or less; calyx > 1 cm and keel > 1.2 cm in length; flowers yellow and indumentum dense; inflorescence peduncle about as long as subtending leaf - shorter or equal, seldom longer; throughout (N Pirin)	*O. urumovii*
5*	Stipules adnate to petiole for half length or more; calyx < 1 cm, keel < 1.2 cm; flowers yellow or purple; indumentum sparse except occasionally on calyx	6
6	Hairs on calyx long and dense, especially at apex, exceeding calyx teeth in length; inflorescences exceeding leaves; flowers purple (one site in N Pirin)	*O. kozhuharovii*
6*	*Calyx with short sparse indumentum, or nearly glabrous; inflorescences not exceeding leaves (peduncle about as long as subtending leaf) flowers yellow or purple	7
7	Leaflets usually > 9 pairs; flowers yellow (widespread alpine)	*O. campestris*
7*	Leaflets 6-8 pairs; flowers purple; raceme 1-4-flowered, peduncle and legume with short, appressed hairs (Dinaric Alps, Bosnia-Herzegovina)	*O. prenja*

Oxytropis prenja was ignored by Chrtek & Chrtkova (1983) and seems to be little known outside its native area. The description in Leins & Merxmüller (1968) would not distinguish it from *O. kozhuharovii*. Its image has twice appeared on postage stamps for Bosnia-Herzegovina, as a painting (1997) and a photograph (2003). Accompanying website texts claim it as an endemic of the Dinaric Mts, Prenj Planina, Čvrsnica, and Vran, where it occurs in limestone crevices at altitudes of 1900-2228 m. This taxon has also been said to occur in Albania and Macedonia (Greuter, 1989), where it may have been confused with *O. halleri* subsp. *korabensis*.

Although the similarities of the pollen grains between *Oxytropis* and *Astragalus* species are the reason to regard all of them as one pollen type Astragalus, some slight differences among members *Oxytropis* are described. The tectum which is very restricted, is predominantly perforate in *O. pilosa, O. purpurea* and finely reticulate in *O. campestris*, *O. urumovii*, *O. kozhuharovii*, (Pavlova, 2013). Also although the group is rather homogeneous regarding the pollen grains' morphology and the differences between species are insignificant, a cluster diagram shows that *O.urumovii* is more similar to *O. dinarica* and *O. kozhuharovii* is more similar to *O. campestris* (Pavlova, 2013).

We conclude that each of the localised Balkan endemic *Oxytropis* are distinct species. In our view, they are best differentiated as shown in the key (Table 3).

MOLECULAR ANALYSIS

Neither ITS nor TrnL were particularly variable. For both markers, only two polymorphic sites were detected out of 452 base pairs sequenced (ITS) and 454 base pairs (TrnL). A neighbour-joining tree of the TrnL polymorphisms (two insertion/deletions) differentiated *O. urumovii* from the other species, but did not differentiate *O. kozhuharovii* from *O. campestris*. The ITS polymorphisms (two base substitutions) did not differentiate any of the species. Only the RAPD data provided enough resolution to adequately examine variation between the sampling locations.

The results from the neighbour-joining unrooted tree based on RAPD presence/absence data (Figure 9) and from the principal component dendrogram analysis of the RAPD band matrix (Figure 10) are very similar. In the neighbour-joining unrooted tree based on RAPD presence/absence data

(Figure 9), the four species examined remained distinct with good bootstrap support.

The molecular analysis revealed the following: *O. urumovii* proved to be the most distinct species of the four. As it is diploid, it could be argued that *O. urumovii* is ancestral to this group and a palaeoendemic element of the flora of the Pirin Mts.

Compared to Bulgarian *O. campestris* and Scottish *O. halleri, O. kozhuharovii* is about equally related to each. Both analyses would be consistent with the hypothesis that *O. campestris* could have arisen from the allohexaploid union between *O. urumovii* and *O. kozhuharovii,* thus agreeing with the morphological data (unfortunately, material of *O. dinarica* was received too late to be included in this analysis).

