**UNIVERSITY OF SÃO PAULO
SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO**

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**Chemical and biological studies of tannins and anthraquinones
acting on the gastrointestinal tract**

**Estudos químico e biológico de taninos e antraquinonas com
atuação no sistema gastrointestinal**

Doctoral thesis presented to the Graduate Program of School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences.

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RESUMO

DEMARQUE, D. P. **Estudos químico e biológico de taninos e antraquinonas que atuam no sistema gastrointestinal**. 2017. 164f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2017.

Diversas plantas com atuação no trato gastrointestinal – ricas em taninos e antraquinonas – ainda deixam muitas dúvidas quanto a sua caracterização estrutural, o seu mecanismo de ação e toxicidade. O presente trabalho objetivou o estudo químico e biológico de duas plantas utilizadas para doenças relacionadas ao sistema gastrointestinal: barbatimão (*Stryphnodendron rotundifolium*), rico em procianidinas e taninos (utilizada para combater gastrite); e cáscara sagrada (*Rhamnus purshiana*), rica em antraquinonas (utilizada para constipação). Inicialmente foi realizado um estudo químico com as plantas envolvendo isolamento e caracterização de componentes do extrato por RMN (^1H , ^{13}C , DEPT e NOESY), espectrometria de massas (MS), infravermelho e dicroísmo circular vibracional (VCD). Essas substâncias foram utilizadas para a padronização dos extratos utilizados em estudo biológico. O estudo biológico envolveu testes *in vitro* para avaliar a toxicidade de diferentes componentes do extrato de cáscara sagrada e teste *in vivo* com barbatimão, visando, através do tratamento de animais e remoção do estômago para estudo em microscopia confocal, entender o mecanismo de proteção gástrica. Com o desenvolvimento do trabalho foi possível desenvolver novas metodologias para a identificação de antraquinonas utilizando espectrometria de massas e provar a veracidade de regras empíricas utilizadas para a determinação da configuração absoluta de moléculas dessa classe. Os testes *in vitro* com antraquinonas isoladas e padrões comerciais indicam a possibilidade de aprimoramento dos perfis químicos utilizados para a produção de medicamentos a base de extratos de cáscara sagrada, visando à redução de toxicidade. Quanto ao barbatimão, o estudo químico possibilitou a aplicação da técnica de VCD para a diferenciação de procianidinas diastereoisoméricas. Os estudos *in vivo* confirmaram a eficácia do uso de taninos para combater gastrite através da formação de um revestimento protetor.

Palavras-chave: taninos, antraquinonas, gastrite, laxativo, farmacognosia.

ABSTRACT

DEMARQUE, D. P. **Chemical and biological studies of tannins and anthraquinones acting on the gastrointestinal tract.** 2017. 164f. Thesis (Doctoral). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2017.

Several plants with action in the gastrointestinal tract - rich in tannins and anthraquinones - cause many doubts concerning their chemical characterization, mechanism of action and toxicity. The present work aimed to develop a chemical and biological study of two plants used for gastrointestinal system related diseases: barbatimão (*Stryphnodendron rotundifolium*), rich in procyanidins and tannins (used to treat gastritis); and cascara sagrada (*Rhamnus purshiana*), rich in anthraquinones (used for constipation). Initially, a chemical study with plants was performed comprising isolation and characterization of components of the extract through NMR (^1H , ^{13}C , DEPT and NOESY), mass spectrometry (MS), and infrared and vibrational circular dichroism (VCD). These substances were used for extract standardization used in biological studies. The *in vitro* tests were performed in order to evaluate the toxicity of different components of the cascara extract. The *in vivo* tests with barbatimão aimed to better understand the mechanism of gastric protection, by treating animals with the extract, removing their stomachs, and analyzing them with fluorescence microscopy. Through this work we were able to develop new methods for identifying anthraquinones by mass spectrometry and to prove empirical NMR rules used to determine the absolute configuration of molecules of this class. The tests with isolated anthraquinones indicated the possibility of improving chemical profile to further reduce cascara sagrada toxicity. The barbatimão chemical study allowed the application of VCD technique for the differentiation of diastereoisomeric procyanidins, and confirmed the effectiveness of using tannins for gastritis treatment through the formation of a protective coating.

Keywords: tannins, anthraquinones, laxative, gastritis, pharmacognosy.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABIFISA	<i>Associação Brasileira das Empresas do Setor Fitoterápico</i> (Brazilian Association of Companies of the Phytotherapeutic Sector)
AC	Absolute Configuration
ANVISA	<i>Agência Nacional de Vigilância Sanitária</i> (Brazilian Health Regulatory Agency)
BTMCrEx	Barbatimão Crude Extract
BTMEtAcPh	Ethyl Acetate phase of barbatimão
CAS	Cascaroside
CAD	Collisionally-activated dissociation gas
CasCrEx	Cascara sagrada Crude Extract
CasEtAcPh	Ethyl Acetate partition Phase of cascara sagrada
CasMWPh	Methanol/water partition Phase of cascara sagrada
Cat	Catechin
CCC	Classic Chromatography Column
CD	Circular Dichroism
CE	Collision Energy
CEP	Collision Cell Entrance Potential
Conf	Confirmation
CUR	Curtain Gas
CXP	Collision Cell Exit Potential
Da	Dalton
DAD	Diode Array Detector
DHB	Dihydroxybenzoic acid
DNA	Deoxyribonucleic Acid
DP	Declustering Potential
DT	Dwell Time
ECD	Electronic Circular Dichroism
EP	Entrance Potential
ESI	Electrospray Ionization
GA	Gallic Acid
Galloc	Gallocatechin
GAS1	Nebulization Gas
GAS2	Turbo Heater Gas
GIT	Gastrointestinal Tract
GOT/AST	Glutamate-pyruvate transaminase/Aspartate aminotransferase
HDL	High Density Lipoprotein
HM	Herbal Medicines

HPLC	High Pressure Liquid Chromatography
HR-MS	High Resolution Mass Spectrometry
IR	Infrared
IS	Ionization Voltage
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled with Mass Spectrometry in tandem
MD	Molecular Dynamics
MS	Mass spectrometry
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
ORD	Optical Rotatory Dispersion
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PCY	Procyanidin
PDE	Prodelphinidin
PNASP	Polyphenols not Absorbed on Skin Powder
QT	Quantification
RDA	Retro-Diels Alder
RIP	Receptor-interacting protein
RT	Retention Time
SGPT/ALT	Glutamate-pyruvate Transaminase/Alanine Transaminase
SS	Standard Solution
SUS	<i>Sistema Único de Saúde</i> (Brazilian Public Health System)
TFA	Trifluoroacetic acid
THP	Tradicional Herbal Products
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
TOF	Time of Flight analyser
TP	Total Phenolics
UV	Ultraviolet
VCD	Vibrational Circular Dichroism
VLDL	Very Low Density Lipoprotein

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INTRODUCTION

1. INTRODUCTION

Medicinal plants are the oldest disease treatments known to man and are still important and useful therapeutic tools. According to the Brazilian Association of Companies from the Phytotherapeutic Sector (ABIFISA, in Portuguese, *Associação Brasileira das Empresas do Setor Fitoterápico*) herbal medicines are responsible for about US\$ 20 billion in profits, globally (GUIA DA FARMÁCIA, 2016). For the Brazilian market the continuous growth of the herbal medicines – 6.1% in 2013 and 8.0% in 2014 compared to the previous years – is an outlier in the industry's general growth trend.

Due to the importance of this sector for the Brazilian and global market and the effectiveness of adherence to therapeutic treatments using herbal medicines, the Brazilian Public Health System (SUS, in Portuguese, *Sistema Único de Saúde*) has supported them as alternatives to classic treatments. Furthermore, SUS also recommends increasing the research development of herbal medicines for the most commonly used plants (BRASIL, 2006).

The resumption of herbal medicine treatments in the last 10 years, especially by SUS makes this treatment official, mobilized the Brazilian Health Regulatory Agency (ANVISA, in Portuguese, *Agência Nacional de Vigilância Sanitária*) to create two different product categories in 2014: Traditional Herbal Products (THP) and Herbal Medicines (HM), where vegetable drugs have to undergo the same scientific tests as common medicines to be classified as HM (BRASIL, 2014). Consequently, the efficacy, safety, and mechanism of action of the HM should be proved.

In light of these regulatory modifications, the variability of secondary metabolism, the chemical complexity and the deficiency of scientific evidence about the pharmacological and toxicological mechanism of some widely used plants still compromises the vegetable drug's efficacy and safety to become an HM. This can affect even commonly used and known species, such as those used for constipation and gastritis treatment. Species that act in the gastrointestinal tract (GIT) have a chemical complexity which includes a huge range of compounds with wide stereochemical variations. In addition, the toxicological aspects of some plants that are used as laxatives are still not completely elucidated, nor the mechanism of action of plants used for gastritis treatment.

The changes in Brazilian regulatory system point toward the requirement of scientific studies for HM development. In order to produce scientific information to include THP in HM classification, important chemical, pharmacological and toxicological issues that have been neglected for plants used as laxatives and to treat gastritis need to be solved. Therefore,

this thesis focuses on the chemical and biological problems concerning some plants with GIT action.

1.1. Plants acting on the gastrointestinal tract (GIT)

The GIT is a collection of organs that are responsible for converting food into energy. The dysfunctions which medicines act on include: dyspepsia, gastroesophageal reflux, irritable bowel syndrome, inflammatory conditions of the upper tract (ulcers, esophagitis, gastritis), chronic gastrointestinal infections, dysbiosis and constipation (BONE; MILLS, 2013). Between them, dyspepsia and constipation are very common disturbances treated with medicinal plants, since most cases are not particularly severe.

The non-severity of some gastrointestinal problems, along with the popular common sense of non-toxic herbs contributes to drug abuse and, consequently, side effects emerge. For instance, even a natural tea to treat constipation may cause severe toxic effects neglected by lay people.

This drug abuse is related to several reasons. Individuals who abuse laxatives can be divided into the following groups: individuals with eating disorders (ROERIG et al., 2010; TOZZI et al., 2006), individuals who use laxatives when constipated and continue to overuse them to the point that their intestine becomes refractory (BAKER; SANDLE, 1996), and individuals who use them to lose weight or for sports reasons (MARQUART; SOBAL, 1994).

Regarding the drugs used for gastric dysfunctions, such as dyspepsia, the main concern is an empirical explanation for their action. The tissue binding property of some chemicals is the reasonable explanation how some phenol-rich species improve gastric problems. Considering the necessity to convert THP into HM, the science of natural products should better understand how gastric problems are improved by these medicines, how this process happens, and which characteristics of the chemicals are necessary to them became part of an HM to treat gastric problems. Furthermore, this same concern applies to the toxicological aspects of laxative plants, where understanding the role of each compound can help to develop safer medicines.

1.2. Anthraquinones-rich plants

1.2.1. Mechanism of action of anthraquinones-rich plants as laxatives

Anthraquinones are extensively present in nature, found in plants, bacteria, fungi and insects (GESSLER; EGOROVA; BELOZERSKAYA, 2013; PANKEWITZ et al., 2007). They are widely used as pharmacological drugs for constipation and non-prescription dietary supplements for weight loss. Currently, these compounds are used for a variety of biological activities, including anti-inflammatory, antifungal, antibacterial, antiviral and antiarthritic (MALIK; MULLER, 2016). For instance, the drug Artrolyt® (diacerein), obtained by rhein semisynthesis (natural origin), is widely prescribed to treat several joint disorders, such as osteoarthritis of the hip and knee. Due to the cytotoxic action of some components of this class, several medicines have been developed to treat cancer, as doxorubicin (natural), daunorubicin (natural) and valrubicin (semisynthetic) (CANEQUE et al., 2015; MALIK; MULLER, 2016; SONG, G. P. et al., 2015). The main mechanism of action of these drugs is apoptosis induction, which explains the action of these molecules as cytotoxic agents. Although apoptosis induction is desired in cytotoxic agents used for cancer treatment, this mechanism is intrinsically

Figure 1: Emodin laxative action (Adapted from Srinivas et al. 2006).

linked to the side effects caused by overdose and long-term use of anthraquinone-rich plants.

The mechanism of action and toxicity of anthraquinones used as laxatives is illustrated by studies with the active ingredient emodin. These molecules, when present in the intestinal lumen, can undergo hydrolysis by bacteria (when glycosylated), which releases the aglycone and the glycoside moieties (Figure 1). The aglycone changes the sodium and potassium pump (causing liquid retention) and leads to increased calcium in the smooth muscle of the lamina propria (causing increased peristalsis) (SRINIVAS et al., 2007). Afterwards, there is direct action in the stimulation of chloride channels by enterocytes (YANG, H. et al., 2011).

1.2.2. Toxicological concerns

Medicinal herbs used as laxatives and some of the Medicinal Plants on SUS's Interest List, such as cascara sagrada (*Rhamnus purshiana*), have side effects ranging from dehydration to darkening of colon mucosa, with indications of cancer risk (ABENDROTH et al., 2009). The mucosa darkening, also known as pseudo-melanosis coli, is a pigment present in macrophages from colonic mucosal cells in lamina propria. The melanosis coli has been linked to the chronic use of laxative/purgative anthranoid-rich plants (BOCKUS; WILLARD; BANK, 1933; WALKER; BENNETT; AXELSEN, 1988). Despite effective laxative action of anthraquinone-rich plants, clinical studies demonstrated that 73.4% of patients who chronically used anthranoids laxatives had melanosis coli, whereas for patients who used other types of laxatives this number was reduced to 26.6%. These results show a clear association of this class of compounds with colon darkening (BADIALI et al., 1985). Histological studies show that a large number of apoptotic bodies is not due to natural renewal, but to laxative action, suggesting that melanic substances are formed by anthraquinones action (BENAVIDES et al., 1997).

In fact, it has been proven that some anthraquinones can disturb the mechanism of apoptosis in cells. Apoptosis is programmed cell death, an important mechanism for cell renewal and to get rid of infected or damaged cells (PORTT et al., 2011). Apoptosis can be classified in three types: type I or apoptotic cell death, with autophagy receiving the most attention in the past years; type II, which is caspase-independent cell death (paradoxically autophagy is also known as type II); and type III, which was originally known as a catastrophic form of cell death related to necrosis. For type III the stimulation of tumor necrosis factor (TNF- α) receptors can induce cell death called necroptosis. Although authors have tried to classify and separate this complex process for didactic purposes, this mechanism is all interrelated and some parts are still unknown.

A better classification sorts the apoptotic signaling pathways in two groups: extrinsic (also called death receptor) and intrinsic (also called mitochondrial) pathway. The extrinsic pathway is triggered by external stimulation, which activates death receptors (TNF is the most famous) and the caspase cascade, resulting in rapid cell death. The intrinsic apoptotic pathway, which is often deregulated in cancer, is related to cytokine deprivation, DNA damage, and endoplasmic reticulum stress. These pathways also converge to a common pathway resulting in caspase cleavage and cell death (ICHIM; TAIT, 2016). Consequently, for apoptosis induction to occur a compound or extract must activate a caspase pathway.

In a recent study, Chen et al. (2011) proposed a melanosis-forming mechanism that correlated the accumulation of pigments to the long-term use of these natural compounds. When such compounds enter the colon, they produce a laxative effect and damage the epithelial cells. These cells release TNF- α for the cell renewal induction mechanism through apoptosis. Furthermore, they are phagocytized by macrophages, which migrate to lamina propria of the epithelium. In the lamina propria the apoptotic bodies become lipofuscin, giving rise to the black patches that darken the colon. With chronic use, this damage causes the immune system to deregulate and consequent inflammation.

The chemical complexity of anthraquinone-rich plants makes the elucidation of mechanisms of action and toxicology challenging. Regarding the scientific literature, several compounds present in this species, as aloe-emodin (DONG, X. et al., 2017; WU, Y. Y. et al., 2017), emodin, (DONG, X. X. et al., 2016; SU et al., 2005; ZHANG et al., 2017), aloin (BUENZ, 2008; LEE et al., 2014), chrysophanol (LIM et al., 2017), present apoptosis induction for a huge variety of mechanisms. Understanding the toxicity of these compounds at the molecular level suggests anticancer activities and applications. However, decreased laxative herbal medicines toxicity has not progressed, since the extracts are still commercialized as crude extracts. Additionally, the toxicity of some chemical markers that are used due to their abundance in the extract is still unknown.

This is especially true for cascara sagrada, where cascaroside A is the chemical marker used to standardize the crude extract. Anthraquinone glycosides can act as weak promoters in rat colon carcinogenesis (MERETO; GHIA; BRAMBILLA, 1996), suggesting a weaker toxicity compared to the correspondent aglycone. Studies report that after intestinal bacteria remove the sugar moiety, the aglycone has strong laxative effects (SCHORKHUBER et al., 1998), and – in theory – are more toxic.

