Universidad de La Frontera
Facultad de Ingeniería y Ciencias
Programa de Doctorado en Ciencias de Recursos Naturales

Calafate (*Berberis microphylla* G. Forst): Characterization of alkaloids and biological activities against human pathogenic bacteria and agricultural insect pests

Doctoral thesis in fulfillment of the requeriments for the Degree Doctor in Sciencies in Natural Resources By

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TEMUCO – CHILE 2017

Calafate (*Berberis microphylla* G. Forst): Characterization of alkaloids and biological activities against human pathogenic bacteria and agricultural insect pests

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Todo lo puedo en Cristo que me fortalece
Filipenses 4:13

Agradecimientos

En esta tesis no solo están los resultados de una investigación, sino que está el amor y pasión por mi trabajo, momentos de frustración y superación de obstáculos. Sin embargo, esto no sería posible sin el apoyo de todas aquellas personas que me han permitido poder llevar a cabo de buena manera mi trabajo. De este modo, a continuación escribo en estas líneas mis agradecimientos más sinceros a quienes han hecho de esto una realidad.

Agradezco al programa de Doctorado en Ciencias de Recursos Naturales de la Universidad de La Frontera. A su director el Dr. Francisco Matus, por su gestión en la última etapa de mi tesis y a la Dra. María de la Luz Mora, por su apoyo en mi desempeño académico durante estos años.

A los integrantes de la comisión examinadora: Dra. Graciela Palma, Dr. Eduardo Fuentes y Dr. Alejandro Urzúa, por sus comentarios y aportes durante el desarrollo de esta investigación. En especial quisiera agradecer al Dr. Alejandro Urzúa, quien contribuyó generando importantes aportes en los escritos de mis publicaciones.

A mi tutor, Dr. Andrés Quiroz y cotutor Dr. Víctor Fajardo por sus conocimientos entregados, así como por la confianza que han depositado en mí, la cual espero poder mantener en el tiempo.

A mi profesor, Fernando Pardo, quien me apoyó en las primeras etapas de mi formación en postgrado no sólo como investigador y profesional, sino también como persona.

A todos los miembros del Laboratorio de Química Ecológica y Laboratorio de Productos Naturales, por su disposición y buena voluntad.

A los funcionarios del Departamento de Ciencias Químicas y Recursos Naturales, por su amabilidad y cooperación.

A las becas CONICYT, Apoyo Tesis Doctoral Nº 24121407 y Pasantía Doctoral Nº 75120095, por los recursos otorgados para el desarrollo de las diversas actividades vinculadas con la presente tesis.

A mis queridos amigos, Ana Mutis, Aixa González, Gustavo Curaqueo, Marcelo Panichini, Sebastián Meier y Rubén Palma, en quienes encontré un apoyo incondicional en todo momento, en especial en los más difíciles, donde me demostraron que en esos momentos es donde uno conoce a los amigos de verdad.

A mi querida amiga Ana Mutis, quien estuvo a mi lado para darme la fortaleza necesaria para continuar con mi trabajo.

A mi madre, Mónica Carrasco, por su amor y apoyo incondicional, por inculcarme que nunca hay que rendirse y que siempre debe estar el deseo de ser mejor, tanto como profesional como persona.

A mi padre, Eduardo Manosalva, por su amor y preocupación, gracias por tu apoyo en todo momento.

A mi Hermana, Paulina Manosalva, por confiar en mí y aunque la distancia nos mantenga separadas, sabemos que siempre nos llevamos presentes en el corazón.

A mi esposo, Daniel Groves, por cada momento vivido y darme esa alegría que se llama Tomás, quien nos enseño a ver la vida de forma más feliz y agradecida.

¡Gracias!

Summary and outline of this thesis

Berberis microphylla, is a native plant commonly known as “calafate" that grows wildly in southern of Chile and used by aboriginal ethnic groups in traditional medicine. In recent years, some species of *Berberis* from Chile have been studied due to interesting biological properties related to their alkaloids content. The aim of this thesis was to evaluate the presence of isoquinoline alkaloids in different parts of *B. microphylla* and to evaluate the biological activity against bacteria and insects elicited from the respective alkaloidal extracts. The alkaloids compounds presents in leaf, stem and root extracts from *B. microphylla* such as allocryptopine, berberine, calafatine, isocorydine, jatrorrhizine, palmatine, protopine, reticuline, scoulerine, tetrahydroberberine and thalifendine were identified by high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC ESI-MS/MS). Antibacterial assays of alkaloid plant extracts of *B. microphylla* were carried out by using inhibition zone diameters. The results demostrated significant inhibition activities elicited by leaves, stems and roots extract against Gram-positive bacteria, whereas only roots extracts showed similar activity against some bacteria compared to commercial antibiotics (ampicillin and cephalothin). Pure berberine was found to be active only against *Staphylococcus aureus* and *Staphylococcus epidermidis,* with similar activity to that of the roots extracts. Moreover, using the Fractional Inhibitory Concentration (FIC) index it was possible to determine that the same alkaloid extracts in combination with conventional antibiotics exhibited synergistic effects against the microorganisms tested. Finally, antifeedant activity of stem and root extracts against the 3rd instar of *Plutella xylostella* larvae was studied using non-choice leaf disc assay. The results showed that stem and root extracts strongly reduced food consumption of larval, except when stem extracts was applied at

the lowest concentration. Two pure alkaloids, berberine and palmatine, also were assayed and only berberine at the highest concentration exhibit antifeedant activity against *P. xylostella* larvae. The findings of the present research show that this native plant is a source for antibacterial agents against Gram-positive human pathogenic bacteria and also could be utilized to control *P. xylostella* and eventually other phytophagous pests.

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CHAPTER I

GENERAL INTRODUCTION

1.1. General Introduction

The genus *Berberis* has gained an important place in various traditional medical systems worldwide for their efficacious medicinal properties. In general, pharmacological and biological properties are attributable to the presence of alkaloids in plants, including antimicrobial, antiinflammatory, anticancer, antidiabetic, cytotoxic and insecticide activity (Tewary et al., 2005; Li et al., 2007; Das et al., 2009; Upwar et al., 2011; Patel et al., 2012; Ali et al., 2013). Therefore, research has been focused on identification and isolation of new compounds in the *Berberis* genus with prominent biologycal activity (Stermitz et al., 2000b; Yeşilada et al., 2002; Musumeci et al., 2003; Fatehi et al., 2005; Ho et al., 2009).

1.1.1. Distribution of the Berberis genus

Berberis genus (Berberidaceae) commonly known as "barberry", includes about 500 native species from Asia, Europe, North Africa, North America and South America (Ahrendt, 1961). Members of this genus comprise evergreen and semi-evergreen shrubs and small trees reaching from 1 to 5 m in height, often spiny, with smooth or sulcate branches, glabrous or puberulas leaves (Landrum, 2003). Flowers are found not only in asraceme and umbellate inflorescences, but also as, solitary and auxillary flowers, all in yellow, orange to red-orange auxillary corymbus or clusters (Moore, 1983) growing under a wide range of ecological conditions (Landrum, 1999). The fruits are always dark purple, bluish and black, and sometimes can be covered by a layer of wax (Landrum, 2003). These species can be propagated by seed or by vegetative methods (Arena and Martínez-Pastur, 1994). For this reason, although species can cover large

areas, only low numbers of individuals are found forming populations (Bottini et al., 2002).

According to the geographical distribution of species belonging to the *Berberis* genus, two important diversification groups have been described: Septentrional and Austral (Landrum, 1999). The Septentrional group (Old World) with about 300 species occurs mainly in Eurasia but extends to North America and North Africa. This group is characterized by red berries and deciduous leaves (Ahrendt, 1961). The Austral group (New World) contains ca. 200 species distributed in South America with only a few members in Central America (Landrum 1999). Many Austral species have foliaceous spines, dentate stamens and deep orange flowers (Ahrendt, 1961).

In the South American Andes, the *Berberis* genus is well represented by species having a wide geographical distribution in southern Chile and Argentina (Patagonia) (Landrum, 2003). In the latter habitat, *Berberis* species are known as "calafate" or "michay" and grow under a wide range of ecological conditions from forest to steppe (Correa, 1984; Botinni et al., 2000).

The knowledge about the traditional use of these native plants in general is inherited from older relatives and learned among people of the same community. For this reason, records on the use of austral plants is scarce (Cuadra and Fajardo, 2008). Some reports inform that calafate plants were also used by indigenous people for the treatment of fevers, inflammations, stomachaches and diarrhea (Montes and Wilkomirsky, 1987; Muñoz and Wilkomirsky, 2004). Additionally, the Aonikenk people used yellow scraping of the bark as tobacco, due to its hallucinogen effect probably caused by the alkaloid berberine, whereas the fruit of the plant was used by Kawésqar people as a source of food (Dominguez et al., 2012).

In addition, these especies are known to have diverse applications. For instance, their blue-black, fleshy and juicy bittersweet fruits are eaten fresh or cooked as jams, jellies and syrups (Bottini et al., 1993; Correa, 1984). The fruits of *B. microphylla* are a rich source of nutrients and compounds with significant antioxidant activity (Ruiz et al., 2010). *Berberis* members have beneficial effects on wind speed reduction for seasonal breeding roosts in sheep (Bustos, 1995), and in steppe play a crucial role in the control of desertification avoiding soil erosion, while in forests have become the refuge for valuable plant species (Bottini et al., 1993). For example, *Berberis heterophylla* plays an important role in desertification control in Patagonia (Arena and Vater, 2003).

On the other hand, some *Berberis* species from Chile and southern of Argentina, such as *Berberis chilensis, Berberis darwinii* and *Berberis heterophylla* have shown interesting pharmacological and antimicrobial properties based on the presence of alkaloids (Morales et al., 1993; Martinez et al., 1997; Freile et al., 2003; Enriz and Freile, 2006; Freile et al., 2006; Alarcon et al., 2014).

1.1.2. Chemical constituents of the Berberis genus

The chemical constituents isolated and identified from *Berberis* species include alkaloids, anthocyanins, flavonoids, terpenoids, lignans, lipids, sterols, proteins and vitamins (Karimov, 1993; Mazzuca et al., 2005; Ruiz et al., 2010; Ruiz et al., 2014; Ragasa et al., 2015). However, the most bioactive constituents in the *Berberis* genus are the isoquinoline alkaloids (Tewary et al., 2005; Gulfraz et al., 2007; Li et al., 2007; Das at al., 2009; Bhardwaj and Kaushik, 2012). These compounds are identified primarily in stems and roots containing higher concentrations and variety of isoquinoline alkaloids (Hussaini and Shoeb, 1985; Khamidov et al., 1997a; Stermitz et al., 2000b; Singh et al.,

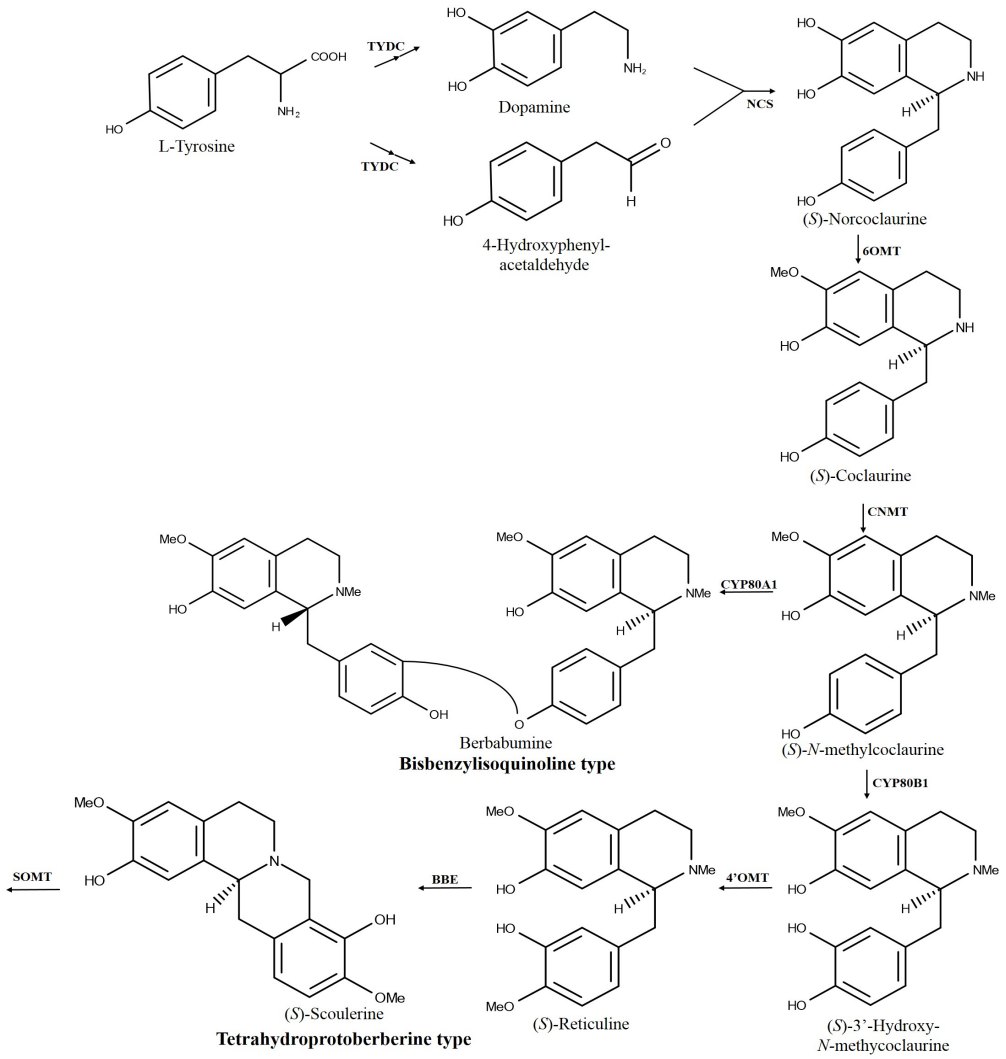
2007; Meeran et al., 2008; Kakar et al. 2012). Some of the major alkaloids reported from various *Berberis* species are berberine, berbamine, columbamine, isotetrandrine palmatine, jatrorrhizine and oxyacanthine (Bhardwaj and Kaushik, 2012; Srivastava et al., 2015). Furthemore, berberine is the most representative compound of the genus because its presence in all *Berberis* species and playing an important role in biological activities (Chandra and Purohit, 1980; Ivanovska and Philipov, 1996; Kosalec et al., 2009; Adb El-Wahab et al., 2013).

The presence of alkaloids is dependant on environmental conditions (altitude, soil nutrition) and plant related factors (species, genotype, stage and organ development) (Andola et al., 2010 a, b). For example, multifactor correlations analysis suggest that the berberine content in *Berberis asiatica* is influenced by edaphic features, altitude, and season. The authors concluded that: (i) root and stem bark from lower altitude contained more berberine, (ii) berberine concentration was the highest in summer and the lowest in rainy season and (iii) higher berberine concentrations was detected in samples obtained from low soil moisture levels and high soil potassium levels (Andola et al., 2011).

