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Cecropia catarinensis Cuatrecasas (*Urticaceae*): Chemical and biological studies

Dissertação do 2º Ciclo de Estudos Conduncente ao grau de Mestre em Controlo de Qualidade na Área de Especialização em Fármacos e Plantas Medicinais

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i. Resumo

O reino das plantas tem disponibilizado vários agentes terapêuticos para o tratamento de um grande número de doenças. Parte do seu interesse advém do conhecimento que tem sido adquirido ao longo dos séculos, o que justifica o seu uso na medicina tradicional. Embora o cancro seja uma doença pouco descrita na medicina tradicional, as plantas têm contribuído largamente para o arsenal terapêutico atual daquela doença. No entanto, o mesmo não poderá ser dito dos agentes antimicrobianos, uma vez que derivam habitualmente de metabolitos secundários produzidos por bactérias e fungos saprófitos e não diretamente pelas plantas.

As plantas do género *Cecropia* têm sido utilizadas na medicina tradicional, especialmente em países da América Latina, o que explica o grande número de estudos relativos à sua atividade biológica. No entanto, são poucos os estudos dedicados aos seus efeitos anticancerígenos e antimicrobianos. De facto, a espécie *Cecropia catarinensis* nunca foi investigada nestas áreas de pesquisa.

Este trabalho relata o estudo fitoquímico das folhas da *Cecropia catarinensis*, que inclui a avaliação *in vitro* dos efeitos inibidores do crescimento de células do adenocarcinoma da mama (MCF-7), de células do cancro do pulmão (NCI-H460) e de células do melanoma (A375-C5) pelo extrato bruto metanólico da planta (CME) e por duas frações semipurificadas (CME1 e CME2), utilizando-se o ensaio de SRB; e a avaliação da atividade antimicrobiana do extrato e das frações da planta (CME, CME1 e CME2) e dos compostos isolados a partir de CME1 sobre as bactérias *Staphylococcus aureus* ATCC 25923, uma estirpe clínica de *Staphylococcus aureus* resistente à meticilina, *Escherichia coli* ATCC 25922 e *Pseudomonas aeruginosa* ATCC 27853, bem como sobre os fungos *Cândida albicans* ATCC 10231, *Aspergillus fumigatus* ATCC 46645 e uma estirpe clínica de *Trichophyton rubrum*, utilizando o método de microdiluição em caldo.

O extrato bruto metanólico inibiu o crescimento das três linhas celulares utilizadas neste ensaio. A fração CME1 apresentou a maior atividade inibitória de entre as três amostras testadas. A fração CME2 apresentou uma atividade moderada sobre as células MCF-7 e NCI-H460 e foi inativa para as células A375-C5. A fração CME1 foi escolhida para ser purificada, fornecendo quatro compostos. Foram propostas as estruturas de dois triterpenóides pentacíclicos, o ácido ursólico e o uvaol, e de dois esteróides, a *β*-sitostenona e o *β*-sitosterol. O composto proposto como sendo ácido ursólico demonstrou ser seletivo sobre as bactérias gram-positivas.

Palavras-chave: *Cecropia catarinensis*, metabólitos secundários, triterpenóides pentacíclicos, atividade antitumoral, atividade antimicrobiana.

ii. Abstract

Plants have a long history of affording therapeutic agents for the treatment of a large number of diseases. Part of their interest comes from the knowledge that has being acquired over the centuries, which justifies their use in traditional medicine. Although cancer is a poorly described disease in traditional medicine, plants have widely contributed to the current therapeutic arsenal of that disease. However, the same cannot be said about antimicrobial agents as they have usually derived from secondary metabolites that are produced by soil bacteria and fungi and not directly by plants.

Plants of the genus *Cecropia* have been used in traditional medicine, especially in Latin American countries, which explains the large number of studies concerning their biological activity. However, there are few studies dedicated to their anticancer and antimicrobial effects. Indeed, the specie *Cecropia catarinensis* was never investigated concerning this area of research.

This work reports the phytochemical study of the leaves of *Cecropia catarinensis*, which includes the *in vitro* evaluation of the growth inhibitory effects of the methanolic crude extract CME and two semi purified fractions (CME1 and CME2) towards human breast adenocarcinoma cells (MCF-7), human non-small cell lung cancer cells (NCI-H460) and human melanoma cells (A375-C5), using the SRB assay; and the antimicrobial activity evaluation of the crude extracts (CME, CME1 and CME2) and the compounds isolated from CME1 against the bacteria *Staphylococcus aureus* ATCC 25923, a clinical strain of MRSA, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, and the fungi *Candida albicans* ATCC 10231, *Aspergillus fumigatus* ATCC 46645 and a clinical strain of *Trichophyton rubrum*, using the broth microdilution method.

The crude methanolic extract inhibited the growth of the three human tumour cell lines used in this biological assay. CME1 was not only active against the panel of cancer cells but presented the highest inhibitory activity among the three tested samples. CME2 showed moderate activity against MCF-7 and NCI-H460 cells and was inactive towards A375-C5 cells. CME1 was chosen to be further purified, which afforded four compounds. It was proposed the structures of two pentacyclic triterpenoids, uvaol and ursolic acid, and two steroids, *β*-sitostenone and *β*-sitosterol. The compound proposed as ursolic acid demonstrated to be selective against gram-positive bacteria.

Keywords: *Cecropia catarinensis*, secondary metabolites, pentacyclic triterpenoids, antitumor activity, antimicrobial activity.

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vi. Abbreviations and symbols

A375-C5 – Human malignant melanoma IL-1 insensitive cells
A549 – Human breast carcinoma cells
ACE – Angiotensin I converting enzyme
B16 – Murine melanoma cells
Caco-2 – Human colon adenocarcinoma cells
CLSI – Clinical and laboratory standards institute
^{13}C NMR – Carbon-13-Nuclear magnetic resonance
CNS – Central nervous system
DMSO – Dimethyl sulfoxide
EBV-EA – Eipstein-Barr virus – Early antigen
EC_{50} – Half maximal effective concentration
FBS – Fetal bovine serum
GC-MS – Gas chromatography - Mass spectrometry
HCT-8 – Human colon carcinoma cells
HEp-2 – Human larynx carcinoma cells
HHV-1 – Human herpes virus type 1
HHV-2 – Human herpes virus type 2
HL-60 – Human promyelocytic leukaemia cells
^{1}H NMR – Hydrogen-1-Nuclear magnetic resonance
HPLC – High-performance liquid chromatography
HPLC-DAD – High-performance liquid chromatography - Photo-diode array
HSCCC – High-speed countercurrent chromatography
IC_{50} – Half maximal inhibitory concentration
K562 – Human acute myelocytic leukaemia cells
LC-ESI-MS – Liquid chromatography - Electrospray ionization - Mass spectrometry
LC-MS – Liquid chromatography - Mass spectrometry
Lucena-1 – Multidrug resistant leukaemia cells
MCF-7 – Human breast adenocarcinoma cells
MHA – Muller-Hinton agar
MHB – Muller-Hinton broth
MIC – Minimum inhibitory concentration
MLC – Minimum lethal concentration
MRSA – Methicillin-resistant *Staphylococcus aureus*
NCI – National cancer institute (USA)
NCI-H460 – Human non-small cell lung cancer cells

SRB – Sulforhodamine B

RAS – Renin-angiotensin-system

ROS – Reactive oxygen species

RP-HPLC – Reversed phase - High-performance liquid chromatography

SDA – Sabouraud dextrose agar

TLC – Thin-layer chromatography

Chapter 1

Introduction

Catharanthus roseus: http://www.biolib.cz/en/taxonimage/id54089/;
Taxus brevifolia: http://www.dereilanatureinn.ca/woodlands/berry-patch/;
Camptotheca acuminata: http://www.chinahorticulture.net/wp/en/2013/02/16/camptotheca-acuminata/;
Podophyllum peltatum: http://www.missouribotanicalgarden.org/gardens-gardening/your-garden/plant-finder/plant-details/kc/l800/podophyllum-peltatum.aspx.

1. Introduction

Since ancient times, humans have been using nature as a source of therapeutic agents and plants have particularly sustained all sophisticated traditional medicine systems, such as the Egyptian medicine (documented in "Ebers Papyrus"), the traditional Chinese medicine (with the Chinese Materia Medica) or the Indian Ayurvedic system. On the Western side of the world, both Greeks and Romans developed the rational use of herbal drugs and the Arabs preserved the Greco-Roman expertise and included the use of their own sources, together with Indian and Chinese herbs that were unknown to the Romans and Greeks [Cragg and Newman, 2013; Ji *et al.*, 2009].

Indeed, approximately 65% of the world population predominately relied on plant traditional medicines for primary healthcare. Moreover, plants have an indirect role in the healthcare systems, since plants have been affording lead compounds for the development of drugs that are now in clinical practice. For example, **galegine (1)** was originally isolated from *Galena officinalis* L. and was used as a model for the synthesis of the oral antidiabetic agent **metformin (2)**; or **papaverine (3)** that was obtained from *Papaver somniferum* and that led to **verapamil (4)**, a calcium channel blocker used to treat hypertension; or otherwise, the same plant *Papaver somniferum* that afforded the painkillers morphine and codeine; or even the antimalarial **quinine (5)** that constituted the basis for the synthesis of other antimalarial agents **chloroquine (6)** and **mefloquine (7)** and that was originally isolated from the bark of *Cinchona* species, which had been used by Amazonian indigenous groups for the treatment of fevers [Cragg and Newman, 2013].

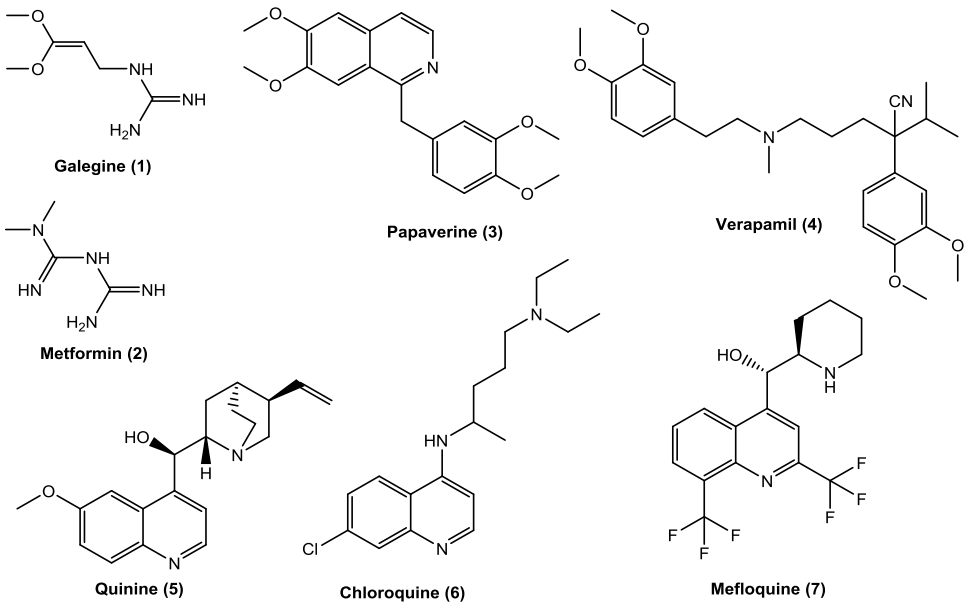


Figure 1 – Strutures of galegine (1), metformin (2), papaverine (3), verapamil (4), quinine (5), chloroquine (6) and mefloquine (7)

Although cancer had been a poorly described disease in folk or traditional medicine, plants have a long history of affording anticancer agents to the current therapeutic arsenal. Between them, *Vinca* alkaloids are some of the best well-known plant-derived anticancer drugs [Cragg and Newman, 2013]. **Vincristine (8)** and **vinblastine (9)** are naturally occurring *Vinca* alkaloids, isolated from the periwinkle plant *Catharanthus roseus*, formerly known as *Vinca rosea*. The Vinca alkaloids bind specifically to the β subunit of tubulin dimmers at the so-called "*Vinca* domain", inducing a conformational change in tubulin which leads to the formation of paracrystalline aggregates. The pool of available free tubulin dimmers is decreased, which shifts the equilibrium and leads to microtubule depolymerisation and mitotic spindle destruction. Therefore, the actively dividing cells are inhibited to progress through mitosis, which results in metaphase arrest that then triggers apoptosis [Ng, 2011; Nobili *et al.*, 2009].

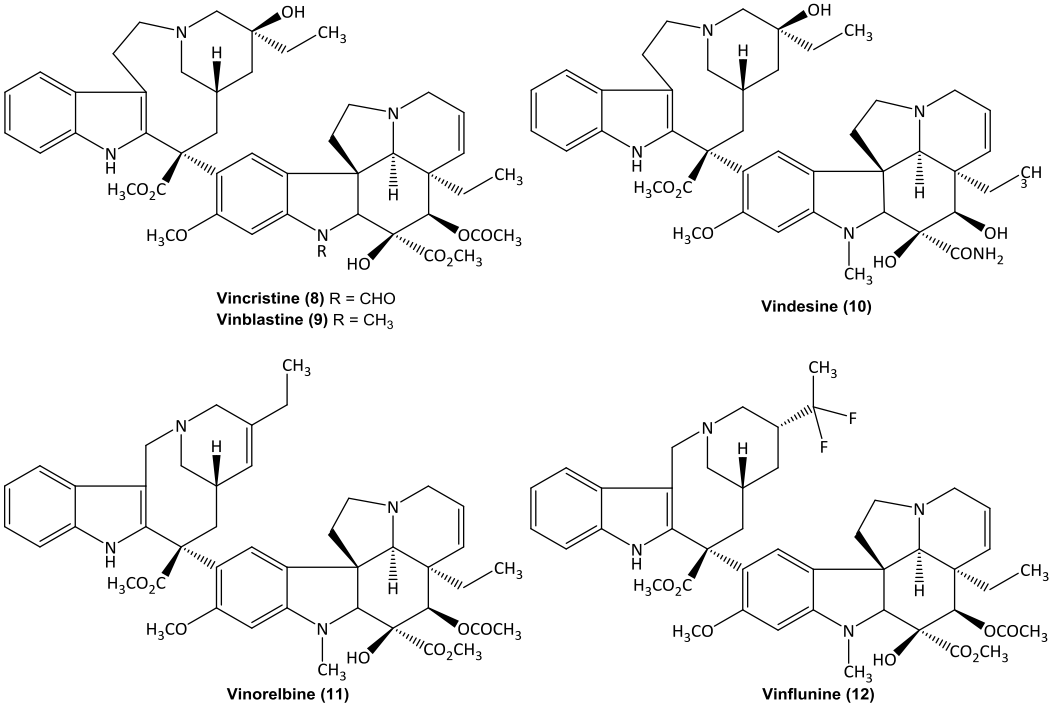


Figure 2 – Strutures of vincristine (8), vinblastine (9), vindesine (10), vinorelbine (11) and vinflunine (12)

Vindesine (10) and **vinorelbine (11)** are semisynthetic analogues that were obtained from structural modifications of vinblastine. Those derivatives interact more weakly with tubulin that the naturally occurring *Vinca* alkaloids, affecting the microtubule dynamics in a different way [Nobili *et al.*, 2009]. Moreover, the recently approved **vinflunine (12)** is a fluorinated analogue of vinorelbine that has the lowest overall *in vitro* affinity to tubulin among the *Vinca* alkaloids family, being such interaction the most readily reversible. However, this weaker interaction does not correlate to a lower efficacy because vinflunine

has shown to be the most active against a number of murine and human tumour xenografts. The exact mechanism is not fully understood but, apart from the microtubule disturbance effects, vinflunine seems to inhibit the vascular endothelial cell growth, possessing antiangiogenic activity [Ng, 2011].

The natural terpene **paclitaxel (13)** is the most interesting plant-derived anticancer drug discovered in recent years [Cragg and Newman, 2013]. The compound is a microtubule-stabilizing agent that was isolated from the bark of the Pacific yew *Taxus brevifolia* and is widely used to treat breast, ovarian and lung carcinomas [Nobili *et al.*, 2009]. Several supply problems were overcome by its semisynthesis from 10-deacetylbaccatin III that is extracted from the twigs and needles of the European yew *Taxus baccata*. **Docetaxel (14)** is also obtained from 10-deacetylbaccatin III by semisynthesis [Nicolaou *et al.*, 2009; Nobili *et al.*, 2009]. Both drugs arrest cell cycle at G2/M stage by binding to specific sequences of the β-tubulin subunit, the so-called “taxane binding site”, which ultimately leads to apoptosis [Nobili *et al.*, 2009].

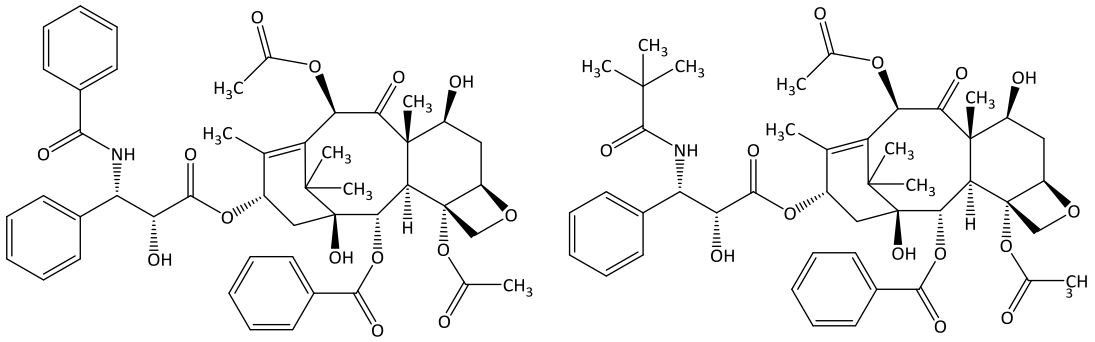


Figure 3 – Strutures of paclitaxel (13) and docetaxel (14)

Another example of the role of plants in the anticancer drug discovery is the camptothecins family. Actually, the enzyme topoisomerase I was identified as a target for cancer chemotherapy by camptothecin, which was isolated from the Chinese ornamental tree *Camptotheca acuminata*. However, severe bladder toxicity prevented camptothecin to become clinically approved. Less toxic semisynthetic analogues, such as **irinotecan (15)** and **topotecan (16)**, were approved for the treatment of colorectal and ovarian cancer respectively [Cragg and Newman, 2013; Nobili *et al.*, 2009]. Camptothecins derivatives share a common five-ring structure with a chiral centre located in the terminal lactone, where its S-conformation and hydroxyl group are essential for biological activity. As topoisomerase I bind covalently to the DNA double-strand by a reversible trans-esterification reaction, camptothecins stabilize this covalent DNA-topoisomerase I complex, preventing relegation during the catalytic cycle [Nobili *et al.*, 2009].