Although some studies suggest that glycosides-anthraquinones are less toxic than the correspondent aglycone, these studies do not characterize the toxicity of cascarosides present in cascara sagrada. Furthermore, it remains unknown if these compounds are responsible for melanosis coli or trigger the apoptosis mechanism in the cells. Understanding the level of toxicity and potency of different classes of compounds may be an alternative for developing more effective medicines with fewer side effects.

1.2.3. Chemical concerns regarding anthraquinones

Anthraquinones are part of quinone compounds, characterized by a cyclic diketone ring between the two aromatic rings. Besides that, the substitution in one of the ketone groups for H₂, hydroxyl, or sugar generates the anthrone (substitution for H₂) and oxanthrone (substitution for hydroxyl) classes.

These compounds are colorful and important dyes used in the food and cosmetics industries. Such color property made this class the first colorants to be used. Alizarin, which is an anthraquinone extracted from the *Rubia* species (*Rubia tinctorum*), was the first industrialized dye produced and synthesized in the year 1869. Nowadays, industrialized food colors are among the most important dyes.

In order to avoid drastic toxic effects, quality control is an important step before releasing an herbal medicine on the market. Some of these anthraquinone-rich plants need to be stored for at least a year to ensure safe levels of anthrones, oxanthrones and anthraquinones. Hence, the ratio of these compounds can be used as chemical markers for quality and safety, such as cascaroside A (TSUKIDA; SUZUKI, 1954).

Due to their importance for quality control, detailed structure elucidation of these compounds has been a concern among the natural product chemists. The structural variations of these compounds comprise sugar moieties, hydroxyl and alkoxyl groups attached to the aromatic rings and different substituents in the side chains. Additionally, the stereochemical assignment of anthrone and oxanthrone compounds is particularly important, as the introduction of the C₁₀-stereocenter by the plant organism is not stereospecific, so that often both (10S) and (10R)-diastereomers are found in the same plant (Figure 2) (MANITTO, PAOLO; MONTI; SPERANZA, 1990b; MANITTO, PAOLO et al., 1993a).

In light of the non-selectivity C₁₀-stereocenter formation, the determination of the correct absolute configuration (AC) is a crucial part of the structure elucidation process for anthrones and oxanthrones. Nevertheless, until now, such AC determinations of newly discovered anthrones and oxanthrones are based on crystal structures of aloin A and B (RAUWALD; LOHSE; BATS, 1989), from which empirical correlations for electronic circular dichroism (ECD) spectra and Nuclear Overhauser Effect (NOE) contacts were derived (MANITTO, PAOLO et al., 1990b; MANITTO, PAOLO et al., 1993a). The ECD approach basically relies on the similarities of ECD spectra of unknown compounds with either aloin A (10S configuration) or aloin B (10R configuration), assuming that substituent effects will not affect the ultra violet/circular dichroism (UV/CD) spectral signatures. The AC

determination with NOE NMR data is based on the assumption of a most favored conformation. As a result, to ensure correct AC of these compounds extra optical technique and conformational analysis have to be applied to confirm if this rule can be generalized for the entire class of compounds.

Figure 2: Anthraquinones found in *R. purshiana*.

In summary, anthranoid-rich plants used as laxatives are still commercialized as crude extracts and, considering the scientific studies available, there is no evidence for the possibility of reducing the toxicity by enriching the extract with less toxic compounds. The presence of O-glucosyl and C-glucosyl anthraquinones and free aglycones regarding the induction of side effects as melanosis coli is not completely understood. Furthermore, considering the importance of quality control of *R. purshiana*, new techniques should be applied to help correct structural elucidation and avoid drastic diarrhea, since differentiation between small structural variations to stereochemical variations should be based on more trustworthy techniques than NOESY.

1.3. Tannin-rich plants

1.3.1. Mechanism of action of anti-ulcer medicines

Limited information exists about the mechanism of action for other plant species that act on the gastrointestinal tract, such as the species that contain tannins. The mechanism of action which explains why tannins improve the symptoms of diarrhea and gastritis is not fully understood and the hypotheses for their action are related to the ability to chelating metals, antioxidant activity and their power of complexation with other molecules (HASLAM, E., 1996; HASLAM, E. et al., 1989). In fact, the presence of tannins is associated with the gastroprotective activity, where the previous precipitation of these compounds neutralizes the extract activity (PRADO et al., 2014).

The gastroprotective activity is important in peptic ulcer treatment. Peptic ulcer is a break of stomach or duodenum mucosa. There are several pathophysiologic mechanisms which often require multiple physiological approaches. The emergence of

peptic ulcer can be related to emotional causes, anti-inflammatory use, *Helicobacter pylori* infection and other causes that increase acid secretion. Thus, understanding the cause is an important step in the pharmacotherapy approach.

The available antiulcer agents for ulcer treatment involve drugs that decrease acid secretion, neutralize acid content, modify risk factors and promote mucosal defense (Figure 3). Among the agents that decrease acid secretion are H₂ receptor antagonists and proton pump inhibitor, which are the most commonly used drugs (ranitidine and omeprazole). Agents that neutralize acid content are often bases that neutralize the stomach pH. Agents that modify risk factors include antibiotics, which eliminate *H. pylori* and lead to peptic ulcer cure. The last agent class used in ulcer peptic pharmacotherapy is the mucosal defense promoter. These drugs are coating agents, which form a viscous gel and adhere to gastric epithelial cells, including damaged areas (GOLAN; ARMSTRONG; ARMSTRONG, 2017; GOLAN et al., 2009).

Figure 3: Therapeutic targets of antiulcer drugs.

The proposed mechanism of tannin action is based on the complexation power of these molecules, which are assumed to be acting as coating agents. Although other derivative constituents can be absorbed by the degradation of the monomers performed by the microflora (as occurs with proanthocyanidins that are absorbed in dimers and trimmers) (DEPREZ et al., 2000; DEPREZ et al., 2001; KOLECKAR et al., 2008), polymeric molecules are limited to the intestinal lumen, reinforcing the gastroprotective mechanism of action. Although the binding property is well established and has been applied to tan leather for many years, the requirements for use as an herbal medicine are much more complex. Therefore, understanding the molecular size needed for drug activity and how long drugs exert their effects are some of the questions that need to be answered in order to turn tannin-rich plants into herbal medicines for ulcer treatment. Furthermore, there is no proof of their action as coating agents and the explanation for their action has been limited to theoretical beliefs.

1.3.2. Tannin as gastritis treatment alternatives

The mechanism of tannin-containing plants action should be better understood to develop HM with native species from the Brazilian *cerrado*, such as barbatimão, exemplified by these most commonly known species: *Stryphnodendron adstringens*, *S. obovatum*, *S. polyphyllum*, *S. coriaceum* and *S. rotundifolium* (LOPES et al., 2008; LOPES et al., 2009; LOPES et al., 2005). All these tannin-rich species are used indistinctively (or succedaneous) to treat diarrhea, gastritis, and skin lesions. Barbatimão is on SUS's plants of interest list and is part of the Phytotherapeutic Form of the Brazilian Pharmacopoeia (MARTINS; LIMA; RAO, 2002). This plant is similar to other tannin-rich species that are used for stomach problems, as the explanation concerning its mechanism of action limited to the chemical properties is attributed to its constituents.

Audi et al. (1999) studied the antiulcerogenic activity of barbatimão (*S. adstringens*) and found that the induction of ulceration occurred due to acute stress (rats fasted for 24 hour) and acidified alcohol (induction of lesions due to the disruption of the mucosal protection system). The authors reported a significant antiulcer activity, but did not establish the nature of the compounds responsible for the antiulcer activity nor the mechanism of their antiulcerogenic effects.

Skin products using barbatimão extract are available in the Brazilian market; however, oral medications to treat gastric problems have not yet been made available for public use. Considering the wide ethnopharmacological use of tannin-rich plants for gastric

problems and the restricted explanation about their activity associated with their chemical properties, further studies are needed to better understand their action.

1.3.3. Chemical concerns regarding tannins

The term "tannins" arose in 1796 by Seguin to designate substances found in plant extracts that were able to combine with proteins from animal skins, converting the skins into leather and preventing their putrefaction. Currently, they are defined as a class of polyphenols of high molecular mass (500-3000 Daltons), and can reach up to 5000 Da, originating from the polymerization of simple polyphenols. This high molecular weight is directly related to the astringent activity of this group of molecules, which is a common sensation when eating a green banana. Ecologically, astringency functions as a defense mechanism, by increasing the concentration of tannins to protect itself from natural predators (HASLAM, EDWIN, 1989; HASLAM, E. et al., 1989).

They form insoluble complexes with proteins, which explains why they can be used in the leather tanning process. Extracted tannins were extensively combined with animal skin proteins, which prevented putrefaction, and rendered skins resistant to boiling water by turning them into leather. There is a tendency to return to using tannins for leather making so to decrease the use of heavy metals, which reduces the environmental impacts (HASLAM, EDWIN, 1989).

Tannins are classified into 2 groups that differ according the phenolic core and connection. These are: condensed tannins (proanthocyanidins) and hydrolysable tannins. There are also complex tannins, which consist of a union of condensed tannins with hydrolysable tannins.

The hydrolysable tannins are products of esterification of sugar with gallic acid units (Figure 4, compound **(15)**). The condensed tannins are two or more units of catechin (**(16)** derivatives (until 3 units can be also called procyanidins – **(18)** and **(19)**), which polymerize and form complex structures, that are usually very difficult to purify **(20)**.

Figure 4: Examples of hydrolysable tannin (15), catechin (16), gallocatechin (17), procyanidin dimer linked C4-C8 (18), procyanidin dimer linked C4-C6 (19) and condensed tannin (20).

The diversity of tannins can be verified based on the structures exemplified in Figure 4. For condensed tannins, the structural elucidation is challenging, since small differences between units are very difficult to establish. These differences can be related to position and number of hydroxyl groups in the B-ring, the linkage between the units (usually C4-C8 or C4-C6) and the stereochemistry of C-2, C-3 and C-4 of each unit. The stereochemical aspects are particularly puzzling, since a simple procyanidins dimer can have 32 (2^5) stereoisomers. Besides usual C-3/C-4 trans configuration, some examples of cis configurations can be found in the scientific literature (SCHLEEP; FRIEDRICH; KOLODZIEJ, 1986).

The importance of stereochemistry in pharmaceutical drugs has been known for years, especially after Thalidomide's tragedy (CANER et al., 2004). In the phenols class stereochemistry is particularly important due to evidence of different biological activities of stereoisomers. This is highlighted by some authors, as Ottaviani et al. (2011), who report that (-)-epicatechin is the only stereoisomer capable of mediating a significant arterial dilation between some tested catechins. Other activities of these molecules when present in biological systems have been reported (DONOVAN et al., 2006; SAEZ-AYALA et al., 2013; SIRK et al., 2009; TAYLOR; HAMILTON-MILLER; STAPLETON, 2005).

Considering the importance of AC establishment, natural product scientists used to determine the chiral centers of these phenol compounds through degradation of the molecule and comparison with sugar (BIRCH; CLARKLEWIS; ROBERTSON, 1957). The general methodology for structural elucidation of tannins involves previous derivatization, usually applying acetylation, in an attempt to make the atoms distinguishable between the units. Considering typical small sample sizes and time-consumption synthesis processes, new techniques of structural elucidation could be applied to make the process less arduous.

In light of the chemical and biological issues of turning herbal products into herbal medicines, some modern techniques can be applied to solve these problems.

1.4. Techniques applicable to chemistry and biological studies of tannins and anthraquinones

Due to structural elucidation challenges regarding tannins and anthraquinones and biological issues regarding their action and toxicology, modern techniques can be applied to simplify this process. In this thesis we focus on mass spectrometry, fluorescence microscopy, and vibrational optical activity as an effort to solve issues concerning these plants recommended by SUS.

1.4.1. Mass Spectrometry

Nowadays, mass spectrometry (MS) is an indispensable technique for chemistry, biochemistry, medicine, and pharmacy. It is crucial for structural elucidation, sequencing biomolecules, quantification of metabolites, and drug quality control. The field of application that MS has achieved is broad and modern examples can be found for future use even in surgeries (FATOU et al., 2016).

The development of MS had a crucial advance with electrospray ionization (ESI) development. This technique allowed thermolabile and high-molecular-weight compounds, such as biopolymers and proteins, to be ionized and transferred to the gas phase, thus developing new applications in molecular biology, medicine, and plant metabolomics (ERNST et al., 2014; FENN, 2003; FENN et al., 1989). The influence of ESI in chemistry was recognized in 2002 when John B. Fenn (one of the scientists behind ESI development) won the Nobel Prize in Chemistry. Since then, the coupling of liquid chromatography to mass

spectrometry (LC–MS), which was possible because of the nature of the ESI process (an atmospheric pressure ionization method), has expanded the diversity of molecules that can be analysed by mass spectrometry (DIAS; MELO; CROTTI, 2012).

The electrospray ionization process occurs at ambient pressure when high voltage is applied to a capillary where the solution is passing through the analyte. Droplets form at the tip of the capillary and are released into the space between the source and the inlet in the analyser (nebulization). The presence of gases and heating causes the droplet to divide and dry, forming droplets with only 1 ion (applied theory for large molecules) or expelling ions into the gas phase (applied theory for small molecules).

Other ionization techniques were developed to increase the range of compounds that can be analyzed by MS. The laser ionization method used in matrix-assisted laser desorption/ionization (MALDI) has been shown to be a good resource for natural product chemists, especially for analysis of big molecules. The ionization process consists of solubilizing the sample mixed with matrix, which is applied to a plate and dried. The MALDI ionization process is not fully understood, however it is believed that the laser heats the crystals by accumulation of a large amount of energy, which sublimates the matrix with the analyte. In gas-phase, the matrix can transfer the charge to the analyte, although it is accepted that the ionization process can occur during the mixture, especially with the presence of additives.

Considering the importance of mass spectrometry as an analytical and structural elucidation tool, understanding the fragmentation reactions of the precursor ion is essential for the structural characterization of organic compounds using ESI-MS/MS data (DEMARQUE et al., 2016). Initial studies have devoted special attention to standardize the reactions under CID conditions, primarily those from atmospheric pressure ionization techniques (HOLCAPEK; JIRASKO; LISA, 2010; LEVSEN; SCHWARZ, 1983). These studies showed major reactions, and the mechanisms were derived from the functional groups, which are the fundamentals to the mechanisms. However, for studies with natural and synthetic products the overall system must be considered, so that the mechanism involved is not based solely on the functional groups. In the structure elucidation process based on ESI-MS/MS data, the knowledge of the fragmentation reactions involved in the formation of the peaks of the mass spectrum may provide important information about the molecular structure and the connectivity between the atoms. (SCHWARZENBERG et al., 2014).

1.4.2. Fluorescence microscopy

Fluorescence microscopy is an excellent tool to determine tissue distribution of molecules in cellular structures, especially for natural product-derived compounds. Molecules from natural sources have intrinsic fluorescence and have been used as fluorescent probes attached to molecules that do not display this property. This practice shows the efficiency of natural products as probes and the possibility of applying this technique to determine the natural compound distributions in tissues (ALEXANDER et al., 2006).

The phenomenon of fluorescence is linked with absorption of energy in the form of photons of visible or ultraviolet light. Considering the need to better understand the mechanism of tannins action and the property of natural products fluorescence along with the theoretical beliefs for their action as local coating agent, fluorescence microscopy can be a powerful tool to clarify some of these issues.

1.4.3. Chiroptical techniques

The AC is often the most challenging part in the structural elucidation process. Considering that it is not always suitable to grow crystals for crystallographic techniques, such as X-ray, modern chiroptical techniques have been widely used for natural product chemists.

The term chiroptical spectroscopy can be used as the study of chiral systems using optical spectroscopy methods. These methods are based on the optical activity of the substance, which is the property of a molecule to change the light of a certain wavelength (λ). When this phenomenon changes the rotation of linear polarized light, it is called optical rotatory dispersion (ORD). If the incident light is circularly polarized, the differential absorption between left and right polarized light is called circular dichroism (CD). The incident visible light (in the UV region) is the electronic circular dichroism (ECD), while the incident light in the mid-infrared spectral region is called vibrational circular dichroism (VCD) (NAFIE, 2011).

Although VCD is much more sensitive to solvent-induced structural changes than its electronic counterpart (MERTEN, CHRISTIAN, 2017), it has become increasingly popular in the natural products chemistry community for AC determinations of complex molecules (BATISTA JR; BLANCH; BOLZANI, 2015). This is also due the development of quantum

theories parallel and computers, where the spectra can be predicted and compared with the experimental.