The biosynthesis of the isoquinoline alkaloids (Figure 1) in begins with the conversion of tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde via a sequential decarboxylation, *ortho*-hydroxylation and deamination pathway. Among these early steps, tyrosine/dopa decarboxylase (TYDC; an aromatic L-amino acid decarboxylase), converts tyrosine and dopa to their corresponding amines (Sato and Yamada, 2008). Subsequently, dopamine and 4-hydroxyphenylacetaldehyde are condensed by norcoclaurine synthase (NCS) to yield (*S*)-norcoclaurine (Sato and Kumagai, 2013), which is the central precursor to all isoquinoline alkaloids (Sato and Yamada, 2008; Hagel and Facchini, 2013). The (*S*)-norcoclaurine is later converted to

(*S*)-coclaurine by *S*-adenosyl methionine (SAM)-dependent norcoclaurine 6-*O*-methyltransferase (6OMT) which is in turn converted to *N*-methylcoclaurine by SAM-dependent coclaurine *N*-methyltransferase (CNMT) (Choi et al., 2002; Sato and Yamada, 2008). Finally, *N*-methylcoclaurine is converted to 3′-hydroxy-*N*-methyl coclaurine by P450 hydroxylase (CYP80B1) and then converted to (*S*)-reticuline by SAM-dependent 3′-hydroxy *N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT) (Sato and Kumagai, 2013). The (*S*)-reticuline is the central intermediate in branch pathways that lead to different structural types of isoquinoline alkaloids in *Berberis* species such as: protoberberine alkaloids (e.g. berberine), aporphine alkaloids (e.g. isoboldine), and protopine alkaloids (e.g. allocryptopine) (Karimow, 1993; Dewic, 2002;Takemura et al., 2010; Singh et al., 2015).

In the case of dimeric bisbenzylisoquinoline alkaloids, such as berbamunine, these are produced from the intermediates of the (*S*)-reticuline pathway by the action of a phenol coupling P450-dependent oxidase (berbamunine synthase, CYP80A1) (Kraus and Kutchan, 1995). Other bisbenzylisoquinoline derived from the intermediates of the (*S*)-reticuline pathway is calafatine, which has an unusual oxidation model (Fajardo et al., 1979b) (Figure 2).


L-Tyrosine
TYDC
TYDC
Dopamine
4-Hydroxyphenyl-
acetaldehyde
NCS
(S)-Norcoclaurine
6OMT
(S)-Coclaurine
CNMT
(S)-N-methylcoclaurine
CYP80A1
Berbabumine
Bisbenzylisoquinoline type
CYP80B1
(S)-3'-Hydroxy-
N-methycoclaurine
4'OMT
(S)-Reticuline
BBE
(S)-Scoulerine
Tetrahydroprotoberberine type
SOMT

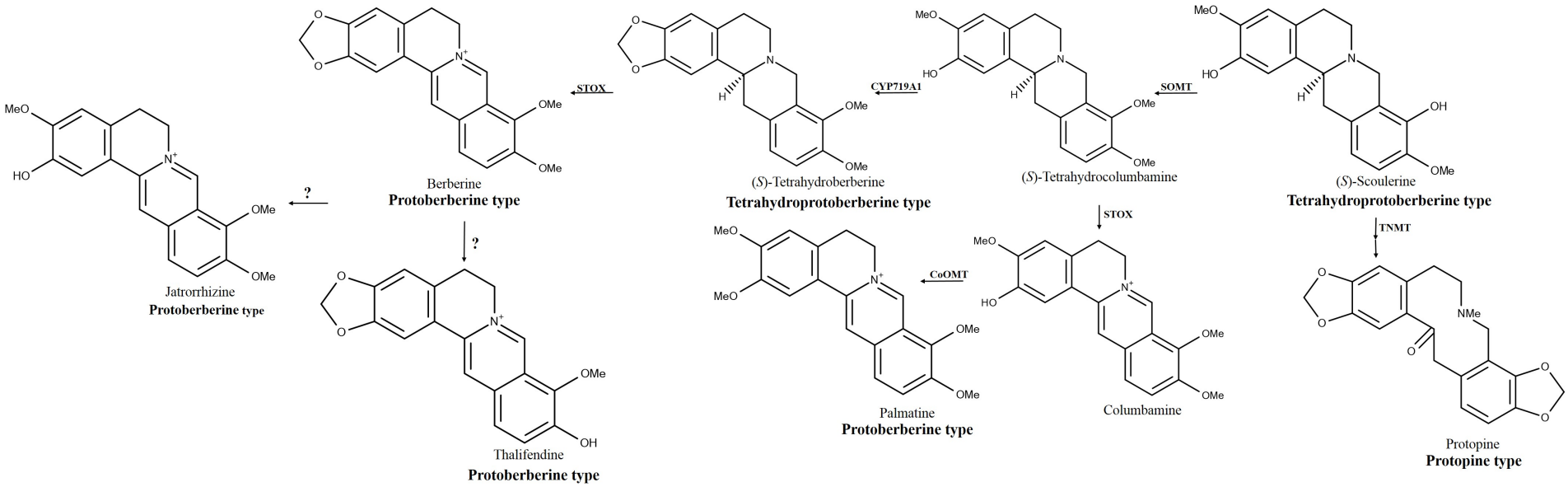


Figure 1. Biosynthetic patways of some isoquinoline alkaloids identified in *Berberis* species. Unbroken arrows indicate single enzymatic conversions. TYDC, tyrosine/dopadecarboxylase; NCS, norcoclaurine synthase; 6OMT, norcoclaurine 6-O-methyltransferase; CNMT, coclaurine *N*-methyltransferase; CYP80A1; CYP80B1, *N*-methylcoclaurine 3′-hydroxylase; 4′OMT, 3 hydroxy *N*-methylcoclaurine 4′-O-methyltransferase; BBE, berberine bridge enzyme; SOMT, scoulerine 9-*O*-methyltransferse; TNMT; STOX, tetrahydroprotoberberine oxidase; CYP719A1, canadine synthase; CoOMT (Adapted from Sato and Yamada; 2008,Li et al., 2011; Sato et al., 2013).

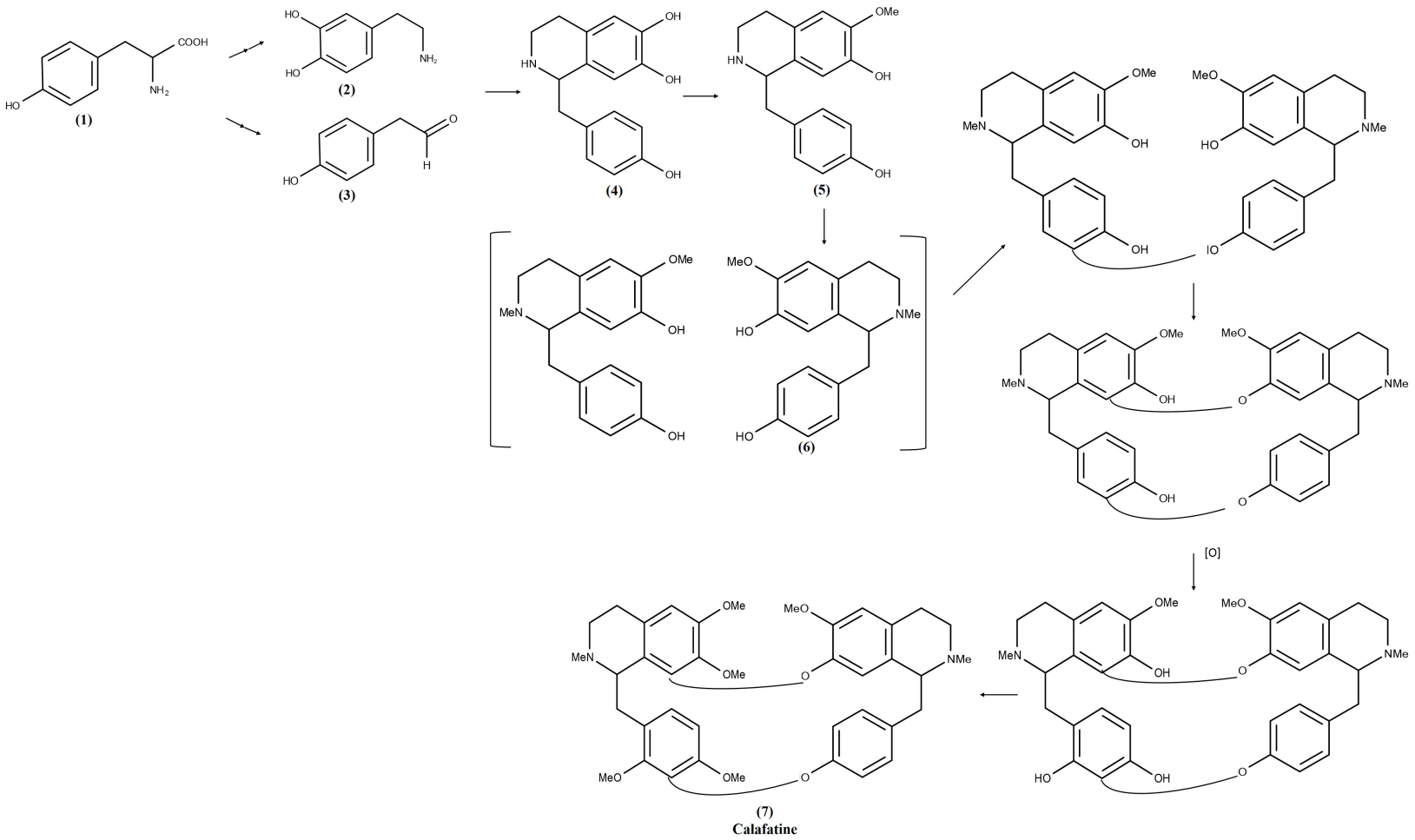


Figure 2. Hypothetical biogenesis of calafatine (Adapted from Fajardo et al., 1979b). (1) L-Tyrosine; (2) Dopamine; (3) 4-Hydroxyphenylacetaldehyde; (4) Norcoclaurine; (5) coclaurine; (6) *N*-methylcoclaurine; (7), Calafatine.

1.1.3. Pharmacological activities of the Berberis genus

Medicinal plants research has recently increased due to its importance as a source of biologically active substances. These plants contain secondary compounds such as alkaloids that have been described as useful antimicrobial phytochemical (Orhan et al., 2007; Bhattacharjee et al., 2010). The phytochemical analysis of *Berberis* species indicated that berberine, berbamine, jatrorrhizine and palmatine alkaloids are the most biologically active compounds (Cordell et al., 2001; Yeşilada and Küpeli, 2002; Imanshahidi and Hosseinzadeh, 2008; Campisi et al., 2011).

In this context, Küpeli et al. (2002) observed that out of the eight alkaloids detected in the roots of Turkish *Berberis* species (berberine, palmatine, jatrorrhizine, columbamine, berbamine, oxyacanthine, aromoline and magnoflorine), only berberine, berbamine and palmatine showed the main antiinflammatory activity. Other studies have found that extracts from *Berberis brevissima* and *Berberis parkeriana* show antimicrobial properties, in which jatrorrhizine was the most active alkaloid against resistant *Staphylococcus aureus* strain (Ali et al., 2013). Nevertheless, it is important to note that the biological activity of *Berberis* spp is usually attributed to berberine (Stermitz et al., 2000 a,b) compound present across all species of the genus (Chandra and Purohit, 1980; Kosalec et al., 2009; Končič et al., 2010). A study by Adb El-Wahab et al. (2013) demostrated the potential of the *Berberis vulgaris* extract and its active alkaloid, berberine, on suppressing lipid peroxidation, suggesting a promising use in the treatment of hepatic oxidative stress, Alzheimer and idiopathic male factor infertility. Moreover, the anti-inflammatory properties of *Berberis crataegina* might be attributable to berberina, main active ingredient isolated from roots of plants (Yeşilada and Küpeli, 2002). Iauk et al. (2007) reported an significantt antifungal activity against *Candida*

species showed alkaloidal fraction of *Berberis aetnensis* and berberine. In another study, the authors concluded that the antimicrobial activity of *Berberis aristata* against *Staphylococcus aureus*, *Bacillus subtilis* and *Aspergillus niger* was due to the presence of berberine in the extract (Anubhuti et al., 2011).

Additionally, *Berberis* species from southern Chile and adjacent Argentina (Patagonia) have shown interesting pharmacological (Morales et al., 1993; Martinez et al., 1997; Alarcon et al., 2014) and antimicrobial (Enriz and Freile, 2006; Freile et al., 2006; Freile et al., 2003) properties based on the presence of isoquinoline alkaloids. For example, *Berberis buxifolia* (s.n. *B. microphylla*) showed important antimicrobial activity against *Escherichia coli and S. aureus* (Pitta and Alvarez *et al.,* 2008). Moreover, this species produced a particular arrangement of isoquinoline alkaloids identified as dymers (proaporphines-benzyisoquinolines, bisbenzylisoquinoline), (Fajardo et al., 1979; Fajardo et al., 1986; Fajardo, 1987) attributed to these plants grown under environmental stress conditions (Cuadra and Fajardo, 2008). Another native species exhibiting pharmacological properties is *Berberis darwinii,* where root extracts showed potential antiinflammatory action by inhibiting the production of superoxide anion, the expression of tumor necrosis factor-alpha (TNFα), and interleukin-1-beta (IL-1β) in monocytes activated by lipopolysaccharide (Alarcon et al., 2014).

1.1.4. Insecticide and antifeedant activities of the Berberis genus

Chemical pesticides have been used for several decades in controlling pests that reduce both quality and quantity of crop yield (Ferry et al., 2004; Aktar et al., 2009). However, their indiscriminate use resulted in several problems such as pesticides

resistance, elimination of natural enemies, toxic residues in food, water, air and soil with subsequent negative effects on human health and ecosystem disruption (Zettler, 1991; Gupta, 2004; Bale et al., 2008; Opit et al., 2012). Under such scenario, plants compounds and plant derived products offered an advantage over synthetic pesticides as pests control agents in agriculture (Rajapake and Ratnaseka, 2008; Mwine et al., 2011) due to their biodegradability and low environmental persistence (Isman, 2006; Sarwar, 2015).

Alkaloids have therefore been reported as an important group of natural substances, that play a major role in the ecology of organisms and have been suggested to constitute part of the plant defense system against phytophagous herbibores (Wittstock and Gershenzon, 2002; Bustamante et al., 2006). Those compounds have been widely investigated for their antifeedant, insecticidal and deterrent properties (Brem et al, 2002; Jeyasankar et al., 2012; Acheuk and Doumandji-Mitiche, 2013; Jeyasankar et al., 2014; Ge et al, 2015).