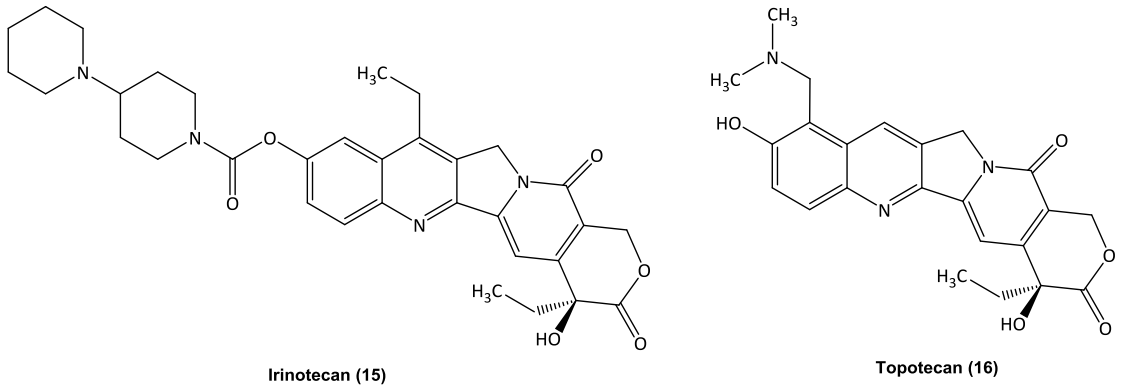


Figure 4 – Strutures of irinotecan (15) and topotecan (16)

Root extracts of the Indian plant *Podophyllum peltatum* have been used as folk medicines for centuries. **Etoposide (17)** and **teniposide (18)** are semisynthetic derivatives of 4-epipodophyllotoxin and are cell cycle dependent and phase specific, affecting the S and G2 phases of cell division. Being topoisomerase II poisons, these drugs form a stable drug-DNA-topoisomerase II ternary complex which prevents the releasing from the DNA and results in DNA double strand-breaks [Nobili et al., 2009].

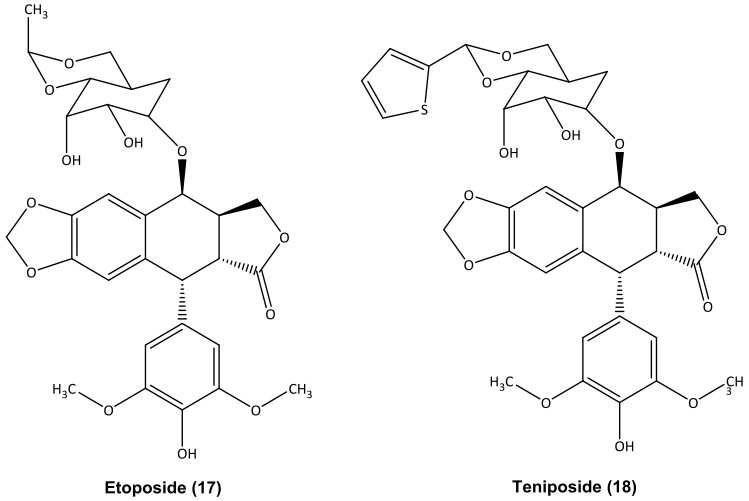


Figure 5 – Strutures of etoposide (17) and teniposide (18)

Unlike the successful plant-derived antitumor agents, almost all antibacterial agents derived from secondary metabolites that were produced by soil bacteria and fungi and not directly by plants. However, there has been an enormous effort to exploit the plant kingdom in the past, without a notable success to date. Curiously, the use of traditional plant-based medicines is common in some countries, but the intrinsic value of plant extracts as therapeutic concoctions does not follow the modern perceptions of efficacy and safety that could be determined by controlled clinical trials. Indeed, plant extracts may disadvantageously interact with modern medicines and may be harmful to users [Taylor,

2013]. However, this not means that plants extracts may not be considered as a starting point for the purification of bioactive chemical constituents for the development of antibiotic agents.

Nonetheless, the *in vitro* antibacterial activity of plant crude extracts, which is usually determined by the minimum inhibitory concentration (MIC), is generally high in a considerable number of studies that have used unfractionated extracts of roots and aerial parts of plants. However, there are an extensive number of plant-derived compounds with antimicrobial activity, such as aromatic secondary metabolites that are responsible for plant defence and constitute plant pigments and odours, or alkaloids that have long been known to possess antiparasitic activity, or plant-derived coumarins that have shown good activities against Gram-positive bacteria due to be inhibitors of bacterial DNA gyrase, or flavonoids and isoflavonoids that display weak antibacterial activity because they intercalate into biomembranes and favourably destabilize and modify the membrane properties of some Gram-positive pathogens, or even some terpenoids and isoprene-based constituents of essential oils that have good-to-moderate activities against Gram-positive bacteria. A final amazing example is the plant *Berberis fremontii* that synthesises the alkaloid berberine (a bacterial DNA intercalator) and also synthesises the compound 5'-methoxyhydnocarpin (an inhibitor of multidrug resistance pump), preventing extrusion of berberine from bacterial cytoplasm. Indeed, there are plant-derived products that could have a potential value to modify complex bacterial phenotypes and the paradigms of virulence and bacterial resistance [Taylor, 2013].

The objective of this work was to proceed to the chemical and biological studies of the leaves of *Cecropia catarinensis*, which included: the isolation, purification and elucidation of secondary metabolites from the plant crude extracts; the biological evaluation of the crude extracts and the isolated compounds for the antimicrobial activity against the bacteria *Staphylococcus aureus* ATCC 25923, a clinical strain of MRSA, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 and the fungi *Candida albicans* ATCC 10231, *Aspergillus fumigatus* ATCC 46645 and a clinical strain of *Trichophyton rubrum*, using the broth microdilution method; the biological evaluation of the plant extracts and the isolated secondary metabolites for the *in vitro* growth inhibitory activity towards a panel of human tumour cell lines (MCF-7, NCI-H460 and A375-C5 cells) using the SRB assay.

Chapter 2

The genus *Cecropia* as a potential source of therapeutic agents

Cecropia glaziovii: http://sites.google.com/site/biodiversidadecatarinense/plantae/magnoliophyta/urticaceae/cecropia-glaziovii-embauba;
Cecropia hololeuca: http://orienteocidente.wordpress.com/2013/07/20/embauba-branca-cecropia-hololeuca-3/;
Cecropia obtusifolia: http://wildlifeofhawaii.com/flowers/416/cecropia-obtusifolia-trumpet-tree/;
Cecropia pachystachya: http://www.natureloveyou.sg/Cecropia%20pachystachya/Main.html.

2. The genus *Cecropia* as a potential source of therapeutic agents

The genus *Cecropia* is constituted approximately by 60 species that were originally included in the family *Moraceae* and that were lately reclassified and included in the new family *Cecropiaceae*. However, recent taxonomic studies based on morphological and phylogenic approaches included the genus *Cecropia* in the family *Urticaceae*. These plants exist in the tropical and subtropical regions of the South and Central America [Costa *et al.*, 2011].

Species of the genus *Cecropia* have been used in folk medicine for the treatment of the most common chronic diseases such as Diabetes Mellitus, hypertension or asthma. Indeed, their extracts are included in Pharmacopoeias of various South American countries which demonstrate their use as phytomedicines. For this reason, there are several biological studies with standardized extracts of these plants for the evaluation of their hypoglycaemic or hypotensive properties, among others, which also included an attempt to understand their mechanisms of action. However, there are few reports on the isolation and on the chemical characterisation of the secondary metabolites present in their extracts, as well as, on the biological evaluation of their antifungal, antibacterial or cytotoxic activities.

The next sections correspond to a brief literature review of the chemical and the biological profiles of some species of the genus *Cecropia*. Our plant *Cecropia catarinensis* will be considered as individual specie as it was classified by Professor Jorge Pedro Carauta. Indeed, there are disagreements in the classification of *C. catarinensis* plants that occur in the South of Brazil, since these plants have been considered as *C. pachystachya* by some authors [Costa *et al.*, 2011], who in some extent support the idea that *C. catarinensis* is no more than a synonymous of *C. pachystachya*. They argued that the chemical fingerprint of extracts belonging to plants collected in the South and the Midwest of Brazil were compared by TLC and by HPLC, showing that there were no qualitative differences between samples. They also claimed that there are taxonomic studies that indicate that the plants belong to the same species but they did not detail the nature of those studies. Phylogenetic studies would be necessary to support the findings on the chemical fingerprints of the samples examined.

2.1. *Cecropia glaziovii*

The plant *Cecropia glaziovii* Sneth is a characteristic tree of the South-eastern Brazilian coasts that have been used in folk medicine as a cardiotonic, antihypertensive and antiasthmatic remedy [Rocha *et al.*, 2002]. In fact, in several ethnopharmacological

reports, *C. glaziovii* has been described for the control of high-blood pressure and as a diuretic [Costa *et al.*, 2011].

These ethnopharmacological features should be based on the chemical composition of the plant. Firstly, a chemical investigation of a standardized aqueous extract of the dried leaves of *C. glaziovii* revealed the presence of **chlorogenic acid (19)**, catechins [**(+)-catechin (20)** and **(-)-epicatechin (21)**], procyanidins {**procyanidin B2 [epicatechin-(4β→8)-epicatechin] (22)**, **procyanidin B3 [catechin-(4α→8)-*ent*-catechin] (23)**, **procyanidin B5 [epicatechin-(4β→6)-epicatechin] (24)** and **procyanidin C1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin] (25)**} and flavonoids [**isoorientin (26)** and **isovitexin (27)**] by HPLC analysis. The aqueous extract was partitioned with n-butanol and the resulting butanolic fraction was further purified by preparative HPLC columns, yielding 1.5% of chlorogenic acid, 12.6% of catechins, 22% of procyanidins and 23% of flavonoids. These substances were chemically identified by ^{13}C-NMR, ^{1}H-NMR and LC-MS and their structures are represented in the figure 6 [Tanae *et al.*, 2007].

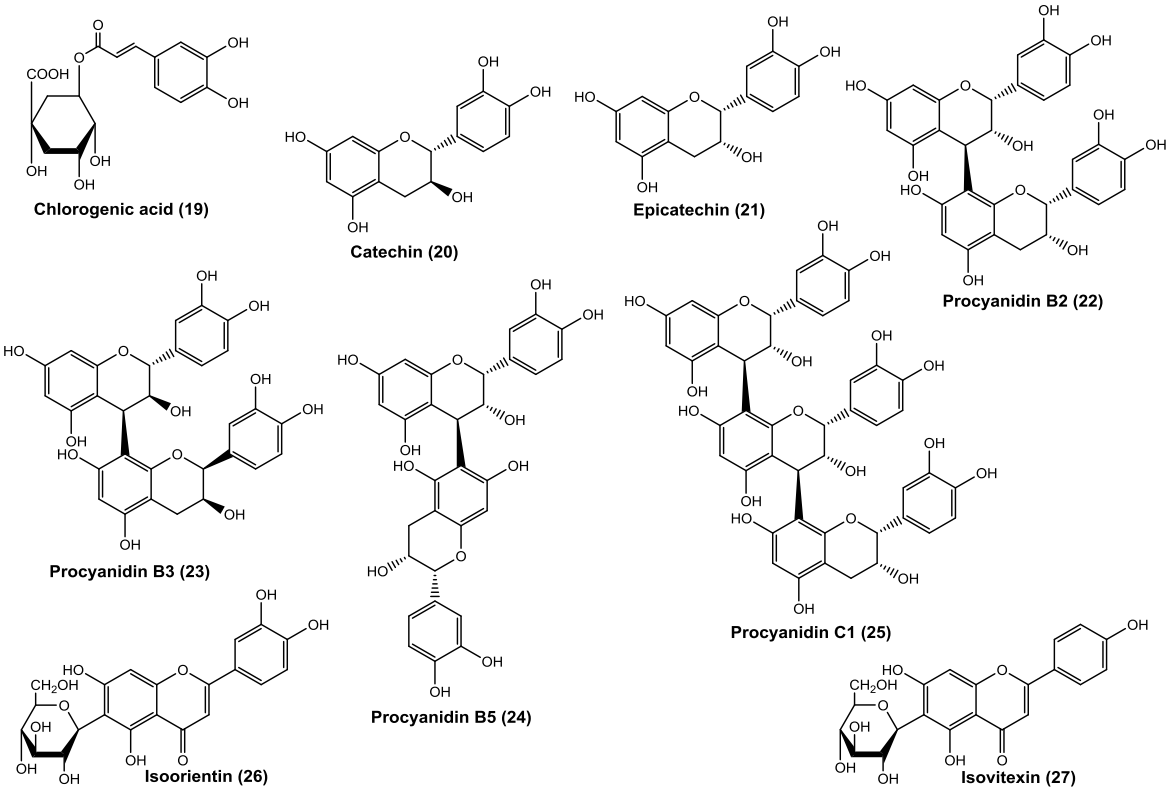


Figure 6 – Structures of chlorogenic acid (19), (+)-catechin (20), (-)-epicatechin (21), procyanidin B2 (22), procyanidin B3 (23), procyanidin B5 (24), procyanidin C1 (25), isoorientin (26) and isovitexin (27)

Secondly, it is also important to mention an experimental design of a method that evaluated the contents of chlorogenic acid and **caffeic acid (28)** extracted from *C. glaziovii* Sneth. The detection of caffeic acid in this specie was firstly reported in this paper. The HPLC-DAD method was validated and enabled the separation and quantification of the two phenolic acids [Arend *et al.*, 2011].

However, before these two studies, the flavonoids **orientin (29)** and the *O*-heteroside **isoquercetrin (30)** were also reported for *C. glaziovii* [Costa *et al.*, 2011].

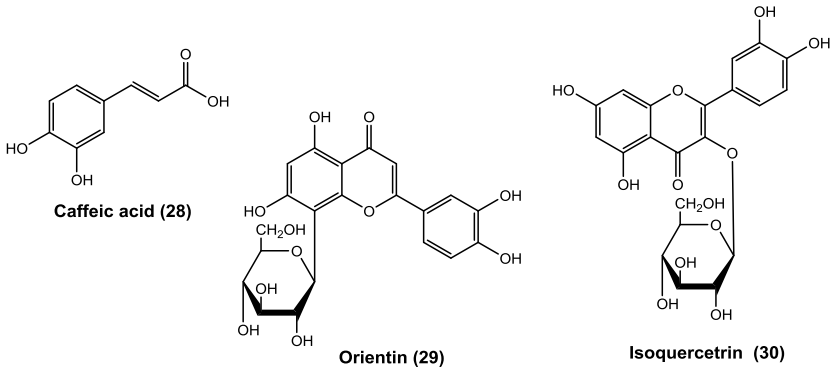


Figure 7 – Structures of caffeic acid (28), orientin (29) and isoquercetrin (30)

The already described standardized aqueous extract and its purified fractions were tested for its biological activity *in vivo* on vital physiological systems such as the cardiovascular, the respiratory, the gastrointestinal and the central nervous system (CNS), and were reported by different members of the same group of researchers [Tanae *et al.*, 2007]. Therefore, the antihypertensive activities of the aqueous extract and the butanolic fraction were compared on normotensive rats and on some validated experimental models of hypertensive rats in order to understand the hypotensive mechanism of action. The results indicated that the treatment of normotensive rats induced blood pressure reduction after repeated oral administration and this effect was probably produced by low polarity compounds as the hypotensive activity was more pronounced with the butanolic fraction. Furthermore, both extracts decreased the blood pressure of hypertensive rats and this study indicated that the plant extracts did not seem to interfere with the renin-angiotensin-system (RAS). Additionally, when hypertension of normotensive rats was induced by the prior administration of an inhibitor of nitric oxidase synthase, the subsequent oral administration of the aqueous extract caused a significant and persistent reduction in blood pressure, suggesting that the hypotensive effect may not be related to nitric oxide synthesis. The hypotensive mechanism of *C. glaziovii* might be related to specific blockade of calcium channels on smooth muscles [Lima-Landman *et al.*, 2007].

In a parallel study, the angiotensin I converting enzyme (ACE) activity of the standardized aqueous extract was evaluated in rats, in a comparative study with captopril. The known ACE inhibitor was used as a positive control on the same rats, which were also treated with a single dose of the aqueous extract and with repeated doses for 60 days. The plasma ACE activity during acute or prolonged treatment did not changed and was not correlated with the simultaneously recorded hypotension, unlike when rats were treated with captopril [Ninahuaman *et al.*, 2007].

Those antihypertensive properties that were found *in vivo* contrasted with the results obtained for other researchers who described the *in vitro* reduction of ACE activity by the dichloromethane/methanol (1:1) extract of *C. glaziovii* [Costa *et al.*, 2011; Ninahuaman *et al.*, 2007].

Even before those two aforementioned studies reported by Lima-Landman *et al.* and Ninahuaman *et al.*, the cardiovascular interest of the plant has been scientifically investigated for some time and it has been observed that animals were much calmer in the experiments when they were chronically treated with *C. glaziovii* extracts. These observations prompted the group to investigate the effects of *C. glaziovii* in the Central Nervous System (CNS). In a period prior to the chemical standardization of the aqueous extract, the group analysed the anxiolytic-like effect of the aqueous extract and two semi-purified fractions obtained from the leaves of *C. glaziovii* in mice. The behavioural data showed that the effects were not evident after a single-dose treatment but were significantly observed after the repeated treatment. They also found that the anxiolytic-like effects were 5 to 10 times greater in the semi-purified fractions [Rocha *et al.*, 2002].

The last investigation guided the group for the future biological evaluation, the antidepressant activity of *C. glaziovii*, because a dual anxiolytic and antidepressant-like activity is generally described for medicines. The standardized aqueous extract and the purified fraction were evaluated on *in vivo* forced swimming test in rats and the increased animal mobility indicated an antidepressant-like effect. This effect might be correlated with the hypotensive activity of the plant. However, in the *ex vivo* evaluation of hippocampal monoamines levels test, the neurotransmitters biochemical analysis of rats treated with the butanolic fraction demonstrated a significant increase in monoamines levels. Additionally, the study also aimed to search for the active compounds presented in plant extracts and their underlying mechanism of action by *in vitro* tests using synaptosome preparations of different brain regions necessary for the evaluation of the monoaminergic uptake. Among the isolated catechins and procyanidins, the results showed that procyanidin B3, catechin and procyanidin B2 significantly inhibited the monoamines uptake in brain synaptosomes. However, flavonoids did not blockade the monoamines

uptake. These results suggest that catechins and procyanidins may contribute to the antidepressant-like effect of *C. glaziovii* by blocking the monoamine reuptake, but pharmacokinetic studies are still needed to establish the correlation of these *in vitro* results and the *in vivo* antidepressant-like activity of *C. glaziovii* extracts [Rocha *et al.*, 2007].

As already referred, these researchers also evaluated the effects of the standardized aqueous extract and the butanolic purified fraction on the respiratory system, using unrestrained guinea pigs challenged with histamine by recording the changes in the respiratory pressure and rate. Although the plant extract effectively inhibited the histamine-induced broncospasm, this effect was mild when compared to the protection provided by ketotifen, a known H_1-histamine receptor antagonist. Furthermore, the *in vitro* assays with guinea pig tracheal preparations showed that the plant extract was non-competitive toward histamine. Therefore, the *C. glaziovii* broncospasm protection seemed to be related to other mechanism. In order to find that mechanism, the group pre-treated animals with propanolol, which resulted in the inhibition of the tracheal relaxation induced by the butanolic fraction. This β-adrenergic agonist effect was also observed *in vitro* at high concentration of the purified fractions due to have induced the relaxation of the cholinergic tonus in guinea pig tracheal preparations [Delarcina *et al.*, 2007].