Before the possibility of spectra calculation, empirical rules were applied to AC using ECD. The octant rule was the most used to predict the signal in ECD spectra. This rule is based on a three dimensional representation of the molecule, where three dimensional coordinate axes are superimposed onto the molecule with the origin at the carbonyl carbon and one of axes coinciding with the C=O bond in the carbonyl group. These are the octants, where molecule is viewed from the O atom toward the C atom of the carbonyl group. Based on the most steric bulk octant, the ECD signal is predicted according Figure 5.

Figure 5: The octant rule: diagram to establish the octant signal.

This technique can be applied to molecules in which the most favorable conformation of the molecule and the distribution of the bulk are unambiguous. For anthraquinones, a conformational study is needed to confirm if the NOESY contact found for aloins is a rule that can be applied for any anthrone, regardless of the substituents. For procyanidins, the problem relies in the absence of carbonyl group (present in flavonoids), implicating ineffective ECD analysis. So, VCD is a feasible alternative for both anthraquinones and procyanidins.

In summary, considering the need to frame THP in HM, along with the importance of proving their effectiveness and reducing toxic effects, this thesis focuses on solving some issues that may appear during the release of HM made with plants that act on the gastrointestinal tract. The aim of this research is to help make HM safer, more effective, and scientifically proven.

OBJECTIVES

2. OBJECTIVES

- To extract, isolate, and identify the chemical constituents of barbatimão (*Stryphnodendron rotundifolium*) and cascara sagrada (*Rhamnus purshiana*);
- To assess the absolute configuration of the isolated compounds using VCD;
- To apply mass spectrometry in order to analyze extracts;
- To quantify and standardize the dose of barbatimão and cascara sagrada extracts;
- To treat animals with barbatimão extract in order to evaluate the tannin distribution in gastric tissue and correlate them with possible mechanism of action;
- To assess biochemical parameter alterations in animals treated with barbatimão;
- To evaluate the cytotoxicity of different anthraquinones and caspase induction of the cytotoxic compounds;
- To propose a structure-activity relationship between the anthraquinones regarding the cytotoxicity.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

Chemicals and reagents

HPLC-grade methanol (J.T. Baker), trifluoroacetic acid (TFA, J.T. Baker), formic acid (J.T. Baker) and ultrapure water (Millipore, MA, USA) were used in HPLC and mass spectrometry analysis. For other analyses P.A. grade solvents were used. Silica gel 35-75 Mesh (Merck®) was used for classic chromatography column (CCC) and Alugram Xtra Sil G/UV254 (Macherey-Nagel) was used for thin layer chromatography (TLC) analyses. The standards gallic acid, catechin, aloin, emodin, and anthralin were acquired from Sigma-Aldrich.

Animals

The biological test was performed on male rats, weighing between 200 to 250g. The experimental procedures were approved by the Ethics Committee of Ribeirão Preto campus, University of São Paulo (Protocol 14.1.722.53.2). The animals were kept in collective cages (41 length x 32 width x 18 height), and maintained under controlled temperature ($22 \pm 2^{\circ}\text{C}$) and lighting (12/12 light/dark cycle), with water and food *ad libitum*. The animals were fasted a maximum of 12 hours before the treatment. Rats were euthanized with anesthesia thiopental overdose (150 mg/kg – intravenous).

3.1. Chemical and biological study of cascara sagrada

3.1.1. Extraction and HPLC-MS analysis

We obtained commercial powder of cascara sagrada (*R. purshiana*) bark from Santos Flora (lot number CASC01/1210) and extracted it through percolation with ethanol. The crude extract was dried (yield 34 %), resuspended (50 g) in methanol:water (1:1), and partitioned with hexane (yield 18 %) and ethyl acetate (CasEtAcPh; yield 10 %). The resulting methanol:water phase (CasMWPh; yield 26%) and ethyl acetate phase (CasEtAcPh) were analyzed in TLC plates (ethyl acetate 95:5 methanol) and later analyzed in HPLC-MS (Figure 6).

The HPLC-MS analysis was performed using a Shimadzu LC-6AD coupled with mass spectrometer ESI-TOF (Bruker Daltonics). For the chromatographic separation a C_{18} column was used (ODS2 - Sigma-Aldrich 25 cm $\times$ 4.6 mm; 5 μm of particle size). The

applied flow was 1 ml/min and injection volume of 20 μ l. The mobile phase was ultrapure water (solvent A) and methanol (solvent B) with formic acid 0.1% (v/v) was added to both.

The gradient elution method started with 20% of B, was kept isocratic for 20 min and increased to 30% of B until 26 min. Another isocratic period with 30% of B was kept for 50 min and was then increased to 50% until 66 min, reaching 100% (B) at 86 min. Eight more minutes were used to wash and stabilize the column.

Figure 6: Partitions made with Cascara Sagrada bark. (Subtitle: CasCruEx – Crude extract obtained from percolation of the plant bark powder; MeOH – methanol; Hx – hexane; EtAc – Ethyl Acetate; CasHxPh – hexane phase; CasEtAcPh – Ethyl acetate phase; CasMWPh – methanol/water phase)

3.1.2. Isolation of cascarosides

The purification of cascarosides enriched fraction – CasMWPh (1.56 g) – was performed using silica gel in CCC, eluted with ethyl acetate and methanol (95:5). After 50 fractions (50 ml), the mobile phase proportion was modified to ethyl acetate and methanol (8:2) and 97 fractions were collected. After TLC analysis, the fractions were gathered in ten fractions [(CasFr01-12; 0.89%), (CasFr13-15; 0.25%), (CasFr16-25; 1.47%), (CasFr26-45; 0.76%), (CasFr46-90; 7.37%), (CasFr91-100; 6.09%), (CasFr101-123; 15.95%), (CasFr124-145; 43.12%), (CasFr146; 14.17%) and (CasFr147; 0.44%)]. HPLC-MS analyses of the fractions were performed.

The CasFr124-145 fraction (672 mg) was solubilized in 20 ml of methanol and submitted to isolation process in HPLC-semipreparative (Shimadzu LC10A) using a column Shimadzu Shimpack ODS(H) (Kit 200 mm x 20 mm, 5 μ m particle size). The flow rate was 10 ml/min and injection volume was 1 ml. The mobile phase was composed of water (A) and methanol (B), with TFA 0.01% (v/v) added to both. The elution profile started with 20 % of

B, kept isocratic for 39 min, and was then increased to 30 % of B until 49 min and kept isocratic for 55 min. A gradient was used to elevate until 90 min, and 15 min were used to wash and equilibrate the system.

The fraction CasFr124-145 lead to 11 fractions CasPrepFr1 (retention time (RT) 10.9 min; yield 1.9%), CasPrepFr2 (RT 24.6 min; yield 2.1%), CasPrepFr3 (RT 33.7 min; yield 2.1%), CasPrepFr4 (RT 52.5 min; yield 2.1%), CasPrepFr5 (RT 59.3 min; yield 0.6%), CasPrepFr6 (RT 74.3 min; yield 0.9%), CasPrepFr7 (RT 76.8 min; yield 1.3%), CasPrepFr8 (RT 77.9 min; yield 0.5%), CasPrepFr9 (RT 81.5 min; yield 0.3%), CasPrepFr10 (RT 83.3 min; yield 1.5%) and CasPrepFr11 (RT 86.6 min; yield 0.8%). The fractions were dried using a rotary-evaporator and freeze-dryer. Structural elucidation was performed based on ^1H and ^{13}C NMR, DEPT 135°, NOESY (Bruker, 400 MHz and Bruker 500 MHz) and High Resolution Mass Spectrometry (HR-MS, Bruker, Ultra Q-ToF) data.

3.1.3. Stereochemical analysis

The vibrational spectra were recorded on a Bruker Vertex 70 equipped with a PMA 50 unit for polarization modulated measurements. Solutions of the isolated compounds (~40mg/ml) were held in a transmission cell with BaF_2 windows with 100 μm pathlength. Both infrared (IR) and VCD spectra were recorded at 4 cm^{-1} spectral resolution by accumulating 32 and ~85000 scans (20 hours accumulation time), respectively. Baseline correction of the VCD spectra was done by subtracting the spectra of solvent recorded under identical conditions.

A systematic conformational analysis of the isolated compounds was carried out on force field level (MMFF (HALGREN, 1996)) using the Spartan 14 (Spartan 14, 2014) software package by evaluating about 4000 structures for each molecule. The 100 lowest energy conformations of CASPrepFr1, CASPrepFr2, and the other 30 lowest energy conformations were further optimized at B3LYP/6-31G(2d,p)/IEFPCM(methanol) level of theory using Gaussian 09 Rev E.01 (FRISCH et al., 2013). Based on these optimized structures, the vibrational spectra were simulated as described above using the $\Delta G_{298\text{K}}$ -based Boltzmann factors. Vibrational line broadening was simulated by assigning a Lorentzian band shape with half-width at half-height of 6 cm^{-1} to the calculated dipole and rotational strength. Finally, the calculated wavenumbers were scaled by a factor of 0.98. The molecular dynamics simulations were carried out using the AMBER software package (CASE et al., 2015) at the general Amber force field (GAFF). The molecules were each placed in a cubic box and

solvated with about 1400 methanol molecules. Following the typical equilibration procedure, the final production run simulated solvation shell around CASPrepFr2 and CASPrepFr6 for 5 ns.

3.1.4. Direct infusion mass spectrometry

Direct-infusion mass spectrometry was performed using electrospray ionization coupled with ion trap mass spectrometer analyzer (AmazonSL, Bruker). The parameters to acquire the sequential fragmentation (ESI-MS³) were: capillary 3500V, end plate offset 500V, nebulizer 15 psi, dry gas 5 L/min, dry temperature 250 °C and pump flow 5 µL/min. Nitrogen was used as dry and nebulizer gases. The isolated compounds CasPrepFr1, CasPrepFr2, CasPrepFr3, CasPrepFr4, CasPrepFr6, and CasPrepFr10 were analyzed and the fragmentation mechanisms were proposed.

3.1.5. Extract standardization

Doses administered to the animals were standardized through quantification of the isolated compounds in the extracts. For this, a HPLC Shimadzu (LC-20AD) coupled with an ESI triple quadrupole mass spectrometer (ABSciex API 3200) was used. Epigallocatechin was used as an internal standard. For chromatographic separation a column Supelco Ascentis Express C₁₈ (5 cm x 2.1 mm, 2 µm of particle size) was used, coupled with a pre-column with the same material. The mobile phases were optimized to obtain the best signal/noise ratio and water and methanol (both with 0.1% of formic acid) were adopted. The elution method (method 03) initiated with 5% of B, kept isocratic for 0.7 min, which was then increased to 35% for 1 min and to 45% for 5 min. After this period, a new gradient until 100% was adopted for 7.5min. 3.5 min were added to the method to wash and stabilize. The injection volume was 15 µL, the column temperature was 45°C, and the autosampler temperature was 5°C.

The negative ionization mode was employed and the ionization source parameters were CUR 12 (*Curtain Gas*), source temperature 450 °C, ionization voltage (IS) -4500 V, CAD gas (*Collisionally-activated dissociation gas*) 8, Gas1 (nebulization gas) 50 and Gas2 (turbo heaters gas) 50. The parameters DP (*declustering potential*), EP (*entrance potential*), CEP (*collision cell entrance potential*), CE (*collision energy*), CXP (*collision cell exit*

potential) and DT (*Dwell time*, monitoring time for each transition keeping around 18 scans/peak) were optimized for each monitored transition and are shown in Table 1.

Table 1: MS parameters optimized for cascarosides quantification (QT – quantification; Conf – confirmation).

		Q1	Q3	DT	DP	EP	CEP	CE	CXP
Casc A/B	QT	579.0	297.0	15	-61	-10	-54	-51	-8
	Conf	579.0	459.0	15	-61	-10	-54	-39	-7
Casc 10-H A/B	QT	595.0	269.0	15	-60	-10	-54.5	-64	-6
	Conf	595.0	432.0	15	-60	-10	-54.5	-34	-15
Casc C/D	QT	563.0	281.0	15	-53	-10	-53.5	-52	-52
	Conf	563.0	443.5	15	-53	-10	-53.5	-41	-13
Epi	QT	457.0	169.0	20	-40	-4	-50.6	-28	0
	Conf	457.0	125.0	20	-40	-4	-50.6	-52	0

The quantification methodology was validated for precision (curve with 9 points and 6 replicates each), inter-run accuracy (6 replicates in 9 points), selectivity, and linearity. The assumed concentrations were 10, 20, 50, 100, 125, 250, 375, 500, 1000 ng/ml. The extracts and phases were diluted in the 25 mg/ml concentration and then diluted in the same curve conditions.

3.1.6. Biological tests *in vitro*

For viability assay, cell line NOK-SI (normal oral keratinocyte immortalized spontaneously) was grown in DMEM (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS-GIBCO) in a humid atmosphere containing 5% CO₂ at 37 °C. The cells were plated in 96-well plates (1.10⁴ cells/well) and after 24 hours the cells were treated with increasing concentrations (0.1, 1, 10, 20, 50, 100 and 200 µM for the cascarosides A and B and 0, 10, 50, 100, 200, 300, 500 µg / ml for CasCruEx, CasEtAcPh, Aloin, Emodin and Anthralin standards). After 48 hours cytotoxicity was determined using the Alamar Blue[®] method. The dye was added at the final concentration of 0.01mg/ml and after 3 hours of incubation at 37°C containing 5% of CO₂, the plate fluorimeter was read in the emission lengths 530/25 and excitation 590/35. The results were expressed as percentages of viable cells taking control of each compound (diluent) out of 100%. One-way Anova was used and only differences with p < 0.05 were considered statistically significant.

For caspase assay, cells of the NOK-SI lineage were plated (9.10⁵ cells in 100mm³ plates) and treated with 300 µg/ml of each compound for 48 hours. Cellular protein extracts were obtained with cell lysis reagent and extraction of CellLytic proteins (Sigma-Aldrich),

with protease inhibitors and phosphatases (Sigma-Aldrich). The protein concentration was determined using the Bradford protein assay system (Bio-Rad). Thirty micrograms of protein were electrophoresed on 10% sodium-dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (GE HealthCare). The membranes were blocked with a 5% solution of skimmed milk powder in Tris buffer containing 10% Tween-20 (TBST). Antibodies against Caspase 3 (Caspase-3 (8G10) Rabbit mAb # 9665) and as constitutive calnexin (Calnexin (C5C9) Rabbit mAb # 2679) were added. Reactions were revealed using a chemiluminescent ECL Western blotting system (GE HealthCare).

3.2. Chemical and biological study of barbatimão

3.2.1. Acquisition, extraction and HPLC-MS analysis

Barbatimão (*S. rotundifolium*) bark was collected in Campo Grande, Mato Grosso do Sul state, in april/2015 and identified by Dr. Flávio Macedo Alves. A voucher specimen was deposited under registration number 64224 (GPS 20°30'13.5' S; 54°35'20.6'' W) in the CGMS herbarium, Campo Grande, MS, Brazil. The bark was dried in a circulating air stove (40°C) and grounded in knife mill (20 mesh). The extraction was made by maceration: 1kg was immersed in acetone:water (7:3) and left for 48 hours. The solvent was removed and the process was repeated 4 times. The crude extract obtained (BTMCrEx; yield 20 %) was dried and lyophilized. The BTMCrEx (20 g) was submitted to partition with ethyl acetate (BTMEtAcPh). The BTMEtAcPh was dried and analyzed in HPLC-MS (Shimadzu LC-6AD coupled with mass spectrometer UltrOTOF; ESI-TOF; Bruker Daltonics) and HPLC-MS/MS (Shimadzu LC-6AD coupled with ESI-Ion Trap, AmazonSL, Bruker). The column used was ODS2 - Sigma-Aldrich (C₁₈, 25 cm × 4.6 mm, 5µm of particle size), flow 1 ml/min and injection volume of 20µL. The mobile phases were ultrapure water (Millipore, MA, USA) and methanol (J. T. Baker), with 0.1% formic acid (J. T. Baker). The analytical elution method started with 5% organic phase, increasing to 25% until 50 min and to 35% until 70 min. 8 minutes were added to column wash and stabilize.

