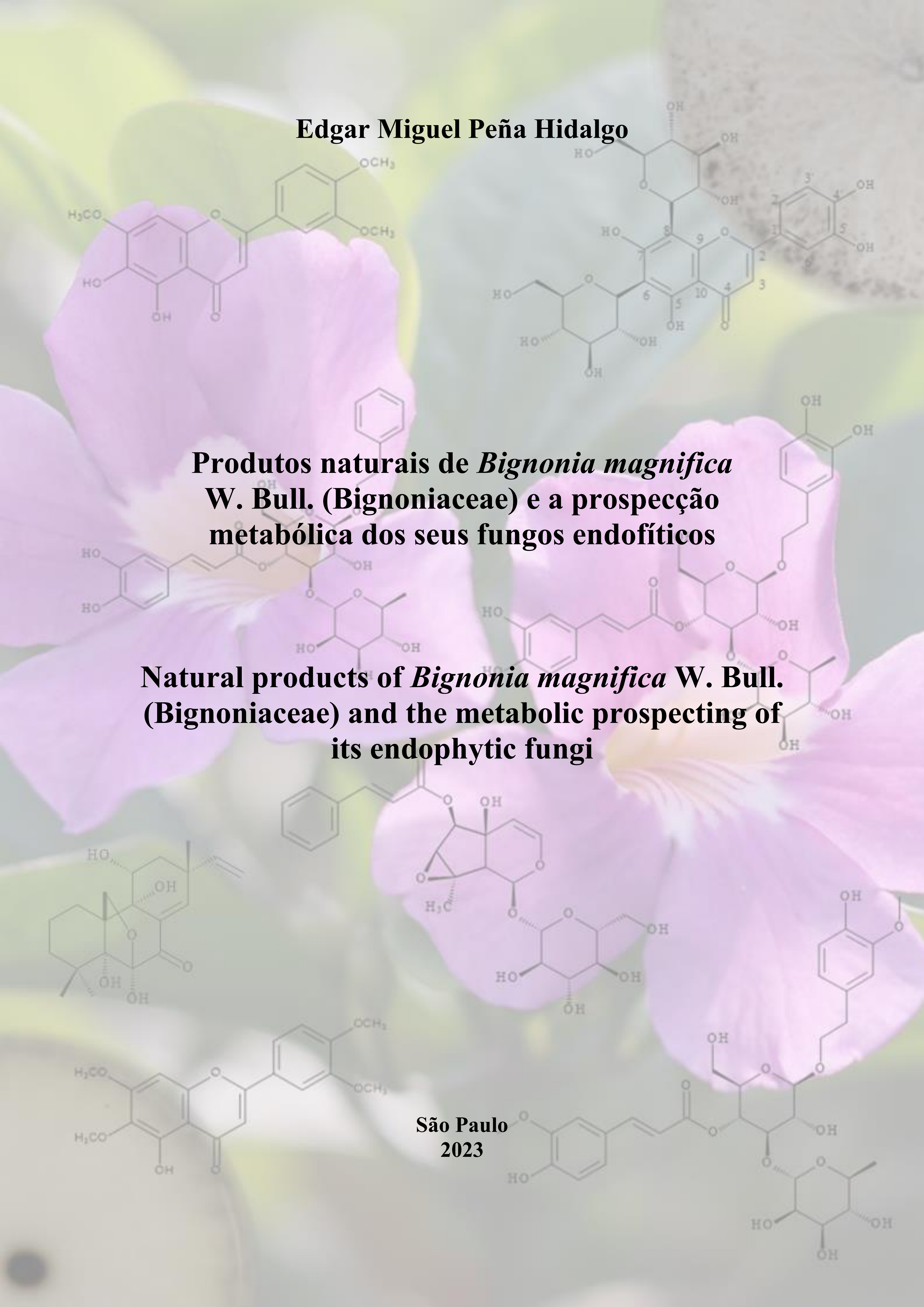

Edgar Miguel Peña Hidalgo

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Botânica.

Orientador: Marcelo José Pena Ferreira

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Prof(a). Dr(a).

Prof(a). Dr(a).

Prof. Dr. Marcelo José Pena Ferreira
Orientador

“Work it harder, make it better
Do it faster, makes us stronger
More than ever, hour after hour
Work is never over”

Bangalter,T. Guy, M.