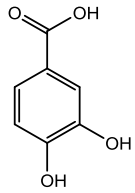
The last study with the standardized aqueous extract and the butanolic fraction of *C. glaziovii* corresponded to the assay on gastric acid secretion of pylorus-ligated mice and on acute models of gastric mucosal lesions. The results demonstrated that the intraduodenal injection of the aqueous extract decreased the volume and the total acidity of gastric secretion but without changing its pH. In turn, the butanolic extract not only reduced the volume and total acidity of the gastric secretion, but also its pH because the organic fraction was more concentrated than the aqueous extract in active compounds. Furthermore, the assays with the acute models of gastric mucosal lesions demonstrated the antiulcer activity of the butanolic fraction, since pre-treatment of animals with the butanolic fraction protected against gastric ulcers induced by stress, ethanol and indomethacin. Additionally, the group performed an *in vitro* assay with rabbit gastric H^+, K^+-ATPase preparations in order to verify the inhibitory effect of the butanolic fraction and the isolated compounds. The butanolic fraction inhibited the gastric proton pump as well as all the isolated compounds with IC_{50} values similar to the obtained with the butanolic fraction. Therefore, the antacid and antiulcer effects of *C. glaziovii* may be related to the inhibition of the gastric proton pump by the polyphenolic active constituents [Souccar *et al.*, 2008].

Lastly, the *in vitro* antiviral activity of *Cecropia glaziovii* Sneth was investigated using the crude extract obtained from the leaves of the plant and their related fractions, a butanolic fraction and a C-glycosylflavonoid-enriched fraction (confirmed by HPLC-DAD). These samples were tested on human herpes virus types 1 and 2 (HHV-1, HHV-2) replication and all the samples were active but the C-glycosylflavonoid-enriched fraction was the most active. For this reason, the group decided to evaluate the viral multiplication steps with the C-glycosylflavonoid-enriched fraction and then to elucidate the mechanism of action. The results seemed to indicate that the C-glycosylflavonoid-enriched fraction only reduced the viral infectivity of HHV-2. However, the fraction inhibited the entry into cells, inhibited the viral cell-to-cell spread and impaired envelope proteins of both viruses, as the envelope plays important rules in viral attachment and penetration. Furthermore, the group tested isovitexin and isoorientin separately and found that these compounds were both inactive against HHV-1 and HHV-2 replication, which suggested that the C-glycosylflavonoid-enriched fraction has synergistic effects that need to be studied in more detail [Silva *et al.*, 2010].

2.2. *Cecropia hololeuca*

According to Costa *et al.*, the plant *Cecropia hololeuca* Miquel was listed in the first edition of the Brazilian Pharmacopeia [Costa *et al.*, 2011] and Botsaris referred in his research article that the antimalarial proprieties of *C. hololeuca* were considered for the first time in ethnobotanical studies. Indeed, the general indications found in ethomedicinal studies were diuretic, antihypertensive, sedative, anti-inflammatory, expectorant or antiasthmatic, among others, being lately indicated as an adjuvant in the treatment of malaria that was characterised by very high fever or neurological symptoms. However, no scientific data on antimalarial activity was referred [Botsaris, 2007].

The C-glycosylflavonoids (orientin and isoorientin) and the proanthocyanidins (catechin, epicatechin, procyanidin B2 and procyanidin C1) were identified in *C. hololeuca* as these chemical groups have been described as the main constituents of the plant. Additionally, other phenolic compounds were also described, namely chlorogenic acid and **protocatechuic acid (31)** [Costa *et al.*, 2011].



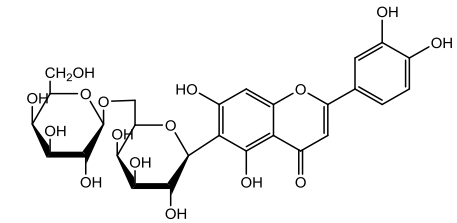
Protocatechuic acid (31)

Figure 8 – Structure of protocatechuic acid (31)

In an in *vitro* biological study, the ethanolic extract obtained from the leaves and barks of *C. hololeuca* was examined for its ACE inhibition, showing that the extract inhibited the ACE action at 0.33 mg/mL. This activity may result by a possible synergism of the compounds that constituted the extract, as C-glycosylflavonoids and proanthocyanidins were inactive when tested separately [Costa *et al.*, 2011].

2.3. *Cecropia lyratiloba*

The plant *Cecropia lyratiloba* Miquel afforded a new apigeninglycoside [**apigenin 6-*C*-galactosyl-6''-*O*-*β*-galactopyranoside (32)**], isoorientin and a mixture of orientin and isovitexin. The plant leaves were extracted with methanol and the crude extract was then partitioned with ethyl acetate in order to obtain a fraction rich in flavonoids. This fraction was then submitted to a countercurrent chromatography which demonstrated to be an effective method to resolve the flavonoid enriched fraction, enabling the isolation of the aforementioned C-glycosylflavonoids [Oliveira *et al.*, 2003].



Apigenin 6-*C*-galactosyl-6''-*O*-*β*-galactopyranoside (32)

Figure 9 – Structure of apigenin 6-*C*-galactosyl-6''-*O*-*β*-galactopyranoside (32)

In another study, the methanolic extract obtained from the dried leaves of *C. lyratiloba* was treated with different solvents and then chromatographed on a silica gel column, yielding three fractions (a terpenoid, a flavonoid and a tannin fraction). The flavonoid fraction was treated twice by HSCCC, resulting in the separation of isoorientin and a mixture of orientin and isovitexin. The effects *in vitro* of the methanolic extract and the flavonoid fraction were afterwards investigated on the contractibility of cardiac, vascular and tracheal smooth muscles from rats. Unlike the flavonoid fraction that reduced the isometric tension of the electrically stimulated papillary muscles, the methanolic extract did not affect the amplitude of contraction of these muscles, suggesting that only the flavonoid fraction may alter the cardiac contractibility. In turn, the effects on the vascular muscles were investigated in the aorta and both samples produced vascular smooth muscle relaxation in a dose-dependent manner and with the same magnitude. The role of the endothelium in the vascular contractibility was also evaluated and the group founded that vasodilatation was dependent on functional endothelium. Additionally, the mechanism of vasodilatation was investigated and the effect might be mediated by the release of nitric

oxide by the endothelium as the aortic rings were pre-incubated with a nitric oxide inhibitor. Regarding the tracheal smooth muscle, the samples had no effect in the contractile response. Finally, the isolated flavonoids were also tested on the vascular smooth muscle for the attempt to identify the active compounds. Isoorientin and the mixture of orientin and isovitexin produced lower effects than the flavonoid fraction, which may suggest a synergism in the flavonoid fraction. The group concluded that vasodilatation may contribute to the antihypertensive effect of *C. lyratiloba* [Almeida *et al.*, 2006].

Rocha *et al.* reported the isolation and the cytotoxic evaluation of four pentacyclic triterpenoids. Therefore, **euscaphic acid (33)**, **tormentic acid (34)**, **2-*α*-acetyl tormentic acid (35)** and **3-*β*-acetyl tormentic acid (36)** were isolated from the barks of *C. lyratiloba* using HSCCC and the chemical characterisation of the compounds was determined GC-MS, $_{1}$H and $_{13}$C NMR [Rocha *et al.*, 2007].

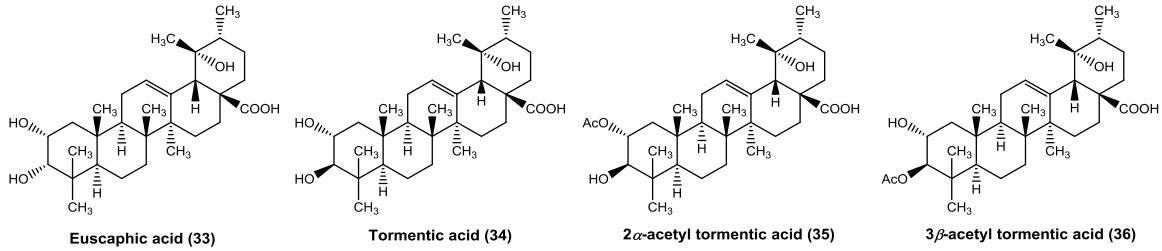


Figure 10 – Structures of euscaphic acid (33), tormentic acid (34), 2-*α*-acetyl tormentic acid (35) and 3-*β*-acetyl tormentic acid (36)

The cytotoxicity of those isolated compounds was then evaluated *in vitro* against a sensitive (K-562) and a multidrug resistant (Lucena-1) leukaemia cell lines, which overexpress the efflux pump glycoprotein P (P-gp), reducing the intracellular amount of anticancer drugs. The cytotoxic activities of the triterpenes are presented in the table 1 and all the compounds were active against both sensitive and multidrug resistant cells [Rocha *et al.*, 2007].

Table 1 – Cytotoxic activities of the pentacyclic triterpenes isolated from *C. lyratiloba*

Compound name	Cytotoxic activity (μM)		Reference
	K-562	Lucena-1	
Euscaphic acid	IC_{50} = 76.71 ± 8.23	IC_{50} = 83.79 ± 4.17	Rocha *et al.*, 2007
Tormentic acid	IC_{50} = 89.36 ± 2.23	IC_{50} = 80.25 ± 8.68	
2-*α*-Acetyl tormentic acid	IC_{50} = 38.35 ± 5.29	IC_{50} = 41.38 ± 4.16	
3-*β*-Acetyl tormentic acid	IC_{50} = 56.61 ± 9.95	IC_{50} = 72.87 ± 4.71	

The group commented the structure/activity relationship of tormentic acid since acetylation at C2 increased the activity and acetylation at C3 resulted in a milder effect. Furthermore, the mechanism of action was determined using euscaphic acid by inducing apoptosis in k-562 cells. The cell cycle analysis indicated that DNA fragmentation was dose dependent and was mediated by activation of caspase-3, which ultimately led to apoptosis. Euscaphic acid also reduced the viability of other human tumour cell lines, namely HEp-2 (human larynx carcinoma cells), Caco-2 (human colon adenocarcinoma cells) and A-549 (human breast carcinoma cells), with the IC_{50} values of 105.7 ± 0.39 μM, 104.1 ± 0.68 μM and 95.58 ± 5.14 μM respectively [Rocha *et al.*, 2007].

Lately, the same group of researchers investigated the mechanism of antineoplastic action of 3-β-acetyl tormentic acid, this time using Lucena-1 cells, and they found that the compound induced apoptosis by activation of the intrinsic apoptotic pathway, which included DNA fragmentation, caspase-3 activation and cytochrome C release from mitochondria. Interestingly, the compound did not affect the expression of the efflux pump P-gp or its activity, and beyond this, the compound was not a substrate for the transporter since 3-β-acetyl tormentic acid was able to induce apoptosis in Lucena-1 cells. These findings together with the ability of other naturally occurring triterpenes to bypass resistance and to be active against multidrug resistant leukaemia cells suggested that these compounds may represent a potential therapeutic application in chronic myeloid leukaemia [Rocha *et al.*, 2012].

2.4. *Cecropia obtusifolia*

The plant *Cecropia obtusifolia* Bertol has been traditionally used for the treatment of type 2 diabetes mellitus, especially in Mexico where it is widespread along both coasts [Andrade-Cetto and Heinrich, 2005]. Indeed, this traditional use justified the fact that the majority of *C. obtusifolia* reports have being related to its hypoglycaemic activity [Costa *et al.*, 2011].

Interestingly, there are two published clinical trials that evaluated the clinical effect of the aqueous extract of *C. obtusifolia*, in order to confirm the folk therapeutic application of the plant. In the first clinical trial, the effects of the aqueous extract of *C. obtusifolia* were compared to the effects of the extract of *Marrubium vulgare* in type 2 diabetic people, whom were monitored the blood glucose and the plasma lipids levels. The results suggested that the aqueous extract of *C. obtusifolia* may be an adjunct treatment for type 2 diabetic people with poor metabolic control, since it decreased the total serum cholesterol and triglycerides and it presented only mild and limited side effects. The trial

also indicated that *C. obtusifolia* was more effective than *Marrubium vulgare* for reducing the fasting blood glucose [Herrera-Arellano *et al.*, 2004].

In the second clinical trial, the hypoglycaemic effect of *C. obtusifolia* was evaluated in recently diagnosed type 2 diabetic patients who were only controlled with diet and exercise. The trial demonstrated a significant and sustained hypoglycaemic effect of the plant aqueous extract after 18 weeks of daily administration. The extract was not hepatotoxic, had no effect on the cholesterol or triglyceride levels and did not induce the insulin secretion [Revilla-Monsalve *et al.*, 2007].

In addition to chlorogenic acid and isoorientin that have been reported as the most abundant compounds in *C. obtusifolia* extracts, being chlorogenic acid the acclaimed responsible for the hypoglycaemic activity, the compounds ***β*-sitosterol (37)**, **stigmasterol (38)** [Andrade-Cetto and Heinrich, 2005], **stigmast-4-en-3-one or *β*-sitostenone (39)**, **4-cholestene-3,24-dione (40)**, **4,22-cholestadien-3-one (41)**, **vanillic acid (42)**, **4-vinyl-2-methoxy-phenol (43)**, **2-methylbenzaldehyde (44)**, **2,3-dihydrobenzofuran (45)** and **3'-methoxyacetophenone (46)** were also identified in *C. obtusifolia* [Guerrero *et al.*, 2010].

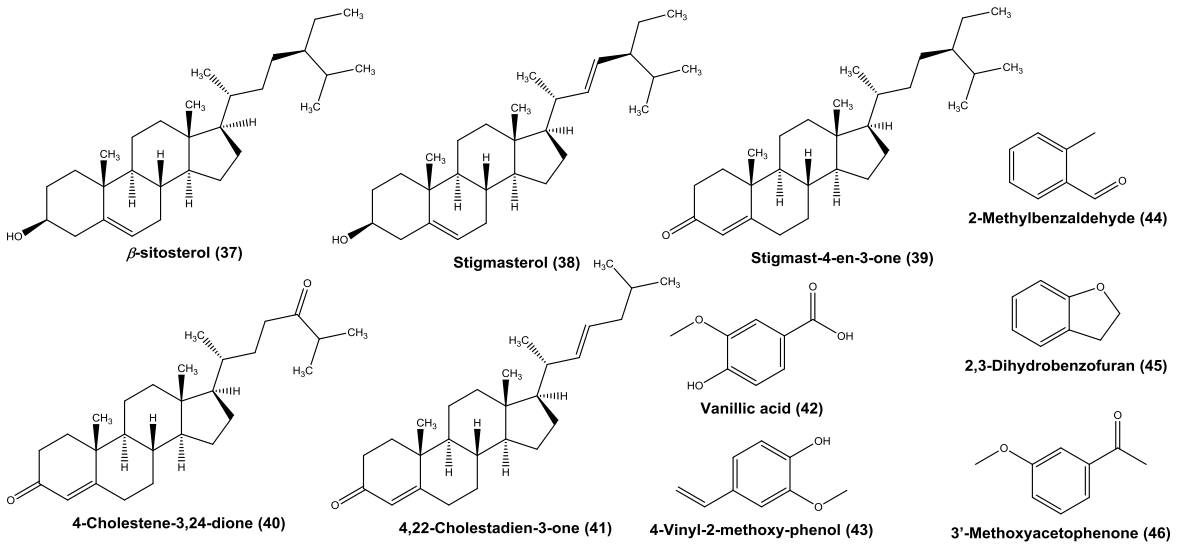


Figure 11 – Structures of *β*-sitosterol (37), stigmasterol (38), stigmast-4-en-3-one (39), 4-cholestene-3,24-dione (40), 4,22-cholestadien-3-one (41), vanillic acid (42), 4-vinyl-2-methoxy-phenol (43), 2-methylbenzaldehyde (44), 2,3-dihydrobenzofuran (45) and 3'-methoxyacetophenone (46)

There are several biological studies that aimed to confirm the phytomedicinal value of *C. obtusifolia* and that tried to elucidate the mechanism of hypoglycaemic action. For example, in one study, the aqueous and butanolic extracts obtained from the leaves and the two identified and isolated chemical constituents, chlorogenic acid and isoorientin,

were tested in streptozotocin induced diabetic rats, showing significant hypoglycaemic effects 180 minutes after oral intake [Andrade-Cetto and Wiedenfeld, 2001].

In another study, Nicasio and colleagues investigated the hypoglycaemic activity of the methanolic extracts of *C. obtusifolia* and *C. peltata* and correlated this activity with the chlorogenic acid content of the extracts. They found that the hypoglycaemic effect was significantly different between the two species, since *C. peltata* showed a greater reduction in glucose level in non-diabetic rats and it was assumed that this effect was correlated to the highest content of chlorogenic acid in the methanolic extract of *C. peltata* [Nicasio *et al.*, 2005].

None of the two mentioned studies have focussed on the elucidation of the mechanism of action, although some hypotheses have been suggested. In the attempt to understand the mechanism of action, Andrade-Cetto and co-workers tested the effect of *C. obtusifolia* extract on α-glycosidase activity *in vitro* and demonstrated that the extract presented a higher inhibitory activity than acarbose, a known α-glycosidase inhibitor. The authors assumed that one or more compounds existing in the extract inhibited the enzyme besides other possible mechanism [Andrade-Cetto *et al.*, 2008].

In another study, the antidiabetic mechanism of action of *C. obtusifolia* aqueous extract and its active compound chlorogenic acid were investigated *in vitro* for the glucose uptake in insulin-sensitive and insulin-resistant murine adipocytes. The results indicated that both extract and chlorogenic acid may exert their hypoglycaemic effect by mimicking or potentiating the action of insulin which stimulates the glucose uptake in adipocytes. The authors did not rule out the possibility of other compounds present in the aqueous extract may have the same action as chlorogenic acid [Alonso-Castro *et al.*, 2008].

In addition, the gluconeogenesis inhibitory effects of extracts from *C. obtusifolia* and *C. peltata* were tested *in vivo* by a pyruvate tolerance test performed in 18 hours-fasted rats and *in vitro* by inhibiting glucose-6-phosphate translocase in intact rat liver microsomes. On one hand, after a fasting period, the extracts of both plants and also chlorogenic acid were able to block the glucose production by administrating pyruvate, a source for hepatic glucose production. On the other hand, the IC_{50} values that were obtained for the extracts of both plants were similar, which suggested that the same compounds may be responsible for the enzyme inhibition and then for the blockade of gluconeogenesis. The group analysed the content of extracts by HPLC-DAD and they confirmed that chlorogenic acid and isoorientin were the main components on both plants [Andrade-Cetto and Vázquez, 2010].

As demonstrated, the interest of *C. obtusifolia* has been centred on its antidiabetic properties. However, other pharmacological effects were also reported for this specie. Indeed, for example in Panamá, *C. obtusifolia* has been traditionally used for the treatment of cardiovascular diseases like hypertension. Caballero-George and colleagues reported the *in vitro* effects of extracts obtained from leaves and stems of *C. obtusifolia* in the angiotensin II AT_1 receptor, neuropeptide Y Y1 receptor and endothelin-1 ET_A receptor by radioligand-binding techniques. All the receptors are involved in the vasoconstrictor phenomena when activated for endogenous ligands. As diverse extracts were tested, the AT_1 receptors were competitively inhibited by the methanol:dichloromethane (1:1) extract obtained from the leaves (inhibition of 80 ± 16% at 100 µg/mL) and also by the methanol:dichloromethane (1:1) extract obtained from the stems (inhibition of 58 ± 2% at 100 µg/mL). The tannin content was then removed from the stem extract in order to eliminate the nonspecific binding of these compounds. However, the effect was reduced to 20 ± 5% of inhibition at 100 µg/mL. The plant had almost no effect to Y1 receptors and the inhibition of ET_A receptors for the ethanolic extracts was 51 ± 12% and 63 ± 7% when obtained respectively from the stems and the leaves [Caballero-George *et al.*, 2001].