Berberis species are characterized by synthesizing alkaloids in their leaves, stems and roots, having an interesting spectrum of insecticide and antifeedant properties. For example, *Berberis lycium* was found during a screening for pesticidal activity of five medicinal plants against agriculturally important pests (adults of *Aphis cracccivora*, *Tetranychus urticae* and larvae of *Spodoptera litura*, *Plutella xylostella* and *Helicoverpa armigera*). The results indicated that the type of extracting solvent of particular plant/parts affected their activity because the non-polar extracts of the roots of *B. lycium* were more active against *A. craccivora* than that of the polar extracts (Tewary et al., 2005). Quevedo et al. (2007) reported that stem extracts from *Berberis tabiensis* have shown toxic activity against third-instar larvae of *Culex quinquefasciatus* (Say), and the crustaceous *Artemia salina* (Leach). Other studies have reported that

bisbenzylisoquinoline alkaloids identified in stems and leaves extracts of *Berberis glauca* showed an antifeedant activity on larvae 3rd y 4th stage of the moth *Spodoptera sunia* (Lepidoptera), as well as toxic effects (Moreno-Murillo et al., 1995).

Berberine has elicited a decreasing of larvae growth activity of *Lepidoptera* species, *Papilio polyxenes*, *Parides bunichus*, *Battus polydamas* and *Eurytides marcelllls* (Miller and Feeny, 1989), as well as, insecticide activity against *Drosophila melanogaster* larvae for berberine and palmatine (Miyazawa et al., 2002). Shields et al. (2008), evaluated feeding deterrents of nine alkaloids (acridine, aristolochic acid, atropine, berberine, caffeine, nicotine, scopolamine, sparteine, and strychnine) against *Lymantria dispar* larvae. Results suggested that berberine and aristolochic acid were the two most potent feeding deterrents (Shields et al., 2008). The results obtained for berberine are interesting because this compounds is a major constituent in stems and roots of *Berberis* plants (Ivanovska et al., 1996).

In this regard, research should focus on the identification of bioactive compounds in the *Berberis* genus, given its medicinal properties. At present, there are no reports concerning the antimicrobial and antifeedant and insecticide activity of alkaloids from *Berberis microphylla*, and based on the evidence described, extracts of this plant could be a source for the development of new compounds that could serve for the treatment of infections diseases and for pest control.

1.2. Hypothesis

Berberis microphylla (Syn. *B. buxifolia* and *B. heterophylla*) is a native species with medicinal properties attributed to the presence of isoquinolinic alkaloids. To date there is just one work showing antibacterial activitis of *B. buxifolia* against two microorganisms, *S. aureus* and *E. coli*, but there a no reports related to insecticidal activity elicited by *B. microphylla*. However, the literaturae is plenty of reports showing interesting biological activities of isoquinoline skeleton on a number of organisms. On ther other hand, the information about the alkaloidal richness of *B. microphylla* is scarce. Considering the above, this work propose two hypothesis:

Hypothesis 1

Extracts of different parts of *B. microphylla* show qualitative and quantitative chemicals differences in their alkaloid content.

Hypothesis 2

Alkaloidal extracts from different parts of *Berberis microphylla* show antimicrobial activities against human pathogenic bacteria and can modify the behavior of agricultural insect pests.

1.3. General objetive

To evaluate the presence of isoquinoline alkaloids in different parts of *B. microphylla* and to evaluate the biological activity against bacteria and insects elicited from the respective alkaloid extracts.

1.4. Specific objetives

(i) To determine qualitative and quantitative alkaloids content in leaf, stem and root extracts of *B. microphylla* from southern region of Chile.

(ii) To evaluate the *in vitro* antibacterial activity of alkaloid extracts from *B. microphylla* against bacterial strains and study the synergistic effect between alkaloid extracts with standard antibiotics.

(iii) To evaluate the insecticide and antifeedant activity of alkaloid extracts from *B. microphylla* against agricultural insect pest.

CHAPTER II

IDENTIFICATION OF ISOQUINOLINE ALKALOIDS FROM *Beberis microphylla* By HPLC ESI-MS/MS

Published in Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas

(2014) 13 (4): 323-334

Identification of isoquinoline alkaloids from *Berberis microphylla* by HPLC ESI-MS/MS

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Abstract

Berberis microphylla (G. Forst) is a native plant growing in Patagonia. In recent years Patagonia *Berberis* are becoming important due to their interesting biological properties related to their alkaloids content. The aim of this study was determine the distribution and proportion of isoquinoline alkaloids in leaves, stems and roots of *B. microphylla* collected in two different climatic zones from Chilean Patagonia. Using by HPLC ESI-MS/MS isocorydine, jatrorrhizine, palmatine, reticuline, scoulerine, tetrahydroberberine and thalifendine were detected for the first time in this specie, and the presence of allocryptopine, berberine, calafatine and protopine, previously isolated in *B. microphylla* was corroborated. The alkaloids profile showed differences of compounds in samples collected in two climatic zones, where more compounds were detected in plants from Lago Deseado than Cerro Sombrero. Furthermore, a greater number of alkaloids were found in stem and root extracts and berberine and thalifendine were detected in higher proportion in these structures.

Keywords: *Berberis microphylla*, Patagonia, isoquinoline alkaloids, HPLC ESI-MS/MS

2.1. Introduction

Berberis microphylla G. Forst, (Berberidaceae) locally called "calafate" or "michay" is a native shrub that grows wildly in southern cone of South America (Chilean and Argentinean Patagonia) (Moore, 1983; Orsi, 1984). The plants had been used in traditional medicine as a treatment of different diseases such as, fevers, inflammations and diarrhea (Muñoz et al., 2004). In recent years, Patagonia *Berberis* has also gained prominence importance due to their interesting pharmacological (Morales et al., 1993; Martinez et al., 1997; Alarcon et al., 2014), antifungal (Enriz and Freile, 2006; Freile et al., 2006; Pitta-Alvarez et al., 2008) and antibacterial properties (Freile et al., 2003) based on the presence of alkaloids.

To date, few chemical studies in *B. microphylla* (known also *B. buxifolia* and *B. heterophylla*) in relation of the presence of isoquinolinoline alkaloids have been reported (Fajardo et al., 1979a; Leet et al., 1983). Alkaloids are naturally secondary metabolites and its biosynthesis in some family plants is influenced for abiotic factors in order to tolerate and survive under harsh weather conditions (Bustamante et al., 2006; Cuadra and Fajardo, 2008). *B. microphylla* grown in Patagonia is exposed to stressful habitats such as strong wind, cloudy and cold days and arid soils (Garredau et al., 2013). Moreover, the altitude, soil nutrients and phenological stage of the plant affects the alkaloids content in *Berberis* species (Chandra and Purohit, 1980; Andola et al., 2010a; Echeverria and Niemeyer, 2012; Niemeyer, 2014). The literature indicate that the presence of berberine (major alkaloid in *Berberis* plant) could be restricted to a specific organ or distributed in different organs of plants; including leaves, stem and roots (Khamidov et al., 2003; Končić et al., 2010). However, in *B. microphylla* only has been studied the presence of alkaloids but its distribution in the plant is unknown.

In the last decades, several methods have been report for the determination of alkaloids in plant, included colorimetry (Shamsa et al., 2008), thin layer chromatography (TLC) (Patel et al., 2012), capillary zone electrophoresis (CZE) (Gong et al., 2003) and nuclear magnetic resonance (H-NMR, C-NMR) (Quevedo et al., 2008; Fajardo et al., 2009). In some cases, the traditional methods mentioned above require sample extractions and long time for completing the whole analysis (Chan et al., 2007). In the recent years, high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC ESI-MS/MS) has demonstrated to have an advantage over other methods for the high sensitivity, specific and rapid analysis for the identification of components in complex alkaloid mixtures (Chen et al., 2000; Fabre et al., 2000; Luo et al., 2005; Ding et al., 2007).

This work reported a qualitative and semi-quantitative study of the isoquinoline alkaloids found in leaves, stems and roots of *B. microphylla* collected in two different climatic zones from the Patagonia determined by HPLC ESI-MS/MS. Furthermore, the results have been discussed with the purpose to find some relation between the variation of alkaloids in the plant and the habitat that they grow.

2.2. Materials and methods

2.2.1. Plant material

Representative samples of leaves, stems and roots of *B. microphylla* were collected during flowering season at Cerro Sombrero (52°48’26.7’’S; 68°52’50.1”W) and Lago Deseado (54°22’12.4”S; 68°45’45.0”W) in November and December 2011. Sample collections were performed in the Province of Tierra del Fuego in two climatic zones corresponding to cold steppe (Cerro Sombrero) and submontane (Lago Deseado).

In cold steppe the climatic data indicate that the rainfall gradient is low (250-350 mm), mean annual temperature is 6.8° C, soils present a pH 6-7 and erode readily for strong winds. Further south, submontane zone is characterized by more acid soil (pH 4 - 5.2), rainfall increases (338-852 mm) and the temperature decreases to 2.7° C (annual mean) (Moore, 1983). Voucher specimens (178056-Cerro Sombrero; 178057-Lago Deseado) were deposited in the Herbarium of the Universidad de Concepción, Concepción, Chile.

2.2.2. Alkaloids extraction

Extraction was carried out according to the methodology described by Cabezas et al. (2009) with some modifications. Oven dried and powdered leaves (100 g), stems (300 g) and roots (300 g) of *B. microphylla* were sequentially extracted (24, 48 and 72 h) with methanol at room temperature. Methanolic extracts were evaporated *in vacuo* at 40° C, and the residue was reconstituted with 200 mL 10% HCl for 1 h under agitation (orbital shaker, MS-NOR, Taiwan), and allowed to stand for 12 h at 10° C and then filtered. The filtrate was washed with $CHCl_3$ (5 x 100 mL). The aqueous phase was adjusted to pH 10 with NH_4OH and extracted with $CHCl_3$ (5 x 100 mL). The solvent was evaporated for obtaining the extract containing alkaloids.

2.2.3. Alkaloid standards

Berberine (purity, >90%), palmatine (purity, >97%) and jatrorrhizine (purity >95%) were purchased from Sigma Aldrich (St. Louis, USA) and all solvent used for extraction were analytical grade. Calafatine was obtained from Laboratorio de Productos Naturales, Universidad de Magallanes (Punta Arenas, Chile). HPLC grade

acetonitrile, methanol water and formic acid were purchased from Merck (Darmstadt, Germany).

2.2.4. HPLC ESI-MS/MS

The chromatographic separation was carried out using a RP-C18 BioSuite column (2.1 x 150 mm, 3μm), injecting 10 μL at 0.2 mL/min and 35º C. 0.01 g of standards and sample extracts were dissolved in 10 mL of methanol and submitted to LC–MS/MS. The chromatographic separation was performed using a linear gradient solvent system consisting of 0.1% formic acid (A) and acetonitrile (B). The linear gradient was composed of 0–3 min 10% B, 3-35 min 10-70% B, 35-40 min 70% B, 40-50 min 70-10% B, then again, under the initial conditions (10% B) for 10 min. Each standard was injected with an electro spray ionization (ESI) source into the mass spectrometer (LC-MS MS Shimadzu Prominence coupled at mass spectrometer Applied Biosystems/MDS Sciex3200 Qtrap, Massachusetts, USA). The ion source temperature was set to 400º C, and the capillary voltage was 5.5 kv. For alkaloids determination, data were collected as positive-ion spectra by means of Enhanced Mass Scan (EMS) over a *m/z* 100-1000 Da range at 1000 Da/s and Enhanced Product Ion (EPI) over a *m/z* 50-1000 Da range at 4000 Da/s. The CUR gas was 20 psi, GS1 30 psi and GS2 60psi.

In addition, the content of alkaloids was performed calculating the relative amounts of the individual alkaloids present in the plant extracts. The ion intensities were extracted at the m/z values of the molecular (M^+) or pseudo-molecular $(M+H)^+$ ions of the corresponding detected compounds. The relative ion peak area of each compound from the sample was compared to the relative ion peak area of the total alkaloids.

2.3. Results

2.3.1. Identification of alkaloids by HPLC ESI-MS/MS

The identification of isoquinoline alkaloids in samples of *B. microphylla* were analysis using HPLC ESI-MS/MS. The ESI-MS spectra recorded in the positive ionization mode exhibited the $[M+H]^+$ ion for the tertiary bases, and $[M]^+$ ion for the quaternary salts that allowed the determination of molecular weight. All compounds were identified by comparison retention time and MS spectra with those authentic standard or referring the literature and base data. Table 1 shows the spectral data and retention time of each alkaloid identified in *B. microphylla* collected in Patagonia. Different structural type of isoquinoline alkaloids identified in the samples of *B. microphylla* can be classified as: a) aporphine: isocorydine; b) benzylisoquinoline: reticuline; c) bisbenzylisoquinoline: calafatine; d) protopine: allocryptopine, protopine; e) protoberberine: thalifendine, jatrorrhizine, palmatine and berberine; f) tetrahydroprotoberberine: scoulerine, tetrahydro-berberine (Figure 1).

Compounds were characterized by MS spectra showing their expected parent ion and MS/MS spectra and characteristic fragment ions (Table 1). In the case of aporphine-type alkaloids the MS spectra of $[M+H]^+$ ion *m/z* 342 was tentatively identified as isocorydine (Jeong et al., 2012). The rupture of $[M+H]^+$ for isocorydine produce a characteristic fragment ion at *m/z* 311, this could be formed by the loss of CH_3NH_2. The ion *m/z* 311 could lead to the formation of the *m/z* 279 fragment because due to loss of CH_3OH, and by lose of OCH_3 and CH_3 from the ion at *m/z* 279, the ion at *m/z* 248 and *m/z* 264 are generated respectively. A benzilisoquinoline-type alkaloid eluting at 13.1 min was assigned as reticuline because the rupture $[M+H]^+$ at *m/z* 330 producing the

characteristic fragments ions at *m/z* 192 and *m/z* 137. The ion *m/z* 192 could lead to the formation of the *m/z* 177 fragment due to loss of CH_3 (Schmidt et al., 2005). Calafatine, a bisbenzylisoquinoline-type alkaloids was identified comparing the MS spectra with authentic standard. The $[M+H]^+$ at *m/z* 653 yielded ions product *m/z* 610 and *m/z* 622 corresponding to losses of CH_2NCH_3 and OCH_3 respectively.