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Resumo geral

Bignonia magnifica W. Bull (Bignoniaceae) é uma liana nativa da Colômbia, conhecida por seu apelo ornamental. Este estudo visou desvendar sua composição química, entender suas adaptações ecológicas e explorar o potencial biotecnológico de seus fungos endofíticos. O capítulo inicial aborda a diversidade química de flavonoides em Bignonieae, tribo a qual pertence a espécie estudada. Posteriormente, são investigados os produtos naturais produzidos por *B. magnifica*. Técnicas analíticas revelam a predominância de flavonoides metoxilados e *n*-alcanos nas ceras epicuticulares, além da identificação de compostos fenólicos, flavonoides glicosilados e iridoides no material macerado. Uma análise comparativa examinou *B. magnifica* em jardins ornamentais versus ambientes não ornamentais na paisagem tropical australiana. Enquanto as ceras cuticulares permanecem consistentes, as plantas não ornamentais exibem um acúmulo significativo de verbascosídeo, mostrando o investimento da planta na acumulação desse metabólito no novo ambiente. Por fim, de *B. magnifica* foram isolados 80 fungos endofíticos, com uma cepa, #888, se destacando devido ao seu potencial citotóxico. Dessa cepa, a substância diaphortein B exibe notável citotoxicidade contra as linhagens celulares HCT116 e CaCo-2, sugerindo seu potencial em estudos futuros de terapia do câncer. Juntas, essas pesquisas abrangem a variedade química do *B. magnifica*. Elas revelam suas complexidades químicas, destacam sua adaptabilidade a diferentes ambientes e identificam agentes citotóxicos em seus endofíticos. Essas descobertas destacam a importância da planta em contextos ecológicos e seu potencial em biotecnologia.

Palavras-chave: Verbascosideo, diaportheina B, plantas invasoras, iridoides, flavonoides metoxilados.

General abstract

Bignonia magnifica W. Bull (Bignoniaceae) is a native Colombian liana renowned for its ornamental significance. This study aims to explore its chemical composition, understand its ecological adaptations, and investigate the biotechnological potential of its endophytic fungi. The initial chapter describes the chemical diversity of flavonoids found in Bignonieae, the tribe in which the studied species is classified. Subsequently, the natural products of *B. magnifica* are investigated. Analytical techniques revealed a predominance of methoxylated flavonoids and *n*-alkanes in the epicuticular waxes. Phenolic compounds, glycosylated flavonoids, and iridoids were identified in the macerated material. A comparative analysis was carried out examining *B. magnifica* in ornamental gardens versus non-ornamental environments in the Australian tropical landscape. While cuticular waxes remained consistent, non-ornamental plants exhibited a significant accumulation of verbascoside, indicating the investment of the plant in the accumulation of this compound in the new environment. Finally, 80 endophytic fungi were isolated from *B. magnifica*, and the cytotoxic potential was highlighted for strain #888. Diaphortein B from this fungus showed notable cytotoxicity against HCT116 and CaCo-2 cell lines, suggesting a need for more in-depth studies in cancer therapy. The comprehensive set of these studies demonstrated the chemical diversity of *B. magnifica*, unveiling its chemical intricacies, highlighting its adaptability to different environments, and identifying cytotoxic agents within its endophytes. These findings underscore the plant's importance in both ecological contexts and its potential in biotechnology.

Keywords: Verbascoside, diaporthein B, alien plants, iridoids, methoxylated flavonoids.

General introduction

Bignoniaceae

The Bignoniaceae Juss. is a group of flowering plants found in the Lamiales order, consisting of approximately 850 species distributed among 85 genera (Fonseca et al., 2023). These plants have a pantropical distribution, with the Neotropical region being home to about 72% of the species, while only three species are found in North America. These include the native *Bignonia capreolata* L. and the introduced species *Dolichandra unguis-cati* (L.) LG Lohmann and *Amphilophium buccinatorium* (DC.) L. G. Lohmann (Lohmann & Taylor, 2014).

The family comprises trees, shrubs, and lianas with petiolate leaves, which are usually oppositely arranged and compound, although they may occasionally be simple, as in *Catalpa* Scop. Additionally, domatia are reported in 14 genera, manifesting as pockets or hair tufts. The campanulate hermaphrodite flowers are irregular, solitary, or aggregated in terminal cymes or racemes and are pollinated by insects, birds, or bats. The fruit is typically a winged, dehiscent capsule (Watson, L. and Dallwitz, M.J., 1992 DELTA).

Bignoniaceae has a diversity center in Brazil, with 33 genera and 412 species (Lohmann & Taylor, 2014). The plants occur in various habitats, including dry environments and humid forests (Olmstead et al., 2009). The family is divided into eight clades: Bignonieae, Catalpeae, Jacarandeae, Oroxyleae, Tourrettieae, Tecomeae, Paleotropical clade, and Tabebuia alliance.