In another study, the vasoactive effects of *C. obtusifolia* collected in Panamá were also evaluated, this time, on endothelium-intact aortic preparations precontracted with phenylephrine. The results showed that the acid and neutral fractions of the methanolic and the dichloromethane extracts obtained from the aerial parts of the plant produced vasorelaxant effects, being about 50% of relaxation for the acid methanolic, neutral methanolic and acid dichloromethane fractions and about 20% of relaxation for the neutral dichloromethane fraction. In parallel, the group evaluated the inhibition of contractile responses to angiotensin II on endothelium-intact aortic rings isolated from spontaneously hypertensive rats, where they tested the same fractions. The group found that none of the fractions reduced the angiotensin II aortic contraction, which agreed with the *in vivo* findings of Lima-Landman *et al.* for *C. glaziovii* but contrasted with the reports of Caballero-George *et al.* in the *in vitro* blockade of AT_1 receptors for *C. obtusifolia*. The differences may result from the extractive processes that may afford extracts with substantially different chemical contents [Guerrero *et al.*, 2010].

Finally, the aqueous extract of *C. obtusifolia* was evaluated for its analgesic, anti-inflammatory and SNC depressant effects in different experimental models. This study was based on the traditional uses of *C. obtusifolia* in El Salvador, since the plant leaves are used as sedative as well as anti-inflammatory and pain-killer for the treatment of arthritis and rheumatism. The aqueous leaf extract showed a substantial central depressor effect because it reduced the animal exploratory capacity, it suppressed the escape

capacity and produced significant motor incordination. The analgesic activity was only confirmed for the chemical stimuli tests and not for the hot plate test, which suggested the peripherial analgesic effect rather than the central analgesic effect. The study also demonstrated the topical and the systemic anti-inflamatory effects of the aqueous leaf extract of *C. obtusifolia* [Pérez-Guerrero *et al.*, 2001].

2.5. *Cecropia pachystachya*

As some of the aforementioned species of *Cecropia*, the plant *Cecropia pachystachya* Trécul has been also traditionally used in folk medicine as a cardiotonic and a diuretic herbal medicine, as well as for the treatment of cough and asthma [Consolini *et al.*, 2005]. The leaves and barks of *C. pachystachya* are also used for its hypoglycaemic effects [Aragão *et al.*, 2010]. The plant grows in the Neotropical regions of the Northeast of Argentina, Paraguay and the Southern region of Brazil. The plant also grows in the temperate hilly grasslands of central Argentina. The accepted phytomedicinal applications allowed the plant to be described in the sixth edition of the Argentinean National Pharmacopeia [Consolini and Migliori, 2005].

The effects of *C. pachystachya* were reported by Consolini and colleagues in two different papers, using samples collected in two phytogeographical regions of Argentina (Neotropical and temperate areas) in order to understand the impact of growing in different geographical areas on the pharmacological profile. In the first study, the group compared the *in vivo* cardiovascular effects on rats of the aqueous extracts obtained from the different geographical samples. The sample collected in the Neotropical area possessed stronger hypotensive effect than the sample collected in the temperate area. Another difference between the samples was their effects on heart rate, which was only increased by the temperate sample. However, none of the samples produced a diuretic effect on rats [Consolini and Migliori, 2005].

In the second study, the group evaluated the cardiac and the sedative effects of the plant. The aqueous extract obtained from the temperate area was tested on isolated cardiac preparations because this extract showed a positive chronotropic effect in the previous study. The group found a transitory positive inotropic effect on the isolated heart by measuring contractility at constant rate. Additionally, both aqueous extracts collected in the different geographical areas were evaluated *in vivo* on the open-field test, where they observed the exploratory behaviour and the spontaneous locomotion of mice. The Neotropical sample potentiated the anxiolytic dose of diazepam but not its sedative dose. However, both extracts showed a similar sedative effect that was not antagonized by

flumazenil, which suggested that the extracts may act in the GABA-A receptors but they not bind to the alosteric site of benzodiazepines [Consolini *et al.*, 2006].

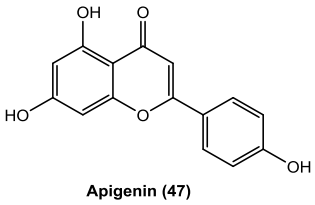
The authors also performed a qualitative comparison in the chemical composition of both geographically distant extracts. The HPLC analysis did not reveal qualitative differences between the chromatographic profiles of the samples but it revealed quantitative differences as the areas of some peaks differed between chromatograms. By using a specific reagent in the TLC analysis, the group indicated that the chromatogram of the temperate sample presented six spots of flavonoids while the chromatogram of the Neotropical sample had only four spots of flavonoids [Consolini *et al.*, 2006]. Despite the evidence presented, the content differences may be responsible for the differences in the pharmacological profiles of the samples. It is true that the chemical evaluation of the extracts was not the principal objective for the authors as they had referred, but further chemical investigation would be necessary to better understand the observed differences between the two geographical areas and to know the compounds that presented those activities.

Unlike the previous study, the methanolic extract obtained from the leaves of *C. pachystachya* was initially analysed by TLC in another study, allowing the detection of two characteristic flavonoid colour spots. The HPLC-DAD analysis revealed the presence of chlorogenic acid and the flavonoids, orientin and isoorientin. The UV spectroscopic identification of the first compound was carried out by comparing with the reference compound and the flavonoid peaks were identified by comparing UV-DAD spectra with data found in literature. In this study, the researchers also evaluated the hypoglycaemic effect of *C. pachystachya* in normal and alloxan induced diabetic rats. The hypoglycaemic effect of the extract was observed and no significant difference was observed between animals treated with the extracts and those treated with oral antidiabetic drugs (metformin and glibenclamide) [Aragão *et al.*, 2010].

The group of researchers Uchôa *et al.* evaluated several extracts of *C. pachystachya* obtained from different parts of the plant and conducted a bioassay-guided fractionation, testing their antimalarial activity against *Plasmodium berghei* in malaria-infected mice. The ethanolic extracts obtained from woods, roots and leaves reduced parasitemia of malaria-infected mice. Because the root extract showed the strongest activity, this extract was further partitioned in subfractions and analysed *in vivo*. This bioguided assay resulted in the isolation of β-sitosterol and tormentic acid, which were in turn evaluated *in vivo* and also *in vitro* against the human parasite *Plasmodium falciparum*. The former compound showed a borderline activity whereas the latter compound effectively inhibited the parasite

growth, which indicated that this terpenoid may be a good candidate for the development of an antimalarial drug [Uchôa *et al.*, 2010].

The antiparasitic properties of *C. pachystachya* were also reported in another study. This time, the antileishmanial activity of the plant was evaluated by the inhibition of arginase, an enzyme that plays an important role in the biosynthesis of polyamines which are therefore required for cell proliferation. The *Leishmania (Leishmania) amazonensis* promastigote survival/proliferation and the *in vitro* arginase activity were investigated using the ethanolic extract of the plant and the subsequent subfractions obtained by the treatment with different solvents. The ethyl acetate fraction presented the best results for the inhibition of promastigote growth and the arginase inhibition, as well as, it changed the promastigote cellular organization which was observed by qualitative ultrastructural analysis. The major bioactive molecules of the ethyl acetate fraction were characterized as glycoside flavonoids using LC-ESI-MS. The group identified **apigenin (47)**, orientin and isovitexin, being orientin the main component of the ethyl acetate fraction [Cruz *et al.*, 2013].



Apigenin (47)

Figure 12 – Structure of apigenin (47)

The specie *C. pachystachya* was also studied for the anti-inflammatory, antinociceptive and cytotoxic effects. The anti-inflammatory activity of the methanolic leaf extract was tested *in vivo* in mice, both for oral and topical administrations. Therefore, the extract was not only effective *per os* and statistically similar to indomethacin, but also it presented an effect similar to dexamethasone when used topically. Additionally, the tests performed *in vivo* to evaluate the antinociceptive effect of the extract demonstrated that this effect might be achieved by decreasing the sensitivity of peripheral nociceptive receptors, which is characteristic of the lipoxygenase and cyclo-oxygenase inhibitors. Finally, the cytotoxic activity of the extract was evaluated against Jurkat cells (human T cell lymphoblast-like cells) and HL60 cells (human promyelocytic leukaemia cells), two wild-type leukaemia cells lines, along with HL60.Bcl-2 cells which are HL60 cells with ectopic expression of *Bcl-2*, known to be resistant to anti-cancer drugs. The effects of the extract on the viability of the panel of cancer cell lines are presented in the table 2, where are also presented the results for etoposide. This drug was not able to reduce the viability of HL60.Bcl-2 cells as

the extract did, which suggested that the extract may be composed for pro-apoptotic substances that interfere with the expression of the *Bcl-2* family in HL60 cells [Aragão *et al.*, 2013].

Table 2 – Effect of the methanolic extract from *C. pachystachya* on the viability of the panel of cancer cell lines

Extract / Compound	% Inhibition of viability			Reference
	HL60 cells	HL60.Bcl-2 cells	Jurkat cells	
Methanolic extract	56.9 ± 11.6	54.6 ± 2.5	5.1 ± 14.3	Aragão *et al.*, 2013
Etoposide	80.8 ± 6.3	27.1 ± 12	69.2 ± 8.2	

There was another report on the cytotoxic activity of *C. pachystachya*. The leaf methanolic extract was tested towards B16 (murine melanoma cells), HL60, MCF-7 (human breast adenocarcinoma cells) and HCT-8 (human colon carcinoma cells). The table 3 presents the cytotoxic activity data of the extract, which inhibited the cellular proliferation of all the cell lines above 90% but the obtained IC_{50} values were above 125 µg/mL except for HL60 cells [dos Santos Júnior *et al.*, 2010].

Table 3 – Cytotoxic activity data of the methanolic extract from *C. pachystachya* Trécul

Cytotoxic activity	Panel of tumour cell lines				Reference
	B16 cells	HL60 cells	MCF-7 cells	HCT-8 cells	
Cell growth inhibition percentage (GI%) (%)	92.2	106.7	97.5	97.4	dos Santos Júnior *et al.*, 2010
50% inhibitory concentration (IC_{50}) (µg/mL)	> 125	95	> 125	> 125	

Besides the aforementioned studies with references to the chemical composition of the extracts, the specie also afforded **pomolic acid (48)**, **ursolic acid (49)**, **oleanolic acid (50)**, ***α*-amyrin (51)**, **19-*α*-hydroxy-*α*-amyrin (52)** and *β*-sitostenone. The flavonoid *O*-heteroside isoquercetrin was also reported in the *C. pachystachya* specie. Addiitonally, the phenolic compounds chlorogenic acid and protocatechuic acid were also described for *C. pachystachya*. Finally, the proanthocyanidins catechin, epicatechin, procyanidin B2 and procyanidin C1 were also identified [Costa *et al.*, 2011].

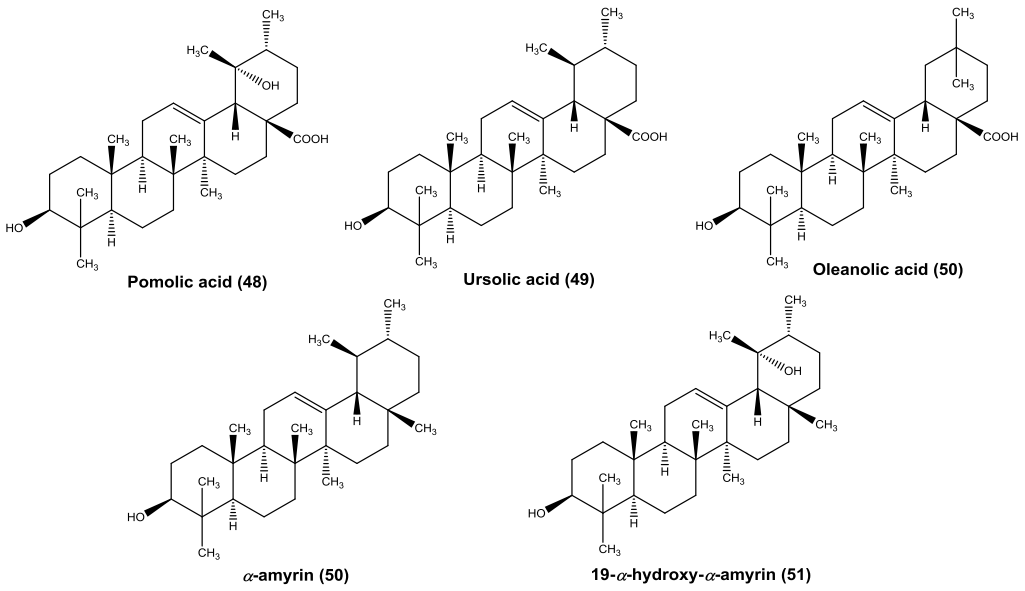


Figure 13 – Structures of pomolic acid (48), ursolic acid (49), oleanolic acid (50), α-amyrin (51) and 19-α-hydroxy-α-amyrin (52)

2.6. *Cecropia peltata*

The hypoglycaemic effect of *Cecropia peltata* L. was already mentioned in the corresponding section of *C. obtusifolia*, where that effect was compared between the plants in two different studies. Summarily, on one hand, the hypoglycaemic effect of *C. peltata* was superior to the effect of *C. obtusifolia*, which was correlated with the chlorogenic acid contents of the extracts [Nicasio et al., 2005]. On the other hand, extracts of both plants seemed to inhibit gluconeogenesis, which was evaluated *in vivo* and *in vitro* [Andrade-Cetto and Vázquez, 2010].

Besides its hypoglycaemic effect, the specie *C. peltata* is also traditionally used for wound healing. The Nayak's study regarded the wound-healing properties of the aqueous and ethanolic extracts, which where tested by topical and oral administration using the excision wound model in rats. The data showed that both extracts administrated by both routes reduced the wound areas. Additionally, the histopathologycal evaluation of the healing tissue obtained from the treated animals indicated a good organization of the tissue, which may indicate that some constituents of *C. peltata* extracts stimulate epithelialisation [Nayak, 2006].

Finally, the antimicrobial activity of *C. peltata* was investigated *in vitro* against four bacteria (*Staphylococcus aureus*, *Streptococcus β hemolitic*, *Bacillus cereus* and *Escherichia coli*) and against yeast (*Candida albicans*). As showed in the table 4, the aqueous, the ethanolic and/or the hexane extracts of the plant were tested against the

refered microorganisms. The results corroborated with antimicrobial activity of *C. peltata* which is used in Colombian folk medicine to treat infections [Rojas *et al.*, 2006].

Table 4 – Antimicrobial activity of *C. peltata* L.

Extract	Minimum inhibitory concentration (µg/mL)					Reference
	Staphylococcus aureus	***Streptococcus β hemolitic***	***Bacillus cereus***	***Escherichia coli***	***Candida albicans***	
Aqueous extract	NT	NT	NT	54.7 ± 6.8	NT	Rojas *et al.*, 2006
Ethanolic extract	16.6 ± 0.8	NT	14.9 ± 3.4	3.6 ± 0.2	NT	
Hexane extract	NT	20.2 ± 2.9	NT	2.9 ± 0.6	21.5 ± 6.3	

NT: Not tested

2.7. *Cecropia schreberiana*

In this section, it will be presented a phytochemical investigation on the specie *Cecropia schreberiana* Miquel. The plant was identified by one of the authors of the original article, which reports the first chemical characterization of the phytochemical constituents of the specie *C. schreberiana*. The acetone crude extract obtained from the leaves was partitioned with different solvents and the resulting fractions were treated by diverse methods such as chromatography on silica gel open columns and semi-preparative RP-HPLC. The plant afforded four triterpenoids that were pomolic acid, tormentic acid, α-amyrin and **arjunolic acid (53)**. The isolated flavone-C-glycosides were **vitexin (54)**, orientin and isoorientin. **Cinchonain Ia (55)** and **cinchonain Ib (56)** were firstly isolated in the family *Urticaceae*. Finally, catechin and epicatechin were also isolated from the plant as well as the procyanidins B2 and B5. All the isolated compounds were identified by few methods, namely specific rotation, $_{1}$H and $_{13}$C NMR spectra, whose experimental data were compared with the data already reported [Li *et al.*, 2013].

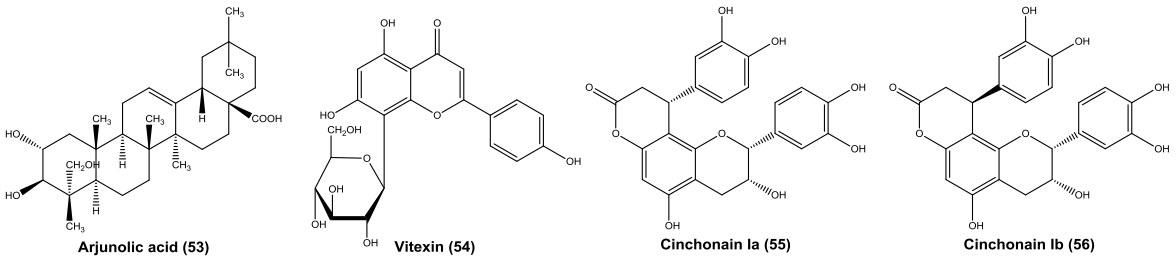


Figure 14 – Structures of arjunolic acid (53), vitexin (54), cinchonain Ia (55) and cinchonain Ib (56)

2.8. *Cecropia catarinensis*

Unlike other species of the genus *Cecropia*, there are few descriptions on the traditional use of the specie *Cecropia catarinensis* Cuatrecasas. However, the authors Almeida *et al.* mentioned that the specie *C. catarinensis* has been used for the treatment of asthma and high blood pressure and it is also used as a diuretic and an antispasmodic agent [Almeida *et al.*, 2006]. Additionally, the specie has not been much studied, which indicates that the folk medicine applications of the plant have not been scientifically checked yet. In fact, concerning the evaluation of the cytotoxic and the microbial activities of the plant, we are the first group to investigate the extracts, fractions and the pharmacologically active components of the specie for the aforementioned activities. However, before moving on to the study of our plant, it is important to identify a study that focussed on the chemical investigation of the plant, which was conducted Machado and colleagues.

Therefore, the article reported the phytochemical study of roots stems and leaves of the plant, which afforded a total of eight compounds. The roots were successively extracted with hexane, dichloromethane and ethyl acetate. The hexane extract yielded β-sitosterol and 2α-acetyl tormentic acid. The compounds **2*α*-acetyl euscaphic acid (57)**, **2*α*-acetoxy-3*β*,19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide (58)** and **3*β*-acetoxy-2*α*,19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide (59)** were isolated from the dichloromethane extract as well as tormentic acid and an inseparable mixture of ursolic and oleanolic acids. The ethyl acetate extract afforded further amounts of tormentic acid, 2*α*-acetyl tormentic acid, 2*α*-acetoxy-3*β*19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide, 3*β*-acetoxy-2*α*,19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide and 2*α*-acetyl euscaphic acid and also euscaphic acid, pomolic acid and **2*α*,3*α*,23-trihydroxy-olean-12-en-28-oic acid (60)**. The stems were similarly treated as the roots were, yielding the same compounds except 2*α*-acetoxy-3*β*19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide and 3*β*-acetoxy-2*α*,19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide. Finally, the leaves were extracted with methanol and then partitioned between hexane and a mixture of methanol and water (9:1). This last fraction was evaporated and extracted with chloroform, giving a mixture of triterpenes, and then with butanol, affording a mixture of *C*-flavonoid glycosides (vitexin, isovitexin, orientin and isoorientin) [Machado *et al.*, 2008].