In addition, the molecular evidence suggests that *O. kozhuharovii* could be the allotetraploid derivative of the sympatric diploid endemic *O. urumovii* and the widespread diploid (and tetraploid) *O. halleri,* the nearest populations of which occur today in Mt Korab, some 220 km away. Today, this disjunction seems significant, but during the Late Glacial stadials mountain steppe vegetation (*Artemisia-Chenopodiaceae-Poaceae*) in which they occur dominated at high and mid-altitudes (Wijmstra, 1969; Florschütz et al., 1971; Bottema, 1974, 1979; Lang, 1994; Tzedakis, 1994, 1999; Bozilova, 1996; Bozilova et al., 1989; Bozilova & Tonkov, 2000; Tonkov et al., 2002, 2006; etc). At that time species that now occur at a higher altitude were more widely distributed at lower altitudes.

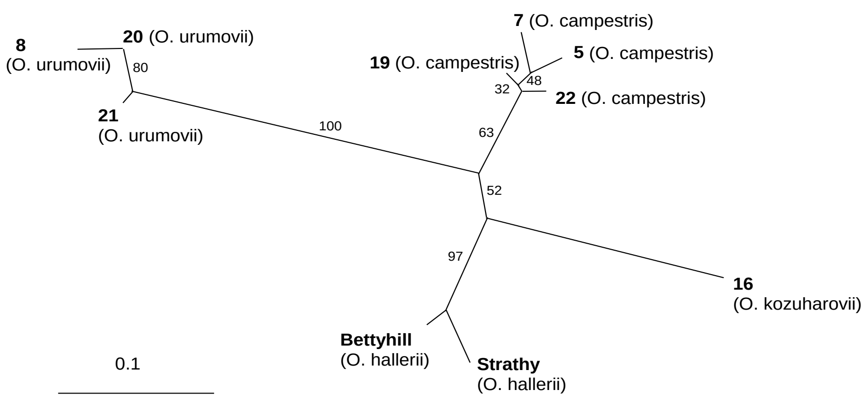


Figure 9. Nei’s genetic distance between sampling locations calculated from the RAPD presence/absence data with 1000 bootstrap replications. A consensus neighbour-joining tree was calculated from the bootstrap replicate Nei’s distance matrices and visualised using TREEVIEW.

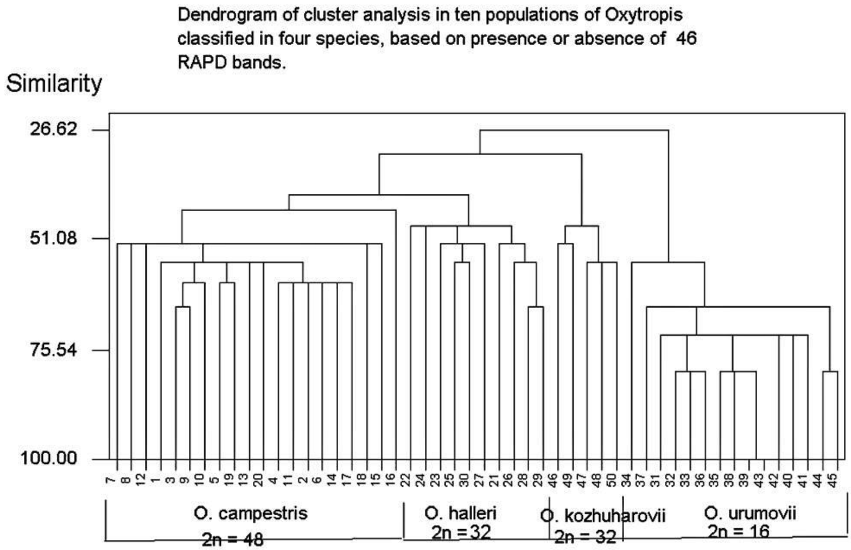


Figure 10. Dendrogram of cluster analysis in ten populations of *Oxytropis* classified in four species, based on presence or absence of 46 RAPD bands.