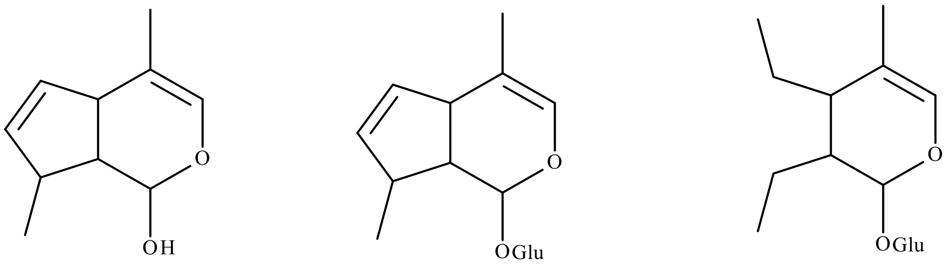
Bignonieae is the largest clade, with about half of the species described. This tribe is the major group of lianas in the forests of the New World and comprises 393 species and 21 monophyletic genera (Lohmann & Taylor, 2014; Olmstead et al., 2009). This clade was organized in a generic classification by Lohmann and Taylor (2014) which recognized 395 species in 21 genera: *Adenocalymma* Mart. ex Meisn. (82 species), *Amphilophium* Kunth. (47 spp.), *Anemopaegma* Mart. ex Meisn. (45 spp.), *Bignonia* L. (30 spp.), *Callichlamys* Miq. (1 spp.), *Cuspidaria* DC. (19 spp.), *Dolichandra* Cham. (8 spp.), *Fridericia* Mart. (67 spp.), *Lundia* DC. (13 spp.), *Manaosella* J. C. Gomes (1 spp.), *Mansoa* DC. (12 spp.), *Martinella* Baill. (2 spp.), *Neojobertia* Baill. (2 spp.), *Pachyptera* DC. ex Meisn. (4 spp.), *Perianthomega* (1 spp.), *Pleonotoma* Miers (17 spp.), *Pyrostegia* C. Presl (2 spp.), *Stizophyllum* Miers (3 spp.), *Tanaecium* Sw. (17 spp.), *Tynanthus* Miers (15 spp.), and *Xylophragma* Sprague (7 spp.).

Natural products in Bignoniaceae

Bignoniaceae is often used in landscaping and is an essential source of wood and bioactive compounds (A.H Gentry, 1991). Chemically, the family is characterized by terpenoids, especially iridoids and quinones, with expressive production of naphthoquinones, flavonoids, phenylpropanoid derivatives, and to a lesser extent, alkaloids (Cipriani et al., 2012).

Iridoids

Iridoids are classified as monoterpenoids and are acetal derivatives of iridodial. Due to the inherent instability of the hydroxyl group at C-1, iridoids frequently undergo reactions with sugars to form glycosides. Based on the integrity of the cyclopentane unit, they can be categorized into two types: iridoid glycosides and secoiridoid glycosides (Figure 1) (Wang et al., 2020).



Iridoid alcohol

Iridoid glycosides

Secoiridoids

Figure 1. Skeletal types of iridoids in plants.

Iridoids are predominantly present in angiosperm species and occasionally in monocotyledons. These compounds can be found in various plant parts such as leaves, fruits, roots, and sprouts and play a protective role against predators (Ndongwe et al., 2023).

Iridoids in Bignoniaceae are predominantly present in species belonging to the tribe Tecomeae, characterized by decarboxylated iridoid glucosides and C-4 formyl iridoids. The biosynthesis of glycosylated iridoids involves several enzymatic reactions, typically starts with the formation of the iridoid skeleton. This skeleton is derived from a precursor compound called isopentenyl diphosphate (IPP) (Figure 2). The enzyme geranyl pyrophosphate synthase catalyzes the condensation of IPP molecules to form geranyl pyrophosphate (GPP). GPP is further converted into iridodial, the basic structure of iridoids, from this, oxidation of aldehyde to acid and glucosylation transform the hemiacetal into an acetal. The addition of sugar (glycosylation) to iridoids is crucial for their stability, solubility, and bioavailability. (Dewick, 2009)