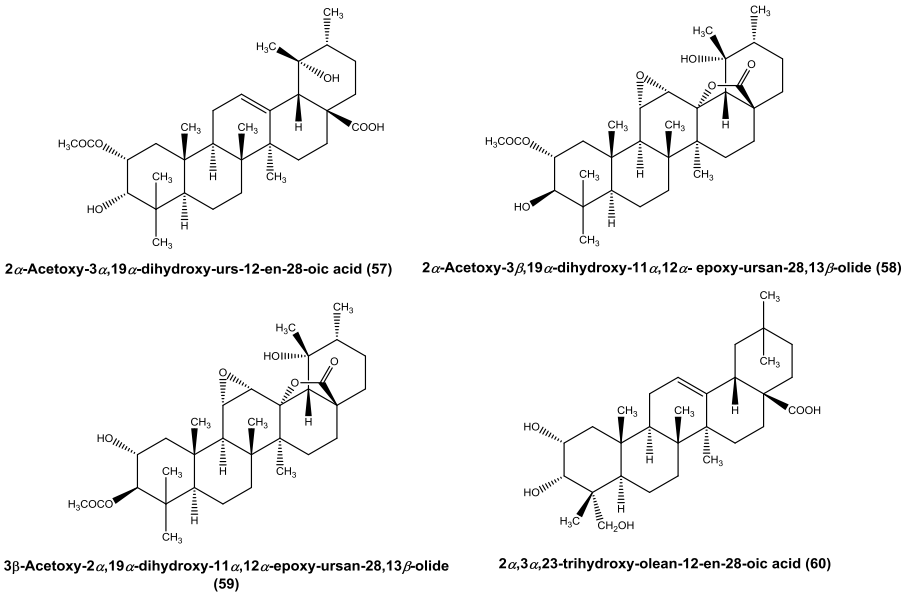


Figure 15 – Structures of 2*α*-acetyl euscaphic acid (57), 2*α*-acetoxy-3*β*,19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide (58), 3*β*-acetoxy-2*α*,19*α*-dihydroxy-11*α*,12*α*-epoxy-ursan-28,13*β*-olide (59) and 2*α*,3*α*,23-trihydroxy-olean-12-en-28-oic acid (60)

As noted throughout this chapter, it is possible that some of the mentioned compounds may be isolated or identified in our plant. However, the specie *C. catarinensis* is still poorly studied, which increases the hypothesis of finding unpublished secondary metabolites for this specie. The next chapter corresponds to the experimental procedures of the present biological and chemical studies of our plant material.

Chapter 3

Experimental procedures



http://www.ls.wisc.edu/research-ors.html;
http://www.picstopin.com;
http://www.dreamstime.com/stock-image-96-well-plate-beaker-image5595471;
http://www.bacto.com.au/culture-media/.

3. Experimental procedures

3.1. Materials and methods

Silica gel column chromatography (0.04 – 0.063 mm, Mn silica gel 60 M, Machery-Nagel) was used to purify the CME1 and the groups G4 and G5.

For preparative TLC, chromatographic plates (20 x 20 cm, 0.25 mm thickness) were prepared in the laboratory with silica gel 60 GF_{254} (Merck) as stationary phase.

For analytical TLC, those prepared chromatographic plates were also used, as well as, the pre-coated aluminium sheets (20 x 20 cm) Alugram® Sil G/UV_{254} (Machery-Nagel).

To elute both analytical TLC and preparative TLC, it was added 6 six drops of formic acid to each 100 mL of eluent.

Both analytical TLC and preparative TLC were physically revealed with UV lamps at 254 nm and 365 nm and were chemically developed by spraying a mixture of sulphuric acid and methanol (4:1); the plates or the pre-coated sheets treated with the referred mixture were then heated at 100ºC for 5-6 minutes. In the preparative TLC, the chemical development was only executed in a thin portion of the left border of the plate.

The used solvents were petroleum ether 60-80º (Merck), dichloromethane (VWR), chloroform (VWR), acetone (Atom Scientific; Carlo Erba) and methanol (VWR, Sigma-Aldrich). All the solvents possessed the “pure” or “pro-analysis” degree of purity.

Solvents were evaporated at reduced pressure, using a waterbath Büchi B-480 at 40ºC, a rotavapor Büchi R-114 and a vacuum controller Büchi V-850.

Melting points were obtained on a Köfler microscope and were uncorrected.

GC-MS analysis was performed in a Finnigan GC-MS system. The chromatographer was a Trace GC 2000 Series (ThermoQuest CE Instruments), the mass spectrometer coupled was a GCQPlus Finnigan (ThermoQuest) and the software for data acquisition and treatment was Xcalibur® 1.0. The capillary column was Rtx® - 5MS (Restek Crossbond® 5% diphenyl-95% dimethylpolysiloxane), with 30 metres, 0.25 mm of internal diameter and 0.25 µm df, and with an intermediate polarity. The vector gas was helium, scoring 1.5 ml/min. The chromatographer operated in the following conditions: injection in a *splitless* mode; volatilisation temperature at 250°C; oven’s initial temperature of 80°C for 5 minutes, increasing 10°C/min until the final temperature of 300°C. For the mass spectrometer, the transference line temperature was 275°C and the ion source temperature was 210°C; the ionization was achieved by electronic impact at 70eV and the

analyser was an “ion trap” type. For the qualitative analysis, the detector was used in TIC (total ion chromatogram) mode, with a mass interval m/z = 50-650.

^{1}H and ^{13}C NMR spectra were recorded in deuterated chloroform or deuterated DMSO (Deutero GmbH) at room temperature on a Bruker Avance-300 MHz instrument operating at 300.13 and 75.47 MHz, respectively. Chemical shifts were represented as ppm (δ). The coupling constants (J) were represented in Hertz (Hz).

The antifungal and antibacterial activities were evaluated according to the clinical and laboratory standards institute (CLSI) for the determination of MIC (minimum inhibitory concentration) and MLC (minimum lethal concentration) by a broth microdilution method.

The *in vitro* growth inhibition activities were evaluated on three human tumour cell lines (MCF-7, NCI-H460 and A375-C5 cells), according to the procedure adopted in the NCI for the “*In vitro* anticancer drug screening” - SRB assay. The absorbance measurement at 492 nm was performed in the microplate reader BIORAD 680 model and the absorbance values were retrieved using Gene5 *software* (Biotek).

3.2. Plant material

The leaves of *Cecropia catarinensis* were collected in Mendes (Rio de Janeiro, Brazil) by Professor Douglas Siqueira de Almeida Chaves from the Universidade Federal Rural do Rio de Janeiro (Brazil) and were identified by Professor Jorge Pedro Carauta. A voucher specimen was deposited in the Museu Nacional do Rio de Janeiro (Brazil) under the number R212.173.

3.3. Chemical studies

3.3.1. Extraction and treatment of the leaf crude extract

The leaves of *C. catarinensis* were dried during 78 hours at room temperature and the dried material was then mechanically triturated. The extractive procedure is schematized in the figure 16. The crude methanolic extract (CME) was obtained using 1 kg of the triturated material of *C. catarinensis* by exhaustive extraction with 5 L of methanol at room temperature. The obtained macerate was filtered and concentrated at reduced pressure by evaporating the solvent, which yielded 152.22 g of CME.

In the next step, 150.29 g of CME were treated with 10 L of dichloromethane and were exhaustively extracted at room temperature. The resulting soluble fraction was then filtered and concentrated at reduced pressure, which yielded 11.60 g of a fraction identified as F1. The resulting insoluble fraction was in turn treated with 11 L of ethyl acetate, which afforded a soluble fraction that was also filtered and concentrated at

reduced pressure, yielding 28.13 g of a fraction identified as F2. The fractions F1 and F2 were analysed by TLC and had a similar chromatographic profile, which allowed the combination of both fractions to give a less polar semi-purified fraction named CME1 (38.20 g).

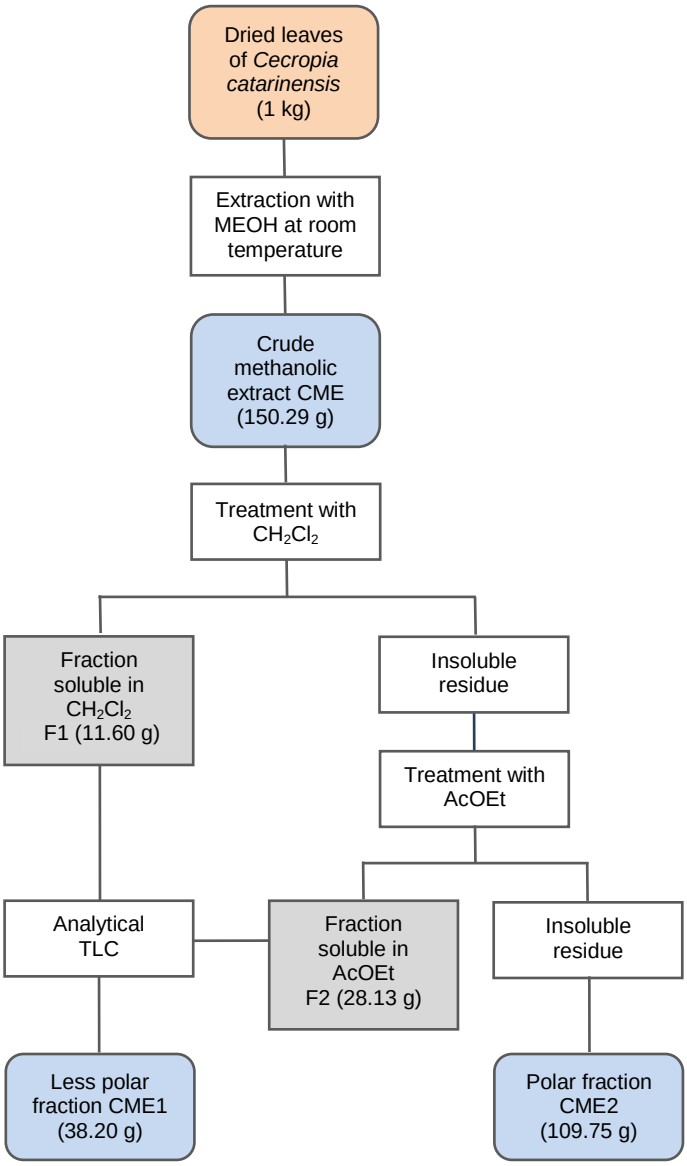


Figure 16 – Extractive process of the dried plant material and treatment of the methanolic crude extract

The insoluble residue that resulted from the treatment and extraction with ethyl acetate was finally concentrated at reduced pressure, yielding 109.75 g of a polar semi-purified fraction that was identified as CME2.

3.3.2. Purification of the non-polar extract CME1

The semi-purified fraction CME1 with lower polarity was chemically investigated in order to isolate active compounds. The purification process started with a chromatography on silica gel column which was eluted with organic solvents (petroleum ether, dichloromethane, acetone and methanol) and mixtures of them by increasing the gradient of polarity. The table 5 resumes the fractionation process of CME1.

Table 5 – Fractions obtained from the purification of CME1

Fractions	Eluents
F1 – F28	Petroleum ether 60-80º
F29 – F52	Petroleum ether 60-80º + dichloromethane (9:1)
F53 – F83	Petroleum ether 60-80º + dichloromethane (7:3)
F84 – F109	Petroleum ether 60-80º + dichloromethane (1:1)
F110 – F143	Petroleum ether 60-80º + dichloromethane (3:7)
F144 – F183	Petroleum ether 60-80º + dichloromethane (1:9)
F184 – F208	Dichloromethane
F209 – F242	Dichloromethane + acetone (9:1)
F243 – F270	Dichloromethane + acetone (7:3)
F271 – F296	Dichloromethane + acetone (1:1)
F297 – F324	Dichloromethane + acetone (3:7)
F325 – F337	Acetone
F338 – F346	Acetone + methanol (9:1)
F347	Methanol

This column chromatography produced 347 fractions of 250 mL that were then gathered in 14 groups (G1 to G14) according to the chromatographic profile of the fractions evaluated by TLC analysis. The table 6 identifies the groups, the fractions and the weight of each group. The yield of the process was 88.38%.

Table 6 – Groups obtained from the fractionation of CME1

Groups	Fractions	Weight (g)
G1	F1 – F15	1.890
G2	F16 – F45	0.145
G3	F46 – F106	1.115
G4	F107 – F140	0.645

Table 6 (continued) – Groups obtained from the fractionation of CME1

Groups	Fractions	Weight (g)
G5	F141 – F182	0.566
G6	F183 – F208	0.216
G7	F209 – F214	2.020
G8	F215 – F231	1.864
G9	F232 – F242	0.680
G10	F243 – F296	8.920
G11	F297 – F324	2.910
G12	F325 – F337	1.080
G13	F338 – F345	1.160
G14	F346 – F347	10.550

3.3.2.1. Purification of group G2

Since the group G2 weighted 0.145 g, the purification of G2 started with a first preparative TLC. The sample was dissolved using a mixture of petroleum ether and dichloromethane (3:7) and the chromatographic eluent was a mixture of petroleum ether and dichloromethane (1:9). After revelation with UV lamp (254 nm), the target fraction (Rf 0.23) was collected and extracted with a mixture of petroleum ether and dichloromethane (3:7), yielding 0.053 g.

According to the analytical TLC of that first purification step, the target fraction needed to be re-purified. The re-purification of that fraction was carried out by another preparative TLC that was performed with a mixture of petroleum ether, dichloromethane and acetone (1:8:1) as eluent. After revelation with UV lamp (254 nm), the target fraction (Rf 0.82) was then extracted with a mixture of petroleum ether and dichloromethane (3:7). This second putrification step resulted in the isolation of **compound 1** (0.013 g).

3.3.2.2. Purification of group G4+5

A previous analytical TLC of the groups G4 (0.645 g) and G5 (0.566 g) demonstrated that both groups could be combined in the same purification process. Therefore, the groups were fractionated together in the same column as a new fraction G4+5 (1.211 g), using 160 g of silica gel. The sample was dissolved in a mixture of petroleum ether and dichloromethane (3:7) and the column was eluted with different mixtures of solvents in an increasing gradient of polarity. The fractions were eluted from the column as indicated in the table 7 and 516 fractions were collected with 50 mL each one.

Table 7 – Fractions obtained from the purification of group G4+5

Fractions	Eluents
F1 – F90	Petroleum ether 60-80º + dichloromethane (3:7)
F91 – F267	Petroleum ether 60-80º + dichloromethane (1:9)
F268 – F317	Dichloromethane + acetone (9:1)
F318 – F348	Dichloromethane + acetone (7:3)
F349 – F376	Dichloromethane + acetone (1:1)
F377 – F392	Dichloromethane + acetone (3:7)
F393 – F409	Acetone
F410 – F448	Acetone + methanol (7:3)
F449 – F472	Acetone + methanol (1:1)
F473 – F491	Acetone + methanol (3:7)
F492 – F516	Methanol

The collected fractions were monitored by analytical TLC and the fractions were combined according to their chromatographic profile as indicated in the table 8. The yield of the process was 78.53%.

Table 8 – Combined fractions from the purification of group G4+5

Combined fractions	
Identification	**Weight (g)**
F1 – F33	0.039
F34 – F44	0.006
F45 – F99	**0.051**
F100 – F118	0.012
F119 – F178	0.030
F179 – F194	0.015
F195 – F274	0.101
F275 – F298	0.206
F299 – F324	0.227
F325 – F479	0.240
F480 – F516	0.024

The analytical TLC indicated a target compound between fractions 45 to 99 (0.051 g). Since the compound was insoluble in acetone, we proceeded to a solid-liquid extraction with acetone and the supernatant was separated from the compound. This procedure produced a pure compound that was codified as **compound 2** (0.048 g).

3.3.2.3. Purification of group G6

The group G6 was purified by a preparative TLC using a mixture of dichloromethane and acetone (9:1) as eluent. After development with sulphuric acid, the target fraction (Rf 0.60) was extracted with pure dichloromethane, yielding 0.043 g.

The TLC analysis of that fraction indicated that it needed to be re-purified. The fraction was subjected to another preparative TLC using the same eluent and the same extractive solvent as the first purification step. Unfortunately, the resulting fraction (0.015 g) required an additional purification step.

The third preparative TLC was eluted with a mixture of dichloromethane and acetone (19:1) and pure dichloromethane was once again used to extract the target fraction (Rf 0.76). Therefore, the purification of G6 was performed with three consecutive preparative TLC that resulted in the isolation of **compound 3**, yielding 0.008 g of this compound.

3.3.2.4. Purification of group G8

In a small aliquot (0.1 g) of G8, it was observed a deposition of a white solid. This solid was firstly treated with petroleum ether (5 x 2 mL) and the supernatant was separated from the solid. The solid was then treated with a mixture of dichloromethane and acetone (1:1) (5 x 2 mL) and the supernatant was again separated from the solid. The resulting solid was codified as **compound 4** (0.010 g).

The same procedure was carried out on a larger scale (5 x 30 mL) of G8 (1.863 g), which yielded 0.149 g of **compound 5**.

The figure 17 consists in a scheme that resumes the methodology employed for the purification of the groups G2, G4+5, G6 and G8.

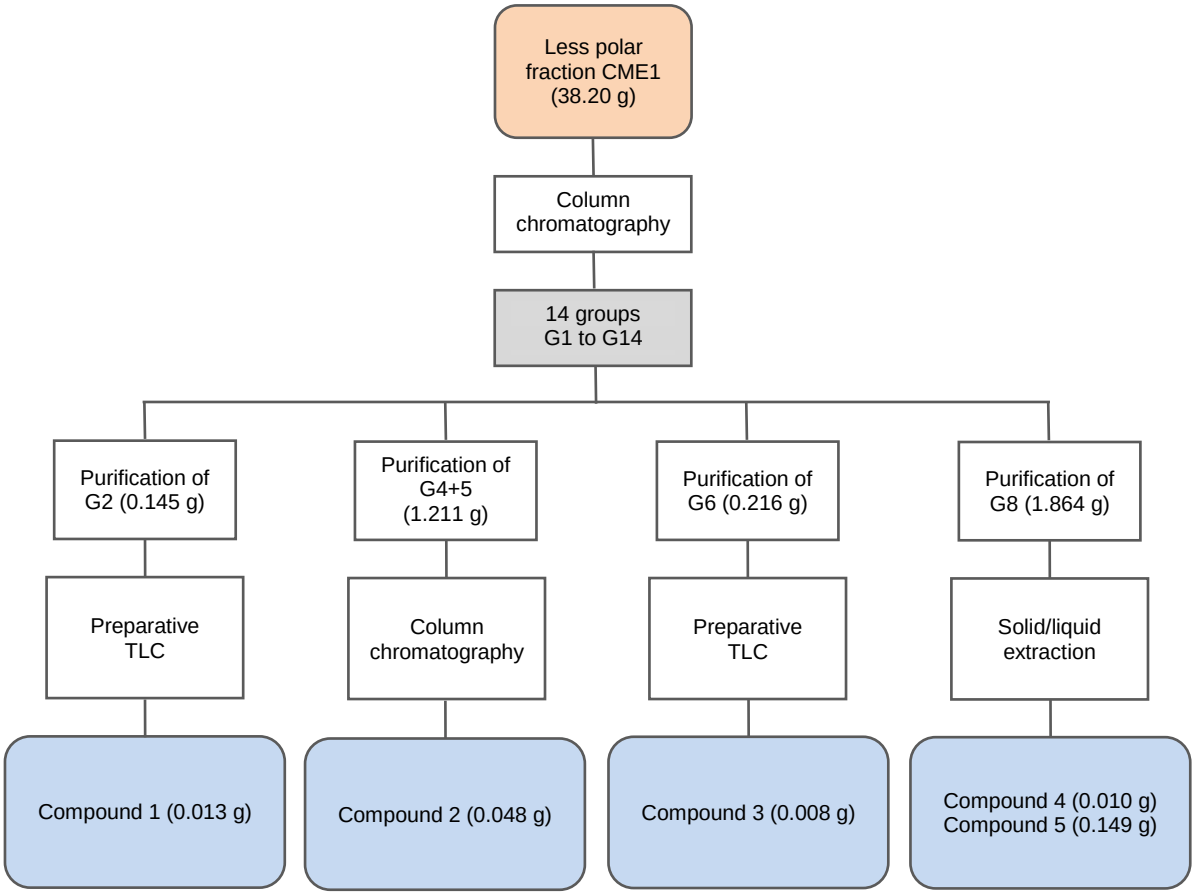


Figure 17 – Purification procedures of groups G2, G4+5, G6 and G8

3.4. Biological studies

3.4.1. Evaluation of the antimicrobial activity

The antimicrobial activities of the plant extracts and the isolated compounds were performed against the microorganisms identified in the next sections.