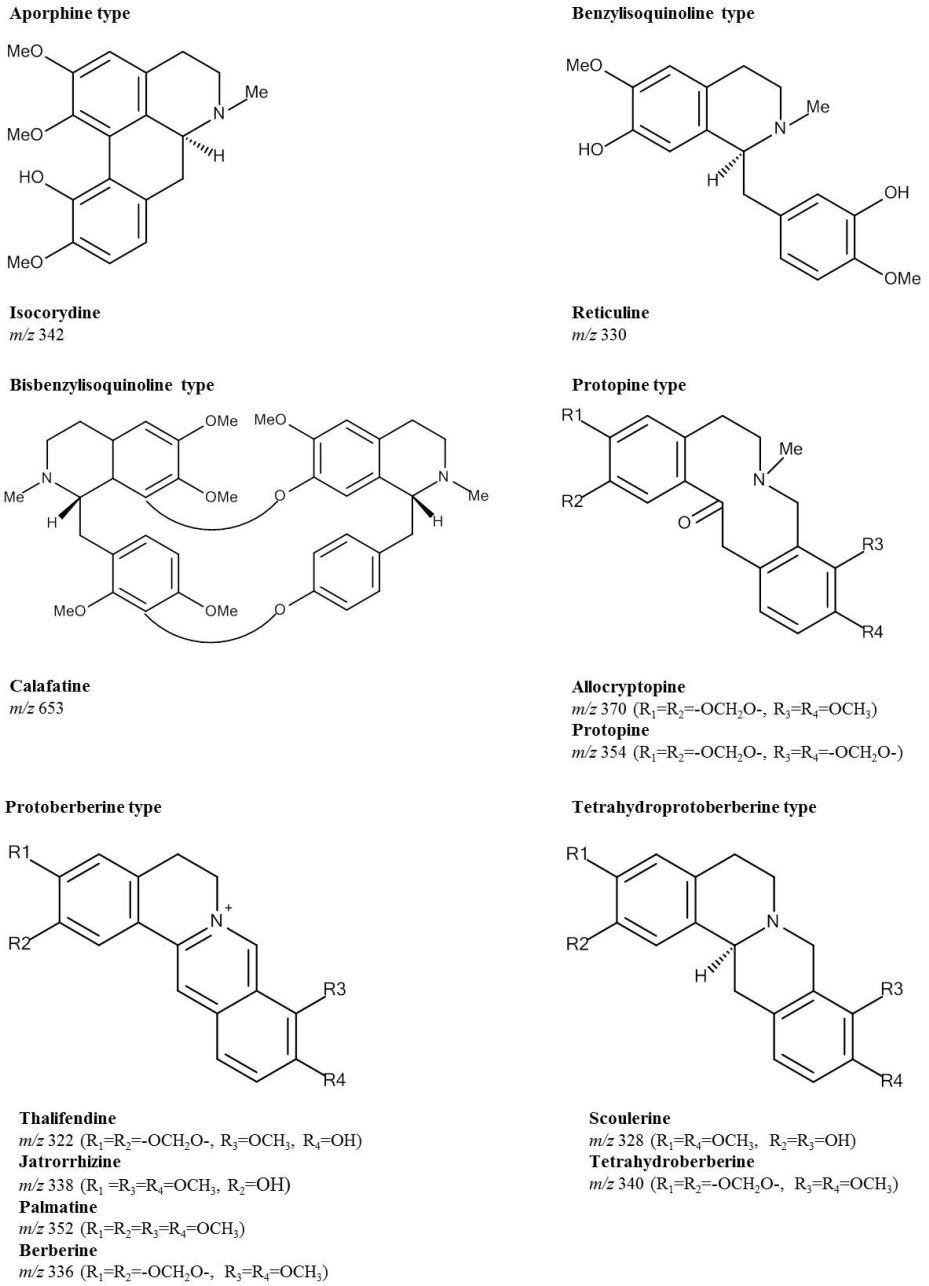

Figure 1. Structure of type of isoquinoline alkaloids identified in *B. microphylla.*

Four protoberberine-type alkaloids thalifendine, jatrorrhizine, palmatine and berberine eluting at 18.1, 18.7, 19.6 and 20.0 min respectively, were identified in base of their MS/MS spectral with authentic standard and previous report (Jeong et al., 2012). The fragmentation of $[M]^+$ at *m/z* 322 corresponding to thalifendine yielded the characteristic ion at *m/z* 307 by the loss of CH_3. Then the ion fragment due to loss of CO (Wu et al., 2005). The alkaloid palmatine, $[M]^+$ *m/z* 352, was identified due to losses of CH_4, $2CH_3$ and C_2H_4O producing the fragments *m/z* 336, 322 and 308 respectively. The ion *m/z* 322 could lead to the formation of the *m/z* 294 fragment due to loss of CO (Wu et al., 2005). The presence of $[M]^+$ at *m/z* 336 in accordance with authentic standard corresponded to berberine. The $[M]^+$ produced the characteristic fragments ions at *m/z* 320, 306, 292, 278 by the losses of CH_4, $2CH_3$, C_2H_4O and $2CH_3$-CO respectively (Wu et al., 2005). Two tetrahydroprotoberberine-type alkaloids scoulerine (13.8 min) and tetrahydroberberine (18.5 min) showed characteristic tandem MS spectra as reported in the literature (Jeong et al., 2012; Tang et al., 2013). The $[M+H]^+$ at *m/z* 328 was identified as scoureline and the rupture of this ion produced a characteristic fragments ions at *m/z* 178 and *m/z* 151 formed by RDA (retro Diels-Alder) or B-ring cleavage (Jeong et al., 2012). The alkaloid tetrahydroberberine displayed $[M+H]^+$ ion at *m/z* 340 and the losses of $C_{10}H_{12}O_2$ and CH_3-$C_{10}H_{10}O_2N$ assigned to the fragments ions *m/z* 176 and *m/z* 149 respectively (Tang et al., 2013).

Protopine and allocryptopine eluting at 14.2 and 17.4 min respectively, are protopine-type alkaloids identified in samples of *B. microphylla*. The $[M+H]^+$ ion at *m/z* 354 and $[M+H]^+$ was tentatively identified as protopine by comparing with literature data (Tang et al., 2013).

The $[M+H]^+$ ion at *m/z* 354 produced a fragment ion at *m/z* 275 that could result from the loss of CH_3-NH_2-$CH_2(OH)_2$. The rupture type RDA from $[M+2H]^+$ at *m/z* 355

produced two fragments ions at *m/z* 149 and *m/z* 206. The ion *m/z* 206 could lead to the formation of the *m/z* 189 fragment due to loss of OH (Tang et al., 2013). Other protopine-type alkaloid was putatively identified as allocryptopine on the basis of previous report (Tang et al., 2013). The $[M+H]^+$ ion at *m/z* 370 produced a several fragments ions at *m/z* 290, 206 and 188 by the losses of $2CH_3O$-OH, $C_{10}H_{12}O_2$ and $C_{10}H_{12}O_2$-H_2O respectively (Tang et al., 2013).

Table 1. Mass Spectral data and retention times (tR) of isoquinoline alkaloids identified from *B. microphylla* by HPLC ESI-MS/MS.

Compounds	t_r (min)	$[M+H]^+$	$[M]^+$	*m/z* fragment ion (% base peak)
Aporphine-type				
Isocorydine[b]	13.08	342		190(54), 207(36), 248(18), 222(27), 264(27),265(72), 279(100), 296(36), 311(27)
Benzylisoquinoline-type				
Reticuline[a,b]	13.1	330		137(100), 192(92), 177(28), 207(14)
Bisbenzylisoquinoline-type				
Calafatine[c]	16.6	653		610(62), 622(100)
Protopine-type				
Allocryptopine[b]	17.4	370		188 (100), 206 (28), 290 (28)
Protopine[a,b]	14.2	354		149(3), 177(12), 189(100), 206(18),247(18), 275(37)
Protoberberine-type				
Thalifendine[b]	18.1		322	251(6), 279(44),292(6),307(100)
Jatrorrhizine[c]	18.4		338	280(54), 294(100), 307(72), 308 (36) 322(100)
Palmatine[c]	19.6		352	279(2), 294(18), 308(57), 322(47), 336(100)
Berberine[c]	20.0		336	205(1), 234(2), 263(9), 275(11), 278(62), 292(78), 320(100), 306(59)
Tetrahydroprotoberberine-type				
Scoulerine[b]	13.8	328		151(11), 178(100)
Tetrahydroberberine[b]	18.5	340		149(21), 176(100), 324(2)

tR = Retention time

Compounds identified using a MS database (http://spectra.psc.riken.jp/); b MS data of literature; c authentic standard

2.3.2. Analysis of alkaloids in plant extracts by HPLC ESI-MS/MS

Table 2 shows the proportion of isoquinoline alkaloids in leaves, stems and roots extracts of *B. microphylla* collected in Cerro Sombrero and Lago Deseado analyzed by HPLC ESI-MS/MS. The results indicate the presence of seven alkaloids (isocorydine, jatrorrhizine, palmatine, reticuline, scoulerine, tetrahydroberberine and thalifendine) not reported before in *B. microphylla* and confirmed the presence of four alkaloids (allocryptopine, berberine, calafatine, protopine) previously reported for this specie (Podesta et al., 1987). Furthermore, a greater number of compounds were observed in stem and root extracts, stand out berberine and thalifendine in stems and roots, and tetrahydroberberine in leaves. Also, there were found differences in alkaloid content between samples collected in two climatic zones differences. Protopine only was identified in samples collected in Cerro Sombrero whereas, isocorydine, tetrahydroberberine, scoulerine and reticuline only were identified in sample from Lago Deseado.

Table 2. Identification and proportion of isoquinoline alkaloids in *B. microphylla* from Patagonia

Compounds	Cerro Sombrero			Lago Deseado		
	Leaves (%)[a]	Stems (%)[a]	Roots (%)[a]	Leaves (%)[a]	Stems (%)[a]	Roots (%)[a]
Allocryptopine	-	0.32	1.29	-	1.36	1.58
Berberine	-	78.50	76.43	24.60	85.49	79.82
Calafatine	-	-	0.23	-	-	1.10
Isocorydine	-	-	-	-	1.19	-
Jatrorrhizine	-	-	0.16	-	2.55	2.68
Palmatine	-	-	6.27	-	-	3.05
Protopine	-	2.18	3.05	-	-	-
Reticuline	-	-	-	-	-	1.22
Scoureline	-	-	-	-	1.87	-
Tetrahydroberberine	-	-	-	75.36	-	-
Thalifendine	-	18.93	12.55	-	4.49	10.51

-: not detected

a: Proportion (%) of each compound with a total alkaloids identified in the samples

2.4. Discussion

The identification of major and minor compounds in herbs is of great importance to understand its quality and biological properties (Liang et al., 2004; Deevanhxay et al., 2009). Phytochemical analyses of *B. microphylla* showed the presence of different structural type of isoquinoline alkaloids distributed in different organs of the plant. *Berberis* species contains alkaloids with antimicrobial, antiviral, insecticide and pharmacological activities (Moreno-Murillo et al., 1995; Ivanovska and Philipov, 1996; Küpeli et al., 2002; Yeşilada and Küpeli, 2002; Singh et al 2007; Quevedo et al., 2007; Shahid et al., 2009; Maliwichi-Nyirenda et al., 2011; Potdar et al., 2012).

The alkaloid profiles in *B. microphylla* showed the presence of isocorydine, jatrorrhizine, palmatine, reticuline, scoulerine, tetrahydroberberine and thalifendine not reported before in *B. microphylla.* These compounds have been isolated from different organs in other species of *Berberis*. Khamidov et al. (1997a; 1997b) described the presence of isocorydine as minor alkaloid in leaves and stems of *B. thumbergii* and leaves of *B. densiflora*, whereas Yosupov et al. (1993) found this compound in young shoots and leaves of *B. heteropoda*. Jatrorrhizine and palmatine were identified in aerial part, seed and root-barks of *B. julianae*, *B. thunbergii* and *B. vulgaris*, respectively (Brázdovičová et al., 1975; Brázdovičová et al., 1980; Suau et al., 1998). Reticuline has been isolated from *B. heteropoda* in young shoots and leaves extract from *B. integerrima* (Karimov et al., 1993). Furthermore, tetrahydroberberine and thalifendine also was identified in *Berberis* plants (Karimov, 1993). Other alkaloids present in the extracts as allocryptopine, berberine, calafatine and protopine, have been previously reported in *B. microphylla* (Podesta et al., 1987). Even more, berberine is present in all species of *Berberis* and it can be found in leaves, fruit, stem and roots (Hussaini and Shoeb, 1985; Weber et al., 1989; Karimov et al.,

1993; Khamidov et al., 1997a; Andola et al., 2010b), and it has been reported together with as the compounds more active biologically (Bhardwaj and Kaushik, 2012).

Pharmacological studies indicated that berberine have extensive properties, such as antimicrobial, antiinflamatory, antioxidant, hypoglycemic and hypolipidemic effects, and effects against cancer and the cardiovascular system (Serafim et al., 2008). In this regard, Bandyopadhyay et al. (2013) reported antibacterial activity of berberine against drug resistant *Escherichia coli* strain, probably due to its intercalation with nucleic acid inhibiting the multiplication of cells. This mechanism together with apoptosis induction in cells could be explaining the anticancer activity reported for berberine (Meeran et al., 2008; Abd El-Wahab et al., 2013). Moreover, antiinflamatory activity has been associated to content of protoberberine alkaloids, palmatine, berberine and jatrorrhizine in herb extracts, probably by inhibition of pro-inflammatory cytokine production (Chao et al., 2009; Kim et al., 2009). These compounds also exhibited antifungal effect. Volleková et al. (2003) in a study using in vitro dilution agar plate method against human pathogens showed that jatrorrhizine was the most effective agent against fungal species tested with MIC ranges from 62.5 to 125 μg/mL. In addition, antibacterial activity of jatrorrhizine was reported by Ali et al. (2013) as the most active antimicrobial compound against resistant *Staphylococcus aureus* strain.

Alkaloids reported in this study showed an uneven distribution in plant organs, where the great number of alkaloids was found in stem and roots. Facchini and De Luca (1995) studied the relationships between plant development and alkaloid biosynthesis in specific organs in *Papaver somniferum*, finding accumulation of morphine in aerial organs and roots, whereas sanguinarine only was accumulated in roots, even more, and the biosynthesis organ-specific is regulated by tyrosine/dopa decarboxylase gene family.

Other factors involved in the distribution and accumulation of alkaloids in organs and tissue of plant could be related with environmental conditions, such as light and

temperature. The organs of plant that normally grow in absent of light, such as roots and rhizomes produce alkaloids in largest amount (Cromwell, 1933; Tomè and Colombo, 1995). In addition, in different ecological niches, plants behave differently in terms of biochemical aspects in order to better adapt to their environmental. The altitude, temperature, UV radiation and soils nutrition affect the presence and content of alkaloids (Katoch et al., 2011; Andola et al., 2011; Ghanavi et al., 2013). Variation in the presence of alkaloids in *B. microphylla* could be influenced by the climatic zones where plants were collected. In Lago Deseado was found a greater number of alkaloids in *B. microphylla*. This zone is characterized for low temperature (2.7° C annual mean), acid soils (pH 4 - 5.2), a lower potassium concentration (K) (66 mg/Kg) and phosphorus (P) (2 mg/Kg) of the soil in relation to samples collected in Cerro Sombrero (K, 346 mg/Kg; P, 13 mg/Kg); factors that could cause a stress on the plant and thus to influence the synthesis of compounds. Gremigni et al. (2001) reported the relation about the content of potassium and the production of alkaloids in *Lupinus angustifolius*, finding an increase of the concentration of quinolizidine alkaloids by deficiency of potassium. Similarly, Yaber Grass et al. (2009) reported an increase of pyrrolizidine alkaloids content in *Senecio grisebachii* plants in response to P-deficient treatments, in greenhouse assays.