On the basis of the morphological analysis (see above), it is not impossible that *O. campestris* could have arisen from an allohexaploid union between the yellow- flowered diploid species *O. urumovii* and *O. dinarica* (or even from crosses between a yellow diploid and a purple-flowered tetraploid such as *O. kozhuharovii* or *O. prenja,* if this species also proves to be tetraploid). In that case, the place of origin of the circumpolar *O. campestris* was possibly in the Balkans. *O. campestris* is a key species when the glacial survival of arctic–alpine plants in the Alps is discussed. Patterns of genetic variation detected in mountain plants cannot be explained always by glacial survival within the Alps. Noack (1922) suggested that some plant species with an Arctic–Alpine distribution migrated into the Alps after the Last Glacial Maximum (20 000 BP), following the retreating glaciers. Amplified fragment length polymorphism (AFLP) reveals no genetic divergence of the Eastern Alpine endemic *O. campestris* subsp. *tiroliensis* (*Fabaceae*) from the widespread *O. c.* subsp. *campestris* (Schönswetter et al., 2004). This is in accord with the suggestion that the absence of a distinctive genotype amongst alpine populations could be caused by postglacial immigration from outside the Alps. (Schönswetter et al., 2005, 2006). Schönswetter and co authors (2004) also suggest that there has been a fairly recent colonization of the Alps by *O. campestris*, rather than postglacial expansion from relict populations.

This could be consistent with the idea that *O. campestris* is a relatively new hybrid.

Yurtsev (1999) suggests another hypothesis for the origin of *O. campestris* and its close relatives in Western Europe. According to him, they are derivatives of the South Siberian continental mountain races of *O. sordida*. Section *Orobia* is one of the richest in species within the subgenus *Oxytropis*, and includes 27 species and subspecies within the Arctic. It is one of the most difficult sections with respect to taxonomy. In section *Orobia,* the eastern races of the *O. sordida* aggregate now persist in separate post-glacial refuges in contrast to the East European Taimyr subsp. *Sordida,* which populated the Arctic and Subarctic areas as soon as they became free of ice.

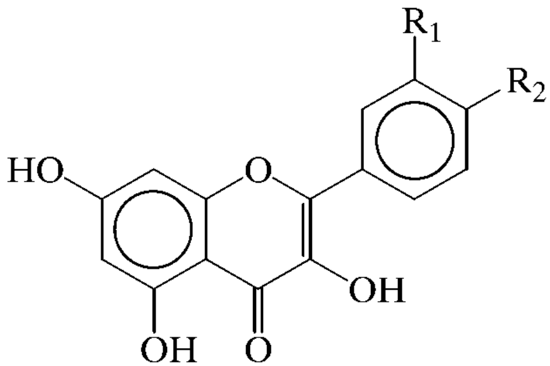
SOME SECONDARY COMPOUNDS

Flavonol aglycons Kaempherol, Isorhamnetin and Quercetin were detected in all species tested from the EtOAc extract by comparison with authentic samples. The same compounds were confirmed by GS/MS analyze Finigram Polaris after methylation as follows:

- Kaempherol retention times 21.00 m/z 285/151
- Isorhamnetin retention times 21.54 m/z 315/300
- Quercetin retention times 19.05 m/z 301/151
- Quercetin-3-O-β-D–(3II-methoxy) - glucopyranoside is detected from *O. kozhuharovii*, retention times 5.77 m/z 301/167/151 (Figure 11).

Our investigation revealed that flavonols are presented in all Bulgarian members of the *Oxytropis campestris* complex. Universal for the three species are flavonols with aglycones Kaempherol (5,7,4’ Tetrahydroxy flavonol), Isorhamnetin (5,7, 4’ Tetrahydroxy 3’ methoxy flavonol), and Quercetin (5,7,3’4’ Tetrahydroxy flavonol). Additionally *O. kozhuharovii* was found to contain Quercetin-3-O-β-D-(3II-methoxy) - glucopyranoside. It is interesting that we have detected a compound in *O. kozhuharovii* not found in the other two species, which suggests that it might have come from the unknown parent that resulted in the purple pigment, e.g. *O. halleri* Bunge ex Koch, *O. dinarica* (Murb.) Wettst or *O. prenja* (Beck) Beck (details in Kožuharova et al., 2007, 2012b).

Even though our data are preliminary they correspond to the results of the phytochemical investigation of the four North American members of *Oxytropis campestris* complex. Elisens and Denford (1982) have isolated and identified thirteen flavone and eight flavonol glycosides from four taxa of *Oxytropis campestris* (L.) DC. s. lat. The authors found that unlike some previously examined polyploid complexes, the component taxa have markedly divergent flavonoid profiles. It appears that considerable phytochemical differentiation has accompanied morphological and cytological diversification within the complex. These data support taxonomic recognition of the component taxa at the specific level (Elisens & Denford, 1982).