3.4.1.1. Antifungal activity

The broth microdilution method is based on CLSI reference protocols M27-A3 for yeasts (*Candida albicans*) and M38-A2 for filamentous fungi (*Aspergillus fumigatus* and dermatophytes) and is used to obtain the MIC and the MLC of the isolated compounds and the plant extracts. The MIC is the lowest concentration of the compound that inhibits the growth of microorganisms and the MLC is the lowest concentration that kills 99.9% of the microorganisms.

The microorganisms used for the antifungal assays were *Candida albicans* ATCC 10231, *Aspergillus fumigatus* ATCC 46645 and *Trichophyton rubrum* FF5 (clinical strain).

The stock solutions of the plant extracts and the isolated compounds were prepared with DMSO and the test solutions were freshly prepared and added to the cell suspensions, obtaining test concentrations that range from 16 to 256 µg/mL. Fluconazole and amphotericin B were used as positive controls. Negative controls corresponded to untreated microorganisms and the effect of DMSO on the microorganisms was also taken into account. It was also employed a sterility control.

The microorganisms were aerobically incubated under the following conditions: 35ºC ± 0.2ºC in atmospheric humidity during 24h/48h for *C. albicans* and *A. fumigatus*, and at 25ºC ± 0.2ºC in atmospheric humidity during 5 days for *T. rubrum*.

The yeast cell suspensions were prepared in 0.85% NaCl and the transmittance of cell density was adjusted to the 0.5 McFarland standards for *C. albicans*. Cell suspensions were diluted with RPMI 1640 in order to achieve an inoculum size of 1 - 5 x 10^3 CFU/mL.

For the filamentous fungi, the spore suspensions were prepared in 0.85% NaCl with tween 80 and the cell density was adjusted at 20-250 conidia/squares (haemocytometer) for *A. fumigatus* and 20 - 60 conidia/squares for *T. rubrum*. Similarly, the spore suspensions were diluted with RPMI 1640 in order to achieve an inoculum size of 0.4 - 5 x 10^4 CFU/mL and 1 - 3 x 10^3 CFU/mL for *A. fumigatus* and for *T. rubrum*, respectively.

MIC values were found as the lowest concentration of the selected compound or extract that revealed 100% growth inhibition. Besides, a quality control was performed by testing the inhibitory activity of fluconazole with the reference strain *C. parapsilosis* ATCC 90018 (CLSI standard for MIC = 0.5 - 2 µg/mL). To evaluate MLC values, after MIC determination, 20 µL samples were taken from each negative well and the growth control was the first well that exhibited growth. The samples were applied on SDA (Sabouraud dextrose agar) plates and incubated at 35ºC ± 0.2ºC for 24h/48h (*C. albicans* and *A. fumigatus*) or at 25ºC ± 0.2ºC for 7 days (*T. rubrum*). MLC values corresponded to the lowest concentration of the selected compounds causing fungal death. All experiments were repeated three times and were always performed in duplicate.

3.4.1.2. Antibacterial activity

Regarding the antibacterial activity, the broth microdilution method was based on CLSI reference protocol M7-A7. The bacteria tested for the plant extracts and all the isolated compounds were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and the methicillin-resistant *Staphylococcus aureus* (MRSA) (clinical isolate). Additionally, the bacteria *Enterococcus faecalis* ATCC 29212 and *Bacillus cereus* NCTC 7587 were also tested for selected compounds.

Cell suspensions were prepared in 0.85% NaCl and the transmittance of cell density was adjusted to the 0.5 McFarland standards in order to achieve an inoculum size of 10^5 CFU/mL. The dilutions of the cells suspensions were obtained adding MHB (Muller-Hinton broth).

The stock solutions of the plant extracts and the isolated compounds were also prepared in DMSO and were diluted in serial two-folds with MHB in order to obtain the final concentrations that ranged from 16 to 256 µg/mL.

Gentamicin was used as positive control. Negative controls, DMSO control and sterility control were also employed. The plates were aerobically incubated at 35 ± 0.2ºC in atmospheric humidity for 16h/20h.

Similarly, MIC values were determined as the lowest concentration of the selected compound or extract that revealed 100% of growth inhibition. Besides, a quality control was performed by testing the inhibitory activity of gentamicin against *E. coli* ATCC 25923 (CLSI standard for MIC = 0.25 - 1 µg/mL) as well. MLC values were obtained after MIC determination, in which 20 µL samples were taken from each negative well and the growth control was the first well that exhibited growth. The samples were also applied on MHA (Muller-Hinton agar) plates and were incubated at 35 ± 0.2ºC for 24h. MLC values corresponded to the lowest concentration of the selected compounds or extracts that caused bacterial death. All experiments were performed in duplicate and were repeated three times, yielding essentially the same results.

3.4.2. *In vitro* growth inhibition of human tumour cell lines

The growth inhibition effects of the methanolic crude extract (CME) and the semi-purified fraction CME1 and CME2 were tested for their *in vitro* growth inhibitory effects against a selected panel of cancer cell lines (MCF-7, NCI-H460 and A375-C5 cells).

The source of MCF-7 and A375-C5 cell lines was the European collection of cell cultures (ECACC) and the NCI-H460 cell lines was an offer from NCI, Bethesda, USA.

The cell lines were routinely maintained in RPMI-1640 medium, which was supplemented with 5% of FBS, at 37ºC in a humidified atmosphere containing 5% of CO_2. The cell number and the viability of the cell lines were routinely determined with Trypan blue exclusion assay (Sigma). All the experiments were performed with cells presenting more than 90% of viability in an exponential growth.

The stock solutions of the plant extracts were aseptically dissolved in DMSO and stored at -20ºC. The appropriated dilutions of those stock solutions were freshly prepared just prior to be used for the assays and were diluted using the growth medium.

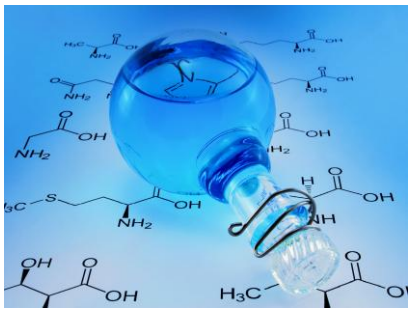
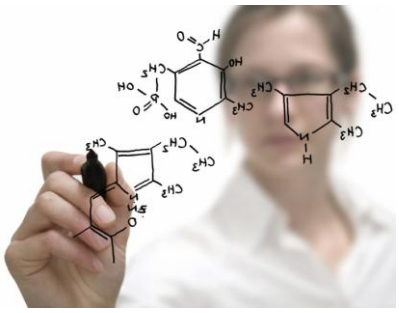
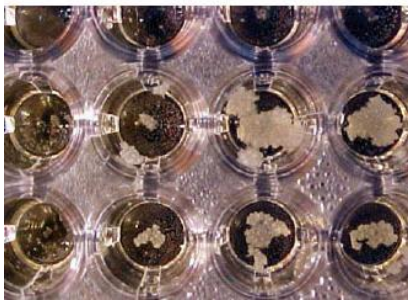
The *in vitro* growth inhibition assays were performed using a procedure adopted in the NCI in the "*In vitro* anticancer drug discovery screen", which employs the protein-binding dye SRB to assess cell growth [Monks *et al.*, 1991]. Therefore, this colorimetric assay indirectly estimates the cell number by staining cellular protein, which is evaluated by the absorbance measurement at 492 nm in the microplate reader.

The cell lines were plated in an appropriate density (MCF-7 and NCI-H460 cells were plated at 5 x 10^3 cells/well and A375-C5 cells were plated at 7.5 x 10^3 cells/well) and were allowed to be attached for 24 hours in the 96 well-plates. The exponential growing cells were further exposed to the selected compounds at increasing concentrations and to the positive control doxorubicin. The cells were incubated for 48 hours at 37ºC and 5% of CO_2. After this incubation period, the adherent cells were fixed *in situ* with 10% trichloroacetic acid, washed with 1% acetic acid and stained with SRB. The protein-SRB complexes were solubilised with 10 mM of Tris buffer and the absorbance was then measured.

For each cell line, a dose-response curve was obtained and the concentration that inhibited cell growth in 50% (GI_{50}) was calculated according to the adopted procedure [Monks *et al.*, 1991]. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was also determined because untreated control cells were exposed to the maximum concentration of DMSO (0.25%) that was used in each assay. All the experiments were repeated at least three times and were always performed in duplicate.

Chapter 4

Results and discussion


http://wallpoper.com/wallpaper/chemistry-343472;
http://www.pzccc.com/competition.php;
http://www.dreamstime.com/stock-image-96-well-plate-image5595411;
http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822009000200002.

4. Results and discussion

4.1. Chemical studies

The isolated compounds were always analysed by TLC before proceeding to additional chemical studies. The table 9 compiles the analytical TLC results obtained for each compound.

Table 9 – Analytical TLC data of compounds1 to 5

Isolated compounds	Analytical TLC data		Plate revelation		
	Eluent	Rf	UV 254 nm	UV 365 nm	H_2SO_4
Compound 1	Petroleum ether + Dichloromethane + Acetone (1:18:1)	0.82	Pronounced quenching	---	---
Compound 2	Dichloromethane + Acetone (9:1)	0.59	---	---	Reddish pink
Compound 3	Dichloromethane + Acetone (19:1)	0.68	---	---	Burgundy
Compound 4	Dichloromethane + Acetone (9:1)	0.48	---	---	Brownish pink
Compound 5 (mixture)	Dichloromethane + Acetone (9:1)	0.48 0.52	---	---	Brownish pink & pink

The compounds were then identified based on their 1D and 2D NMR spectral analysis and/or by GC-MS analysis. In the NMR spectral analysis, the chemical shifts (δ) are expressed in ppm. In the analysis made to the ^{1}H NMR spectra, the corresponding coupling constants (J, Hz) were taken into account to establish the structures of the isolated metabolites.

4.1.1. Structure elucidation of compound 1

Compound 1 was isolated as white solid (13 mg). The chromatographic profile showed one single spot with Rf = 0.82 (petroleum ether + dichloromethane + acetone – 1:18:1). It was identified a pronounced quenching when the compound was observed under UV lamp (254 nm), indicating that there would be a delocalised π system in its molecular structure.

The analysis of the ^{1}H NMR spectrum at 300 MHz in $CDCl_3$ allowed the identification of a singlet of one olefinic proton at δ_H 5.72 and the observation of a complex pattern of signals between 0.5 and 2.5 ppm.

The ^{13}C NMR spectrum revealed the presence of twenty-nine (29) carbon signals, a signal at δ_C 199.79 that was probably related with a carbonyl carbon and two olefinic carbons at δ_C 171.84 and δ_C 123.69. The carbonyl group and the double bond were presumably conjugated, which justify the absorption at 254 nm.

Based on the data above, it was conducted a literature survey that showed the $_{13}C$-NMR data of compound 1 were very similar with those published for *β*-sitostenone as shown in table 10.

Table 10 – ^{13}C NMR data of compound 1 and ^{13}C NMR data described in the literature

Carbons	Compound 1	Prachayasittikul *et al.*, 2009	Carbons	Compound 1	Prachayasittikul *et al.*, 2009
	δ_C ($CDCl_3$ 75 MHz)	δ_C ($CDCl_3$ 125 MHz)		δ_C ($CDCl_3$ 75 MHz)	δ_C ($CDCl_3$ 125 MHz)
C-1	35.63	36.06	C-16	28.17	28.13
C-2	33.96	33.93	C-17	55.81	55.99
C-3	199.79	199.58	C-18	11.94	11.90
C-4	123.69	123.69	C-19	18.66	18.65
C-5	171.84	171.64	C-20	36.08	36.07
C-6	33.80	33.86	C-21	18.97	18.98
C-7	31.99	32.91	C-22	35.91	35.65
C-8	35.58	35.60	C-23	25.92	26.08
C-9	53.74	53.79	C-24	45.73	45.81
C-10	39.55	39.59	C-25	29.03	29.64
C-11	20.97	21.10	C-26	20.91	20.99
C-12	38.56	38.57	C-27	19.81	19.75
C-13	42.33	42.35	C-28	22.99	23.04
C-14	55.92	55.85	C-29	11.92	11.14
C-15	24.15	24.14			

The presented data and the 2D NMR analisys allowed to suggest that compound 1 might be *β*-sitostenone. In order to support this suggestion, it was determined the melting point of compound 1 and the value was in good agreement with that in literature (mp 96-97ºC) [Prachayasittikul *et al.*, 2009].

Nevertheless, the compound was also analysed by GC-MS and the chromatogram presented a single peak at the retention time of 34.27 minutes. The molecular ion corresponded to the 412.6 m/z peak in the mass spectrum. As represented in the figure 18, the characteristic peaks were given at 271.4 m/z (which corresponded to the loss of

the side chain), at 229.4 m/z (which resulted from the fragmentation of the ring D between C13 – C17 and C14 – C15) and at 124.2 m/z (which resulted from the fragmentation of the ring B between C9 – C10 and C6 – C7) [Zaretskii *et al.*, 1967]. However, it is still necessary to determine the optical rotation of compound 1 and to compare with the literature data.

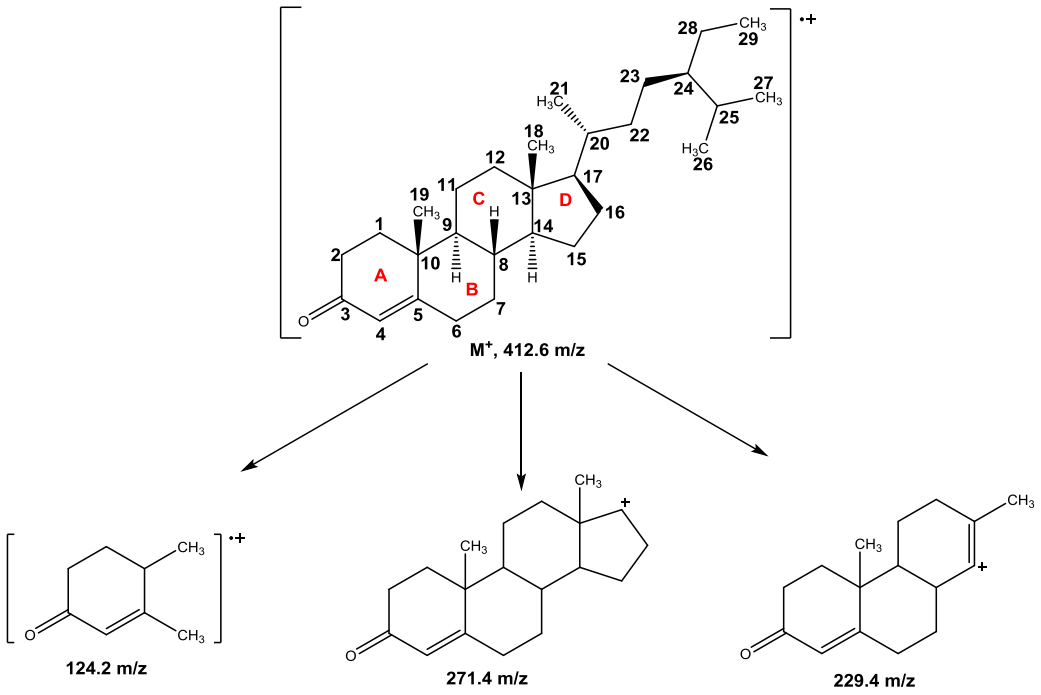


Figure 18 – Fragmentation scheme of *β*-sitostenone

As mentioned in the chapter 2, *β*-sitostenone was already reported within the *Cecropia* genus in the species *C. obtusifolia* [Guerrero *et al.*, 2010] and *C. pachystachya* [Costa *et al.*, 2011]. However, it is the first time that the compound is isolated from *C. catarinensis*.

4.1.2. Structure elucidation of compound 2

Compound 2 was isolated as white solid (51 mg). The analytical TLC of this compound showed that it presented the same retention factor (Rf = 0.59; eluent: dichloromethane + acetone – 9:1) as a sample of *β*-sitosterol used in the laboratory, developing the same reddish pink colour when treated with sulphuric acid. The NMR data of this sample of *β*-sitosterol is given in the annex 1.

In order to confirm that suspicion, the compound was analysed by GC-MS. The gas chromatogram of compound 2 showed a single peak (RT = 32.49 min) and its mass spectrum was screened in the software library, indicating that it was *β*-sitosterol with the highest probability (26.30%).

The molecular mass of *β*-sitosterol is 414.7, which explained the peak at 414.7 m/z. Several characteristic peaks could be identified, depending on the loss of the hydroxyl group from the ring A. Firstly, the peaks 273.4 m/z and 231.4 m/z occurred in the absence of hydroxyl group loss as identified in the figure 19. Secondly, the peaks 255.4 m/z and 213.4 m/z arose if dehydration occurs [Kamboj and Saluja, 2011; Zaretskii *et al.*, 1967].

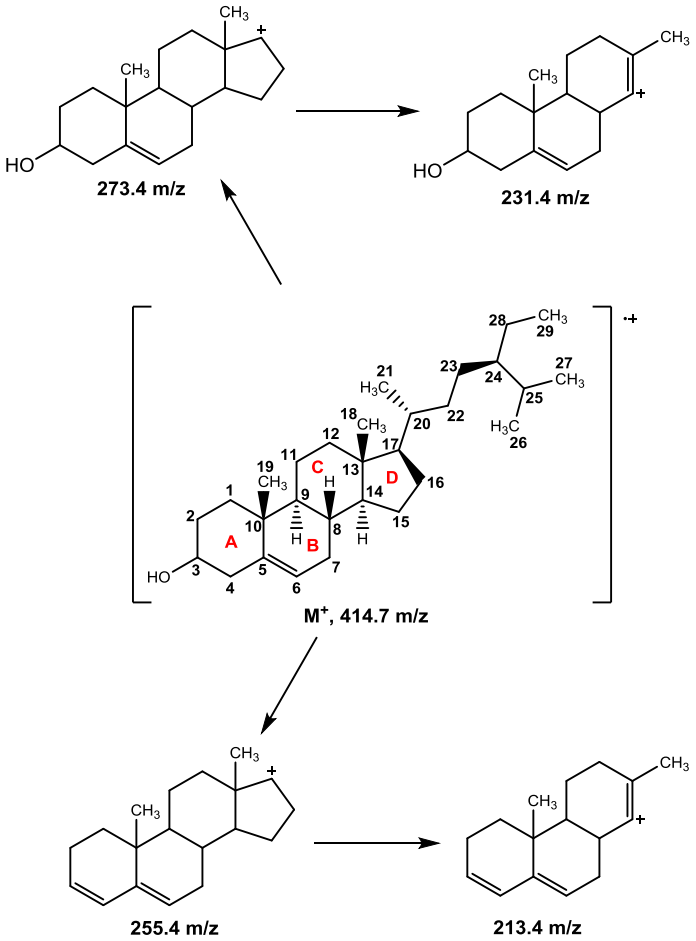


Figure 19 – Fragmentation scheme of *β*-sitosterol

The melting point of compound 2 was 137-139ºC, which was in accordance to literature [Chaturvedula and Prakash, 2012]. In any case, the GC-MS analysis did not provide an absolute structure elucidation of compound 2 but it prevented the determination of NMR spectra since *β*-sitosterol is a trivial compound that has been widely studied.