The content of alkaloids also dependent of the variety, development stage and organ of plant (Furuya et al., 1972; Maknickiene & Asakavičiūtė, 2008). In *B. microphylla* was observed differences in the proportions of alkaloids among the organs; berberine was detected in higher proportion in stems and roots. Similar results in *Berberis* species indicated that berberine is the alkaloid more abundant in stem and root extracts (Andola et al., 2010a). Other reports in *Berberis* indicate that the occurrence of alkaloids is restricted to a specific organ of the plant, whereas other compounds are distributed in the whole plant (Chandra & Purohit 1980; Gorval and Grishkoves, 1999; Khamidov et al., 2003). The results obtained in our study increase the knowledge about the alkaloid composition of *B.*

microphylla. The compounds identified are known to have different bioactivities. This evidence suggests that this plant can be used in future studies of their biological activity.

2.5. Conclusions

An HPLC ESI–MS/MS method was applied to investigate the presence of isoquinoline alkaloids in different organs of *B. microphylla* collected in two locations of the Patagonia. For the first time, seven alkaloids not reported for this specie were identified and also was confirmed the presence of four compounds previously isolated from *B. microphylla*. The presence of alkaloids was associated with organ/plant and could be affected for environmental conditions. Our methodology, carried out in this study, provides sensitivity and specificity for characterization of the alkaloids in *B. microphylla* and other related species.

2.6. Acknowledgements

Authors thanks CONICYT scholarship 21100561 and CONICYT project AT24121407, which provided support for this research. We gratefully acknowledge to Mr. Antonio Cardenas for their assistance in procuring the plant material and for their cooperation.

CHAPTER III

ANTIBACTERIAL ACTIVITY OF ALKALOID FRACTIONS FROM *Berberis microphylla* G. Forst AND STUDY OF SYNERGISM WITH AMPICILLIN AND CEPHALOTHIN

Published in Molecules (2016) 21(1):76

Antibacterial activity of alkaloid fractions from *Berberis microphylla* G. Forst and study of synergism with ampicillin and cephalothin

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Abstract

Berberis microphylla is a native plant that grows in Patagonia and is commonly used by aboriginal ethnic groups in traditional medicine as an antiseptic for different diseases. The present study evaluated the antibacterial and synergistic activity of alkaloid extracts of *B. microphylla* leaves, stems and roots used either individually or in combination with antibiotics against Gram-positive and Gram-negative bacteria. The *in vitro* antibacterial activities of, leaf, stem and root alkaloid extracts had significant activity only against Gram-positive bacteria. Disc diffusion tests demonstrated that the root extract showed similar activity against *B. cereus* and *S. epidermidis* compared to commercial antibiotics, namely ampicillin and cephalothin and pure berberine, the principal component of the alkaloid extracts, was found to be active only against *S. aureus* and *S. epidermidis* with similar activity than the root extract. The minimum inhibitory concentrations (MICs) of the alkaloid extracts ranged from 358 to 83 μg/mL, whereas minimum bactericidal concentrations (MBCs) varied from 717 to 167 μg/mL. In addition, synergistic or indifferent effects between the alkaloid extracts and antibiotics against bacterial strains were confirmed.

Keywords: *Berberis microphylla*, alkaloid extracts, antibacterial activity, synergism

3.1. Introduction

Among Berberidaceae, *Berberis* is represented in Chile and Argentina by 20 species (Landrum, 1999). One representative species of the Patagonia region of both countries is *Berberis microphylla* G. Forst, also known as *Berberis buxifolia* and *Berberis heterophylla*, which grows wild in the under-forest, steppe, and forest–steppe ecotones (Bottini et al., 2000). This species is a perennial shrub with spiny, yellow flowers and black-bluish fruits (Landrum et al., 2003). The leaves, stems, roots and fruits of this species have been used since ancient times in traditional medicine for treating fever, inflammation, stomach ache, diarrhea, urinary tract infection, throat infection, gingivitis, and liver problems (Zin and Weiss, 1998). Moreover, the fruit of the plant has been used by the Kawésqar people as food, whereas the Aonikenk used the yellow scraping of the bark as tobacco for its hallucinogenic effect, which is probably caused by the presence of berberine (Domínguez et al., 2012).

In previous studies, we have reported that *Berberis microphylla* is a rich source of several types of isoquinoline alkaloids. Leaves, stems and roots contain different alkaloids and different proportions of the shared alkaloids. The root has the highest alkaloid yield, and it contains a complex mixture of the following alkaloids: berberine, allocryptopine, calafatine, jatrorrhizine, palmatine, protopine, reticuline and thalifendine. Stems also contain a complex mixture of the following alkaloids: berberine, allocryptopine, isocorydine, jatrorrhizine, protopine, scoulerine and thalifendine. However, the leaf alkaloid extract contains only berberine and tetrahydroberberine (Manosalva et al., 2012) (Figure 1).

Because *B. microphylla* extracts are used in traditional medicine as antiseptics for different diseases, our focus in the present study was to evaluate the antibacterial activity of alkaloid extracts of *B. microphylla* leaves, stems and roots against Gram-positive and Gram-negative bacteria. Furthermore, the fractional inhibitory concentration (FIC) index

was used to determine synergy, antagonism, or indifference against the test organisms as a result of interactions between the alkaloid extracts and antibiotics (Odds, 2003).

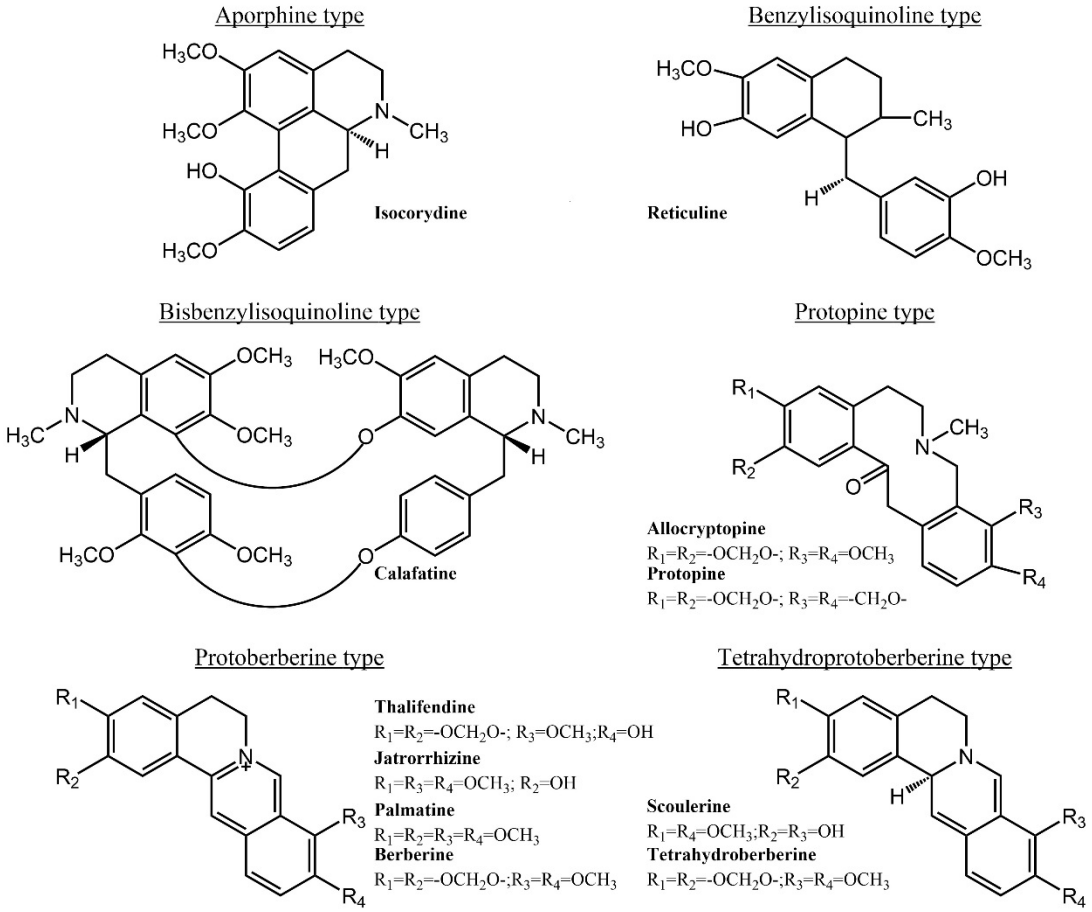

Figure1. *Berberis microphylla* isoquinoline type of alkaloids, from leaves, stems and roots. Adapted from Manosalva et al. (2014).

3.2. Material and methods

3.2.1. Plant material

Representative samples of leaves, stems and roots of *Berberis microphylla* were collected during the flowering season at Lago Deseado, Province of Tierra del Fuego (54°22'12.4'' S; 68°45'45.0''W) in December of 2011. A voucher specimen was deposited at the herbarium of the Universidad de Concepcion (Voucher N° CONC 178057). The plant material was vacuum-packed and stored at -20 °C for further study.

3.2.2. Alkaloids extraction

Extraction was performed according to a previously describedmethod (Cabezas et al., 2009) with somemodifications (Manosalva et al., 2014). In brief, oven-dried and powdered leaves (50 g), stems (50 g) and roots (50 g) of B. microphylla were sequentially extracted (24, 48 and 72 h) withmethanol at roomtemperature. The pooledmethanolic extracts of each plant tissue were evaporated in vacuo at 40 °C, and the residues were agitated with 100 mL of 10% HCl for 1 h, incubated for 12 h at 10 °C and then filtered. The filtrates were washed with $CHCl_3$ (5 x 80 mL). The $CHCl_3$ washings yielded brown non-alkaloidal extracts upon evaporation, which were not investigated. The aqueous phases were adjusted to pH 10 with NH_4OH and extracted with $CHCl_3$ (5 x 80 mL). The solvent was evaporated to obtain the alkaloid extracts of the roots, stems and leaves.The alkaloid compositions of the fractions were identical to those previously published (Manosalva et al., 2014).

3.2.3. Microorganism strains and antibiotics

The alkaloid extracts of *B. microphylla* were tested against representative Gram-negative and Gram-positive bacteria. The following bacteria were tested: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 13311), *Listeria monocytogenes* (ATCC 13932), *Enterobacter aerogenes* (ATCC 13084), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Staphylococcus epidermidis* (ATCC 12228) and *Bacillus subtilis* (ATCC 6633). AMP, CFL and berberine were purchased from Sigma Aldrich (St. Louis, USA), Oxoid Microbiology Products (Basingstoke, Hants, UK) and United States Biological (Swampscott, MA, USA).

3.2.4. Antibacterial assays

The susceptibility tests were performed by the Mueller–Hinton agar-well diffusion method. The bacterial strains were grown in Muller–Hinton broth at 37 °C for 12 h and adjusted to a turbidity of 0.5 McFarland standard (1 x 10^8 CFU/mL). To obtain the inoculum, these suspensions were diluted 100-fold in Muller–Hinton broth to give 1 x 10^6 CFU/mL.

3.2.5. Antimicrobial activity (disc diffusion assay)

The antibacterial activities of the alkaloid extracts of *B. microphylla* were assayed against Gram-negative and Gram-positive bacteria using the disc diffusion method recommended previously by the National Committee for Clinical Laboratory Standard (CLSI 2001) and reported by Konaté et al. (2012) with slight modifications. Briefly, a suspension of the tested microorganism (0.1 mL of 1 x 10^6 CFU/mL) was spread on solid

media plates. The alkaloid compounds of *B. microphylla* were dissolved in 10% dimethyl sulfoxide (DMSO) in water, and the alkaloid solution was filtered through 0.22 µm Millipore Express® (Billerica, MA, USA) membranes for sterile filtration. Filter paper discs (5 mm in diameter) were impregnated with 10 µL of the extracts (equivalent to 500, 1000 and 2000 µg/disc) and placed on the inoculated agar plates. The plates were incubated at 37 °C for 24 h. Discs containing AMP (10 µg) and CFL (30 µg) were used as positive controls, and 10% DMSO was used as a negative control.

3.2.6. Minimum inhibitory concentration (MIC)

Determinations of the MIC for AMP, CFL and *B. microphylla* alkaloid extracts against bacterial strains were performed using the broth dilution method (Ericsson et al., 1971). Briefly, bacterial suspensions were adjusted to 1 x 10^8 CFU/mL, with the aim to achieve 1 x 10^6 CFU/mL. The diluted inoculum (0.1 mL) of each stain was added to 0.9 mL of Mueller–Hinton broth serial dilutions of the antibacterial agents to give a final concentration of approximately 1 x 10^5 CFU/mL. The antibiotics and *B. microphylla* alkaloid extracts were dissolved in sterile distilled water to obtain stock solutions of 100 µg/mL for antibiotics or 1000 µg/mL for the extracts. All tests were performed in triplicate and incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration at which no visible growth was observed (Stefanović et al., 2012).

3.2.7. Minimum bactericidal concentration (MBC)

The MBC was defined as the lowest concentrations of AMP, CFL and B. microphylla alkaloid extracts that inhibit the growth of the inoculum by 99.9% within 24 h of incubation at 37 °C (Fuchs et al., 2002). Each experiment was repeated at least three

times. The MBC values were determined by removing 100 µL of bacterial suspension from the subculture demonstrating no visible growth and inoculating nutrient agar plates. Plates were incubated at 37 °C for a total period of 24 h.

3.2.8 Determination of the Fractional Inhibitory Concentration Index (FICI)

The combined effect of alkaloid extracts of *B. microphylla* with antibiotics against bacterial strains was evaluated by the mean determination of the fractional inhibitory concentration index with a series of combinations corresponding to 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 of the MIC values after incubation at 37 °C for 24 h. The FIC index for all the combinations was determined using the following formula (Zuo et al., 2011):

$$\text{FICI index} = FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$$

where FIC_A and FIC_B represent the fractional inhibitory concentrations of drugs A and B, respectively; MIC_A and MIC_B represent the minimum inhibitory concentrations of drugs A and B, respectively; and [A] and [B] represent the concentrations of drugs A and B, respectively. The FIC index is based on the lower additivity zero interaction theory. This theory is based on the hypothesis that a drug cannot interact with itself and therefore the effect of a self-drug combination will always be additive, with an FIC index of 1. An FIC index lower or higher than 1 indicates, respectively, synergy or antagonism. The FIC index as evaluated by the checkerboard method is interpreted as follows: ≤ 0.5 represents synergy; >0.5 and ≤ 4 represents additivity/indifference; and >4 represents antagonism (Odds et al., 2003).