	R_1	R_2
Quercetine	OH	OH
Kaempferol	OCH_3	OH
Isorhamnetine	H	OH

Figure 11. Flavonol aglycons detected in all three *Oxytropis* species.

BREEDING SYSTEMS

Oxytropis urumovii and *O. kozhuharovii* have restricted ability for vegetative propagation. This affirmation is based on herbarium materials (Figures. 12 and 13) and *ex situ* life span observations. These species are tap rooted and do not branch. Thus the propagation is by seed.

Oxytropis urumovii was found to be capable of spontaneous self pollination (Table 4). The anthers of the stamen sheath have more or less equal filaments. The slightly curved pistil is equal to the stamens so the stigma is situated at the same level with the anthers (90.0% of the flowers, N=50). Spontaneous self-pollination is possible in this case. In some flowers it extends slightly above them (10.0% of the flowers, N=50). This could explain the results presented in Table 4. Spontaneous self pollination possible and probability could be rather high (up to more than 90%) but it may be prevented in some individuals by herkogamy (positional isolation between anthers and stigma). The P/O test indicates that this species has a breeding system between facultative and obligate xenogamy (Table 5).

Oxytropis campestris was found to be capable of spontaneous self pollination, but in fact this is rather rare (Table 4). The slightly curved pistil is equal to the stamens so the stigma is situated at the same level with the anthers (80.0% of the flowers, N = 50). In some flowers the stigma extends slightly above or is situated slightly beneath the anthers (10.0% of the flowers have longer styles and 10.0% of the flowers have shorter styles, N = 50). The P/O test indicates that this species has a breeding system between facultative and obligate xenogamy (Table 5).

O. kozhuharovii was not tested in field experiments with flowers excluded from pollinators. However the result from the indirect test – P/O ratio indicates that this species has a breeding system between facultative and obligate xenogamy (Table 5).

The level of xenogamy is determined by two mechanisms: 1) can the flower self-pollinate? and 2) can the pollen fertilise its own stigma? The results from our field experiments and P/O tests indicate that all these species are potentially self-fertile and do not have self-incompatibility. Self-pollination occurs and seed is set. The prevention of self-pollination is by means of spacial isolation between stigma and anthers of the flower. The process it is more expressed in *Oxytropis urumovii* and less expressed in *O. campestris* (Table 4). Additional proof for a higher ability for self-pollination in *O. urumovii* was obtained *ex situ* as the one and only individual that bloomed with a single inflorescence in 2009 set seed as a result of self pollination. Indirect proof for more restricted self-pollination of *O. kozhuharovii* was obtained *ex situ*. There was a high number of legumes with no seeds (67% in 2007 and 68% in 2008). Thus if we have to rate the self-pollination it would be highest in the diploid *O. urumovii,* followed by tetraploid *O. kozhuharovii* and lowest in hexaploid *O. campestris.*

The populations of the three studied species do not form clones. Inbreeding in *O. urumovii* might be high because the pollinators are scarce and the species posses the ability for self pollination (see below and details in Kožuharova, 2000).

Figure 12. *Oxytropis urumovii*.

Figure 13. *Oxytropis kozhuharovii*.

Table 4. Ability for spontaneous self-pollination: Field experiments with flowers excluded from pollinators

Excluded flowers	Failed to set fruits	Legumes	Percent of fertilization
O. urumovii			
1995 ($N_{flowers}$ = 9, $N_{inflorescences}$ = 3)	7	2	22.2%
2001 ($N_{flowers}$ = 63, $N_{inflorescences}$=13)	5	58	92.1%
O. campestris			
2001 ($N_{flowers}$ = 95, $N_{inflorescences}$ = 10)	92	3	3.3%

Table 5. P/O ratio values correlated to the breeding systems by Cruden (1973, 1976) and Dafni (1992)