4.1.3. Structure elucidation of compound 3

Compound 3 was isolated as white crystal (8 mg). In the TLC analysis, the compound developed a burgundy colour (Rf = 0.68; eluent: dichloromethane + acetone - 19:1) when developed with sulphuric acid.

The analysis of the ^{1}H NMR spectrum at 300 MHz in $CDCl_3$ indicated a complex area between 0.5 and 2.0 ppm. In addition, the following characteristic signals allowed us to propose a pentacyclic triterpene pattern: a triplet of one olefinic proton at δ_H 5.14 (J = 3.5 Hz); a doublet of one proton at δ_H 3.55 (J = 11 Hz) that correlated to another proton at δ_H 3.19 (J = 11 Hz); and a double doublet at δ_H 3.24 (J_1 = 11 Hz and J_2 = 5.5).

The ^{13}C NMR spectra revealed the presence of thirty (30) carbon signals. We highlight the signals at δ_C 125.0 and δ_C 138.7 that could correspond to two olefinic carbons, and the signals at δ_C 69.9 and δ_C 78.9 that might indicate two unprotected carbons, which were probably bonded to a deprotecting element like oxygen. The ^{13}C NMR data suggested an ursane type skeleton, which could probably be the structure of uvaol. In order to verify this suggestion, the ^{13}C NMR data of compound 3 were compared to the ^{13}C NMR data for uvaol published by Zhang and colleagues (table 11).

Table 11 – ^{13}C NMR data of compound 3 and ^{13}C NMR data described in the literature

Carbons	Compound 3	Zhang *et al.*, 2012	Carbons	Compound 3	Zhang *et al.*, 2012
	δ_C ($CDCl_3$ 75 MHz)	δ_C ($CDCl_3$ 125 MHz)		δ_C ($CDCl_3$ 75 MHz)	δ_C ($CDCl_3$ 125 MHz)
C-1	38.7	38.3	C-16	23.2	22.8
C-2	27.2	26.7	C-17	37.9	38.3
C-3	78.9	78.5	C-18	53.9	53.5
C-4	36.8	37.4	C-19	39.3	38.8
C-5	55.1	54.7	C-20	39.4	39.0
C-6	18.3	17.9	C-21	34.0	34.6
C-7	32.8	32.4	C-22	30.6	30.1
C-8	39.9	39.6	C-23	28.1	27.5
C-9	47.6	47.1	C-24	15.7	15.1
C-10	35.1	36.3	C-25	15.3	15.1
C-11	23.3	22.8	C-26	16.7	16.3
C-12	125.0	124.5	C-27	23.3	22.8
C-13	138.7	138.1	C-28	69.9	69.4
C-14	41.9	41.4	C-29	17.3	16.8
C-15	25.9	25.5	C-30	21.3	20.8

Additionally, the data obtained from the 2D NMR spectra were in accordance to our proposal, and the melting point (226-229ºC) was also coincident [Zhang *et al.*, 2012]. The chemical structure of **uvaol (65)** is represented in the figure 20. However, it is still

necessary to determine the optical rotation of compound 3 and to compare with the literature.

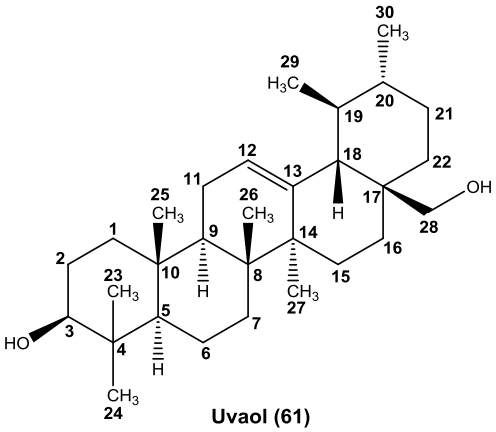


Figure 20 – Structure of uvaol (61)

It is important to note that uvaol has never been isolated within the genus *Cecropia*, so it is the first time that the compound is described in the genus and in the specie *C. catarinensis*.

4.1.4. Structure elucidation of compound 4

Compound 4 was isolated as white solid (10 mg). In the TLC analysis, the compound developed a brownish pink colour (Rf = 0.48; eluent: dichloromethane + acetone - 9:1) when developed with sulphuric acid.

The analysis of the ^{1}H NMR spectrum at 300 MHz in deuterated DMSO allowed us to propose a pentacyclic triterpene pattern: one olefinic proton at δ_H 5.16; one proton at δ_H 4.30 that probably belonged to an hydroxyl group; a proton at δ_H 3.00 that could be bounded to the same oxymethinic carbon as the referred hydroxyl group; and a complex area of signals between 0.68 and 0.9 ppm.

Although the ^{13}C NMR spectra did not immediately allow us to determine the number of carbons of compound 4, its preliminary analysis was able to point the signals at δ_C 126.8 and δ_C 138.6 that could correspond to two olefinic carbons, the signal at δ_C 179.0 that might be related to one carboxyl carbon and the signal at δ_C 76.9 that could indicate one oxymethinic carbon, which was probably bonded to a hydroxyl group. A careful observation of the ^{13}C NMR data suggested an ursane type skeleton, which could probably be the structure of ursolic acid. In order to verify this suggestion, the ^{13}C NMR data of compound 4 were compared to the ^{13}C NMR data for ursolic acid published by Moghaddam and co-workers (table 12).

Table 12 – ^{13}C NMR data of compound 4 and ^{13}C NMR data described in the literature

Carbons	Compound 4	Moghaddam *et al.*, 2007	Carbons	Compound 4	Moghaddam *et al.*, 2007
	δ_C (DMSO-d_6 75 MHz)	δ_C (DMSO-d_6 125 MHz)		δ_C (DMSO-d_6 75 MHz)	δ_C (DMSO-d_6 125 MHz)
C-1	38.4	39.2	C-16	25.9	24.7
C-2	26.4	27.8	C-17	46.7	47.7
C-3	76.9	77.7	C-18	53.2	53.2
C-4	38.1	39.2	C-19	40.0	39.4
C-5	54.8	55.6	C-20	39.0	39.3
C-6	18.1	18.9	C-21	30.7	31.1
C-7	32.7	33.6	C-22	36.6	37.2
C-8	41.0	40.0	C-23	28.2	29.1
C-9	46.9	47.9	C-24	15.1	16.1
C-10	37.3	37.4	C-25	16.0	16.9
C-11	23.9	23.7	C-26	16.3	17.8
C-12	126.8	125.4	C-27	25.1	24.1
C-13	138.4	139.0	C-28	179.0	179.1
C-14	41.4	42.5	C-29	16.6	17.9
C-15	28.1	28.4	C-30	23.1	21.9

However, it would be necessary to repeat the NMR analysis because the signals of DMSO probably overlapped to some of the signals of the compound, hindering a more precise analysis. Although the melting point (287-288ºC) was coincident with ursolic acid on literature [Moghaddam *et al.*, 2007], it is also necessary to determine the optical rotation of compound 4 because this parameter is a criterion of identity and purity.

Nevertheless, it is interesting to note that ursolic acid result from the oxidation of uvaol at C-28. Both compounds have been widely isolated from the aerial part of plants as well as their precursor α-amyrin. The biosynthesis of these nonsteroidal triterpenes with ursane type skeleton is mediated by Cyt P450 monooxigenases as represented in the scheme of the figure 21 [Fukushima *et al.*, 2011].

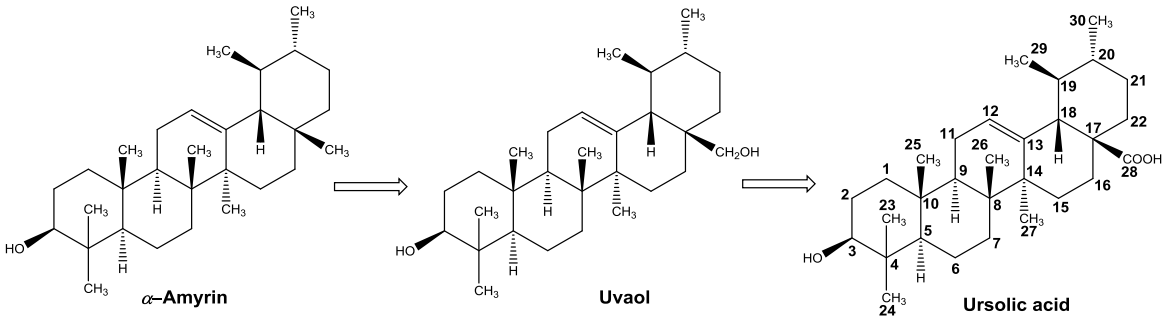


Figure 21 – Pathway for the biosynthesis of ursolic acid

In the last decade, it has been discussed the role of these triterpenoids in plant, which seems to be related to defense and development [Fukushima *et al.*, 2011]. Indeed, these features could explain the observed biological activities that will be discussed later.

Ursolic acid was already described in the specie *C. catarinensis* [Machado *et al.*, 2008].

4.1.5. Structure elucidation of compound 5

Compound 5 was isolated as white solid (149 mg). Actually, compound 5 is a mixture and this feature could be observed by TLC analysis because two different spots (Rf = 0.48 and Rf = 0.52; eluent: dichloromethane + acetone - 9:1) appeared after chemical development. The reason why we kept this mixture in this discussion is related with the biological activity that was observed.

The ^{13}C NMR data indicated that compound 5 was a mixture that could contain ursolic acid as the major compound and another triterpene maybe with similar skeleton, because the signals at δ_C 126.8 and δ_C 138.6 of the two olefinic carbons were doubled (δ_C 124.6 and δ_C 138.2) and the carboxyl carbon signal at δ_C 179.0 was also doubled (δ_C 178.3).

Since the two compounds have different retention factors, we will proceed to its separation by preparative TLC.

4.2. Biological studies

4.2.1. Evaluation of the antimicrobial activities

The crude methanolic extract CME and the semi-purified fractions (CME1 and CME2) were evaluated for their antimicrobial activity by determining their MIC (minimum inhibitory concentration) and MLC (minimum lethal concentration) in some bacteria and fungi, as showed in the table 13.

Table 13 – Antimicrobial activities of CME, CME1 and CME2 expressed as MIC and (MLC) in µg/mL

Microorganisms	Extracts/controls*					
	CME	CME1	CME2	Gentamicin*	Fluconazole*	Amphotericin B*
Staphylococcus aureus ATCC 25923	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	0.5 (0.5)	NT	NT
MRSA Clinical strain	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	640 (640)	NT	NT
Escherichia coli ATCC 25922	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	0.5 – 1 (1)	NT	NT
Pseudomonas aeruginosa ATCC 27853	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	2 – 4 (4)	NT	NT
Candida albicans ATCC 10231	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	NT	1 (> 128)	NT
Aspergillus fumigatus ATCC 46645	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	NT	NT	2 (4)
Trichophyton rubrum Clinical strains FF5	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	NT	16 – 32 (32 – 64)	Not tested

NT: Not tested.

These results did not revealed which extract was more active or a possible candidate for further chemical investigation in order to find active compounds. Indeed, all the extracts presented MIC and MLC values greater than 256 µg/mL for both bacteria and fungi. Anyway, all the isolated compounds were also tested for their antibacterial and antifungal activities as demonstrated in the table 14.

Table 14 – Antimicrobial activities of the isolated compounds expressed as MIC and (MLC) in µg/mL

Microorganisms	Compounds			
	Compound 1 (β-sitostenone)	**Compound 3 (Uvaol)**	**Compound 4 (Ursolic acid)**	**Compound 5 (Mixture)**
Candida albicans ATCC 10231	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)
Aspergillus fumigatus ATCC 46645	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)
Trichophyton rubrum Clinical strain FF5	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)

Table 14 (continued) – Antimicrobial activities of the isolated compounds expressed as MIC and (MLC) in µg/mL

Microorganisms	Compounds			
	Compound 1 (β-sitostenone)	Compound 3 (Uvaol)	Compound 4 (Ursolic acid)	Compound 5 (Mixture)
Staphylococcus aureus ATCC 25923	> 256 (> 256)	> 256 (> 256)	**64 (128-256)**	**32 (128)**
MRSA Clinical strain	> 256 (> 256)	> 256 (> 256)	**32-64 (≥ 256)**	**16 (64-128)**
Enterococcus faecalis ATCC 29212	NT	NT	**32* (64-128)***	**≤ 16* (64-128)***
Bacillus cereus NCTC 7587	NT	NT	**32* (64-128)***	**≤ 16* (32-64)***
Escherichia coli ATCC 25922	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)	> 256 (> 256)
Pseudomonas aeruginosa ATCC 27853	> 256 (> 256)	*> 256 (> 256)*	> 256 (> 256)	> 256 (> 256)

NT: Not tested; * Preliminary results.

Between the isolated compounds, only compound 4 (ursolic acid) and compound 5 (the mixture that probably contains ursolic acid) were active against the Gram-positive bacteria tested. Indeed, the decision of testing the bacteria *E. faecalis* and *B. cereus* was taken in order to confirm the selective activity of ursolic acid towards Gram-positive bacteria. This selectivity of ursolic acid to Gram-positive bacteria was also verified in the data collected from literature that are presented in the table 15. However, it is important to note the study of Fontanay and colleagues, since ursolic acid was inactive against MRSA (*mecA* gene), which is not in accordance with our results.

The study of Fontanay *et al.* was performed using the protocol M7-A6, that is the previous version of the protocol used in our study, but the protocols have not significant differences in the microdilution methodology. Nevertheless, the inoculum size obtained by Fontanay and colleagues was 5 to 50 higher than our inoculum size, which may justify the differences between the results.

Table 15 – *In vitro* activities of ursolic acid in bacteria obtained from literature

Bacterial strains or clinical isolates	Antibacterial activity			References
	Expressed as	Values	Unities	
Dermatophilus congolensis	MIC	15.0	µg/mL	Gbaguidi *et al.*, 2005
Enterococcus faecium FN-1 *Enterococcus faecium* BM4147	MIC	4.0 4.0	µg/mL	Horiuchi *et al.*, 2007
Enterococcus faecalis NCTC 12201 *Enterococcus faecalis* FA2-2	MIC	4.0 4.0	µg/mL	
Streptococcus pneumoniae R6	MIC	8.0	µg/mL	
Staphylococcus aureus OM481 *Staphylococcus aureus* OM584	MIC	8.0 8.0	µg/mL	
Escherichia coli K12	MIC	> 128	µg/mL	
Pseudomonas aeruginosa PAO1	MIC	> 128	µg/mL	
Serratia marcescens NUSM8905	MIC	> 128	µg/mL	
Enterococcus faecalis ATCC 29212	MIC	4.0	mg/L	Fontanay *et al.*, 2008
Enterococcus faecalis (*vanA* gene)	MIC	256.0	mg/L	
Enterococcus faecium (*vanB* gene)	MIC	256.0	mg/L	
Staphylococcus aureus ATCC 25923	MIC	8.0	mg/L	
Staphylococcus aureus ATCC 29213	MIC	8.0	mg/L	
MRSA (*mecA* gene)	MIC	> 256	mg/L	
Pseudomonas aeruginosa ATCC 27853	MIC	256.0	mg/L	
Pseudomonas aeruginosa (overexpression of efflux pumps)	MIC	> 256	mg/L	
Escherichia coli ATCC 25922	MIC	> 256	mg/L	
Penicillinase-producing *Escherichia coli*	MIC	> 256	mg/L	
Staphylococcus aureus ATCC 25923	Zone inhibition at 2 mg/mL at 1 mg/mL at 0.5 mg/mL at 0.25 mg/mL	10.5 9.5 8.5 7.5	mm (diameter)	Wong *et al.*, 2012
Bacillus subtilis NCTC 8236	Zone inhibition at 2 mg/mL at 1 mg/mL at 0.5 mg/mL at 0.25 mg/mL	10.5 9.5 9.0 8.0	mm (diameter)	
Bacillus cereus ATCC 10876	Zone inhibition at 2 mg/mL at 1 mg/mL at 0.5 mg/mL at 0.25 mg/mL	7.0 7.0 7.0 7.0	mm (diameter)	
Escherichia coli ATCC 25922	Zone inhibition at 2 mg/mL at 1 mg/mL at 0.5 mg/mL at 0.25 mg/mL	No activity	mm (diameter)	

Table 15 (continued) – *In vitro* activities of ursolic acid in bacteria obtained from literature

Bacterial strains or clinical isolates	Antibacterial activity			References
	Expressed as	Values	Unities	
Pseudomonas aeruginosa ATCC 17853	Zone inhibition at 2 mg/mL at 1 mg/mL at 0.5 mg/mL at 0.25 mg/mL	No activity	mm (diameter)	Wong *et al.*, 2012
Salmonella typhi (lab. strain)	Zone inhibition at 2 mg/mL at 1 mg/mL at 0.5 mg/mL at 0.25 mg/mL	11.0 10.0 10.0 9.5	mm (diameter)	
Klebsiella pneumoniae (lab. strain)	Zone inhibition at 2 mg/mL at 1 mg/mL at 0.5 mg/mL at 0.25 mg/mL	No activity	mm (diameter)	

Regarding the study of Wong and co-workers, it was found that the compound was active towards the gram negative bacteria *S. typhi*, which indicate that ursolic acid seems to be selective to Gram-positive bacteria but it can also be active towards some Gram-negative bacteria.

Furthermore, the mechanism of action of ursolic acid was already proposed by Kurek and colleagues because the compound inhibits the growth and survival of many bacteria, particularly Gram-positive bacteria. The effect of ursolic acid was tested on the Gram-positive bacteria *Listeria monocytogenes*, demonstrating that the compound affected cell morphology and enhanced cell autolysis by inhibiting peptidoglycan turnover and affecting the mucopeptide profile of bacterial wall [Kurek *et al.*, 2010].

The table 16 compiles the results on the antifungal activity of ursolic acid. Our results were consistent with the results of Liu and colleagues but not conform to the Shai´s results. The reason for these differences is the method employed for MIC determination. Indeed, Liu and colleagues used the same standard method like us, while Shai *et al.* used a non-standardized method which makes difficult to compare the results.