3.2.2. Isolation of catechin derivatives

The BTMEtAcPh was resuspended in methanol and eluted in Sephadex column with methanol. After TLC analysis (eluent ethyl acetate/acetone 7:3), the fractions were gathered in 5 new fractions (BTMFr1-4, 6%; BTMFr5-7, 3%; BTMFr8-10, 11%; BTMFr11-15, 18%; and BTMFr16-31, 34%). To isolate the procyanidins monomers the fraction BTMFr8-10 was submitted to semipreparative HPLC (Shimadzu LC10A) using a Shimadzu Shimpack ODS(H) column (Kit 200 mm × 20 mm, 5 µm particle size). The mobile phase was composed of water (A) and methanol (B), with 0.01% TFA (v/v) added to both. The flow rate was 10 ml/min and injection volume was 1 ml. The elution profile was initiated with 10% of B and was increased to 25% of B until 35 min and kept isocratic until 40 min. A gradient was used to elevate to 33% until 50 min, and 15 min were used to wash and equilibrate the system. The fractions were dried (BTMPrepFr1, 0.5%; BTMPrepFr2, 1%; BTMPrepFr3, 20.7%; BTMPrepFr4, 0.4%; BTMPrepFr5, 0.5%; BTMPrepFr6, 4.4%; BTMPrepFr7, 2.4%;

BTMPrepFr8, 2.4%; BTMPrepFr9, 38.3%; BTMPrepFr10, 1%; BTMPrepFr11, 1.3%; BTMPrepFr12, 2.2%;). After HPLC-MS analysis, the fractions BTMPrepFr3, BTMPrepFr7 and BTMPrepFr9 were sent to NMR (^1H , ^{13}C and DEPT-135°). The NMR data was compared to the literature (LIAO et al., 2014; NOMIZU et al., 2008).

3.2.3. Stereochemical analysis

A systematic conformational analysis of the isolated procyanidins compounds was carried out varying torsional angles of hydroxyl groups. The aromatic hydroxyl groups were always established in the same plane as the aromatic ring (SHIGEMATSU; KOBAYASHI; TANAHASHI, 2001). Therefore, the dihedral angles for non-vicinal hydroxyls α (0° or 180°), β (0° or 180°) and γ (60° , -60° or 180°) were assumed considering all possible combinations (12 conformers). The 12 conformers were inserted in 4 groups, which differ according the dihedral angle of vicinal hydroxyl groups: G1 ($\delta_1 180^\circ$, $\delta_2 180^\circ$, $\delta_3 180^\circ$), G2 ($\delta_1 0^\circ$, $\delta_2 180^\circ$, $\delta_3 180^\circ$), G3 ($\delta_1 0^\circ$, $\delta_2 0^\circ$, $\delta_3 180^\circ$) and G4 ($\delta_1 0^\circ$, $\delta_2 0^\circ$, $\delta_3 0^\circ$). The variations in β angle were considered according to the presence of one hydroxyl group in the A-ring for two compounds (Figure 7).

Figure 7: Conformational analysis developed for isolated procyanidins compounds.

All conformers were optimized at B3LYP/6-311G(2d,p)/IEFPCM(methanol) level of theory using Gaussian 09 Rev E.01. (FRISCH et al., 2013). Based on these optimized structures, the vibrational spectra were simulated as described in item 3.1.3 using deuterated methanol ($\text{CD}_3\text{OD-d}_4$) and mixtures of deuterated methanol ($\text{CD}_3\text{OH-d}_3$) and deuterated acetonitrile ($\text{CD}_3\text{CN-d}_3$).

3.2.4. MALDI-TOF/TOF analysis of fractions

The analysis of fractions and extracts were carried out in MALDI-TOF/TOF UltrafleXtreme (Bruker Daltonics, Bremen, Alemanha). To evaluate the best concentration, the extracts were diluted in the following concentrations: 10 mg/ml, 5 mg/ml and 2.5 mg/ml. Dihydroxybenzoic acid (DHB) plus Na⁺ was used as matrix (20 mg/ml) and a mixture of peptides was used for external calibration (peptide calibration standard of Bruker: angiotensin I and II, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39 and somatostatin 28). An equal amount of 5 µl of matrix and extracts were mixed and applied in MALDI plate. After spectra acquirement, the fragmentation pattern of tannins was analyzed (GUARATINI et al., 2014).

3.2.5. Extract standardization

3.2.5.1. Procyanidins quantification

Procyanidins in BTMCrEx were quantified through quantification of isolated compound gallo catechin and the analytical standards catechin and gallic acid. For this, a HPLC Shimadzu (LC-20AD) coupled with an ESI triple quadrupole mass spectrometer (ABSciex API 3200) was used. 10-hydroxycascarside A was used as internal standard. For chromatographic separation a column Supelco Ascentis Express C18 (5 cm x 2.1 mm, 2 µm of particle size) was used, coupled with a pre-column with the same material. The mobile phases were optimized to obtain the best signal/noise ratio and water and methanol (both with 0.1% of formic acid) were adopted. The elution method initiated with 15% of B, kept isocratic for 0.7 min, was then increased to 35% until 1 min and to 45% until 2.5 min. After this period, a new gradient of 100% was adopted until 3.0 min. To wash and stabilize 5 min were added to the method. The injection volume was 10 µL, the column temperature was 45°C, and the autosampler temperature was 5°C.

The negative ionization mode was employed and the ionization source parameters were CUR 12, source temperature 450 °C, IS -4000 V, CAD gas 8, Gas1 50 and Gas2 50. The parameters DP, EP, CE, CXP and DT were optimized for each monitored transition and are shown in Table 2.

Table 2: Mass spectrometer conditions (DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: collision cell exit potential, DT: Dwell time) to quantification of Catechin (CAT), Gallocatechin (GCT), Gallic Acid (GC) using 10-hydroxycascaroside A (10Casc-A) as internal standard.

		Q1	Q3	DT	DP	EP	CE	CXP
Cat	QT	289.0	109.0	20	-40	-4	-30	0
	Conf	289.0	125.1	10	-40	-4	-28	-2
Galloc	QT	305.0	167.1	110	-40	-4	-26	0
	Conf	305.0	125.0	10	-40	-4	-26	0
GA	QT	169.0	125.0	110	-30	-10	-24	-2
	Conf	169.0	106.8	10	-30	-10	-18	0
10H-Casc-A	QT	595.0	269.0	90	-60	-10	-64	-6
	Conf	595.0	432.0	10	-60	-10	-34	-15

The quantification methods were validated for precision (curve with 9 points and 6 replicates each), inter-run accuracy (6 replicates in 9 points), selectivity, and linearity. The assumed concentrations were 20, 50, 100, 125, 250, 375, 500, 1000, and 2000 ng/ml. The extract administered to the animals was weighed in triplicate and injected using the validated methodology conditions.

3.2.5.2. Tannins quantification

The total tannins quantification of crude extract was performed using the pharmacopeial method (BRASIL, 2010). An aliquot (0.25 g) of the crude extract was solubilized in distilled water and transferred to a 250 ml volumetric flask (“initial solution”).

The **sample solution to quantify total phenolics (TP)** was prepared by diluting 5 ml of the initial solution in a volumetric flask with distilled water to 25 ml. From this solution, 2 ml was added in a 25 ml volumetric flask with 1 ml of phosphomolybdotungstic reagent and 10 ml of distilled water. The volume was completed with 29% sodium carbonate solution (p/ V). After 30 minutes the absorbance in spectrophotometer was determined using wavelength 760 nm and water as blank reference.

The **sample solution for polyphenols not absorbed on skin powder (PNASP)** was prepared using 10 ml of the initial solution with 0.1 g of skin powder and was stirred for 60 minutes. After that, the solution was filtered and transferred to a volumetric flask (25 ml) with distilled water. From this solution, 2 ml was transferred to another 25 ml volumetric flask and 1 ml of phosphomolybdotungstic reagent and 10 ml of distilled water were added. The volume was completed with 29% sodium carbonate solution (p/ V). After 30 minutes the absorbance in spectrophotometer was determined using wavelength 760 nm and water as blank reference.

The **standard solution (SS)** was prepared diluting 50 mg of pyrogallol in 100 ml volumetric flask with distilled water. From this solution, 5 ml were transferred to another 100 ml volumetric flask and filled with distilled water. From this, 2 ml was transferred to a 25 ml volumetric flask, 1 ml of phosphomolybdotungstic reagent and 10 ml of distilled water were added. The volume was completed with 29% sodium carbonate solution (p/ V). After 30 minutes the absorbance in spectrophotometer was determined using wavelength 760 nm and water as blank reference.

The total tannin content (TT) was calculated according to the formula:

$$TT = \frac{62.5 \times (TP - PNASP) \times m_2}{SS \times m_1}$$

m_2 = pyrogallol mass in grams
 m_1 = sample mass in grams

3.2.6. Biological test

The Barbatimão CrEx was administered by oral gavage (400 mg/kg of animal) (MELO, J. O. D. et al., 2007). The experimental groups were divided randomly: 6 animals without treatment (blank) and 6 animals treated 30 minutes before euthanasia (for 3 of these animals the organs were washed 3 times with 5 ml of PBS and the 3 remaining the organs were immersed in phosphate buffer saline (PBS) in order to remove the blood excess). The third group consisted of 3 animals treated 1.5 h before euthanasia. The organs were frozen in a nitrogen chamber and kept in a freezer (-80°C) until analysis procedures.

For blood collection, the animals were anesthetized with ketamine (100 mg/kg) and xylazine (10mg/kg, intraperitoneally), 1.5 h after Barbatimão CrEx administration (ADINORTEY et al., 2013; AL ASMARI et al., 2014). The time for blood collection was chosen according to the T_{max} reported for the compounds in the literature (HUO et al., 2016). The blood was collected through the femoral artery. After coagulation, it was centrifuged for 20 minutes (4°C, Boeco M-240R, 400 x g). The serum was stored at -70°C and sent to Setor de Análises Clínicas of Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo (FCFRP-USP) to evaluate: creatinine, uric acid, urea, HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), VLDL (Very Low Density Lipoprotein), GOT/AST (Glutamate Oxaloacetate Transaminase/ Aspartate Aminotransferase), SGPT/ALT (glutamate-pyruvate transaminase/ Alanine transaminase), LDH (Lactate Dehydrogenase), alkaline phosphatase, glucose, cholesterol, and triglycerides. The evaluation was done by comparing treated specimens to non-treated animals and the literature (MELO, M. G. D. et

al., 2012). T-tests were used to calculate and evaluate the difference between averages in the software STATISTICA 8.0.

The organ sections were made using a Leica CM1860 cryostat. Tissue-Tek was used to fix the stomach to the plate and position it for transverse cuts. The temperature used in cryostat was -15°C and the thickness of confocal microscopy was 30 µm. The confocal laser scanning microscope used was Leica TCS SP8, the excitation was performed using 488 nm laser with 28.9% intensity. Absorption was adjusted to 575nm - 634nm.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Results and discussion – cascara sagrada

4.1.1. Chemical profile

Cascarosides are anthrones extensively present in *Rhamnus* species and are key chemicals used for quality control of laxative herbal products (KRENN et al., 2004; MANITTO, P. et al., 1993;1995). To obtain these compounds, partition was carried out using methanol/water and ethyl acetate, considering that glycosylated anthrones would have a different partition coefficient than the free aglycones. As a result, the partition methodology applied to cascarosides-enrichment in CasMWFas was effective, since the presence of sugar moieties provides high polarity and tends to keep them in the most polar phase (Figure 8). This is also confirmed through the quantification analysis of these extracts, applied in cellular biology studies (Section 4.4.5). Figure 8 shows chromatograms of CasMWFas and cascarosides are indicated by the RT. The MS analyses of the chromatographic peaks (Figure 9), compared to the isolated cascarosides, confirmed cascarosides A and B for peaks at RT 24.6 and 10.9 min (m/z 603.1697 and 603.1667 [$C_{27}H_{32}O_{14}+Na^+$]). Cascarosides C and D are related to chromatographic peaks at RT 35.3 and 45.9 min (m/z 587.1726 and 587.1701 [$C_{27}H_{32}O_{13}+Na^+$]). Peaks at RT 4.0 min and 7.6 min suggest cascarosides A and B oxidized derivatives (m/z 619.1606 and 619.1625 m/z , [$C_{27}H_{32}O_{15}+Na^+$]). The purity was confirmed by HPLC-DAD-HRMS and NMR analysis.

Figure 8: Comparison between CasMWFas and CasEtAcFas.

Figure 9: LC-MS analysis of CASMWFas. A) Chromatogram at 298nm. B) MS spectra in positive mode of the chromatographic peaks.

4.1.2. Structural elucidation of isolated compounds

In order to study chemical and toxicological aspects of the anthraquinones present in cascara sagrada bark, the cascarosides were isolated. Figure 10 shows the chromatogram of the compounds after the isolation process, confirming, together with NMR, the purity.

Figure 10: Chromatogram of the isolated compounds from CasMWFas.

CasPrepFr1 was isolated as an amorphous yellow powder and identified as a structural derivative of cascaroside B. The mass spectra of CasPrepFr1 indicated an additional oxygen (m/z 619.1642 [$M+Na^+$]) when compared to cascaroside B and was compatible with molecular formula $C_{27}H_{32}O_{15}Na^+$ (error = -0.5 ppm). In Figure 11 is it possible to notice the structural differences between the cascaroside B and the structure proposed for CasPrepFr1 (10-hydroxycascaroside B). NMR data is presented in Table 3 (Spectra in appendix Figures A3-10, pages 95-103). In the downfield NMR spectra, singlet signals at 6.86 ppm and 7.40 ppm were attributed to H-2 and H-4, respectively. The H-5 (7.26 ppm; 8.0 Hz, d), H-6 (7.66 ppm; 8.0 Hz; t) and H-7 (7.33 ppm; 7.6 Hz, d) presented vicinal coupling - doublets and triplet. Hydrogens attached at C-11 were assigned to singlet at 4.62 ppm. The additional oxygen presence was confirmed by the absence of H-10 signal in 1H spectra and absence of C-10 signal in DEPT 135°. Furthermore, C-10 is present in a deshield region (76.9 ppm) in comparison to cascaroside-B (46.2 ppm) and its H-10 couples with H-1'. In NOESY spectra

correlations between H-4 (6.88 ppm) with H-1' (3.21 ppm) and H-5 (7.26 ppm) with H-2' (2.99 ppm) the same structural configuration proposed by Manitto et al. (1993) for cascaroside B was confirmed (stereochemical aspects were also confirmed and discussed in section 4.1.3).

Figure 11: Comparison between 10-hydroxycascaroside B and Cascaroside B.

Table 3: NMR data (^1H , ^{13}C and Dept-135; D_2O) of 10-hydroxycascaroside B (Comparison with Cascaroside B (MeOD; 300MHz)(MANITTO, PAOLO et al., 1993a)

	^1H -Experimental	^1H -Literature	^{13}C - Experimental	^{13}C -Cascaroside B	DEPT-135
1	-----	-----	160.8	162.0	-----
1a	-----	-----	118.5	120.2	-----
2	6.86 (s)	6.83 (s)	115.3	114.0	(CH)
3	-----	-----	150.7	151.0	-----
4	7.40 (s)	6.98 (s)	117.3	117.2	(CH)
4a	-----	-----	147.3	143.4	-----
5	7.26 (8.0 Hz; d)	7.27 (d; 7.4Hz)	121.7	124.6	(CH)
5a	-----	-----	146;7	145.4	-----
6	7.66 (8.0 Hz; 7.6Hz; dd)	7.53 (dd; 8.3Hz; 7.4Hz)	135.7	134.6	(CH)
7	7.33 (7.6 Hz; d)	7.36 (d; 8.3Hz)	115.5	116.7	(CH)
8	-----	-----	158.4	159.0	-----
8a	-----	-----	123.0	125.0	-----
9	-----	-----	190.9	192.1	-----
10	-----	4.59 (d; 1.9Hz)	76.8	46.1 (CH)	-----
11	4.62 (s)	4.62 (s)	64.6	64.5	(CH ₂)
1'	3.21 (d; 9.6 Hz)	3.31 (dd; 9.6 Hz; 1.9 Hz)	83.9	85.6	(CH)
2'	2.99 (9.6 Hz; 9.2 Hz; dd)	2.99 (dd; 9.6 Hz; 8.7 Hz)	70.8	71.7	(CH)
3'	3.27 (m)	3.26 (dd; 8.7 Hz)	79.0	79.8	(CH)
4'	2.84-2.93 (m)	2.84 – 2.93 (m)	73.1	72.0	(CH)
5'	2.84-2.93 (m)	2.85 – 2.93(m)	81.2	81.3	(CH)
6'	3.45-3.60 (m)	3.51 (dd; 11.9 Hz; 2.1 Hz); 3.36 (dd; 11.9 Hz; 4.9 Hz)	62.5	63.0	(CH ₂)
1''	5.02 (d; 8.0Hz)	4.95 (d; 7.7 Hz)	102.5	103.2	(CH)
2''	3.66 (m)	3.65 (dd; 7.7 Hz; 8.7 Hz)	74.4	74.7	(CH)
3''	3.45-3.60 (m)	3.41-3.47 (m)	77.5	77.8	(CH)
4''	3.45-3.60 (m)	3.41-3.47 (m)	70.8	71.2	(CH)
5''	3.45-3.60 (m)	3.41-3.47 (m)	77.9	78.5	(CH)
6''	3.89 (d; 12.2 Hz); 3.73 (dd; 12.2 Hz; 4.6 Hz)	3.93 (dd; 12.1 Hz; 1.8 Hz); 3.72 (dd; 12.1 Hz; 5.4 Hz)	62.1	62.6	(CH ₂)

CasPrepFr2 was isolated as an amorphous yellow powder and identified as a structural derivative from cascaroside A. The mass spectra was compatible with molecular formula $C_{27}H_{32}O_{15}Na^+$ (error = 0.9 ppm), which indicated an additional oxygen in comparison to cascaroside A. In 1H NMR data (Table 4; Spectra in appendix, Figures A11-18, pages 104-112) we observed five signals in the aromatic region (H-2, H-4, H-5, H-6 and H-7). H-2 and H-4 signals were singlets, and H-5 and H-7 were doublets, coupled with H-6 in ortho (t , $J = 8Hz$). These signal multiplicities and J confirmed the substitution pattern. The assignment of diastereotopic hydrogens attached at C-11 was 4.68 ppm (AB system). As its epimer 10-hydroxycascaroside-B, this compound did not have the signal of H-10 and C-10 and was displaced to 77 ppm, which confirmed the presence of the hydroxyl in this position. Besides, H-1' was observed as a doublet and there was no signal relative to C-10 visualized in DEPT 135° spectrum. Thus, CasPrepFr2 is a structural derivative of cascaroside A with additional oxygen in C-10 (Figure 12). In NOESY spectra, we detected correlations between H-4 (7.35 ppm) with H-2' (2.96 ppm) and H-5 (7.45 ppm) with H-1' (3.26 ppm). According to Manitto et al. (1993) this compound has the same configuration as cascaroside A. According to Chemical Abstracts database, this is the first report of 10-hydroxycascaroside-A and 10-hydroxycascaroside-B.