3.2.9. Statistical analysis

All tests were performed in triplicate, and the bacterial activity was expressed as the mean of inhibition diameters (mm) produced (excluding disc diameter of 5 mm). Inhibition data were checked for normal distribution and variance homogeneity, and the data were analyzed by ANOVA followed by HSD-Tukey's test for mean separation ($p < 0.05$) using R-commander 2.0.3.

3.3. Results and discussion

3.3.1. Antibacterial activity of alkaloid extracts of B. microphylla

Berberis microphylla leaf, stem and root alkaloid extracts showed significant antibacterial activity against Gram-positive bacteria but not against Gram-negative bacteria. Susceptibility of Gram-positive bacteria was dependent on the alkaloid extracts tested and the bacterial strain. Pure berberine was found to be active only against *S. aureus* and *S. epidermidis* with similar activity than the roots extracts (Table 1). It is well known that Gram-negative bacteria have a complex barrier system that can regulate and sometimes prevent the passage of biocides through the cytoplasmic membrane into the cytoplasm (Denyer et al., 2002).

Although the preparation of extracts, bacterial strains, fraction concentrations and microbiological techniques used in the literature for determining antimicrobial activity are not standardized, our results are in agreement with these reports. Previous reports have shown that extracts of *Berberis spp*. Have antibacterial activities that may be principally associated with the presence of alkaloids in leaves, stems and roots. The root and stem hydroalcoholic extracts of four *Berberis* spp. (*B. aristata, B. asiatica, B. chitria and B.*

lycium) are effective against 11 Gram-positive bacterial strains, with berberine as the probable component responsible for antimicrobial activity (Singh et al., 2007).

Another study showed that the methanolic extract of *B. lyceum* is active against nine of 11 Gram-positive and four of seven Gram-negative bacterial strains (Gulfraz et al., 2007). Different extracts of the stem bark of *B. asiatica* were previously tested against 19 bacterial strains; these extracts were active against *Staphylococcus aureus* and *Enterococcus faecalis* but had very little activity against the 11 Gram-negative bacterial strains. In this same study, pure berberine was found to be less active than the tested extracts (Bhandari et al., 2000).

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *B. microphylla* alkaloid extracts are shown in Table 2. The differences in the MIC and MBC values suggest that the *B. microphylla* alkaloid extracts had promising antimicrobial activity. The susceptibility of test organisms was dependent on the alkaloid extracts of the different plant tissues and the bacterial strain. The stem and root alkaloid extracts showed lower MIC and MBC values for *S. epidermidis*. These results may be related to the complexity of the mixture of alkaloids (synergistic effect) in the stem and root as well as the presence of the inactive antimicrobial tetrahydroberberine in the alkaloid extract of leaves (Iwasa et al., 1996; Denyer at al., 2002; Manosalva et al., 2014). This is the first report on the antibacterial properties of the alkaloid extracts of *B. microphylla*.

Table 1. Inhibition zone diameters (mm) of alkaloids extract of *Berberis microphylla* against pathogenic bacteria [A].

	Microorganisms							
	Gram-negative				**Gram-positive**			
	E. aerogenes **ATCC 13084**	***E.coli*** **ATCC 25922**	***L. monocytogenes*** **ATCC 13932**	***S. typhimurium*** **ATCC 13311**	***S. aureus*** **ATCC 25923**	***B. cereus*** **ATCC 11778**	***S. epidermidis*** **ATCC 12228**	***B. subtilis*** **ATCC 6633**
Plant extracts								
Leaves								
500 µg/disc	I	i	i	i	2.9 ± 0.2^{h}	2.8 ± 0.2^{f}	6.4 ± 0.5^{e}	3.8 ± 0.2^{g}
1000 µg/disc	i	i	i	i	4.7 ± 0.6^{g}	4.7 ± 0.5^{e}	7.7 ± 0.6^{e}	4.9 ± 0.2^{ef}
2000 µg/disc	i	i	i	i	7.7 ± 0.2^{e}	6.9 ± 0.2^{c}	9.7 ± 0.6^{d}	5.7 ± 0.6^{e}
Stems								
500 µg/disc	i	i	i	i	5.7 ± 0.2^{f}	3.9 ± 0.2^{e}	7.00 ± 0.0^{e}	3.8 ± 0.4^{fg}
1000 µg/disc	i	i	i	i	8.9 ± 0.2^{d}	5.7 ± 0.6^{d}	10.7 ± 0.6^{d}	5.3 ± 0.6^{e}
2000 µg/disc	i	i	i	i	9.7 ± 0.6^{d}	7.0 ± 0.5^{c}	13.0 ± 0.5^{c}	6.9 ± 0.2^{cd}
Roots								
500 µg/disc	i	i	i	i	5.7 ± 0.6^{f}	11.0 ± 0.0^{b}	9.7 ± 0.2^{d}	3.8 ± 0.4^{fg}
1000 µg/disc	i	i	i	i	8.9 ± 0.2^{d}	12.0 ± 0.5^{b}	13.0 ± 0.6^{c}	5.8 ± 0.3^{de}
2000 µg/disc	i	i	i	i	11.1 ± 0.1^{c}	13.9 ± 0.2^{a}	15.3 ± 0.6^{b}	7.9 ± 0.20^{c}
Berberine								
500 µg/disc	I	i	i	i	6.9 ± 0.2^{e}	i	12.7 ± 0.2^{c}	i
1000 µg/disc	I	i	i	i	7.7 ± 0.2^{e}	i	14.3 ± 0.6^{b}	i
2000 µg/disc	I	i	i	i	9.7 ± 0.6^{d}	i	14.9 ± 0.2^{b}	i
Antibiotics								
Ampicillin 10 µg/disc	10.0 ± 0.0	21.0 ± 0.0	30.0 ± 0.0	19.0 ± 0.0	34.0 ± 0.5^{b}	11.0 ± 0.06^{b}	16.7 ± 0.5^{b}	27.0 ± 0.06^{b}
Cephalothin 30 µg/disc	20.0 ± 0.0	16.0 ± 0.0	20.0 ± 0.0	18.0 ± 0.0	35.6 ± 0.06^{a}	2.6 ± 0.0^{f}	25.9 ± 0.2^{a}	36.0 ± 0.06^{a}

Discount the diameter of a sterile disc (5mm); i : inactive. ; [A] mean of triplicates ± standard deviation of three replicates; [a-g] different letters indicated significant differences according to Tukey test ($p < 0.05$).

Table 2. Minimum inhibitory concentrations (MICs)[a] and minimum bactericidal concentrations (MBCs)[a] of alkaloids extract of *Berberis microphylla* and berberine against Gram positive bacteria.

	Gram-positive bacteria							
	S. aureus ATCC 25923		*B. cereus* ATCC 11778		*S. epidermidis* ATCC 12228		*B. subtilis* ATCC 6633	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Plant Extract								
Leaves	250	661	358	717	124	250	333	550
Stems	167	334	125	250	83	167	274	500
Roots	83	167	125	250	83	167	187	366
Berberine	167	334	i	i	167	334	i	i

[a] MIC and MBC values given as μg/mL,means of triplicate ± standard deviation of three replicates; i: inactive

Other antibacterial studies of plant alkaloid extracts have shown similar MIC and MBC values with variances according to bacteria and plant compounds. Extracts are classified as antimicrobials on the basis of MICs in the range of 100–1000 μg/mL (Tegos et al., 2002). Alkaloid extracts of the aerial part of *Sida acuta* had MIC and MBC values against different clinical isolates of *Staphylococcus* that varied from 80 to > 400 μg/mL (Karou et al., 2006). Berberine, jatrorrhizine and the crude extract of *Mahonia aquifolium* showed activity against 20 clinical isolates of *Propionibacterium acnes*. The alkaloid extracts of *Mahonia aquifolium* showed MIC values that varied from 100 to 500 μg/mL against *Staphylococcus epidermidis* and from 250 to 500 μg/mL against *S. hominis* (Slobodníková et al., 2004).

In addition to the known antibacterial activity of berberine, jatrorrhizine and tetrahydroberberine (Iwasa et al., 1996; Denyer et al., 2002; Manosalva et al., 2014), the antibacterial activity of other pure alkaloids found in *B. microphylla* alkaloid fractions have been previously reported. Protopine and allocryptopine, isolated from aerial parts of *Hypecoum erectum L.*, showed significant antibacterial activity (MIC 125 μg/mL) against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and

low antibacterial activity (MIC > 500 µg/mL) against the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus cereus* and *B. subtilis* (Su et al., 2011). Tetrandrine and demethyltetrandrine, two bisbenzylisoquinoline alkaloids isolated from *Stephania tetrandra* S. Moore, related to calafatine, showed in vitro anti-MRSA and antibiotic synergistic effects with four antibiotics: ampicillin, azithromycin, cefazolin and levofloxacin (Zuo et al., 2011). Also, reticuline isolated *from Annona salzmanni* D.C. tested against the Gram-positive and Gram-negative bacteria: *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* showed to be inactive (Paulo et al., 1992). Protopine, allocryptopine, calafatine and reticuline are present in low proportion in root alkaloid extracts, and stem alkaloid extracts contain only protopine and allocryptopine (Manosalva et al., 2014). The presence of these compounds in the root and stem alkaloid extracts of *B. microphylla* is not reflected in significant changes of the MIC values of these fractions (Table 2).

3.3..2. Fractional Inhibitory Concentration (FIC) *Index*

Synergistic interactions are a result of a combined effect of active compounds from extracts and antibiotics (Stefanovic et al., 2011). Antimicrobial compounds from plants may inhibit bacteria by several alternative mechanisms vs. antibiotics, enhancing the activity of the latter.

Synergy research in phytomedicine has established synergy as being important (Konaté et al., 2012). Several examples of synergistic activity between natural plant compounds and conventional antibacterial agents as an alternative way of overcoming resistance of pathogenic bacteria to current antibiotics have been reported (Chung et al., 2011; Olajuyigbe et al., 2012; Basri et al., 2014).

Based upon FIC index calculations, the combination of alkaloid extracts of *B. microphylla* and ampicillin (AMP) and cephalothin (CFL) showed synergistic and indifferent effects against pathogenic bacteria, respectively (Tables 3 and 4). A synergistic effect was observed against *B. cereus*, *B. subtilis* and *S. epidermidis* with the leaf alkaloid extract/AMP combination. This effect was also observed against *S. aureus* and *S. epidermidis* with the stem alkaloid extract/AMP combination, and the root alkaloid extract/AMP combination showed the same effect against *B. subtilis* (Table 3). The synergistic effect caused by alkaloid extracts and CFL was observed with the following combinations: leaf alkaloid extract/CFL against *S. aureus*, *B. cereus* and *S. epidermidis*; stem alkaloid extract/CFL against *S. aureus* and *B. subtilis*; and root alkaloid extract/CFL against *B. cereus* and *S. epidermidis*.

The synergistic effects of *B. microphylla* alkaloid extracts in combination with AMP and CFL against Gram-positive bacterial strains were in agreement with previous studies on isoquinoline alkaloids. The isoquinoline alkaloid-rich extracts of *Stephania suberosa* also show synergistic effects when combined with AMP against AMP-resistant *Staphylococcus aureus* (Teethaisong et al., 2014). The synergistic and additive antimicrobial activities of the bisbenzylisoquinoline alkaloids, namely tetrandrine and demethyltetrandrine, show that both compounds enhance the inhibitory efficacy of cefazolin antibiotics against methicillin-resistant *S. aureus* in vitro (Zuo et al., 2011). Berberine, which is the principal component of *B. microphylla* alkaloid extracts, has been shown to enhance the antibacterial activity of selected antibiotics against coagulase-negative *Staphylococcus strains* in vitro (Wojtyczka et al., 2014).

Table 3. Fractional inhibitory concentration index (FICI); combination of alkaloid extracts of *Berberis microphylla* with ampicillin.

	Gram-positive																			
	S. aureus ATCC 25923					*B. cereus* ATCC 11778					*S. epidermidis* ATCC 12228					*B. subtilis* ATCC 6633				
	MIC_a	MIC_b	FIC	FICI	Effect	MIC_a	MIC_b	FIC	FICI	Effect	MIC_a	MIC_b	FIC	FICI	Effect	MIC_a	MIC_b	FIC	FICI	Effect
Leaves extracts (µg/mL)																				
Leaves	250	125	0.5	1	I	358	89.5	0.25	0.5	S	124	31.0	0.25	0.5	S	333	83.25	0.25	0.5	S
Ampicillin	0.06	0.03	0.5			4.0	1.0	0.25			1.6	0.4	0.25			0.03	0.0075	0.25		
Stems extracts (µg/mL)																				
Stems	167	41.7	0.25	0.5	S	125	125	1	2	I	83	41.5	0.5	1	I	274	68.5	0.25	0.5	S
Ampicillin	0.06	0.015	0.25			4.0	4.0	1			1.6	0.8	0.5			0.03	0.0075	0.25		
Roots extracts (µg/mL)																				
Roots	83	41.5	0.5	1	I	125	125	1	2	I	83	41.5	0.5	1	I	187	46.7	0.25	0.5	S
Ampicillin	0.06	0.03	0.5			4.0	4.0	1			1.6	0.8	0.5			0.03	0.0075	0.25		

MICa: MIC of sample alone; MICb: MIC of the combination.
FIC of alkaloid extracts: MIC of alkaloids extract in combination with antibiotic/MIC of alkaloids extract.
FIC of antibiotic: MIC of antibiotic in combination with alkaloid extracts/MIC of antibiotic.
FICI index: FIC of alkaloids extract + FIC of antibiotic.
S: Synergistic; I: Indifferent.

Table 4. Fractional inhibitory concentration index (FICI); combination of alkaloids extract of *B. microphylla* with cephalothin.

	Gram-positive																			
	S. aureus ATCC 25923					*B. cereus* ATCC 11778					*S. epidermidis* ATCC 12228					*B. subtilis* ATCC 6633				
	MIC_a	MIC_b	FIC	FICI	Effect	MIC_a	MIC_b	FIC	FICI	Effect	MIC_a	MIC_b	FIC	FICI	Effect	MIC_a	MIC_b	FIC	FICI	Effect
Leaves extracts(µg/mL)																				
Leaves	250	62.5	0.25	0.5	S	358	89.5	0.25	0.5	S	124	31.0	0.25	0.5	S	333	166.5	0.5	1	I
Cephalothin	0.06	0.015	0.25			50	12.5	0.25			0.2	0.05	0.25			0.01	0.005	0.5		
Stems extracts (µg/mL)																				
Stems	167	41.7	0.25	0.5	S	125	62.5	0.5	1	I	83	41.5	0.5	1	I	274	68.5	0.25	0.5	S
Cephalothin	0.06	0.015	0.25			50	25	0.5			0.2	0.1	0.5			0.01	0.0025	0.25		
Roots extracts (µg/mL)																				
Roots	83	41.5	0.5	1	I	125	31.2	0.25	0.5	S	83	20.7	0.25	0.5	S	187	93.5	0.5	1	I
Cephalothin	0.06	0.03	0.5			50	12.5	0.25			0.2	0.05	0.25			0.01	0.005	0.5		

MICa: MIC of sample alone; MICb: MIC of the combination.
FIC of alkaloid extracts: MIC of alkaloids extract in combination with antibiotic/MIC of alkaloid extracts.
FIC of antibiotic: MIC of antibiotic in combination with alkaloid extracts/MIC of antibiotic.
FICI index: FIC of alkaloid extracts + FIC of antibiotic.
S: Synergistic; I: Indifferent.