Table 16 – *In vitro* activities of ursolic acid in fungi obtained from literature

Fungal strains or clinical isolates	Antifungal activity			References
	Expressed as	Values	Unities	
Sporothrix schenckii	MIC	32.0	µg/mL	Shai *et al.*, 2008
Microsporum canis	MIC	12.0	µg/mL	
Aspergillus fumigatus	MIC	24.0	µg/mL	
Candida albicans ATCC 10231	MIC	63.0	µg/mL	
Cryptococcus neoformans	MIC	63.0	µg/mL	
Candida guilliermondi	MIC	15.6	µg/mL	
Candida spicata	MIC	63.0	µg/mL	
Candida albicans ATCC 90028	MIC	> 200	µg/mL	Liu *et al.*, 2009
Candida parapsilosis ATCC 22019	MIC	> 200	µg/mL	
Candida krusei ATCC 6258	MIC	> 200	µg/mL	

The mixture with ursolic acid was slightly more active than ursolic acid itself, which may suggest that the other compound is more active than ursolic acid or the mixture of both compounds may enhance the activity of each compound through a synergistic effect.

It is important to mention that there were solubility issues with uvaol and β-sitostenone because the samples gelled in contact with the culture medium. These solubility issues may also justify the results obtained with uvaol. In fact, the published studies that evaluated the antibacterial effects of uvaol have also been indicating that the compound does not possess antibacterial activity. The table 17 compiles the data obtained from those studies. However, this solubility issue might be overcome using, for example, nanotechnology in order to increase its solubility in media with higher polarity.

Table 17 – *In vitro* activities of uvaol in bacteria obtained from literature

Bacterial strains or clinical isolates	Antibacterial activity			References
	Expressed as	Values	Unities	
Enterococcus faecium FN-1 *Enterococcus faecium* BM4147	MIC	> 128 > 128	µg/mL	Horiuchi *et al.*, 2007
Enterococcus faecalis NCTC 12201 *Enterococcus faecalis* FA2-2	MIC	> 128 > 128	µg/mL	
Streptococcus pneumoniae R6	MIC	> 128 > 128	µg/mL	
Staphylococcus aureus OM481 *Staphylococcus aureus* OM584	MIC	> 128 > 128	µg/mL	
Escherichia coli K12	MIC	> 128	µg/mL	
Pseudomonas aeruginosa PAO1	MIC	> 128	µg/mL	
Serratia marcescens NUSM8905	MIC	> 128	µg/mL	
Escherichia coli AG100 *Escherichia coli* $AG100_{TET8}$	MIC	> 200 > 200	g/L	Martins *et al.*, 2011
Salmonella enterica serotype Enteritidis 104 *Salmonella enterica* serotype Enteritidis 104_{CIP} *Salmonella enterica* serotype Enteritidis 5408 *Salmonella enterica* serotype Enteritidis 5408_{CIP}	MIC	> 200 > 200 > 200 > 200	g/L	
Enterococcus faecalis ATCC 29212	MIC	200	g/L	
Staphylococcus aureus ATCC 25923 MRSA clinical strain MRSA COL MRSA COL_{OXA} *Staphylococcus aureus* HPV 107	MIC	> 200 > 200 200 100 200	g/L	
Mycobacterium tuberculosis $H_{37}Rv$	MIC	> 200	g/L	

No data was found in the literature regarding the antimicrobial activities of β-sitostenone. However, any approach that improves its solubility in conventional culture media might also provide more conclusive results.

4.2.2. The *in vitro* growth inhibitory evaluation on human cancer cell lines

The plant extracts CME, CME1 and CME2 were tested for the *in vitro* inhibitory activity in the following three human tumour cell lines: breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460) and melanoma (A375-C5). The results of the extracts inhibitory activity are presented in the table 18.

Table 18 – Growth inhibitory effects of CME, CME1 and CME2 in MCF-7, NCI-H460 and A375-C5 cell lines

Extracts/control*	Growth inhibitory activity expressed in GI_{50}		
	MCF-7 cells	NCI-H460 cells	A375-C5 cells
CME	58.8 ± 9.1 µg/mL	68.3 ± 7.2 µg/mL	80.2 ± 4.9 µg/mL
CME1	31.8 ± 6.7 µg/mL	36.1 ± 9.8 µg/mL	42.1 ± 8.8 µg/mL
CME2	80.9 ± 19.2 µg/mL	85.4 ± 27.2 µg/mL	≥200 µg/mL
Doxorubicin*	60.3 ± 1.2 µM	19.6 ±1.9 µM	130.0 ± 25.2 µM

The results are expressed as GI_{50} (µg/mL) values of the tested extracts using MCF-7, NCI-H460 and A375-C5 cell lines by means ± SD of at least three independent experiments performed in duplicate. Doxorubicin was used as a positive control.

Our results showed that the crude extract CME inhibited the growth of the three tumour cell lines. In turn, the less polar fraction CME1 was not only active against the three tumour cell lines used in the assay, but also it presented the highest growth inhibitory activities for the three cell lines between the three samples. CME2 showed the lowest inhibitory activities for breast and lung cancer cell lines, being inactive for melanoma cells.

These results suggested that the more active compounds could be in the less polar fraction CME1 or this fraction could be more concentrated in active compounds. Indeed, the comparative analytical TLC of the three samples showed that there were two violet/red spots in the plate after development with H_2SO_4. These spots (Rf. 0.76 and 0.73; eluent: CH_2Cl_2 + acetone 9:1) were more pronounced in the less polar fraction CME1.

In accordance to this bioassay-guided evaluation, CME1 was selected for further purification procedures in order to isolate and identify the compounds that might be responsible for the activity of CME1. However, for different reasons, none of the isolated compounds were tested against the selected panel of cancer cell lines. Firstly, ursolic acid has been widely isolated and identified within the plant kingdom and it has also been extensively tested for its *in vitro* activity in cancer cell lines (table 19). Secondly, as uvaol and β-sitostenone presented that unexpected solubility issues during the antimicrobial tests, the same problems would be expected to occur in the *in vitro* growth inhibitory evaluation tests.

Table 19 – *In vitro* activities of ursolic acid in cancer cell lines obtained from literature

Cell lines	Cytotoxic activity			References
	Expressed as	Values	Unities	
HL-60 (human promyelocytic leukaemia cells)	IC_{50}	14.63	µM	Cheng *et al.*, 2013
SMMC-7721 (human liver hepatocarcinoma cells)	IC_{50}	19.78	µM	
A-549 (human lung carcinoma cells)	IC_{50}	20.29	µM	
MCF-7 (human breast carcinoma cells)	IC_{50}	> 40	µM	
SW-480 (human colon adenocarcinoma cells)	IC_{50}	21.36	µM	
MGC-803 (human gastric cancer cells)	Inhibition rate at 20 µM	51.70 ± 5.60	%	Liu *et al.*, 2012
Bcap-37 (human breast cancer cells)	Inhibition rate at 20 µM	48.40 ± 5.90	%	
MCF-7 (human breast carcinoma cells)	Inhibition rate at 20 µM	49.40 ± 4.10	%	
PC3 (human prostate cancer cells)	Inhibition rate at 20 µM	57.70 ±1.90	%	
NIH3T3 (murine embryo fibroblast cells)	Inhibition rate at 20 µM	21.70 ± 4.90	%	
HepG2 (human hepatoma cells)	IC_{50}	68.82 ± 3.71	µM	Shao *et al.*, 2011
BGC-823 (human gastric cancer cells)	IC_{50}	66.38 ± 0.66	µM	
SH-SY5Y (human bone marrow neuroblastoma cells)	IC_{50}	54.62 ± 3.87	µM	
HeLa (human cervical adenocarcinoma cells)	IC_{50}	33.12 ± 0.63	µM	
HT-29 (human colon adenocarcinoma cells)	IC_{50}	26.31 ± 2.74	µM	Bai *et al.*, 2011
HEp-2 (human larynx carcinoma cells)	IC_{50}	53.42 ± 4.07	µM	
BGC-823 (human gastric cancer cells)	IC_{50}	22.96 ± 0.99	µM	
GES-1 (human gastric epithelial immortalized cells)	IC_{50}	93.83 ± 8.51	µM	
HL-60 (human promyelocytic leukaemia cells)	Inhibition rate at 10 µM	7.57	%	Wang *et al.*, 2010
BGC-823 (human gastric cancer cells)	Inhibition rate at 10 µM	12.49	%	
HEp-2 (human larynx carcinoma cells)	Inhibition rate at 10 µM	10.77	%	
HeLa (human cervical adenocarcinoma cells)	Inhibition rate at 10 µM	12.36 ± 3.01	%	Meng *et al.*, 2009
SK-OV-3 (human ovarian carcinoma cells)	Inhibition rate at 10 µM	9.06 ± 2.02	%	
BGC-823 (human gastric cancer cells)	Inhibition rate at 10 µM	10.20 ± 1.02	%	
HeLa (human cervical adenocarcinoma cells)	IC_{50}	> 10	µM	
SK-OV-3 (human ovary adenocarcinoma cells)	IC_{50}	> 10	µM	
BGC-823 (human gastric cancer cells)	IC_{50}	> 10	µM	

Table 19 (continued) – *In vitro* activities of ursolic acid in cancer cell lines obtained from literature

Cell lines	Cytotoxic activity			References
	Expressed as	Values	Unities	
Ca9-22 (human gingival carcinoma cells)	IC_{50}	5.90	µg/mL	Chen *et al.*, 2009
HL-60 (human promyelocytic leukaemia cells)	IC_{50}	8.70	µg/mL	
A-549 (human lung carcinoma cells)	EC_{50}	3.71	µM	Lee *et al.*, 2008
SK-OV-3 (human ovary adenocarcinoma cells)	EC_{50}	3.65	µM	
SK-MEL-2 (human skin melanoma cells)	EC_{50}	3.62	µM	
HCT-15 (human epithelial colorectal adenocarcinoma cells)	EC_{50}	5.44	µM	
P-388 (murine leukaemia cells)	IC_{50}	3.50	µg/mL	Chinou *et al.*, 2007
NSCLC-N6 (human bronchial epidermoid cancer cells)	IC_{50}	9.00	µg/mL	
KB (human cervical carcinoma cells)	EC_{50}	8.30	µM	Tatsuzaki *et al.*, 2007
KB-VIN (multi-drug resistant KB sub-line expressing P-glycoprotein)	EC_{50}	8.10	µM	
HepG2 (human hepatoma cells)	IC_{50}	18.00	µM	Tian *et al.*, 2006
R-HepG2 (drug-resistance strain of HepG2 cells)	IC_{50}	15.00	µM	
HL-60 (human promyelocytic leukaemia cells)	EC_{50}	72.00	µg/mL	Ma *et al.*, 2005
BGC-823 (human gastric cancer cells)	EC_{50}	53.70	µg/mL	
Bel-7402 (human hepatocellular carcinoma cells)	EC_{50}	45.00	µg/mL	
HeLa (human cervical adenocarcinoma cells)	EC_{50}	49.40	µg/mL	
A2780 (human ovarian cancer cells)	IC_{50}	8.00	µg/mL	Cao *et al.*, 2004

Some of the studies presented in the table 19 were related to the chemical synthesis of ursolic acid derivatives in order to study the structure-activity relationship of the lead compound and its synthesized derivatives [Bai *et al.*, 2011; Shao *et al.*, 2011; Wang *et al.*, 2010; Meng *et al.*, 2009; Tatsuzaki *et al.*, 2007; Ma *et al.*, 2005]. However, there are also reports on the mechanism of action of ursolic acid: the compound showed antiangiogenic properties and inhibited tumour cell invasion and metastasis, which seemed to be related to its ability to inhibit DNA replication [Resende *et al.*, 2006; Tian *et al.*, 2006]; the compound also induced apoptosis through the activation of caspases and the release of intracellular Ca^{2+} and cytochrome C [Messner *et al.*, 2011; Resende *et al.*, 2006; Tian *et al.*, 2006]; ursolic acid inhibited human topoisomerase I and topoisomerase IIα by competing with DNA for the same binding sites on the enzymes, the compound did not

intercalate with DNA and did not bind to the DNA minor grove as topoisomerase poisons do, therefore, the compound seemed to be a dual catalytic inhibitor of topoisomerase I and topoisomerase IIα, which prevented the interaction between the enzymes and DNA [Syrovets *et al.*, 2000].

It was found only one study with the *in vitro* effects of β-sitostenone against a panel of cancer cells and the results are presented in the table 20. Nonetheless, the antitumour promoting activity of β-sitostenone was determined using EBV-EA assay in Raji cell line, thus 20 µg/mL of β-sitostenone inhibited 97.93% ± 1.5% of cell viability and the inhibition rate was 78.4% ± 1.5% [Habsah *et al.*, 2005].

Table 20 – *In vitro* activities of β-sitostenone in cancer cell lines obtained from literature

Cell lines	Cytotoxic activity			References
	Expressed as	Values	Unities	
HCT-8 (human colorectal adenocarcinoma cell)	IC_{50}	60.60	µM	Gallo *et al.*, 2010
MDA-MB-435 (human breast carcinoma cells)	IC_{50}	60.60	µM	
SF-295 (human glioblastoma multiforme cells)	IC_{50}	60.60	µM	
HL-60 (human acute myelocytic leukemia cells)	IC_{50}	60.60	µM	

The inhibition of EBV-EA activation was also assayed for uvaol, again using Raji cells, which was determined at four different concentrations (1000, 500, 100 and 10 mol ratio) of uvaol. The compound showed an inhibitory effect at 100 mol ratio and exhibited significant activity at the highest concentration, indicating that possess antitumor promoting effects [Ukiya *et al.*, 2002]. Concerning its cytotoxic effects, uvaol was tested against human 1321N1 astrocytoma cell line, as astrocytomas are an aggressive type of primary malignant tumours in the neurological system. The compound affected cell proliferation and induced cell death, modulating the apoptotic response that was characterised by nuclear condensation and fragmentation, ROS production and loss of mitochondrial transmembrane potential [Martín *et al.*, 2009].

Furthermore, the effects of uvaol on parental L5178 cells (mouse lymphoma cells) and *MDR1-transfectes* L5178 cells (mouse lymphoma cells containing the human efflux pump gene *MDR1*) were obtained by Martins and colleagues. The results are presented in the table 21. Additionally, the group found that uvaol was effective in inhibiting the extrusion of rhodamine 123 and ethidium bromide from the *MDR1-transfectes* L5178 cells because these two P-gp substrates were intracellularly accumulated, which indicated that uvaol may be a promising compound for the reversal of multidrug resistance [Martins *et al.*, 2010].

Table 21 – *In vitro* activities of uvaol in cancer cell lines obtained from literature

Cell lines	Cytotoxic activity			References
	Expressed as	Values	Unities	
Parental L5178 cells	IC_{50}	7	mg/L	Martins *et al.*, 2010
MDR1-transfectes L5178 cells	IC_{50}	13	mg/L	

Moreover, uvaol inhibited significantly cell growth and proliferation of MCF-7 breast cancer cell line in a dose and time-dependent manner. The compound showed a significant antiproliferative effect at 100 μM, which involved cell cycle arrest. Although the compound did not show free radical scavenging activity, the compound protected against oxidative DNA damage at 10 μM, reducing intracellular ROS level and preventing H_2O_2-induced oxidative injury [Allouche *et al.*, 2011].

According to the data presented in this section, it is possible that the growth inhibitory activity of CME1 may be justified by its chemical composition on pentacyclic triterpenes, since ursolic acid and uvaol are known to possess that activity. However, the groups with higher polarity remain to be studied, which may be a source of active compounds and with remarkable chemical structures.

Chapter 5

Conclusions

Cecropia catarinensis: http://www.ufrgs.br/fitoecologia/florars/open_sp.php?img=906;
http://www.ufrgs.br/fitoecologia/florars/open_sp.php?img=570;
http://www.ufrgs.br/fitoecologia/florars/open_sp.php?img=8180;
http://www.ufrgs.br/fitoecologia/florars/open_sp.php?img=904.

5. Conclusions

Plants that belong to the genus *Cecropia* have been used in traditional medicine for the treatment of hypertension, Diabetes Mellitus or asthma. The cardiovascular effects have been attributed to the flavonoid content of their aqueous extracts, while their hypoglycaemic effects have been correlated with their content in chlorogenic acid.

The genus *Cecropia* is still poorly investigated in the anticancer drug development field. Indeed, only *C. lyratiloba* and *C. pachystachya* were evaluated in preclinical studies, which means that *C. catarinensis* was tested for the *in vitro* growth inhibitory effects of cancer cell lines for the first time. Our results demonstrated that the crude methanolic extract (CME) and the semipurified fraction with the lowest polarity (CME1) were active against the panel of cancer cell lines tested (MCF-7, NCI-H460 and A375-C5 cells). However, CME1 was more active than CME towards the three cell lines, which indicated that CME1 was probably more concentrated in active compounds.

CME1 was selected for further chemical investigation and purification, affording four compounds. The structures of those compounds were elucidated by spectroscopic and spectrometric methods (NMR and/or MS) and were compared with the data obtained from literature. It is still necessary to proceed to the determination of optical rotation because this parameter is an important criterion of identity and purity. Two known pentacyclic triterpenoids with ursane type skeleton (uvaol and ursolic acid) were indicated as the structures of two of the isolated secondary metabolites, which could explain the cytotoxic activity of CME1. Two known steroids (*β*-sitostenone and *β*-sitosterol) were also indicated as the structures of the other two secondary metabolites that were isolated from CME1.

The antimicrobial effects of extracts obtained from *C. peltata* were described in one study and the results corroborated with the plant use for the treatment of infections in Colombian folk medicine. Despite having been evaluated for the first time, our extracts of *C. catarinensis* were inactive towards the microorganisms tested. However, among the isolated secondary metabolites, the compound that has been suggested as ursolic acid demonstrated to be active and selective for gram-positive bacteria.

For publication purposes, there are still some experiments to perform in order to confirm the structure suggestions of the already isolated secondary metabolites. Additionally, the mixture that seemed to be composed with ursolic acid should be re-purified and re-tested against the bacteria for which it was active. Furthermore, there are other groups that were obtained from the initial fractionation of CME1 that may be purified so that other secondary metabolites can be isolated.

This master also allowed the writing of two articles, one original article and one review, which are currently in the final stage of correction. The provisional title of the original article is “The *in vitro* cell growth inhibition of the crude extract of *Cecropia catarinensis*” and the provisional title of the review is “Soft corals: a potential contribution to enrich the clinical pipeline for anticancer drug discovery”.

Annex 1

^{13}C NMR data of the sample of *β*-sitosterol:

Table 22 – ^{13}C NMR data of the sample of *β*-sitosterol and ^{13}C NMR data described in the literature

Carbons	Sample of *β*-sitosterol	Chaturvedula and Prakash, 2012	Carbons	Sample of *β*-sitosterol	Chaturvedula and Prakash, 2012
	δ_C ($CDCl_3$ 75 MHz)	δ_C ($CDCl_3$ 150 MHz)		δ_C ($CDCl_3$ 75 MHz)	δ_C ($CDCl_3$ 150 MHz)
C-1	37.2	37.5	C-16	28.2	28.5
C-2	29.7	31.9	C-17	56.0	56.3
C-3	71.8	72.0	C-18	36.1	36.3
C-4	42.3	42.5	C-19	19.0	19.2
C-5	140.7	140.9	C-20	33.9	34.2
C-6	121.7	121.9	C-21	26.0	26.3
C-7	31.8	32.1	C-22	45.8	46.1
C-8	31.6	32.1	C-23	23.0	23.3
C-9	50.1	50.3	C-24	12.0	12.2
C-10	36.5	36.7	C-25	29.1	29.4
C-11	21.1	21.3	C-26	19.8	20.1
C-12	39.7	39.9	C-27	19.4	19.6
C-13	42.3	42.6	C-28	18.8	19.0
C-14	56.7	56.9	C-29	11.8	12.0
C-15	26.0	26.3			

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