Figure 12: Comparison between 10-hydroxycascaroside A and Cascaroside A.

CasPrepFr3 was identified as cascaroside B. In mass spectra, the observed ion at m/z 603.1673 $[M+Na^+]$ was compatible with molecular formula $C_{27}H_{32}O_{14}Na^+$ (error = 2.8 ppm) (NMR spectra in appendix, Figures A19-25, pages 113-119). CasPrepFr4 was established as cascaroside A, where the ion m/z 603.1687 $[M+Na^+]$ matched with cascaroside A molecular formula $C_{27}H_{32}O_{14}Na^+$ (error = 0.4 ppm) (NMR spectra Figures A25-33, pages 120-129). Fraction CasPrepFr6 presents ion peak m/z 587.1731 $[M+Na^+]$ in mass spectra correlated to the molecular formula $C_{27}H_{32}O_{13}Na^+$ (error = 1.6 ppm), which was identified as cascaroside D

(NMR spectra Figures A34-37, pages 130-134). CasPrepFr10 was identified as Cascaroside C. In mass spectra the ion m/z 587.1740 $[M+Na^+]$ was compatible with $C_{27}H_{32}O_{13}Na^+$ (error = 0.1 ppm) (NMR spectra Figures A38-44, pages 135-142). All compounds were isolated as an amorphous yellow powder.

Table 4: NMR data (1H , ^{13}C and Dept-135; D_2O) of 10-hydroxycascaroside B (Comparison with Cascaroside B (MeOD; 300MHz)(MANITTO, PAOLO et al., 1993a).

	1H - Experimental	1H -Literature	^{13}C - Experimental	^{13}C -Cascaroside A	DEPT-135
1	-----	-----	161.9	161.9	-----
1a	-----	-----	118.7	120.5	-----
2	6.94 (s)	6.85 (s)	115.2	114.4	(CH)
3	-----	-----	150.5	150.3	-----
4	7.35 (s)	7.00 (s)	119.6	118.6	(CH)
4a	-----	-----	145.1	141.2	-----
5	7.45 (d; 8.0 Hz)	7.26 (dd; 7.7Hz; 1.0Hz)	121.0	123.6	(CH)
5a	-----	-----	149.5	147.2	-----
6	7.65 (t; 8.0Hz)	7.54 (dd; 8.3Hz; 7.7Hz)	135.9	135.7	(CH)
7	7.77 (d; 7.6 Hz)	7.37 (dd; 8.3Hz; 1.0Hz)	116.0	118.5	(CH)
8	-----	-----	158.7	159.1	-----
8a	-----	-----	123.8	125.0	-----
9	-----	-----	191.3	192.4	-----
10	-----	4.58 (d; 2.2Hz)	77.0	46.1 (CH)	-----
11	4.68 (AB syst.; 14.8Hz)	4.60 (AB syst.; 15.2Hz)	64.6	64.5	(CH ₂)
1'	3.26 (d; 8.8 Hz)	3.34 (dd; 9.6 Hz; 2.2 Hz)	83.7	85.0	(CH)
2'	2.96 (t; 9.0 Hz)	2.94 (dd; 9.6 Hz; 8.8 Hz)	71.6	71.7	(CH)
3'	3.20 (d; 9.2 Hz)	3.24 (dd; 8.8 Hz)	79.4	79.8	(CH)
4'	2.8 (t; 8.8 Hz)	2.79 – 2.90 (m)	73.0	72.0	(CH)
5'	2.88 (m)	2.79 – 2.90 (m)	81.6	81.4	(CH)
6'	3.41-3.55 (m)	3.50 (dd; 11.7 Hz; 2.4 Hz); 3.32 (dd; 11.7 Hz; 5.2 Hz)	63.2	63.5	(CH ₂)
1''	4.94 (d; 8.0Hz)	4.91 (d; 7.5 Hz)	105.1	104.9	(CH)
2''	3.61 (dd; 9.2 Hz; 7.6 Hz)	3.60 (dd; 7.5 Hz; 9.1 Hz)	75.0	75.0	(CH)
3''	3.41-3.55 (m)	3.51 (dd; 9.1 Hz)	77.2	77.2	(CH)
4''	3.41-3.55 (m)	3.37-3.47 (m)	71.2	71.2	(CH)
5''	3.41-3.55 (m)	3.37-3.47 (m)	78.7	78.6	(CH)
6''	3.91 (dd; 12.2 Hz; 2.4 Hz); 3.72 (dd; 12.2 Hz; 5.2 Hz)	3.89 (dd; 12.0 Hz; 2.2 Hz); 3.70 (dd; 12.0 Hz; 5.2 Hz)	62.5	62.5	(CH ₂)

Emodin oxanthrone derivatives – which are C-10 oxygenated anthraquinones – have already been characterized in vegetable species and examples include 10-hydroxycascaroside C/D and cassialoin (KRENN et al., 2004). These compounds were isolated from roots of *Rheum emodi*, which is also used to treat constipation. The compounds 10-hydroxycascaroside A and B were identified for the first time and the stereochemistry was attributed by NOE correlation. AC determination with NMR data is based on the assumption

of a most favored conformation, however, previous research without conformational studies consider that all derivatives will not be affected by the substituents in any position.

4.1.3. Stereochemistry determination

In order to confirm if the NOESY contacts established by Manitto et al. (1993) can be applied to all anthrone and oxanthrone derivatives, the IR and VCD spectra of the cascarosides were recorded. Figure 13 shows the cascarosides VCD spectra (above) and IR spectra (bottom) separated in diastereomers pairs.

The experimental spectrum of the fingerprint region taken in methanol- d_4 reveals some remarkable similarities between the sets of diastereomers. In VCD spectrum, the cascarosides A, C, and (10OH)-A show a clearly resolved $(-/+/-)$ -pattern of common spectral feature in the range from $1660\text{--}1525\text{ cm}^{-1}$ (highlighted in Figure 13). The other three cascarosides feature an almost mirror image behavior with a clear $(+/-/-)$ -pattern within the same range. The lower wavenumber regions of the spectra feature VCD bands that are more characteristic of the substitution pattern of the anthrone core, but often show a mirror image relationship. The IR spectra of all compounds are very similar, indicating that neither the change in stereochemistry at C_{10} , the change in the side chain ($R_2=H, OH$), or the substitution of H by OH at C_{10} have a strong effect on the vibrational frequencies. The exception is IR of Cascaroside D (isolated with the same methodology, but another process), which has been shifted due to the presence of some impurities (not detectable in VCD).

Figure 13: Experimental IR and VCD spectra of cascarosides (methanol-d₄, 100 μ m, 70 mM $\approx$ 40 mg/ml). The highlighted range of the VCD spectra is characteristic for the absolute configuration at C10.

In order to analyze the experimental IR and VCD spectra and to confirm if the stereochemistry attributed by the NOE experiment is reliable, a comprehensive conformational analysis was carried out for all six investigated compounds. The lowest energy structures of cascarosides found in this search are shown in Figure 14.

For all six cascarosides, the main conformational features were found to be in good agreement with the crystal structure obtained for aloin B (R₁=H, R₂=OH, OH instead of sugar moiety at C₈) (RAUWALD et al., 1989), which has previously served as a basis for NOE- and ECD-based AC assignments (MANITTO, PAOLO et al., 1993a). The central ring of the 1,8-dihydroxyanthrone core adopts an envelope conformation with the C₁₀ atom raised above the ring plane, and with the glucopyranosyl residue in an axial position. Hydrogen bonds of the carbonyl group C₉=O with the OH groups in C₁ and C_{2'} position introduced additional conformational constraints. In cases of 10-hydroxycascarosides A and B, the rotation of the glucopyranosyl residue at C₁₀ is further constrained by a hydrogen bond between the C_{2'}-OH group and the C₁₀-O group.

Figure 14: Lowest energy conformation of cascarosides.

The spectra were recorded in methanol- d_4 , in order to make all OH-groups undergo H/D-exchange, which is actually an advantage for the present stereochemical investigation of C_{10} , as it shifts all OH-related vibrations into the range of the solvent absorbance. Thereby, not only the spectral contributions of conformationally flexible groups are removed from the spectral range of the VCD measurements, but also the spectral overlap of vibrations for the glucopyranosyl moieties and the (ox-)anthrone core is reduced.

The conformational analysis was carried out without consideration of explicit solvent interactions. However, methanol is a good hydrogen bond acceptor and donor, and may interfere with the conformer stabilizing hydrogen bonds. Therefore, in order to ensure that explicit solvation with methanol does not introduce significant structural changes, we simulated molecular dynamics of Cascaroside A and 10-hydroxycascaroside A. From these simulations, the hydrogen bond dynamics of the solvation shell around the cascarosides could be extracted. Using a classical geometric definition of a hydrogen bond (angle $\geq 135^\circ$, distance $\leq 3\text{\AA}$), the analysis of the molecular dynamics (MD) trajectories revealed that the two aforementioned intramolecular hydrogen bonds of the carbonyl group $\text{C}_9=\text{O}$ with $\text{C}_1\text{-OH}$ (86 % population) and $\text{C}_2\cdots\text{OH}$ (91 % population) were not significantly affected by the solvent environment. In addition to the $\text{C}_1\text{-OH}$ (85 %) and the $\text{C}_2\cdots\text{OH}$ (77 %) hydrogen bond towards $\text{C}_9=\text{O}$, the stabilizing interaction between $\text{C}_2\cdots\text{OH}$ and $\text{C}_{10}\text{-O}$ also remained mainly intact (83 %) when 10-hydroxycascaroside A was solvated with methanol. The hydrogen bonds between the OH groups of the glucopyranosyl units were also mostly oriented towards the adjacent oxygens (average angles around 110°). In fact, the sugar moieties acted as hydrogen bond acceptors towards the solvent with populations ranging from 30-60%. Hence, the MD simulations with explicit methanol confirmed that it was reasonable to assume that the main conformers found in the static conformational search were actually present in solution.

To simulate the IR and VCD spectra, single-conformer spectra were calculated for each of the optimized conformers of cascarosides. Afterwards, population-averaged spectra were prepared using the relative Gibbs free energies $\Delta G_{298\text{K}}$ to calculate Boltzmann factors. Figure 15 shows a comparison of the resulting simulated IR and VCD spectra for cascarosides A and B with the experimental spectra. For cascaroside A the spectra simulations clearly reproduced the experimentally observed $(-/+ / + / -)$ -pattern in the range of $1660\text{-}1525\text{ cm}^{-1}$ (bands 1-4) of the VCD spectrum, which was identified above as a characteristic spectral feature for its configuration. Moreover, almost the entire fingerprint region in both the theoretical IR and VCD spectra provided a good match with the experimental data. Only the IR intensities of bands 3 and 4 were predicted to be more intense than observed in the experimental spectrum. Hence, the overall agreement leaves no doubt about the correct assignment of the AC of C_{10} in cascaroside A and B.

Figure 15: Comparison of the experimental spectra with the simulated IR and VCD spectra of cascaroside A and cascaroside B.

The comparison of experimental and simulated spectra for cascaroside B reveals an equally good agreement. The characteristic range of 1660-1525 cm⁻¹ was again well reproduced, although a slight frequency inaccuracy has to be taken into account. Also the major spectral features of the range below 1500 cm⁻¹ can be easily correlated with simulated features, so that the stereochemistry of C₁₀ in cascaroside B can be unambiguously confirmed by the VCD spectra analysis.

For the other cascarosides investigated, the comparison of the simulated and experimental spectra reveals an equally good agreement, so that all ACs can be easily determined (Figure 16). Considering the remarkable similarity between the experimental data and the robustness of the spectra calculations, the observed spectral pattern appears to be generally characteristic for the stereochemistry at C₁₀.

Figure 16: Comparison of the experimental spectra with the simulated IR and VCD spectra of cascarosides C, D, 10H-A and 10H-B.

In order to prove that this correlation between the spectral pattern and the absolute configuration can indeed be considered a reliable empirical correlation, Figure 17 compares the single conformer VCD spectra of the two most populated conformers of cascaroside A and B in the range of 1670-1400 cm^{-1} . The corresponding line spectra, which represent the frequency and rotational strengths (the VCD intensity) of each contributing vibrational mode, show that the characteristic VCD spectral pattern is generated by only five vibrational modes. A close examination of these five modes reveals that they are fully localized on the anthrone core and best described as coupled C=C and C=O stretching vibrations. The contribution of the glucopyranosyl moieties was not observed and the CH_2 -wagging contributions of the C_3 -side chain were negligibly small. Hence, this VCD spectral pattern is indeed completely independent from the nature and the stereochemistry of the side chains, as long as the hydrogen bonding network to the carbonyl group is present.

Figure 17: Simulated spectra of the lowest energy conformers of cascaroside A (black) and B (red) in the characteristic region from 1650-1500 cm⁻¹ including the corresponding line spectra. The five characteristic vibrational motions are indicated in the molecular structures using red arrows for strong contributions and grey arrows for weak contributions to the overall vibrational motion.

The correct stereochemistry was established for all Cascarosides. The right identification and differentiation between them is essential for quality control, considering that Cascaroside A is used as a reference level in *Rhamnus* herbal medicines.

4.1.4. Cascarosides fragmentation studies

Quality control is an extremely important step in herbal medicine production, especially for *Rhamnus* species. Both the United States Pharmacopeia (USP) and Brazilian Pharmacopeia recommend storing the bark for at least one year before use. This recommendation is based on the presence of high levels of anthrone derivatives in fresh bark, which shows toxic effects and renders the plant unsafe for human consumption (LABADIE, 1970). In fact, storing the bark oxidates emodin-anthrone derivatives in emodin oxanthrone, reducing side effects (TSUKIDA; SUZUKI, 1954). Mass spectrometry can help in this process, as different classes of compounds may follow distinct fragmentation reaction

mechanisms, which enables their differentiation. Due to the importance of quality control in anthraquinone-rich plants it is essential to evaluate anthrone and oxanthrone levels in order to avoid severe side effects, such as diarrhea, dehydration and acceleration of the melanosis coli process. Besides quality control of medicinal plants, differentiating between these two key compound classes can help in pharmacokinetic studies and metabolite identification. Therefore, we performed fragmentation studies herein.