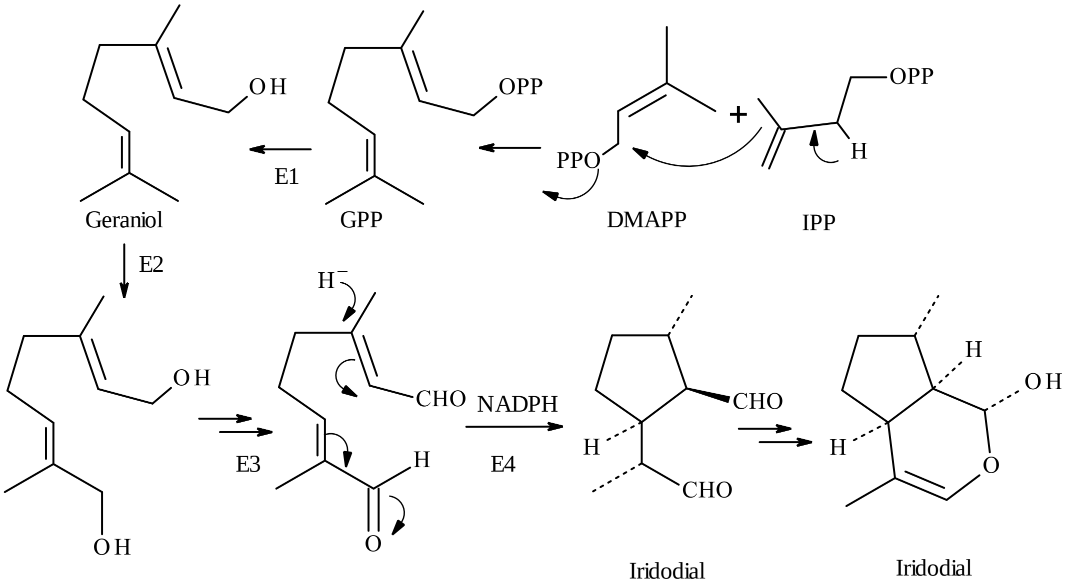
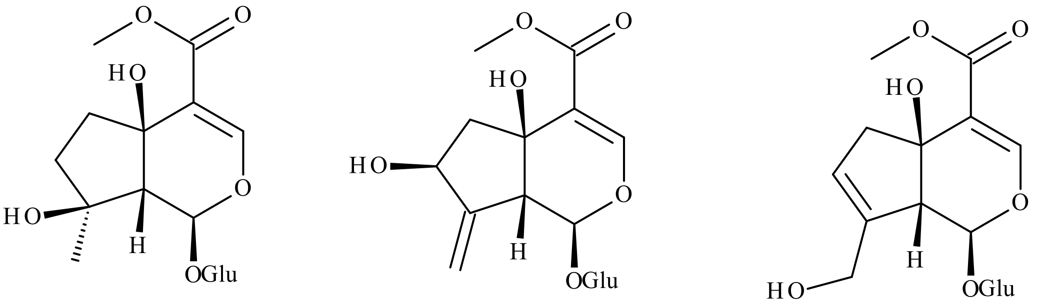


Figure 2. Biosynthesis of Iridodial in plants. E1: geraniol synthase E2: geraniol 10-hydroxylase E3: 10-hydroxygeraniol oxidoreductase E4: monoterpene cyclase.

In Bignonieae, all species are lianas, and the greatest part seems not to present iridoids. However, in the few species where iridoids are present, they are usually C-4 carboxylated as ipolamiide, strictoloside and theviridoside isolated from *Adenocalymma marginatum* (Cham.) DC. (Figure 3) (Schmeda-Hirschmann et al., 2020). Decarboxylated compounds such as 5,7-bisdeoxycynanchoside and cynanchoside are present only in *Dolichandra cynanchoides* Cham (Adriani et al., 1982; Bonini et al., 1981).



Ipolamiide Strictoloside Theviridoside

Figure 3. Iridoids C-4 carboxilated from *Adenocalymma marginatum* (Cham.) DC

Naphtoquinones

Naphthoquinones are a group of highly reactive natural products which have a diverse distribution in nature. Production by higher plants shows that although distribution is widespread, it is confined to certain orders such as Magnoliales, Proteales, Zygophyllales, Malphighiales, Fabales, Rosales, Fagales, Myrtales, Sapindales, Malvales, Caryophyllales, Ericales, Gentianales, and Lamiales.

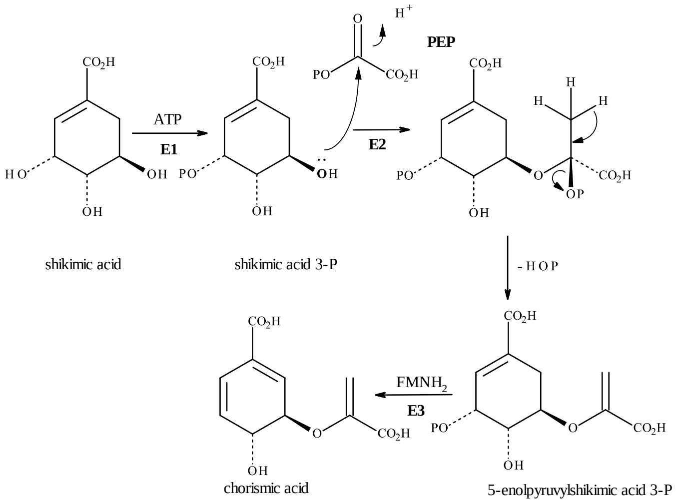


Figure 4. Biosynthesis of chorismic acid in plants. E1: shikimate kinase E2: EPSP synthase E3: chorismate synthase.