	Average	Stdev	Min	Max
O. urumovii ($N_{flowers}$=10)	2532.9	493.7	1666.7	3125.0
O. campestris ($N_{flowers}$=23)	1975.2	976.2	888.9	5671.3
O. kozhuharovii ($N_{flowers}$=5)	1915.2	± 250.8	1574.1	2345.3
Obligate autogamy				
according to Dafni (1992)	28.6	-	18.1	39.0
according to Cruden (1973, 1976)	27.7	± 3.1		
Facultative autogamy				
according to Dafni (1992)	213.9	-	31.9	396.0
according to Cruden (1973, 1976)	168.5	± 22.1		
Facultative xenogamy				
according to Dafni (1992)	1416.4	-	244.7	2588.0
according to Cruden (1973, 1976)	796.6	± 87.7		
Obligate xenogamy				
according to Dafni (1992)	98816.5	-	2108.0	195525.0
according to Cruden (1973, 1976)	5859.2	± 936.5		

POLLINATION ECOLOGY

Pollinator activity at the site of *Oxytropis urumovii* was high. These were *Bombus lapidarius* and *Bombus* sp. workers who visited actively for nectar from the abandoned *Anthyllis vulneraria* (Table 6). Some of them were observed to groom the pollen adhered on their abdomens into their corbiculae.

Table 6. Plant complexes at the study sites flowering simultaneously with *Oxytropis urumovii, O. campestris* and O. *kozhuharovii* and visitation rate = number of visitors/minute (average)

plant species	abundance	available flowers	*B. lapidarius*	*B. terrsestris*	*B. pratorum*	*B. pyrenaeus*
Oxytropis campestris	$Cop._3$	25000	0.18			0.03
Jasione bulgarica	$Cop._3$				0.40	
Onobrychis pindicola	$Cop._3$			0.07		0.12
Cerastium alpinum	$Cop._3$					
Gentiana verna	$Cop._3$					
Thymusthracicus	$Cop._3$					0.03
Genista depressa	$Cop._3$					
Acynos alpinus	$Cop._2$				0.01	
Rhodax canum	$Cop._2$					
Armeria alpina	$Cop._2$					
Anthenariadioica	$Cop._2$					
Alyssum cuneifolium	$Cop._1$					
Aster alpinus	$Cop._1$					
Achillea ageratifolia	$Cop._1$					
Oxytropis urumovii	$Cop._1$	600	-	-	-	-
Anthylis vulneraria	$Cop._3$		0.17			
Linum capitatum	$Cop._3$					
Daphne velenovskyi	$Cop._3$					0.01
Acinos alpinus	$Cop._3$					0.01
Centaurea achtarovii	$Cop._2$					
Thymus thracicus	$Cop._2$					0.01
Helianthemum nummularium	$Cop._2$					
Rhodax canum	$Cop._2$					
Cerastium alpinum	$Cop._2$					
Aster alpinus	$Cop._1$					
Achillea ageratifolia	$Cop._1$					
Oxytropis kozhuharovii	$Cop._1$	500	-	-	-	-
Onobrychis pindicola	$Cop._3$					0.01
Chamaecytisus absinthioides	$Cop._3$					
Daphne oleoides	$Cop._3$					
Saxifraga ferdinandi-coburgii	$Cop._3$					
Anthyllis vulneraria	$Cop._2$		0.01			

Table 7. Pollen load composition of bumblebee individuals (7 workers *Bombus lapidarius* and 2 workers *Bombus pyrenaeus*) collected on the flowers of *Oxitropis campestris* - percent of pollen types

Pollen type	Average (%) In the pollen loads of *Bombus lapidaries*	Number of pollen loads	Average (%) In the pollen loads of *Bombus pyrenaeus*	Number of pollen loads
Oxytropis	98.91% (100%-98.8%)	7	98.65% (99.9%-97.4%)	2
Onobrychis	0.2%	1		
Trifolium			1.4%	1
Thymus			0.7%	1
Potentilla	3.8%	1		
Achillea/Aster	0.2%			
Cirsiu/Carduus	0.6%			
Jasione			0.1	1
Scabiosa				
Cerastium	0.1%	2	0.1%	1
Verbascum	0.1%			
Veronica			0.4%	1
Pinus	0.13%	3		

We did not register flower visitors of *O. urumovii* during the period of observations. Its successful fruit set, however, indicates possible pollinator sharing with the abandoned *Anthyllis vulneraria* among which the few *Oxytropis urumovii* plants were scattered. It is well known that bumblebee individuals have primary foraging specialties (their majors) and secondary specialties (their minors). Minors are often bridges to new majors (Heinrich, 1976a, 1976b). We presume that the two plant species are pollinated by the same pollinators and *Anthyllis vulneraria* is a “major” food species while *Oxytropis urumovii* is a “minor” one for them. Pollinator activity at the study sites of *Oxytropis campestris* was high to very high. The main pollinators were workers of *Bombus lapidarius* and sporadic visits by workers of *B. pyrenaeus* were also observed (Table 6).