3.4. Conclusions

Our results reveal that the alkaloid extracts of *B. microphylla* leaves, stems and roots present selective antibacterial activity against Gram-positive bacterial strains. Pure berberine, the principal component of the alkaloid extracts (Manosalva et al., 2014), was found to be active only against *S. aureus* and *S. epidermidis* with similar activity to that of the root extracts (Table 1). These findings correlate with the use of the plant by aboriginal ethnic groups in traditional medicine as an antiseptic for different diseases. Additionally, based on FIC index calculations, *B. microphylla* alkaloid extracts showed synergistic effects with ampicillin (AMP) and cephalothin (CFL) only on some of the tested bacterial strains. Considering that the synergism observed between alkaloid extracts of *B. microphylla* and antibiotics would reduce the side effects caused by each of these antibacterial agents alone, further research can be focused in the evaluation of toxicity of these alkaloids for eventual future clinical applications.

3.5. Acknowledgments

The authors would like to acknowledge CONICYT scholarship Nº 21100561 awarded to LM and Grant Nº AT24121407. Support for this research at Universidad de Santiago de Chile, Proyectos Basales (Vicerrectoría de Investigación, Desarrollo e Innovación Usach) and FONDECYT Nº 1120037. We are grateful to Mr. Luis Quintana and Mr. Javier Asencio from Seremi de Salud, La Araucanía Region for providing us with the microorganism strains.

CHAPTER IV

BIOLOGYCAL ACTIVITY OF ALKALOID EXTRACTS FROM *Berberis microphylla* G. Forst AGAINST AGRICULTURAL INSECT PESTS

Biologycal activity of alkaloid extracts from *Berberis microphylla* G. Forst against agricultural insect pests

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Abstract

In laboratory by dual choice test, stem and root alkaloid extracts of *Berberis microphylla* showed antifeedant activity against third-instar *Plutella xylostella* larvae. The results indicated that alkaloid extracts showed antifeedant activity against third-instar *P. xylostella* larvae. Stem extracts showed significantly lower leaf consumptions at concentrations of 500 and 1000 mg/L, as illustrated by antifeedant index of 72 and 97% respectively, whereas, root extracts were significantly active at 100, 500 and 1000 mg/L with antifeedant index of 68, 85 and 99 % respectively. In addition, the pure berberine identified as the major alkaloid in the plant extracts showed significant lower food consumption by larvae only at concentration of 1000 mg/L, but the pure palmatine not affect feeding at any concentration. The results suggest that alkaloids extracts of *B. microphylla* have promising antifeedant activity and could by incorporated in a integrated pest management (IPM) programs for *Plutella xylostella*.

Key words: antifeedant activity, alkaloid extracts, *Berberis microphylla*, *Plutella xylostella.*

4.1. Introduction

Insect pests are one of the biotic factors that cause significant crop losses worldwide, where the use of synthetic pesticides (eg. organophosphates and pyrethroids) is a control strategy used traditionally for to pest control (Birch et al., 2011; Zhang et al., 2011; Chareonviriyaphap et al., 2013). However, the chemical control has become less effective in the last decades because of its rapid developing of resistance (Shelton et al., 1993; Bing et al., 2008). In this sense, the indiscriminate use of synthetic pesticide chemicals has given rise to many ecological problems, among which, toxic residues and harm to mammals are the most crucial (Carriger et al., 2006; Pavela, 2008; Aktar et al., 2009; Damalas and Eleftherohorinos, 2011). For this reason, the use of naturally derived plant products emerge as environmentally friendly alternatives to insecticides, with applications as botanical insecticides, antifeedants and insect growth regulators (Isman 1994; Isman, 2006; Sarkar and Kshirsagar, 2014; Kedia et al., 2015).

A number of studies have reported the bioactivity of alkaloid extracts from various plants against important agricultural insect pests (Acheuk and Doumandji-Mitiche, 2013; Ge et al., 2015), acting as contact insecticides (Tang et al., 2008), repellents (Ulubelen et al., 2001) and antifeedants (Gonzalez-Coloma et al., 1998). The presence of alkaloids in some *Berberis* species are believed to be the cause of their insecticidal properties against insects (Tewary et al., 2005; Quevedo et al., 2007). In this regard, *Berberis microphylla*, is a native plant growing abundantly in southern regions of Chile and Argentina (Patagonia) with medicinal values and other bioactive properties attributable to the presence of alkaloids (Pitta-Alvarez et al., 2008; Domínguez et al., 2012; Manosalva et al., 2016). However, studies on the biological activity against insects have not been reported.

In this sense, the aim of this study was to evaluate the insecticide and antifeedant activity elicited from stem and root alkaloidal extracts from *B. microphylla* and pure compounds (berberine and palmatine) against adults of *Tribolium castaneum* (Coleoptera:Tenebrionidae) and *Sitophilus zeamais* (Coleoptera: Curculionidae) and larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) and *Plutella xylostella* (Lepidoptera:Plutellidae).

4.2. Materials and methods

4.2.1. Plant material

Stems and roots samples of *B. microphylla* were collected during the flowering season (December of 2011) near the shores of Lake Deseado, Province of Tierra del Fuego (54°22'12.4"S; 68°45'45.0"W). A voucher specimen was deposited in the herbarium at the Universidad de Concepcion (Voucher N° CONC 178057). The plant material was vacuum-packed and stored at -20 °C for further study.

4.2.2. Alkaloids extraction

Alkaloids extraction was carried out according to the methodology described by Cabezas et al. (2009) with some modifications. Oven dried and powdered leaves (100 g), stems (300 g) and roots (300 g) of *B. microphylla* were sequentially extracted (24, 48 and 72 h) with methanol at room temperature. Methanolic extracts were evaporated in vacuo at 40 °C, and the residue reconstituted with 200 mL 10% HCl for 1 h under agitation (orbital shaker, MS-NOR, Taiwan), and allowed to stand for 12 h at 10 °C

prior to filtering. The filtrate was washed with $CHCl_3$ (5 x 100 mL). The aqueous phase was adjusted to pH 10 with NH_4OH and extracted with $CHCl_3$ (5 x 100 mL). Finally, the solvent was evaporated under reduce pressure for obtaining dried extract containing alkaloids. The solvent was evaporated for obtaining crude extract containing alkaloids.

4.2.3. Alkaloid standards

Berberine chloride (purity, >98%), palmatine chloride (purity, >97%) and all analytical grade solvents used for extractions, were purchased from Sigma Aldrich (St. Louis, USA)

4.2.4. Insects

The insects used in the biossays were *Tribolium castaneum* (Coleoptera:Tenebrionidae) and *Sitophilus zeamais* (Coleoptera: Curculionidae) and larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) and *Plutella xylostella* (Lepidoptera:Plutellidae).

The adults of *T. castaneun* and *S. zeamais* were obtained from colonies permanently maintained in the laboratory. They were reproduced in 1-L glass flasks containing wheat (*Tritricum aestivum*) and maize (*Zea mays*) grains as a source of food for *T. castaneun* and *S. zeamais* respectively. The culture were maintained in total darkness *at* 28–30 °C and 70%–80% relative humidity. Two-weeks-old of adults were selected for the oral toxicity assay.

The larvae of *G. melonella were* obtained from culture maintained in the Entomology Laboratory of Universidad Austral de Chile, Valdivia, Chile and the newly

emerged adults were used to maintain the stock culture. Adults were maintened in 1000 mL glass jars and provided with diet to lay eggs. Neonate larvae were reared in glass jars (1000 mL) filled to one-third with an artificial diet at 28–30 °C and 70%–80% relative humidity as a stock culture. The synthetic diet described by Bronskill (1961) with some modifications was used for rearing *G. mellonella* larvae. The diet contained 100 mg of wheat flour, 100g of wheat bran, 200 g of maize flour, 200 mL of glycerine and 30 mL of distilled water. One-day-old fourth instar larvae were selected for the dipping toxicity assay.

The larvae of *P. xylostella* were obtained from the laboratory of the Biological Chemistry and Crop Protection Department, Rothamsted Research Station (Harpenden, UK). Culture of larvae were maintained on cabbage plants at 25 ± 2°C, under a 16:8 light: dark cycle. One-day-old third instar larvae were selected for antifeedant assay.

4.2.5. Oral toxicity assay

The oral toxicity activity of root alkaloid extracts of *B. microphylla* against adults of *T.castaneum* and *S. zeamais* was determined by ingestion toxicity according to the methodology described by Nenaah (2011) with some modifications. As source of food for the test were used wheat and maize grains for *T. castaneum* and *S. zeamais* respectively. Dilutions of the tested extracts (25, 50, 100, 150 and 200 mg/kg grains) were prepared using acetone as a solvent. Batches of 200 g of sterilized grains without insects were placed in 1-L wide-mouth glass jars. 50 mL of acetonic aqueous dilution that containing the desired quantity of each material was put onto the surface of the grains and acetone alone as the control. Jars were sealed and thoroughly shaked. The grains were then spread and left to dry for 20 min at room temperature. For each

concentration, 50 g of treated grains together with 20 unsexed adults of each insect species were transferred to a 0.5-L wide-mouth glass jars. All samples were maintained at 28–30 °C and 70%–80% relative humidity in a 12:12 light: dark photoperiod. All treatments were set up in three and percentage mortalities were determined 24 ,48 and 72 h post-treatment.

4.2.6. Dipping toxicity assay

The dipping toxicity activity of root alkaloid extracts from *B. microphylla* against *G. melonella* was tested according to the methodology described by Hu et al. (2005) with some modifications. Five concentrations at 0.01, 1, 10, 100, 500 mg/mL of alkaloid extracts were prepared using acetone as a solvent and were tested in three replicates. The 4rd instar larvae were immersed in the corresponding solution for 5 s, then taken out and left to dry. For each concentrations ten treated larvae were transferred to a 0.25 L plastic containers that containing 100 g of artificial diet. The control experiment was carried out by immersing the larvae in acetone. The treatments were held at 24–28 °C and 70–80% relative humidity in a 12:12 light: dark photoperiod. Test insects were considered dead if larvae did not move when prodded with a soft brush. Mortalities were determined after 24, 48 and 72 h post treatment.

4.2.7. Antifeedant assay

The antifeedant activity of stem an root alkaloid extracts of *B. microphylla* and pure compounds (berberine and palmatine) identified in the plant extracts were assessed via dual-choice bioassays using third-instar larvae of *P. xylostella*. In the dual-choice

test, each insect was given a choice between a treated and an untreated leaf. If the substance acted as an antifeedant, insects would then eat a greater amount of the untreated leaves. Concentrations of 100, 500 and 1000 mg/L of each plant extracts and 10, 100 and 1000 mg/L of pure compounds were prepared with ethanol to avoid damaging the leaves, and this solvent used as control. Each treatment was separately applied on half of a fully opened leaf by dipping both sides of the leaf blade up to the midrib in the appropriate solution. The leaf (5 cm diameter disc) was then placed on a filter paper into a tray and kept in the fume cupboard for ca. 20 min to dry. Thereafter, the leaf was placed in a Petri dish padded with a moist filter paper marked on one side to distinguish the treated half of the leaf. Five third-instar *P. xylostella* larve previously starved for 24 hr were then introduced and the dishes kept in the laboratory for 12 h. Each treatment and control was replicated ten times. The eaten leaf area of the treated leaf portion and that of control were recorded separately, and expressed in square centimeters (cm^2). Furthemore, the percentage antifeedant (AFI) of compounds was calculated using an antifeedant index (AFI) (Krishnakumari et al., 2001): AFI (%) = (1– T / C) × 100, where C is the leaf area consumed in control and T is the leaf area consumed in treatments.

4.*2.8. Statistical analysis*

Insecticide and antifeedant activities of alkaloid extracts from *B. microphylla* and pure compounds were statistically analyzed by ANOVA ($P < 0.05$) with Sigma Plot test.

4.3. Results

In this research only antifeedantt bioassay on *P. xylostella* were successful. The results of the activity of alkaloid extracts from *B. microphylla* and pure compounds are presented in Table 1. Stem and root alkaloid extracts at concentration of 500 and 1000 mg/L significantly reduced leaf consumptions by *P. xylostella* larvae. Thus, at 500 mg/L, stems and root extracts elicited a decrease the insect feeding behavior with antifeedant index of 72% and 86% respectively. At 1000 mg/L, stem and root extracts deterrent feeding by larvae of *P. xylostella* with antifeedant index of 97 and 99% respectively.

Also, data clearly revealed that antifeeding activity varied depending on the concentration of the plant extracts and all the activities are dose dependent. In the case of pure compounds, berberine exhibited significant antifeedant activity only at the highest concentration of 1000 mg/L, whereas palmatine did not show such activity.

Table 1. Antifeedant activity of alkaloid extracts from *Berberis microphylla* and pure compounds against the third instar larvae of *Plutella xylostella*

Plant extracts	Mean leaf consumed (cm^2)[a]		AFI (%)[b]	Mean leaf consumed (cm^2)[a]		AFI (%)[b]	Mean leaf consumed (cm^2)[a]		AFI (%)[b]
	100 mg/L	Control		500 mg/L	Control		1000 mg/L	Control	
Stem	0.91 ± 0.12	1.25 ± 0.14	27	0.57* ± 0.15	2.00 ± 0.15	72	0.02* ± 0.00	0.73 ± 0.16	97
Root	0.23* ± 0.07	0.73 ± 0.16	68	0.17* ± 0.05	1.15 ± 0.15	85	0.03* ± 0.00	2.35 ± 0.26	99

Pure compounds	Mean leaf consumed (cm^2)[a]		AFI (%)[b]	Mean leaf consumed (cm^2)[a]		AFI (%)[b]	Mean leaf consumed (cm^2)[a]		AFI (%)[b]
	10 mg/L	Control		100 mg/L	Control		1000 mg/L	Control	
Berberine	0.41 ± 0.08	0.58 ± 0.18	29	0.37 ± 0.11	0.80 ± 0.01	54	0.19* ± 0.02	1.69 ± 0.11	89
Palmatine	0.60 ± 0.12	1.22 ± 0.15	51	0.82 ± 0.21	0.85 ± 0.17	4	0.23 ± 0.04	0.32 ± 0.11	28

[a] Values are mean S.E. (n=10); * indicated significant differences with the control according to Anova test ($p < 0.05$)

[b] Antifeedant Index: AFI (%)= (1- T/ C) x100, C and T represents consumption of control and treated leaf discs, respectively.