This natural product class has an aromatic ring fused with a quinone ring and the compounds are important for many biological processes, including respiration, photosynthesis, and electron transport. The biosynthesis of naphthoquinones begins with the formation of chorismic acid (Figure 4), an intermediate in the biosynthesis of aromatic amino acids (Hook et al., 2014).

One of the most studied plants in terms of natural products in Bignoniaceae is *Handroanthus impetiginosus* (Mart. ex DC.) (Synonym. *Tabebuia impetiginosa* (Mart. ex DC.) Standl., *Tecoma impetiginosa* Mart. ex DC.) a native Amazonian tree known as “ipê roxo” whose traditional use by the peoples of Brazil, northern Argentina, Paraguay, Bolivia, and Peru. The use of this plant by traditional people may date back to pre-Incan times. The name *impetiginosa* comes from the customary use of the bark against impetigo. The inner bark is used in ethnobotanical preparations, used for as antibacterial and antifungal, and to treat fever, syphilis, malaria, trypanosomiasis, as well as stomach and bladder disorders (Gómez Castellanos et al., 2009). In 1858 was isolated from the bark of *H. impetiginosus* the Lapachol (Figure 5) which was identified as a naphtoquinone-type compound with high anticancer activity. The National Cancer Institute (NCI) studied this natural product as a potential drug, but clinical trials in Phase I show no therapeutic response in blood analysis after oral administration of 4000 mg/day. Additionally, some toxicity was observed, leading to the closure of studies in 1970 (Cassady & Douros, 1980).

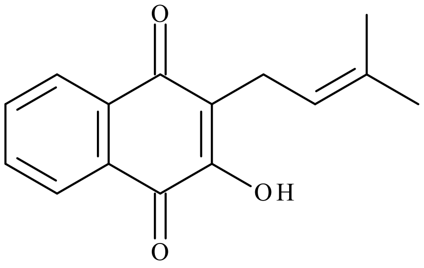


Figure 5. Chemical structure of lapachol, a quinone isolated from *Handroanthus impetiginosus* (Mart. ex DC.)

Flavonoids

Flavonoids are common metabolites in Bignoniaceae occurring either as aglycones, C-glycosyl, and O-glycosyl derivatives. Their structures contain hydroxyl, methoxyl, and glycosyl groups mainly located at C-3, C-5, C-7, C-3’’, and C-4’’.

Several compounds with oxidation at the C-6 position were identified and this feature is characteristic of members of the Bignoniaceae (Alcerito et al., 2002).

Some examples from flavonoids are carajurone, responsible for the red pigment obtained from the leaves of *Fridericia chica* (Bonpl.) L.G. Lohmann. (Violante et al., 2020), cirsimaritin and cirsimarin from *Dolichandra unguis-catii* (L.) L.G. Lohmann (Chen et al., 2017), acacetin and bucegin from *Bignonia callistegioides* Cham. (Castillo et al., 2013). Or dimeric flavonoids like brachydin A isolated from *Fridericia prancei* (A.H.Gentry) L.G. Lohmann. (Figure 6) (Carvalho et al., 2022).