Bombus lapidarius visited strictly the inflorescences of O. campestris. Few of them visited Onobrychis pindicola on the same foraging trip. Highest flower constancy of the pollinators of Oxytropis campestris is connected to their dense patches and suitable food resources. B. lapidarius collected nectar of O. campestris and the pollen was adhered on the hair of their abdominal sternits. Pollen collection was not observed. However about 50% of the workers had full pollen baskets. The pollen analysis revealed that they have broomed the pollen from their body into the baskets and confirmed very high pollinator constancy to O. campestris (Table 7). All bumblebees tended to visit successively more than one flower per inflorescence. The two other plant species in close neighborhood to O. campestris visited actively by bumblebees were Onobrychis pindicola and Jasione bulgarica. They both were visited by other species of bumblebees and were not competitors for pollinators (Table 6).

FRUIT AND SEED SET

The fruit set as a result of open pollination of all three *Oxytropis* species was very high (Table 8). Regarding the results from the breeding systems test the fruit and seed set is a result of xenogamy and partially autogamy. The highest observed pollinator activity is in the population of *O. campestris*, therefore the xenogamy ratio there should be higher.

Table 8. Open pollination fruit set

year of observation	*Oxytropis*	sample-fruits/ flowers	unfertil ized flowers	matured fruits (legums)	percent of fertilezation	average seeds per legumes (min, max)
1995	*O. campestris*	325	47	278	85,5%	6 (2,14)
1996	*O. campestris*	501	22	479	95,6%	6 (2,11)
2002	*O. campestris*	139	19	120	86,3%	6 (1,11)
2005	*O. campestris*	141	1	140	99,3%	9 (2,12)
1995	*O. urumovii*	179	44	135	75,4%	5 (1,11)
1996	*O. urumovii*	182	1	181	99,5%	5 (1,9)
2002	*O. urumovii*	117	8	109	93,2%	5 (1,9)
2005	*O. urumovii*	160	2	158	98,8%	6 (1,10)
2005	*O. kozhuharovii*	868	77	791	91,10%	7 (1,16)

Very important for pollinator activity, pollination success and seed production is the size of the patch or the plant population. The bigger the size is, the higher the pollinator activity (Heinrich, 1979b, Levin, 1978, Handel, 1983, Sih & Baltus, 1987., Pleasants, 1980, Petanidou et al., 1995). This statement we tested with the fruit set of *O. kozhuharovii*. There were no statistically significant differences between group means for the three patches of the population regarding the open pollination fruit set as determined by one-way ANOVA ($F(2,138) = 0.773$, $p = 0.463$). Despite the presumption that the big patch had better chances for pollinator visitation due to the fact that these flowers might be bumblebee pollinator “majors” (Heinrich, 1979b), the fruit set seems to remain unaffected compared to small patches. Possibly the “big” and “small” patches were close enough to obscure differences.

Ex Situ Germinatiion and Ontogenesis

Ex situ seedlings of *Oxytropis urumovii* and *O. kozhuharovii* were grown and seed germination was tested. Most mature seeds of the test plants germinated very well within a couple of days if the seed coat was scarified. Scarification with sand paper was often applied for increasing the germination of legume seed (*Astragalus, Hedysarum, Lupinus, Oxytropis*) and the effect was significant - up to 100% (Kaye, 1997). The seed of four *Astragalus* species germinated at any temperature (13-34˚C) if they were scarified, (Platikanov et al., 2006). Peak germination percentage for *Astragalus australis* var. *olympicus* occurred at 15°/25°C alternating temperatures and at moisture availability with low water potential (distilled water) combined with scarification (Kaye, 1999). Most mature test seeds of *Oxytropis urumovii* and *O. kozhuharovii* germinated within a couple of days if the seed coat was scarified (Kožuharova & Richards, 2009). The hard seed testa replaces chemical primary dormancy of the seed, so that the seeds do not germinate too soon (in the autumn) but only when the weather warms during the following spring when the testa will collapse after e.g. fungal degradation or possibly scarification on the steep slope and rough marble rock and pebble surface. Stratification appeared to be unnecessary. A slight decrease of seed germination was observed with the time (details were presented in Table 1 in another paper: Kožuharova & Richards, 2009).