In positive mode, cascarosides appeared as sodiated adducts. For cascarosides A/B and C/D the sodiated molecule ion was m/z 603 and 587, respectively. When submitted to collisional activation these precursor ions give rise to product ions m/z 441 and m/z 425 for cascarosides A/B and C/D, respectively (Scheme 1). The initial fragmentation is relative to *O*-glucosyl loss by a remote hydrogen rearrangement, which can also occur in the source (DEMARQUE et al., 2016). In the next step, based on MS³ spectrum (Appendix, Figure A1, page 93), the *C*-glucosyl portion dehydrated yielding the fragment ion at m/z 423 and m/z 407 (cascarosides A/B and C/D, respectively) by remote hydrogen rearrangement. The proposed unsaturation position between C-4' and C-5' generated the most substituted alkene. The remote hydrogen rearrangement was the same mechanism used to explain the loss of second glucosyl unit, which produced the anthrone fragments at m/z 279 for cascarosides A/B and m/z 263 for cascarosides C/D. No difference in fragmentation patterns were seen between the known cascarosides with no oxidation in C-10 or even between epimers pairs. The difference of 16 Da between cascarosides A/B and C/D was constant during all fragments, indicating no participation of side chain in fragmentation mechanisms.

The 10-hydroxylcascarosides displayed different fragmentation mechanisms after glucosyl dehydration was observed in MS³ spectra. In the next step, after the first sugar loss, the second sugar unit suffered retro-Diels Alder (RDA) fragmentation followed by McLafferty-type rearrangements to form the quinone group. Traces of aldehyde intermediate m/z 337 can be found in MS³ spectra (DEMARQUE et al., 2016). Differences in fragmentation reactions were not found between the diastereoisomeric 10-hydroxycascarosides, although the additional oxygen in C-10 yielded a different fragmentation in comparison to the known cascarosides in positive mode. In general, the fragment m/z 185 can be found in all anthraquinones.

Scheme 1: Fragmentation mechanisms proposed for cascarosides A-D and 10-hydroxycascaroside A/B in positive mode.

In negative ionization mode cascarosides A/B and C/D appeared as formate adducts m/z 625 and m/z 609, respectively. When submitted to collisional activation, the molecules undid the complex and appeared deprotonated – m/z 579 for cascarosides A/B and m/z 563 for cascarosides C/D (Scheme 2). In MS^3 spectra (Appendix, Figure A2, page 94) we observed a fragment corresponding to *C*-glucosyl partial loss. This step may be preceded by a dehydration followed by a RDA fragmentation. Cycloreversion process without previous dehydration is also possible (DEMARQUE et al., 2016). The fragment was an enol, which possibly tautomerize to the keto form (cascarosides A/B m/z 459 and cascarosides C/D m/z 443). In MS^3 spectra we also observed the fragment without *O*-glucosyl moiety, lost by remote hydrogen rearrangement (m/z 297 to cascarosides A/B and m/z 281 for cascarosides C/D) (WU, X. F. et al., 2013).

The 10-hydroxycascarosides also revealed different fragmentation mechanisms in negative mode compared to cascarosides A to D. After *O*-glucosyl loss (m/z 433 10-hydroxycascarosides A and B) *C*-glucosyl loss occurred through radical fragmentation (α -cleavage). This step violated odd-electron rule and gave rise to fragment m/z 270. The unpaired electron in C-10 was stabilized by both benzene rings and also by the extra oxygen present in these compounds attached to C-10. No differences were found between diastereoisomeric pairs.

Scheme 2: Fragmentation mechanisms proposed for cascarosides A-D and 10-hydroxycascaroside A/B in negative mode.

The fragmentation patterns reported herein, in both acquisition modes, were key-fragmentations to establish the presence of hydroxyl in C-10 position. The diagnostic fragments can aid in the elucidation process by differentiating oxanthrone derivatives from anthrone derivatives. These reactions can also be used to recognize isobars, for example aloins from cassialoins. These two classes differ basically in hydroxyl position: C-11 for aloins and C-10 for cassialoins. The constant fragmentation between cascarosides A/B from C/D points toward a non-participation of C-10 side chains on the mechanism, which enables isobar differentiation.

Through LC-MS analysis of phenolic compounds from *Aloe barbadensis*, Wu et al. (2013) identified the fragment m/z 297 for aloin A and B and m/z 270 for 10-hydroxyaloin A and B in negative mode. The same diagnostic fragments were found in rhubarb extract analysis performed to differentiate these two classes of compounds by Song et al. (2011). Our experimental data concurs with such results and illustrates the applicability of using the present reactions in LC-MS/MS analysis in order to avoid isolation and in NMR studies for structural elucidation.

The reactions proposed for oxanthrone derivatives are important for quality control analysis in anthraquinone-rich plants. Detecting the presence of oxidated anthrone compounds is essential to ensure that the plant was stored long enough to reduce anthrone derivatives. In fact, their presence reduces over the course of time, reducing undesirable side effects like nausea, vomiting, and severe diarrhea (TSUKIDA; SUZUKI, 1954). The additional hydroxyl at C-10 of oxanthrones allows McLafferty-type rearrangements to form the quinone group, while in negative mode the second sugar loss violates the odd-electron rule and forms a radical fragment. The two fragmentation patterns can be used to differentiate anthrones from oxanthrones and can be applied for quality control and elucidation process of anthraquinone-rich plants through mass spectrometry.

4.1.5. Cascarosides quantification in cascara sagrada extract

The analytical method was validated for cascarosides quantification (analytical curves and statistical parameters in appendix, pages 159-164). In Table 5 the cascarosides content of CasEtAcPh and CasCrEx are reported. The cascarosides levels in CasEtAcPh are lower than in CasCrEx.

The world health organization (WHO) recommends contents varying from 0.3 to 1g of crude extract, calculated in 20 to 30 mg of cascaroside A. Therefore, the extract should

have between 2% to 10 % of cascaroside A for a 70 kg person (WHO, 2016). The levels of CasCrEx found in this study are consistent with the international recommendations.

Table 5: Cascarosides content in CasEtAcPh and CasCrEx.

	CasEtAcPh (%)±SD	CasCrEx (%)±SD
Casc A	0.32±0.03	3.31±0.98
Casc B	0.37±0.01	5.78±0.58
Casc C	0.32±0.06	1.15±0.54
Casc D	0.23±0.02	2.79±0.65
10H-A	0.42±0.01	4.54±0.02
10H-B	0.52±0.02	1.63±0.06
Total	2.17±0.17	19.21±2.85

SD – standard deviation

4.1.6. Biological results

In order to understand the contribution of each compound class to the plant toxicity, we performed *in vitro* studies with cells. The first compounds tested were the cascarosides, which did not show any toxicity in the tested concentrations (Figure 18A). The CasEtAcPh and CasCrEx show toxicity. The initial results suggested that more polar the compounds were, the less toxic they would be. We expanded the structures tested, considering studies that show toxicity for the polar compounds when they lose the glucosyl unit. So, emodin, anthralin, and aloin were tested and showed toxicity (Figure 18A).

The structure-activity relationship shows that the presence of glucose units in the molecule reduces the toxicity, while anthrones and anthraquinones with any glucose units show a similar toxic profile.

In order to prove the apoptosis mechanism of cascara compounds, we performed caspase assay. The results are shown in Figure 18B. The activation of apoptosis mechanism can be confirmed through the decreasing of caspase-3, caspase-8 and poly(ADP-ribose) polymerase (PARP); and the increasing of cleaved PARP and Receptor-interacting protein (RIP). These changes indicate apoptosis by extrinsic factors, while changes in calnexin levels would indicated apoptosis by intrinsic pathway (ICHIM; TAIT, 2016). The reduction of RIP and the increases of cleaved PARP levels promoted by CasCruEx were in accordance with toxicity profile, proving its toxicity by triggering apoptosis pathway. Additionally, the high intensity of cleaved PARP bands and the absence of RIP bands for Aloin and Emodin corroborate with the toxic profile and prove to be this class of compounds responsible for the toxic activity of cascara sagrada extract.

Figure 18: (A) Results of percentage of cell viability in different concentrations for extracts and isolated compounds. (B) Western blot result of caspase assay. (-) indicates absence of compound; (+) indicates presence of compound. PARP: poly(ADP-ribose) polymerase; RIP: Receptor-interacting protein (RIP); Casp: caspase.

Despite the more toxic activity, it is known that the anthraquinone aglycones are responsible for laxative activity (KOBASHI; AKAO, 1997; MOREAU; MOREAU; SKINNER, 1985). In fact, glycosylated anthrones – as cascariosides and barbaloin – act as prodrugs, leaving the intestinal flora with the crucial role of producing active metabolites (DE WITTE, 1993; HATTORI et al., 1988). Akao et al. (1996) investigated the purgative action of Barbaloin, which did not induce diarrhea in rats when orally administered. After association with *Eubacterium* sp. – a human intestinal bacteria capable of transforming barbaloin to aloe-emodin – diarrhea was clearly induced. This also occurs with cascariosides (DREESSEN; LEMLI, 1988) and sennosides (DE WITTE, 1993). Furthermore, both C- and O- bonds can be broken by intestinal bacteria, however, only a restrict number of strains can break C- bonds.

The aglycones are the active compounds in anthranoids extracts. For glycosylated anthrones and oxanthrones compounds, the release of aglycone makes the laxative activity effective. Although (ox)anthranoids are the active compounds, they should be avoided in the cascara extract. As already mentioned, cascara bark should be stored for at least one year prior to use, to reduce anthranoid levels (which are oxidated to correspondent anthraquinone). The use of fresh plants causes “gripping” (spasmodic pains in the bowels) and emetic side effects.

The comparison between anthraquinones and (ox)anthrones as laxatives indicates that (ox)anthrones are more potent. This assumption is supported by the polarity concept: glycosylated compounds are more polar and tend to stay in intestinal lumen. Pharmacokinetic studies support that glucuronides remain unaffected during their transit in the gastrointestinal tract, which can lead to microflora releasing the active aglycone in the colon. Assuming that glucosyl (ox)anthrones are less likely to enter the systemic circulation and, to consequently be metabolized, their presence in intestinal lumen allows them to exert a lesser effect than anthraquinones. Additionally, the FDA recommends using 15 to 20 times more concentrated anthraquinones than glycosides anthraquinones (MOREAU et al., 1985).

Besides the lack of laxative activity of glucosyl (ox)anthrones, the intestinal metabolism is capable of releasing the active aglycone. Although it is not possible to have (ox)anthrones directly in the extract due to strong side effects, the use of glycosylated forms as cascariosides is recommend. Furthermore, the present study shows the safety of their use.

Although the most active form was the most toxic as well, the cascariosides safety found in this work should be considered in the development of extracts with cascara sagrada.

As the activity is intrinsically related to toxic effects, the use of less toxic compounds in the extract prevents direct contact with active forms during the intestinal transport. Furthermore, absorption is avoided, leaving the action site unchanged. The need for lower amounts of cascarosides, as well as their slow release of active forms may decrease side effects of long-term use.

In the present thesis, a cascarosides-enrichment in cascara extract is recommended, although clinical experiments should be conducted to support these findings.

4.2. Results and discussion – barbatimão

4.2.1. Chemical profile

Barbatimão is a phenolic-rich plant with a wide diversity of structures (DE MELLO; PETEREIT; NAHRSTEDT, 1999; DEMELLO; PETEREIT; NAHRSTEDT, 1996a;1996b; ISLER et al., 2010; LOPES et al., 2009; LUIZ et al., 2015). The present analysis shows a tannin-enrichment in the last fractions in CCC column, while the small units appeared in the previous fractions. The CCC fraction BTMFr8-10 was submitted to HPLC-prep and three procyanidins were isolated and identified by NMR and MS. We established the absolute configuration using VCD as well. The tannin-rich fraction was analyzed by MS, using MALDI as the ionization method, which is indicated for larger structures.

4.2.2. Procyanidins isolation

The fraction BTMFr8-10 leads to isolation and identification of gallocatechin (BTMPrepFr3), robinetinidol (BTMPrepFr7) and epigallocatechin (BTMPrepFr9) (Figure 19). They were all isolated as amorphous white powder. In high-resolution MS analysis gallocatechin was compatible with molecular formula $C_{15}H_{14}O_7+H^+$ (error = 5.1 ppm); robinetinidol was compatible with molecular formula $C_{15}H_{14}O_6+H$ (error = 5.7 ppm); and epigallocatechin was compatible with molecular formula $C_{15}H_{14}O_7+H$ (error = 5.5 ppm) (Figure 20). The NMR analysis confirms the identification proposed in the present work (Table 6). The NMR data was compared with the literature (LIAO et al., 2014; NOMIZU et al., 2008) (Spectra in Appendix, Figure A45 – S57, pages 143-155). Besides the same molecular formula for gallocatechin and epigallocatechin, differentiation was possible due to the coupling constants in 1H NMR of H-2. Thus, the trans relationship between H-2 and H-3 of BTMPrepFr3 ($J = 7.2Hz$) was confirmed as gallocatechin. The absolute configuration was performed by VCD spectroscopy (Section 4.2.3).

Galocatechin: 2R, 3S; R= OH
Epigallocatechin: 2R, 3R; R= OH
Robinetinidol: 2R, 3S; R= H

Figure 19: Chemical structures of galocatechin, epigallocatechin and robinetinidol isolated from barbatimão.

Figure 20: HPLC-MS analysis of isolated compounds. Top: chromatogram of galocatechin (RT 3.9 min), robinetinidol (RT 5.6 min) and epigallocatechin (RT 5.3 min). Bottom: MS spectra of correspondent compounds.

Table 6: NMR data (^1H and ^{13}C) of galocatechin, robinetinidol and epigallocatechin (MeOD- d_4 ; 400 MHz).

	Robinetinidol		Galocatechin		Epigallocatechin	
	δH^1 (J in Hertz)	δC^{13}	δH^1 (J in Hertz)	δC^{13}	δH^1 (J in Hertz)	δC^{13}
2	4.61, d (6.8)	83.0	4.53, d (7.2)	82.8	4.77, s	79.9
3	3.99, m	64.3	3.97, m	68.8	4.19, m	67.5
4	2.85, dd (5.2, 15.6) 2.66, m	32.7	2.80, dd (16, 5.6) 2.50, dd (16, 7.6)	28.1	2.85, dd (4.8, 16.8) 2.75, dd (3.2, 16.8)	29.1
4a	--	112.5	--	100.8	--	100.1
5	6.84, d (8.4)	131.5	--	157.5	--	157.6
6	6.32, dd (2.4, 8.4)	109.4	5.86, s	96.3	5.94, s	96.4
7	--	157.9	--	157.7	--	157.9
8	6.28, d (2.4)	103.6	5.92, s	95.6	5.96, s	95.9
8a	--	156.1	--	156.8	--	157.2
1'	--	131.5	--	131.5	--	131.6
2'	6.38, s	107.0	6.40, s	107.8	6.54, s	107.0
3'	--	146.9	--	146.9	--	146.7
4'	--	134.0	--	134.0	--	133.6
5'	--	146.9	--	146.9	--	146.7
6'	6.38, s	107.0	6.40, s	107.8	6.54, s	107.0

4.2.3. Absolute configuration of isolated procyanidins

The VCD spectroscopy works in infrared region and can be felt by any chiral molecule (MERTEN, C.; SMYRNIOTOPOULOS; TASDEMIR, 2015). Therefore, it is a useful tool, especially for molecules without chromophores as procyanidins. For procyanidins, the use of VCD in AC determination was effective, presenting a good agreement between the experimental and calculated spectra (Figure 21).

Figure 21: VCD spectra of BTMPrep03 (galocatechol), BTMPrep07 (robinetinidol) and BTMPrep09 (epigallocatechin).

In the VCD spectra comparison between galocatechin, robinetinidol and epigallocatechin we found that they show the same four main bands in 1600 and 1300 cm^{-1} , with the same signal. Considering these four main bands, they are indistinguishable spectra. In deep investigation of conformer's contribution to these bands, in overlapped spectra of all conformers for galocatechol we realized that these bands were independent from conformers variation (Figure 22).

Figure 22: VCD spectra overlap of all galocatechin conformers.

Analyzing the origin of the four main bands in 1600 and 1300 cm^{-1} we found that they are related to C=C stretching of aromatic ring and the out plane bending movements of hydrogen and aromatic rings in C-2 (twisting), as well as for hydrogens in C-4. The investigation of the most populated conformer of galocatechin confirms the signature founded in experimental spectra (Figure 23).

Figure 23: Vibrational modes of four main bands in 1600 and 1300 cm^{-1} .

As shown herein, VCD spectroscopy was only sensitive to C-2 stereocenter (R-configuration) in procyanidins. The lack of C-3 stereocenter contribution in VCD spectra explains why they look similar: all isolated procyanidins have the same configuration in C-2.

As previously discussed (Section 4.1.3.), the change of H/D shifts the vibrations to the solvent absorbance region. In order to increase the sensibility for C-3 stereocenter, the solvent was changed to acetonitrile added to methanol-d₃. As shown in Figure 24, this strategy was not effective to distinguish the diastereomers epigallocatechin and galocatechol.

Figure 24: Comparison of epigallocatechin (black) and galocatechol (red) VCD (above) and IR (bottom) spectra. Calculated spectra are shown in the left and experimental in the right.