4.4. Discussion

Plants produce secondary metabolites in response to herbivorous insects exerting repellent, antifeedant, or toxic effects on them (Howe and Jander, 2008). In the present study, stem and root alkaloid extracts of *B. microphylla* showed only antifeedant activity against *P. xylostella* larvae by reducing leaf consumptions and this activity was dependant on the concentration of the extracts. In the case of *Berberis* species it has only been reported that stems extracts of *B. glauca* had antifeedant activity against *Spodoptera sunia* (Lepidoptera) larvae (Moreno-Murillo et al., 1995). Similar research has reported antifeedant activity of plant extracts containing alkaloids (Mao and Henderson, 2007; Sani et al., 2014). Cornelius et al. (2009) have reported antifeedant activities of seeds, seed pods and flowers extracts from *Erythrina latissima* against *Spodoptera littoralis* larvae. This active fraction showed the presence of erythrina alkaloids in phytochemical tests. Furthermore, alkaloid fractions from *Dioscorea hispida* inhibited larval feeding of *P. xylostella* (Banaag et al., 1997).

It has been well studied that plant-derived antifeedants could be used in pest management as these will reduce the need for synthetic pesticides (Gökçe et al., 2010; Bilal et al., 2012; Jeyasankar et al., 2014). Additionally, plant-derived antifeedants never kill the target insects directly allowing them to be available to their natural enemies and thus help in the maintenance of the natural balance (Jeyasankar et al., 2012). In this sense, it has been reported that quinoline, indole alkaloids, sesquiterpene lactones, diterpenoids, and triterpenoids are the most potent natural insect antifeedants (Schoonhoven, 1982). In a study carried out by Shields et al. (2008) berberine and aristolochic acid were the most potent feeding deterrents alkaloids against *Lymantria dispar* larvae. In addition, Park et al (2000) reported that isoquinoline alkaloids

berberine and palmatine identified in roots of *Coptis japonica* had antifeeding activity against *Hyphantria cunea* larvae when treated by leaf-dipping assay. They suggested that the antifeeding activity was much more pronounced in applications of mixtures of berberine and palmatine, indicating a synergistic effect.

In our study, stems and roots of *B. microphylla* revealed antifeeding activity against *P. xylostella* larvae, and two isoquinoline alkaloids (berberine and palmatine) identified previously in both structures (Manosalva et al., 2014) were tested. Among the alkaloids assays, only berberine reduced larval feeding of the insect. Several investigators have reported that alkaloids possessed feeding deterrent against lepidopteran pests including *P. xylostella* (Brem et al. 2002). Guo et al (2014) found that two alkaloids (7-demethoxytylophorine and 6-hydroxyl-2,3-dimethoxy phenanthroindolizidine), isolated from *Cynanchum komarovii* were active against the third-instar larvae of *P. xylostella* as insecticides, antifeedants and growth inhibitors. Furthermore, Paulraj et al. (2013) reported that leaf fraction from *Adhatoda vasica* contain alkaloids called vasicine acetate and 2-Acetyl benzylamine that present antifeedant activity against *P. xylostella* larve.

Considering that the alkaloid extracts from stem and root of *B. microphylla* are a mixture of isoquinoline alkaloids (Manosalva et al., 2014), future studies will be focused in identifying other bioactive components present in plant extracts of *B. microphylla* against *P. xylostella* larvae to allow the development of botanical insecticides with greater effectiveness than the crude plant extracts evaluated in this study.

4.5. Conclusion

Alkaloid extracts from stems an roots of *B. microphylla* showed antifeedant activity against larvae of *P. xylostella* suggestingextracts have compounds that could be contributing towards feeding suppression against the *P. xylostella* larvae.

4.6. Acknowledgments

The authors would like to acknowledge CONICYT scholarship Nº 21100561 awarded to LM, Grant Nº AT24121407 and Doctoral Internship Nº 75120095. We gratefully acknowledge to Dr. Michael Birkett, Deputy Head of Biological Chemistry and Crop Protection Department, Rothansted Research for their assistance and cooperation in the development of the bioassays.

CHAPTER V

GENERAL DISCUSSION, CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1. General discussion

Medicinal plants contain secondary metabolites that have been a source of novel drugs and pesticides, where in some case have served as an inspiration for the synthesis of non-natural molecules (Baker et al., 2007; Ji et al., 2009; Cantrel et al., 2012; Morsy, 2014). Among these, alkaloids constitute an important class of secondary metabolites with a wide range of pharmacological (Orhana et al., 2007; Kosalec et al., 2009; Kaur and Arora, 2015) and biological properties (Macel et al., 2005; Bustamante 2006; Domínguez et al., 2008; Tang et al., 2008; Ge et al., 2015).

Berberis species from Chile and Argentine have been used in traditional medicine by indigenous people (Zin et al., 1998) and in recent years have gained importance due to have biological activity based on the presence of isoquinoline alkaloids (Morales et al., 1993; Martinez et al., 1997; Mattana et al., 2012; Alarcon et al., 2014). In this sense, *Berberis microphylla* know as "calafate" is representative of southern regions (Patagonia) of these country, but there is little knowledge about their bioactive compounds. For this reason, we have considered the study of chemical profile of alkaloids present in *B. microphylla* and evaluate their biocidal activity against human phatogenic bacteria and agricultural insect pest.

In the present thesis the chemical composition and biological activity of alkaloid extracts from *B. microphylla* were evaluated for potencial use in combination with sinthetic antibioticts on control of human pathogenic microorganis, as well as its complementary alternative to pesticides in the control of agricultural insect pest. This study was carried out following three approaches: (1) identify and quantify the main alkaloids present in extracts of different part of *B. microphylla* by HPLC ESI-MS/MS, (2) evaluate antibacterial activity of alkaloids extracts of *B. microphylla* and pure

compounds against Gram-positive and Gram-negative human pathogenic bacteria, and (3) evaluate insecticide and antifeedant activity of alkaloid extracts of *B.microphylla* and pure compounds against agricultural insect pests, *Tribolium castaneum*, *Sitophilus zeamais*, *Galleria mellonella* and *Plutella xylostella*.

Thus, the chemical analysis in leaves, stems and roots of *B. microphylla* collected in two different climatic zones from Chilean Patagonia showed different types of isoquinoline alkaloids by HPLC ESI-MS/MS methods. Chemical alkaloids profile was different in leaves, stems and roots, with greater number of compounds being identified in root extracts. This fact has also been observed in *Papaver somniferum* in which the biosynthesis of alkaloids is specific to an organ of the plant. For example, morphine accumulates in aerial organs, whereas sanguinarine only accumulates in roots (Facchini and De Luca, 1995). In addition, altitude, temperature, UV radiation and soils nutrition also affect the presence and content of alkaloids (Andola et al., 2011; Katoch et al., 2011; Ghanavi et al., 2013). These factors could cause stress in the plant and thus influence the synthesis of compounds. Also, the environmental factors can be involved in the accumulation of alkaloids in organs and plant tissue. For example, the organs of the plant that normallygrow in absence of light (roots and rhizomes) produce larger amount of alkaloids (Cromwell, 1933; Tome and Colombo, 1995). In this context, we found that compounds synthesized by *B. microphylla* are influenced by the climatic zones of the growing species. Thus, a greater number of alkaloids was found in the plant collected in zone with low temperature (2.7 °C annual mean), acid soils (pH 4 - 5.2) and lower potassium (66 mg/kg) and phosphorus (2 mg/kg) concentration in the soil. Moreover, it were observed differences in the proportions of alkaloids among the different organs of the plant studied, where berberine was detected in higher proportion in stems and roots compared with leaves.

In an attempt to evaluated the biologicall activity of alkaloid extracts from *B. microphylla*, we evaluated *in vitro* antibacterial and antifeedant effects of leaf, stem and root alkaloid extracts. In this way, the antibacterial assays showed that leaf, stem and root alkaloid extracts of *B. microphylla* have significant activity against Gram-positive bacteria and in some cases the antibacterial activity of the roots extract was similar compared to commercial antibiotics. It has been reported that secondary metabolites such as alkaloids, terpenoids and phenolic compounds present in plants are considered important antimicrobial agents against Gram-positive and Gram-negative bacteria (Ding et al., 2009; Kuete et al., 2011; Gobalakrishnan et al., 2013). Also, pure berberine, the principal component of the alkaloid extracts, was found to be active against some bacteria with similar activity to the roots extract. Recent investigations indicate that *Berberis* plant extracts have antibacterial activities that could be associated with the presence of alkaloids particularly, in stems and roots (Li et al., 2007; Kakar et al., 2012; Ghareeb et al., 2013). Singh et al. (2007) found that hydroalcoholic root and stem extracts from four *Berberis* spp (*B. aristata, B. asiatica, B. chitria* and *B. lycium*) were effective against most Gram-positive and Gram-negative bacteria strains indicating that berberine may be responsible for antimicrobial activity.

Additionaly, due to the emergence of multidrug-resistant organisms, combining medicinal plants with synthetic medicines against resistant bacteria is a interesting alternative (Aiyegoro and Okoh, 2009). In this study, the *in vitro* interactions between alkaloid extracts of *B. microphylla* and convencional antibiotics (ampicillin and cephalothin) against Gram-positive pathogens were confirmed on the basis of the fractional inhibitory concentration (FIC) index. Recent studies have report synergistic activity between plant alkaloids and different antibiotics on bacteria of clinical relevance (Zuo et al, 2011; Konate et al., 2012). Extract of S*tephania suberosa* rich in

alkaloids showed synergy in conjunction with ampicillin against ampicillin-resistant *Staphylococcus aureus* (Teethaisong et al., 2014). Also, the alkaloids of *Cienfuegosia digitata* conbined with β-lactams antibiotics (methicillin and ampicillin) showed synergistic effects against multidrug resistance *S. aureus* (Konaté et al., 2012). So, the discovery the synergistic interactions between plant natural compounds and conventional antibacterial agents is of major importance for the development of strategies for the management of microbial infections (Molinari, 2009; Chung et al. 2011; Olajuyigbe and Afolayan 2012; Basri et al. 2014).

Finally, insecticide and antifeedant effects of the stem and root alkaloid extracts of *B. microphylla* were tested against some agricultural insect pest (*G. melonella, P. xylostella, T. castaneum and S. zeamais*), where the results indicated that alkaloid extracts of *B. microphylla* only showed antifeedant activity against *P. xylostella* larvae. In this sense, the antifeedant activity was dependent on the concentration of plant extracts, indicating that alkaloids present in the extracts inhibited larval feeding behaviour or making the food unpalatable. Most defensive plant chemicals discourage insect herbivory by deterring feeding and oviposition or by impairing settling and larval growth, rather than by killing insects outright (Isman, 2002). Thus, various alkaloid plant extracts and pure compounds have worked as feeding inhibitors againt insect pest (Moreno-Murillo et al., 1995; Shields et al., 2008; Sani et al., 2014). In the case of pure compounds, in this study were tested berberine and palmatine, but only berberine exhibited antifeedant activity against *P. xylostella* larvae. Different results were obtaining by Park et al. (2000) because they observed the antifeeding activity with berberine and palmatine against larvae of *Hyphantria cunea*. Other authors reported that alkaloid compounds from plant extracts had significant antifeedant effect against lepidopteran pest. For example, two alkaloids (vasicine acetate and 2-Acetyl

benzylamine) identified in leaf fractions from *Adhatoda vasica* were responsible of the antifeedant activity against *P. xylostella* larve (Paulraj et al., 2013) and the erythrina alkaloids identified in seeds, seed pods and flowers extracts from *Erythrina latissima* had antifeeding activity against *Spodoptera littoralis* larvae (Cornelius et al., 2009). In this sense, utilization of plant compounds as pesticides is one alternative that could be incorporated on agricultural pest control (Isman, 2006; Zapata and Smagghe, 2010).

5.2. Concluding remarks

Alkaloid compositions in different *B. microphylla* organs were determined and the effects of alkaloid extracts and pure compounds against human pathogenic bacteria and agricultural insect pest were evaluated. Thus, isoquinoline alkaloids were identified in leaf, stem and root extracts of *B. microphylla* where the type and amount of alkaloids depend on the organ of the plant and the environmental conditions. Also, stem and root extracts have a great number of alkaloids, and berberine was the more aboundant.

In relation with antibacterial activity of alkaloid extracts it is possible to highlight that leaf, stem and root extracts of *B. microphylla* has antibacterial activities only against Gram-positive bacterial strains and in some cases the extracts have the same or higher activity than commercial antibiotics. Furthermore, the susceptibility of Gram-positive bacteria is dependent on the alkaloid extracts tested and the bacteria. As for the activity of pure compounds it was possible to establish that berberine is only active against some of the tested bacterial strains and the activity of this compound is similar to that of root extracts. Additionally, a synergistic effect is observed between *B. microphylla* alkaloid extracts and antibiotics (ampicillin and cephalothin) against Gram-positive bacteria.

On the other hand, stem and root alkaloid extracts were selected to evaluate their respective insecticidal/antifeedant activity against some agriculturally insect pests. However, these alkaloid extracts of *B. microhylla* only showed antifeedant activity against *P. xylostella*.

The results obtained in this research justify the medicinal uses and the therapeutic values of this native plant as curative agent against infections diseases. Also, this study presente evidence of the antifeedant effects of alkalois of *B. microphylla* against phytophagous insect pest.

5.3. Future directions

Alkaloids are widespread in nature showing a wide range of pharmacological and insecticide activities. Many of the plants that contain alkaloids from the native flora of Chile have been used in traditional medicine, but in spite of it chemical and biological studies have been scarce. Due to *Berberis microphylla*, is a native species from southern of Chile with medicinal properties and is characterized by presence of alkaloids, is an interesting plant to study.

In this sense, our results reveal that *B. microphylla* extracts are a rich source of isoquinoline alkaloids with promising antibacterial and antifeedant activity. Referent to antibacterial activity, it is important to note that, at present, the resistant bacterial strains against drugs is a very serious problem in public health where the medicinal plants are considered as an alternative to synthetic drugs. Thus, these results indicate that *B. microphylla* extracts could have therapeutic value against Gram-positive human pahogenic bacteria.

Furthermore, the alkaloids of *B. microphylla* had antifeedant activitiy against larvae of *Plutella xylostella*. This result is in line with research investigating new botanical insecticides as alternative to synthetic pesticides because are rapidly degraded after application, thereby causing little environmental impact. Thus, the alkaloid extracts of *B. microphylla* could be incorporated into phytophagous insects control as part in the strategy of integrated pest management (IPM).

Nevertheless, despite the important results obtained in the present study is necessary the identification of the active antimicrobial and antifeedant principle present in alkaloid extracts from *B. microphylla* and provide some additional insight into cytotoxic activity of plant extracts and pure compounds.

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