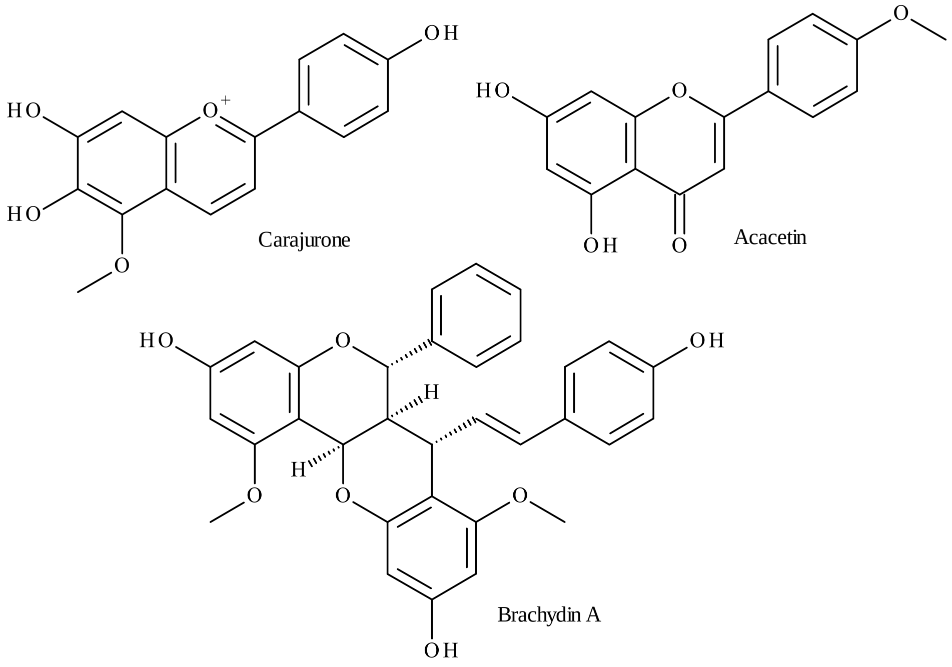


Figure 6. Examples of flavonoid types reported in Bignoniaceae.

Miscellaneous metabolites

There are reports of different types of metabolites from Bignoniaceae, such as the alkaloids of *Martinella iquitoensis A. Samp*, a plant described by different indigenous tribes of the Peruvian and Brazilian Amazon and used for inflammation, infection, and ocular irritations (Alwyn H. Gentry & Cook, 1984). From *M. iquitosensis* A. Samp. was described the occurrence of the pyrroloquinoline alkaloids, martinelin and martinelic acid (Figure 5), proving its antagonistic effects to the bradykinin B1 and

B2 receptor and, consequently, its use as analgesic and anti-inflammatory (Witherup et al., 1995).

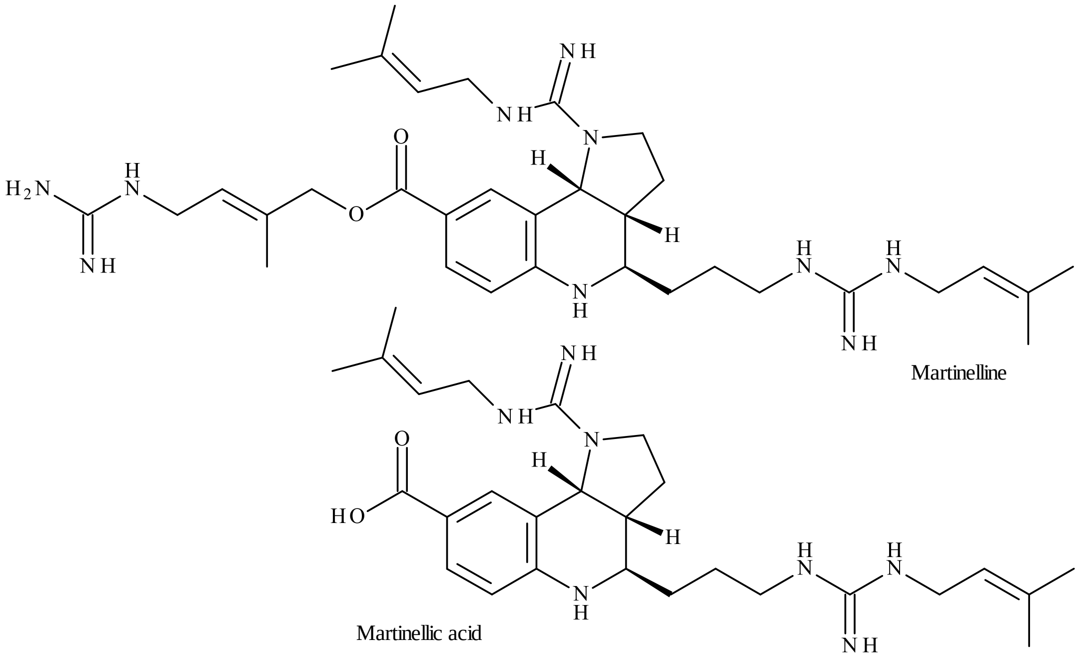


Figure 7. Alkaloids isolated from *Martinella iquitoensis* A. Samp

In *Anemopaegma arvense* (Vell.) Stellfeld ex de Souza were isolated the flavolignans cinchonain IIa and cinchonain IIb (Figure 8). These molecules act against cytotoxicity induced by hydroperoxide (Uchino et al., 2004).