Despite the useful practice of measuring the *J* coupling to establish cis and trans relationships between two chiral centers, it can only be applied to procyanidins monomers. Regarding dimer, for instance, the rotation around interflavanyl units is the main issue for ¹H-NMR spectroscopy. In fact, the reliable information about the relative configuration of C-2 and C-3 involves complex NMR analysis, such as estimations of the relative population of rotational conformers or changes in solvent and temperature. Previous derivatization is commonly adopted as an alternative, even so, ambient temperature NMR may lead to

erroneous determination, especially considering 2,3-cis series. This can be verified in methyl ether acetate derivatives of procyanidins, where ambient temperature NMR leads to line-broadening and duplication of resonances due to intermittent exchange of rotational isomers (KOŁODZIEJ, 1992).

The differentiation of procyanidins monomers can help in the elucidation process of bigger units, avoiding complex NMR analysis. Considering the non-sensibility of the C-3 chiral center, even when small portions of non-deuterated solvent are used, spectra calculation procedures are appealing (DE GUSSEM et al., 2014). Therefore, the differentiation between the diastereomers with VCD was possible only with a subtraction between the trans/cis spectra (Figure 25). This contribution, successfully made for procyanidins, is an effort to make AC of this class a less arduous process and encourage the use of VCD for the high weight condensed procyanidins.

Figure 25: Comparison between BTM03 (gallocatechin) and BTM09 (epigallocatechin) subtracted spectrum with calculated trans-cis subtracted spectrum and cis-trans subtracted spectrum.

4.2.4. MALDI Analysis

The MALDI analysis shows a tannin-enrichment in the last fractions of the CCC column (BTMFr16-31), leading to a better resolution in MALDI analysis. In fact, compounds widely present with tannins – as sugars – affect the MALDI analysis. They can suppress the ionization process due to charge competition, which reduces the analyte desolvation/declustering. This influence is commonly found with ESI and, although MALDI permits more contaminants, reduction favors the ionization process (RUE; RUSH; VAN

BREEMEN, 2017). See Figure 26 for a typical tannin spectrum in MALDI analysis, obtained from BTMFr16-31.

Figure 26: MS spectra of BTMFr16-31 obtained by MALDI.

Mass spectrometry is a powerful tool in the elucidation process of organic compounds. The laser ionization method used in MALDI has been shown to be a good resource for tannins analysis (GUARATINI et al., 2014). The tannins high polymerization degree makes structural elucidation challenging, however MALDI reliably identifies units and degree of polymerization, considering the fragmentation pattern as follows: RDA fragmentation succeeded by the loss of the remaining unit by remote hydrogen rearrangement. Figure 26 shows the fragmentation pattern and the identification of the units according to this resource. The sequential loss of monomers units (loss of 136 Da by RDA and 152 Da by remote hydrogen rearrangement) permits the identification of units even in mixtures. Thus, 4 different tannin series were found with different oxygenation of the 3 initial units (Table 7). The difference in the units proposed according to Table 7 consists of the number of procyanidin (PCY) and prodelphinidin (PDE) units. The first series consist of 7 PCY units, while the second, third and fourth series have one, two and three PDE units, always totalizing 7 units in their oligomers.

Table 7: Tannin series identified in BTMFr16-31. PCY: Procyanidin, PDE: Prodelphinidin.

Series	[M+Na] ⁺ ± error (ppm)	Calculated	MF	Compound
1	889.1955±0.1	889.1955	C ₄₅ H ₃₈ O ₁₈ Na	3PCY
	1041.2266±15.7	1041.2429	C ₅₃ H ₄₆ O ₂₁ Na	4PCY
	1177.2743±13.0	1177.2589	C ₆₀ H ₅₀ O ₂₄ Na	
	1329.2951±8.4	1329.3063	C ₆₈ H ₅₈ O ₂₇ Na	
	1465.3425±13.8	1465.3223	C ₇₅ H ₆₂ O ₃₀ Na	5PCY
	1617.3544±9.5	1617.3696	C ₈₃ H ₇₀ O ₃₃ Na	6PCY
	1753.4000±8.1	1753.3857	C ₉₀ H ₇₄ O ₃₆ Na	
	1905.4227±5.4	1905.4330	C ₉₈ H ₈₂ O ₃₉ Na	
	2041.4539±2.3	2041.4491	C ₁₀₅ H ₈₆ O ₄₂ Na	7PCY
2	905.1923±2	905.1904	C ₄₅ H ₃₈ O ₁₉ Na	2PCY-1-PDE
	1057.2231±13.9	1057.2378	C ₅₃ H ₄₆ O ₂₂ Na	3PCY-1-PDE
	1193.2683±12.1	1193.2538	C ₆₀ H ₅₀ O ₂₅ Na	
	1345.2900±8.3	1345.3012	C ₆₈ H ₅₈ O ₂₈ Na	
	1481.3326±10.4	1481.3172	C ₇₅ H ₆₂ O ₃₁ Na	4PCY-1-PDE
	1633.3557±5.5	1633.3646	C ₈₃ H ₇₀ O ₃₄ Na	5PCY-1-PDE
	1769.3939±7.5	1769.3806	C ₉₀ H ₇₄ O ₃₇ Na	
	1921.4120±8.3	1921.4279	C ₉₈ H ₈₂ O ₄₀ Na	
	2057.4610±8.2	2057.4440	C ₁₀₅ H ₈₆ O ₄₃ Na	6PCY-1-PDE
3	921.1982±13.9	921.1854	C ₄₅ H ₃₈ O ₂₀ Na	1PCY-2-PDE
	1073.2270±5.4	1073.2327	C ₅₃ H ₄₆ O ₂₃ Na	2PCY-2-PDE
	1209.2601±9.4	1209.2487	C ₆₀ H ₅₀ O ₂₆ Na	
	1361.2855±7.8	1361.2961	C ₆₈ H ₅₈ O ₂₉ Na	
	1497.3281±10.6	1497.3121	C ₇₅ H ₆₂ O ₃₂ Na	3PCY-2-PDE
	1649.3518±4.7	1649.3595	C ₈₃ H ₇₀ O ₃₅ Na	4PCY-2-PDE
	1785.3945±10.6	1785.3755	C ₉₀ H ₇₄ O ₃₈ Na	
	1937.4072±8.1	1937.4229	C ₉₈ H ₈₂ O ₄₁ Na	
	2073.4553±6.2	2073.4389	C ₁₀₅ H ₈₆ O ₄₄ Na	5PCY-2-PDE
4	937.1779±2.6	937.1803	C ₄₅ H ₃₈ O ₂₁ Na	3-PDE
	1089.2195±7.5	1089.2276	C ₅₃ H ₄₆ O ₂₄ Na	1PCY-3-PDE
	1225.2540±8.4	1225.2437	C ₆₀ H ₅₀ O ₂₇ Na	
	1377.2896±1.1	1377.2910	C ₆₈ H ₅₈ O ₃₀ Na	
	1513.3150±5.2	1513.3070	C ₇₅ H ₆₂ O ₃₃ Na	2PCY-3-PDE
	1665.3383±9.7	1665.3544	C ₈₃ H ₇₀ O ₃₆ Na	3PCY-3-PDE
	1801.3780±4.2	1801.3704	C ₉₀ H ₇₄ O ₃₉ Na	
	1953.4151±1.4	1953.4178	C ₉₈ H ₈₂ O ₄₂ Na	
	2089.4303±2.4	2089.4338	C ₁₀₅ H ₈₆ O ₄₅ Na	4PCY-3-PDE

4.2.5. Quantification of Barbatimão constituents

The content of tannins in the crude extract was 29.15% ± 0.27. The compounds catechin, gallocatechin and gallic acid represent 0.2, 1.0 and 0.8%, respectively (analytical curves and statistical parameters in appendix, pages 156-158). Thus, the dose of 400 mg/kg administrated to the animals represented around 116 mg of tannins and 8 mg of the other

quantified phenolics. According to Audi et al., (1999) 400 mg/kg dose of the crude extract of Barbatimão inhibited acid-ethanol induced ulceration around 70%, while the cimetidine inhibited only 40%. However, the authors did not quantify the total tannin content. In herbal medicine Fitoscar®, commercialized in Brazil, the total tannin content is standardized at 45%. On the other hand this herbal medicine is recommended for topical use.

The extractive process determines the efficiency of extraction. Brazilian pharmacopoeia specifies that the vegetal drug should contain a minimum of 8% of total tannins (BRASIL, 2010), 0.02% of gallic acid and 0.03% of gallocatechin, when de-cooking extraction is adopted. Although the present extraction yielded a suitable content of other phenolics, the tannin content was estimated at 6% relative to vegetal drugs. Considering that the extractive process was percolation, similar results were found compared to those obtained for the species that were macerated (FIORI et al., 2013).

4.2.6. Biological results

4.2.6.1. Biochemical parameters

The comparison between biochemical parameters for the treated (euthanized 1.5h after the treatment) and non-treated animals only differed in glucose dosage. Animals treated with Barbatimão had their glucose levels reduced (108 ± 16 mg / dL) compared to the non-treated animals (172 ± 18 mg / dL, $p < 0.05$). A diet rich in polyphenols is known to cause reduced glucose levels, considering that these substances affect the absorption of sugar and other components in the diet, micronutrients and drugs (ALZAID et al., 2013; JOHNSTON et al., 2005; MA et al., 2011; SHAN et al., 2016; YANG, Z. F. et al., 2014). These findings should be considered for dosage schedules, in order to avoid interference with nutrition and absorption of other drugs.

4.2.6.2. *In vivo* tests

The action of antiulcer drugs involves blocking H₂ receptors, inhibition of the cholinergic pathway (muscarinic antagonists), inhibition of the proton pump, local antacids and antibacterial and gastric surfactant agents. The proposed mechanism of action of tannins is based on the power of complexation of these molecules on gastric tissue walls, since condensed tannins do not get absorbed. In order to prove the binding ability of polyphenols in gastric tissues, animals were treated and stomachs were removed and analyzed using fluorescence microscopy. Figure 27 shows the images obtained from the biological test. The

stomach of animals treated with barbatimão crude extract (Figure 27C and D) showed a clear distinction with non-treated animals (Figure 27A) observed in fluorescence microscopy. Figure 27A shows that there are no elements in the organ that emit fluorescence, unless some food remains in the lumen, but is not attached to the tissue. On the other hand, organs which were dived in Barbatimão extract (Figure 27B), show fluorescence all over the tissue. This demonstrates a clear permeation and fixation of phenolic compounds throughout the tissue. Organs of the animals euthanized after 0.5 h of treatment, that were not which were washed with PBS showed a distribution of fluorescent substance along the parietal cells (Figure 27C). Such fluorescence is not removed even when the organ was washed with PBS before freezing (Figure 27D), revealing a longstanding effect.

Figure 27: Fluorescence confocal microscopy images of stomach cross sections. First column (A1-D1): visible light image; second column (A2-D2): overlap of visible and fluorescence light images; third column (A3-D3): fluorescence light image. Line A: Stomachs tissues from untreated animals. Line B: Stomach tissues from untreated animals impregnated with *S. rotundifolium* crude extract. Line C: Stomach tissues from treated animals with *S. rotundifolium* crude extract (400 mg/mL); non-washed tissues (euthanized after 0.5 h). Line D: Stomach tissues from treated animals with *S. rotundifolium* crude extract (400 mg/mL); PBS-washed tissues (euthanized after 0.5 h).

Considering sections obtained from several organ parts, we noticed a wide fluorescence distribution, which was related to the presence of phenolic compounds in the tissue surface. Other techniques have been useful for generating images of metabolites in tissues, like MALDI-imaging (SILVA et al., 2014). When compared to fluorescence imaging, the main advantage of MALDI-imaging is the direct identification of compounds attached to the tissue. However the sample preparation and conditions optimization for high quality image acquisition are much more complex and demanding. In the present study fluorescence was an effective tool for demonstrating tannin complexation on the surface of gastric tissue.

Complexation is a surface phenomenon and conformationally and flexibility are important factors that can contribute to the interaction level. In this context, small proteins are better binders than big ones, which have complex secondary and tertiary structures. Considering the size of polyphenols, the binding efficacy increases exponentially from the trimmers to bigger tannin series (HASLAM, E. et al., 1989; SAITO et al., 1998). However, some authors state that large tannins (> 3000Da) make the binding process more difficult, based on molecular diffusion in collagen fibres (HASLAM, EDWIN, 1989). According to the series found in the present work, the tannins content in barbatimão extract is suitably sized for the binding activity.

Tannins that create a layer on the surface of gastric tissue is the main explanation as to why barbatimão improves gastric problems. The present results support barbatimão effectiveness in treating gastric lesions reported in previous work (AUDI et al., 1999). Prado et al. (2014) state that the absence of tannins in *Eugenia dysenterica* extract suppresses antiulcerogenic activity, showing that tannins are responsible for this activity.

The presence of other components in vegetable extracts can also contribute to the improvement of gastric problems added to the cytoprotective action of condensed tannins. Hydrolysable tannins are important substances for treating gastric problems, which is related to the antibacterial activity against *H. pylori*, an important agent that also causes gastritis (FUNATOGAWA et al., 2004). In fact, around 10% of individuals which have *H. pylori* as part of their flora develop peptic ulcers, with its presence a target for pharmacological therapy (HARSHA et al., 2017). Barbatimão is a source of hydrolysable tannins (REGAZZONI et al., 2013) and can also be used when *H. pylori* is present in the gastric tissue.

Herbal medicine use presents considerable advantages when compared to conventional synthetic drugs: synergy and additive effects. Additive effects are the sum of

individual compounds efficiency to improve symptoms. On the other hand, synergy is the concept that the chemical complexity of a vegetable extract is better than the sum of each component separately. Considering the chemical diversity of vegetable extract as barbatimão, the presence of different compounds can act on multiple mechanisms to improve gastric problems.

The complexation process is due to main binding forces derived from hydrophobic effects and hydrogen bonding, in particular the phenolic groups with the hydrogen acceptor bond to the protein (HASLAM, E., 1996). In fact, their presence in the tissue may contribute to more than just as a physical barrier. Sucralfate, which has the same mechanism of action proposed for tannins, has been proven to play an important role in angiogenesis (SZABO et al., 1991). Indeed, the support to re-epithelization is a crucial step to restore gastroduodenal mucosal integrity (SZABO, 2014). Phenols compounds have been studied in vascular protection and studies have shown the importance of maintaining gastric mucosal blood flow. Thus, considering that histopathological studies have proven epithelization, fibroblast proliferation, and angiogenesis by tannins in healed wound tissues, these molecules act in more mechanisms than just physical barrier (LAI et al., 2016).

Using gastroprotective agents for ulcer and gastritis treatment, instead of other pharmacological agents (i.e. proton pump inhibitor) is advantageous because it does not modify the chemical environment necessary for digestion and protection. The gastric juice acts a barrier to avoid microorganism's proliferation and clinical studies have confirmed the increase of infections related to proton pump inhibitors (GIULIANO; WILHELM; KALE-PRADHAN, 2012).

In the present work we observed the gastroprotective barrier formed by tannins. We noticed that even washing the organ with PBS does not remove the layer of tannins in the stomach wall, showing the formation of a long-term layer. Although tannins are water-soluble, their bind forces are strong enough to not become easily detached from the parietal cells. These results combined with the non-acute and chronic toxicity found in the barbatimão (COSTA et al., 2013) extract provide strong indications for developing an antiulcer herbal remedy from the species, since it is already a topical medicine (Fitoscar®, Apsen Laboratory). However, clinical studies are needed for herbal medicine development.

CONCLUSIONS

5. CONCLUSIONS

In this study, we investigated important chemical and biological issues in order to contribute to the development of HM acting on GIT. In the chemical study with cascara sagrada six cascarosides were isolated, two were new chemical structures. The stereochemical analysis proved the correct NOE assignment based on crystal aloin structures and conformational analysis explained why this was possible. Additionally, we achieved the fragmentation profile, allowing differentiation between anthrones and oxanthrones classes. After complete characterization we successfully quantified the cascarosides content in different extracts e submitted them for cellular tests. In the cytotoxicity tests, we delineated a structure-activity relationship for (ox)anthrone versus anthraquinones. In conclusion, we recommend the cascarosides-enrichment due to the less toxic profile found in this work.

For barbatimão, in the chemical study we isolated procyanidins and established their AC configuration using VCD. Also, strategies to differentiate diastereomers using VCD were discussed. Using MALDI analysis several series of tannins were identified. Quantification and standardization were made using the isolated compounds using LC-MS/MS and the pharmacopeial method for the total tannins content. After standardization, in the biological test we verified the reduction in glucose blood levels in the treated animals and, along with fluorescence microscopy studies, their action as coating agents was proven.