The early stage development was estimated as rather vulnerable. Once planted in the experimental plot usually at least half of the planted *O. urumovii* and *O. kozhuharovii* seedlings lived through the first summer (Table 9). Overwintering was the next Rubicon for the seedlings (Table 9). Rather similar the juvenile and immature stages of *Oxytropis chankaensis* are the most vulnerable stages (Artyukova et al., 2012).

Table 9. Seed germination and development of the seedlings

years	2006			2007			2008				2009			2010
	27th May♣	4th Aug	21st Oct	15th April	24th May 16th June♣	10th Aug	3rd May	9th June♣	2nd Aug	11th Sept	25th May	11th June	17th July	25th July
O. kozhuharovii - Number of seedlings														
2006*	25	14	11	10	10	10	2	2	1	1	1	1	1	0
2007*					70	47	18	12	12	12	12	12	12	12
2008*								120	80	40	33	33	33	3
O. urumovii - Number of seedlings														
2006*	14	8	6	5	4	3	1	1	1	1	1	1	0	
2007**					38	20	6	4	4	4	5	4	4	1
2008**								24	19	9	9	9	9	0

Legend: ♣date of planting, *seeds collected in 2005 from wild populations, **seeds collected in 2005 from wild populations.

All plants of *O. urumovii* produced only leaves. In 2009 one individual planted in 2006 bloomed in June and produced mature seeds but then the whole plant died.

The *ex situ* life span observations confirmed the restricted ability for vegetative propagation. This also elucidates the observed *in situ* large patches of *Oxytropis kozhuharovii* with more than 70 flowering stems and thought to be many decades old (Kožuharova et al., 2007). *Ex situ* observations on the ontogenesis for four years revealed that the individual plant does not grow much in size. Explanation for the patches *in situ* is that seedlings can grow densely next to each other as it was observed *ex situ* (Figure 1e). Obviously *in* the cases of dense cover on 0,25m^2 consists of different individuals. *Ex situ* observations on the ontogenesis revealed that the individual plant does not grow much in size and thus they do not make clones (Kožuharova & Richards 2009, Kožuharova et al., 2012a).

Two seedlings of *O. kozhuharovii* grown in 2006 bloomed in 2007 (the next summer after sowing the seed, with 7 and 8 flowering stems respectively). One of them died until the next spring but the other bloomed again in 2008 (with 5 flowering stems). Also three plants grown in 2007 bloomed in 2008 (with 3, 4 and 6 flowering stems). The average number of flowers per head was 8 ± 2.06. The presence of a great number of empty pods (67% in 2007 and 68% in 2008) indicates the reduced ability for spontaneous pollination (possibly positional isolation of the anthers and stigma). In 2007 both plants had fruits. In 2008 the fruits were produced by only one of the five flowering plants, which may mean that the flowers are self-incompatible (details in Kožuharova & Richards, 2009, Kožuharova et al., 2012a).

Some individuals of *O. kozhuharovii in situ* were of considerable size and thought to be many decades old (Kožuharova et al., 2007). *Ex situ* observations on the ontogenesis for four years revealed that the individual plant does not grow much in size. This observation corresponds to the hypothesis that vegetative propagation is not an option as plants do not branch as they send tap roots. But seedlings can grow densely next to each other *ex situ*. Obviously *in situ* the examples of dense cover on 0,25m^2 consist of different individuals.

The flowering period *ex situ* is about a month earlier (Figure 5). The observed earlier blooming *in situ* of *O. urumovii* compared to *O. kozhuharovii* is obviously genetically determined as this flowering pattern is preserved *ex situ* even though a month earlier.

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