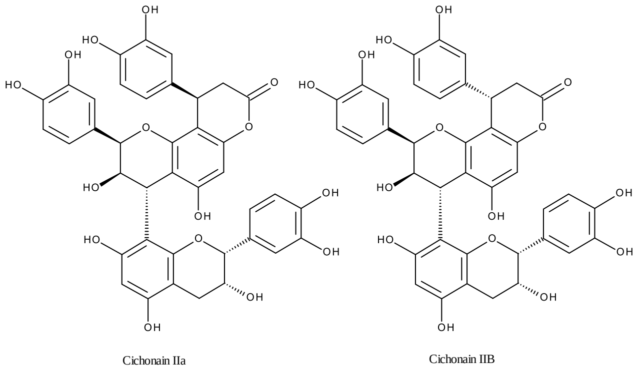


Figure 8. Flavolignanes isolated from *Anemopaegma arvense* (Vell.) Stellfeld ex de Souza

In *Mansoa alliacea* (Lam.) A.H.Gentry, the infusion of the dried aerial parts has been used in Surinam as a vermifuge, to treat fever and rheumatic pains. This plant furnished the polysulfide alliin (Figure 9), and the percentage of this metabolite in the leaves calculated on a dry basis was about 2.15% and is responsible for the smell of garlic (Zoghbi et al., 2009).

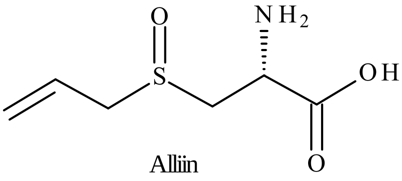


Figure 9. Alliin isolated from *Mansoa alliacea* (Lam.) A.H.Gentry

Terpenoids are also reported in the family, in *Tanaecium jaroba* Sw, plant used in traditional medicine in Bolivia to cure symptoms of malaria. From the bark was isolated the diterpenoid 2α-hydroxy-12β-hydroxy-isopimara-8(14),15-diene and the triterpenoid canophyllol (Mitaine-Offer et al., 2002). Other triterpenoids as ursolic and oleanolic acids (Figure 10) are also described in *Cuspidaria pulchra* (Cham.) L.G.Lohmann (Pauletti, 2012).

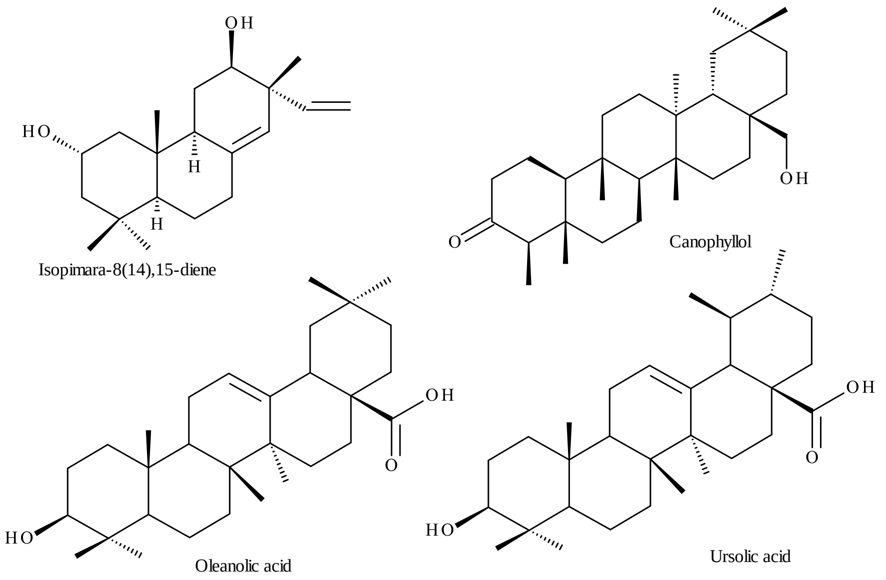


Figure 10. Terpenoids isolated from *Tanaecium jaroba* Sw and Cuspidaria pulchra (Cham.) L.G.Lohmann

Endophytic Fungi in drug discovery

Knowing that natural products continue to be an essential source of new pharmaceuticals and considering that 30% of the most prescribed drugs are of fungal origin and only approximately 5% of fungal diversity has been described, fungi offer enormous potential for search for new products with pharmaceutical potential. It is essential to consider that endophytic fungi are a promising source for the biomonitoring of molecules, considering that the biological activities of these fungi may vary according to the source from which they are isolated due to their interactions with the host organism that modulates its metabolism (Schulz & Boyle, 2005).

Whether or not cultivable, endophytic fungi are isolated from the internal tissues of a host (plant species or marine organism) and do not cause apparent damage to it (Zhang, Song & Tan, 2006). Such microorganisms may produce new bioactive metabolites like the host.

Among the endophytic fungi isolated from Bignoniaceae species, *Botryosphaeria dothidea* (Moug. Ex Fr.) Ces. & De was isolated from *Kigelia africana* (Lam.) Benth. and its extract showed potential as an immunosuppressive agent (Katoch et al., 2015). Extracts of endophytes *Syncephalastrum racemosum* Cohn and *Trichoderma longibrachiatum* Rifai isolated from *Markhamia tomentosa* (Benth.) K. Schum. ex Engl., showed antiproliferative activities against HeLa carcinogenic cell lines with IC_{50} of 43.56 µg. mL^{-1} and 68 µg. mL^{-1} respectively, and antifungal activity against the pathogens *Fusarium oxysporum* Schltdl., *Sclerotinia sclerotiorum* (Lib.) Bary, *Rhizoctonia solani* JG Kühn and *Botrytis cinerea* Pers. (Ibrahim et al., 2017). From *Tabebuia argentea* (Bureau & K. Schum.) Britton, extracts from the endophytes *Aspergillus niger* Tiegh. and *Alternaria alternata* (Fr.) Keissl. showed significant antimicrobial activity against fungi and pathogenic bacteria, also an antioxidant activity calculated as the FRAP (Ferric reducing the ability of plasma) between 4693-4189 µmol. L^{-1} (Sadananda et al., 2011). In *Tabebuia pentaphylla* (L.) Hemsl. the endophytic fungus *Pestalotiopsis pauciseta* (Sacc.) Y.X. Chen, from which taxol was isolated, showed a high cytotoxic activity *in vitro* against the MCF-7 breast cancer cell line with an IC_{50} of 350 µg. mL^{-1} (Vennila et al., 2012).

Objectives and Structure of the Thesis

Bignoniaceae is known for production of a large diversity of classes of compounds with varied biological activities. Given the versatility of these chemical constituents and the observed biological potential within this family, along with the inherent bioactive compound-producing potential of endophytic fungi, this thesis seeks to address the following fundamental questions: What are natural products present in *Bignonia magnifica* W. Bull? What is the cytotoxic potential of the endophytic fungi of this liana?

General conclusion

This study of natural products from *Bignonia magnifica* opens a window into the chemistry of the species. Our findings have revealed a metabolic diversity within this species, adding a nuanced layer to our comprehension of its chemical composition. Within the compounds identified, methoxylated flavonoids, such as eupatiline and 3'-O-Methyleupatorin, are restricted to the leaf's surface. Epicuticular waxes, on the other hand, are predominantly constituted by alkanes, a finding consistent with the wax profile of Bignonieae species. The internal compounds, including glycosylated flavonoids like lucenin 2, phenylethanoid glycosides such as verbascoside, martynoside, and jionoside C, and an iridoid (6-O-trans-cinnamoyl-antirrinoside), shows the plant's inclination towards phenolic compound production.

One noteworthy result is the prominence of verbascoside, which significantly dominates the chemical profile of *B. magnifica*. The presence of this phenylethanoid glycoside opens avenues for understanding its ecological and adaptive roles within the plant. Furthermore, the presence of iridoids signals a distinct pathway of *Bignonia* compared to other Bignonieae genera.

In parallel, the comparative analysis between ornamental and non-ornamental *B. magnifica* specimens in the Australian territory indicate ecological plant responses to the new environment. While the composition of cuticular waxes remains consistent across these plants, a divergence emerges within their internal metabolites. Specifically, non-ornamental plants exhibit a heightened presence of the phenylethanoid glycoside, verbascoside. This suggests that the overproduction of verbascoside in non-ornamental plants may well be a pivotal factor in facilitating their rapid colonization of non-native territories. This phenomenon warrants in-depth investigation to reveal its underlying mechanisms.

The exploration of endophytic fungi within *B. magnifica* provides valuable insights into their biotechnological potential. The isolation of 80 fungal strains from the plant's tissues culminated in the discovery of endophyte #888, characterized by its potent cytotoxic activity. The subsequent isolation and identification of diaphortein B, a known natural product found in *Diaporthe* sp. BCC 6140, caught the attention because of the potent cytotoxic agent against HCT116 and CaCo-2 cell lines. This results underscores the potential of diaphortein B for further action mechanism analyses, positioning it favorably for prospective studies in cancer therapy. Moreover, the compounds currently under structural elucidation hold the promise of expanding our knowledge of the diversity of pimarane diterpenes produced by this endophyte.

In summary, the investigations encapsulated in these thesis offer a multifaceted view of *Bignonia magnifica*, a plant that straddles both chemical and ecological significance. This cumulative body of research not only advances our comprehension of this plant's chemical intricacies and ecological adaptations but also points toward a promising trajectory for future investigations in pharmacology, ecology, and natural product chemistry. In doing so, it lays the foundation for deeper explorations into the rich and multifaceted realm of *Bignonia* species, encompassing both their natural products and ecological adaptations.

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