6. REFERENCES

- ABENDROTH, A. et al. impressive picture of a melanosis coli after chronic anthraquinone laxative use - is there an increased risk for colorectal cancer? **Zeitschrift Fur Gastroenterologie**, v. 47, n. 6, p. 579-582, 2009.
- ADINORTEY, M. B. et al. In vivo models used for evaluation of potential antigastroduodenal ulcer agents. **Hindawi**, v. 2013, 2013.
- AKAO, T. et al. A purgative action of barbaloin is induced by *Eubacterium* sp. strain BAR, a human intestinal anaerobe, capable of transforming barbaloin to aloe-emodin anthrone. **Biological & Pharmaceutical Bulletin**, v. 19, n. 1, p. 136-138, 1996.
- AL ASMARI, A. et al. Gastroprotective effect of minocycline in experimentally induced gastric ulcers in rats. **International Journal of Clinical and Experimental Medicine**, v. 7, n. 3, p. 586-596, 2014.
- ALEXANDER, M. D. et al. A central strategy for converting natural products into fluorescent probes. **Chembiochem**, v. 7, n. 3, p. 409-416, 2006.
- ALZAID, F. et al. Regulation of glucose transporter expression in human intestinal Caco-2 cells following exposure to an anthocyanin-rich berry extract. **Plos One**, v. 8, n. 11, 2013.
- AUDI, E. A. et al. Gastric antiulcerogenic effects of *Stryphnodendron adstringens* in rats. **Phytotherapy Research**, v. 13, n. 3, p. 264-266, 1999.
- BADIALI, D. et al. Melanosis of the Rectum in Patients with Chronic Constipation. **Diseases of the Colon & Rectum**, v. 28, n. 4, p. 241-245, 1985.
- BAKER, E. H.; SANDLE, G. I. Complications of laxative abuse. **Annual Review of Medicine**, v. 47, p. 127-134, 1996.
- BATISTA JR, J. M.; BLANCH, E. W.; BOLZANI, V. D. S. Recent advances in the use of vibrational chiroptical spectroscopic methods for stereochemical characterization of natural products. **Natural Product Reports**, v. 32, n. 9, p. 1280-1302, 2015.
- BENAVIDES, S. H. et al. The pigment of melanosis coli: a lectin histochemical study. **Gastrointestinal Endoscopy**, v. 46, n. 2, p. 131-138, 1997.

BIRCH, A. J.; CLARKLEWIS, J. W.; ROBERTSON, A. V. The relative and absolute configurations of catechins and epicatechins. **Journal of the Chemical Society**, n. 8, p. 3586-3594, 1957.

BOCKUS, H. L.; WILLARD, J. H.; BANK, J. Melanosis coli - the etiologic significance of the anthracene laxatives - a report of forty-one cases. **Journal of the American Medical Association**, v. 101, p. 1-6, 1933.

BONE, K.; MILLS, S. **Principles and Practice of Phytotherapy**. 2nd. United States of America: Elsevier, 2013.

BRASIL. **Política Nacional de Práticas Integrativas e Complementares no SUS**. Brasília – DF: Ministério da Saúde, 2006.

BRASIL. **Farmacopeia Brasileira 5ª Edição**. Brasília - DF: ANVISA 2010.

BRASIL. **RDC nº26, de 13 de Maio de 2014**. Brasília - DF: ANVISA 2014.

BUENZ, E. J. Aloin induces apoptosis in Jurkat cells. **Toxicology In Vitro**, v. 22, n. 2, p. 422-9, 2008.

CANEQUE, T. et al. Synthesis of marmycin A and investigation into its cellular activity. **Nature Chemistry**, v. 7, n. 9, p. 744-751, 2015.

CANER, H. et al. Trends in the development of chiral drugs. **Drug Discovery Today**, v. 9, n. 3, p. 105-110, 2004.

CASE, D. A. et al. **AMBER 2015**. University of California, San Francisco 2015.

CHEN, J. Y. et al. Experimental study on the molecular mechanism of anthraquinone cathartics in inducing melanosis coli. **Chinese Journal of Integrative Medicine**, v. 17, n. 7, p. 525-530, 2011.

COSTA, M. A. et al. Acute and chronic toxicity of an aqueous fraction of the stem bark of *Stryphnodendron adstringens* (Barbatimao) in Rodents. **Evidence-Based Complementary and Alternative Medicine**, 2013.

DE GUSSEM, E. et al. Strength by joining methods: combining synthesis with NMR, IR, and vibrational circular dichroism spectroscopy for the determination of the relative configuration in hemicalide. **Chemistry**, v. 20, n. 52, p. 17385-94, 2014.

DE MELLO, J. C. P.; PETEREIT, F.; NAHRSTEDT, A. A dimeric proanthocyanidin from *Stryphnodendron adstringens*. **Phytochemistry**, v. 51, n. 8, p. 1105-1107, 1999.

DE WITTE, P. Metabolism and pharmacokinetics of anthranoids. **Pharmacology**, v. 47 Suppl 1, p. 86-97, 1993.

DEMARQUE, D. P. et al. Fragmentation reactions using electrospray ionization mass spectrometry: an important tool for the structural elucidation and characterization of synthetic and natural products. **Nat Prod Rep**, v. 33, n. 3, p. 432-55, 2016.

DEMELLO, J. P.; PETEREIT, F.; NAHRSTEDT, A. Flavan-3-ols and prodelphinidins from *Stryphnodendron adstringens*. **Phytochemistry**, v. 41, n. 3, p. 807-813, 1996a.

DEMELLO, J. P.; PETEREIT, F.; NAHRSTEDT, A. Prorobinetinidins from *Stryphnodendron adstringens*. **Phytochemistry**, v. 42, n. 3, p. 857-862, 1996b.

DEPREZ, S. et al. Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. **Journal of Nutrition**, v. 130, n. 11, p. 2733-2738, 2000.

DEPREZ, S. et al. Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. **Antioxidants & Redox Signaling**, v. 3, n. 6, p. 957-967, 2001.

DIAS, H. J.; MELO, N. I.; CROTTI, A. E. M. Electrospray ionization tandem mass spectrometry as a tool for the structural elucidation and dereplication of natural products: an overview. In: PRASAIN, J. K. (Ed.). **Tandem mass spectrometry - applications and principles**: Intech, 2012. cap. 26.

DONG, X. et al. Aloe-emodin induces apoptosis in human liver HL-7702 cells through fas death pathway and the mitochondrial pathway by generating reactive oxygen species. **Phytother Res**, v. 31, n. 6, p. 927-936, 2017.

DONG, X. X. et al. Emodin: A Review of its pharmacology, toxicity and pharmacokinetics. **Phytotherapy Research**, v. 30, n. 8, p. 1207-1218, 2016.

DONOVAN, J. L. et al. (+)-catechin is more bioavailable than (-)-catechin: Relevance to the bioavailability of catechin from cocoa. **Free Radical Research**, v. 40, n. 10, p. 1029-1034, 2006.

DREESSEN, M.; LEMLI, J. Studies in the field of drugs containing anthraquinone derivatives .36. The metabolism of cascariosides by intestinal bacteria. **Pharmaceutica Acta Helvetiae**, v. 63, n. 9-10, p. 287-289, 1988.

ERNST, M. et al. Mass spectrometry in plant metabolomics strategies: from analytical platforms to data acquisition and processing. **Natural Product Reports**, v. 31, n. 6, p. 784-806, 2014.

GUIA DA FARMÁCIA. Fitoterápicos ganham espaço. <http://www.guiadafarmacia.com.br>, 2016. Access in: 13th july, 2017.

FATOU, B. et al. In vivo real-time mass spectrometry for guided surgery application. **Scientific Reports**, v. 6, 2016.

FENN, J. B. Electrospray wings for molecular elephants (Nobel lecture). **Angewandte Chemie-International Edition**, v. 42, n. 33, p. 3871-3894, 2003.

FENN, J. B. et al. Electrospray ionization for mass-spectrometry of large biomolecules. **Science**, v. 246, n. 4926, p. 64-71, 1989.

FIORI, G. M. L. et al. Antimicrobial activity and rates of tannins in *Stryphnodendron adstringens* Mart. accessions collected in the brazilian cerrado **American Journal of Plant Sciences**, n. 4, p. 2193-2198, 2013.

FRISCH, M. J. et al. **Gaussian 09, Rev E.01**. Wallingford CT, USA: Gaussian, Inc. 2013.

FUNATOGAWA, K. et al. Antibacterial activity of hydrolyzable tannins derived from medicinal plants against *Helicobacter pylori*. **Microbiol Immunol**, v. 48, n. 4, p. 251-61, 2004.

GIULIANO, C.; WILHELM, S. M.; KALE-PRADHAN, P. B. Are proton pump inhibitors associated with the development of community-acquired pneumonia? A meta-analysis. **Expert Review of Clinical Pharmacology**, v. 5, n. 3, p. 337-345, 2012.

GOLAN, D. E.; ARMSTRONG, E. J.; ARMSTRONG, A. E. **Principles o pharmacology : the pathophysiologic basis o drug therapy**. Philadelphia, PA: Wolters Kluwer, 2017.

GOLAN, D. E. et al. **Princípios de Farmacologia - A Base Fisiopatológica da Farmacoterapia**. 2^a. Guanabara Koogan, 2009.

GUARATINI, T. et al. Application of matrix-assisted laser-desorption/ionization time-of-flight LIFT for identification of cocoa condensed tannins. **Journal of Mass Spectrometry**, v. 49, n. 3, p. 251-255, 2014.

HALGREN, T. A. Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. **Journal of Computational Chemistry**, v. 17, n. 5-6, p. 490-519, 1996.

HARSHA, C. et al. Antiulcer properties of fruits and vegetables: a mechanism based perspective. **Food Chem Toxicol**, v. 108, p. 104-119, 2017.

HASLAM, E. **Plant polyphenols. Vegetable tannins revisited**. Cambridge: Cambridge University Press, 1989.

HASLAM, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. **Journal of Natural Products**, v. 59, n. 2, p. 205-215, 1996.

HASLAM, E. et al. Traditional herbal medicines - the role of polyphenols. **Planta Medica**, n. 1, p. 1-8, 1989.

HATTORI, M. et al. Metabolism of barbaloin by intestinal bacteria. **Chem Pharm Bull (Tokyo)**, v. 36, n. 11, p. 4462-6, 1988.

HOLCAPEK, M.; JIRASKO, R.; LISA, M. Basic rules for the interpretation of atmospheric pressure ionization mass spectra of small molecules. **Journal of Chromatography A**, v. 1217, n. 25, p. 3908-3921, 2010.

HUO, Y. et al. Development of a UFLC-MS/MS method for the simultaneous determination of seven tea catechins in rat plasma and its application to a pharmacokinetic study after administration of green tea extract. **J Pharm Biomed Anal**, v. 125, p. 229-35, 2016.

ICHIM, G.; TAIT, S. W. G. A fate worse than death: apoptosis as an oncogenic process. **Nature Reviews Cancer**, v. 16, n. 8, p. 539-548, 2016.

ISLER, A. C. et al. Development and validation of a lc-method for the determination of phenols in a pharmaceutical formulation containing extracts from *Stryphnodendron Adstringens*. **Quimica Nova**, v. 33, n. 5, p. 1126-1129, 2010.

JOHNSTON, K. et al. Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. **Febs Letters**, v. 579, n. 7, p. 1653-1657, 2005.

KOBASHI, K.; AKAO, T. Relation of intestinal bacteria to pharmacological effects of glycosides. **Bioscience Microflora**, v. 16, n. 1, p. 1-7, 1997.

KOLECKAR, V. et al. Condensed and hydrolysable tannins as antioxidants influencing the health. **Mini-Reviews in Medicinal Chemistry**, v. 8, n. 5, p. 436-447, 2008.

KOŁODZIEJ, H. ^1H NMR spectral studies of procyanidin derivatives: diagnostic ^1H NMR parameters applicable to the structural elucidation of oligomeric procyanidins. In: HEMINGWAY, R. W. e LAKS, P. E. (Ed.). **PLANT POLYPHENOLS Synthesis. Properties. Significance**. Michigan: Springer Science Business Media New York, 1992.

KRENN, L. et al. Anthrone C-glucosides from *Rheum emodi*. **Chemical & Pharmaceutical Bulletin**, v. 52, n. 4, p. 391-393, 2004.

LABADIE, R. P. The anthracene derivatives in *Rhamnus frangula* L. I. The aglycones. **Pharm Weekbl**, v. 105, n. 7, p. 189-95, 1970.

LAI, J. C. Y. et al. Treatment for diabetic ulcer wounds using a fern tannin optimized hydrogel formulation with antibacterial and antioxidative properties. **Journal of Ethnopharmacology**, v. 189, p. 277-289, 2016.

LEE, M.-C. et al. Aloin-induced cell growth arrest, cell apoptosis, and autophagy in human non-small cell lung cancer cells. **Biomarkers and Genomic Medicine**, v. 6, p. 144-149, 2014.

LEVSEN, K.; SCHWARZ, H. Gas-phase chemistry of collisionally activated ions. **Mass Spectrometry Reviews**, v. 2, n. 1, p. 77-148, 1983.

LIAO, C. R. et al. Studies on cytotoxic constituents from the leaves of *Elaeagnus oldhamii* Maxim. in non-small cell lung cancer a549 cells. **Molecules**, v. 19, n. 7, p. 9515-9534, 2014.

LIM, W. et al. Chrysophanol induces apoptosis of choriocarcinoma through regulation of ROS and the AKT and ERK1/2 pathways. **Journal of Cellular Physiology**, v. 232, n. 2, p. 331-339, 2017.

LOPES, G. C. et al. Chemotaxonomic significance of 5-deoxyproanthocyanidins in *Stryphnodendron* species. **Biochemical Systematics and Ecology**, v. 36, n. 12, p. 925-931, 2008.

LOPES, G. C. et al. Quantitative determination of tannins in three species of *Stryphnodendron* by high efficiency liquid chromatography. **Brazilian Journal of Pharmaceutical Sciences**, v. 45, n. 1, p. 135-143, 2009.

LOPES, G. C. et al. Influence of extracts of *Stryphnodendron polyphyllum* Mart. and *Stryphnodendron obovatum* Benth. on the cicatrization of cutaneous wounds in rats. **Journal of Ethnopharmacology**, v. 99, n. 2, p. 265-272, 2005.

LUIZ, R. L. F. et al. Proanthocyanidins polymeric tannin from *Stryphnodendron adstringens* are active against *Candida albicans* biofilms. **Bmc Complementary and Alternative Medicine**, v. 15, 2015.

MA, Q. et al. Bioactive dietary polyphenols inhibit heme iron absorption in a dose-dependent manner in human intestinal Caco-2 cells. **J Food Sci**, v. 76, n. 5, p. H143-50, 2011.

MALIK, E. M.; MULLER, C. E. Anthraquinones as pharmacological tools and drugs. **Medicinal Research Reviews**, v. 36, n. 4, p. 705-748, 2016.

MANITTO, P.; MONTI, D.; SPERANZA, G. Studies on aloe. Part 6. Conformation and absolute configuration of aloins A and B and related 10-C-glucosyl-9-anthrone. **Journal of the Chemical Society, Perkin Transactions 1**, v. , n. 5, p. 1297-1300, 1990a.

MANITTO, P.; MONTI, D.; SPERANZA, G. Studies on aloe. Part 6. Conformation and absolute configuration of aloins A and B and related 10-C-glucosyl-9-anthrone. **Journal of the Chemical Society, Perkin Transactions 1**, n. 5, p. 1297-1300, 1990b.

MANITTO, P. et al. Conformational studies of natural products. Part 4. Conformation and absolute configuration of cascarosides A, B, C, D. **Journal of the Chemical Society, Perkin Transactions 1**, n. 14, p. 1577-1580, 1993a.

MANITTO, P. et al. Studies on Cascara .2. Structures of Cascaroside-E and Cascaroside-F. **Journal of Natural Products-Lloydia**, v. 58, n. 3, p. 419-423, 1995.

MARQUART, L. F.; SOBAL, J. Weight-loss beliefs, practices and support systems for high-school wrestlers. **Journal of Adolescent Health**, v. 15, n. 5, p. 410-415, 1994.

MELO, J. O. D. et al. Effect of *Stryphnodendron adstringens* (barbatimão) bark on animal models of nociception. **Revista Brasileira de Ciências Farmacêuticas**, v. 43, n. 3, p. 465-469, 2007.

MELO, M. G. D. et al. Valores de referência hematológicos e bioquímicos de ratos (*Rattus norvegicus* linhagem Wistar) provenientes do biotério central da Universidade Federal de Sergipe **SCIENTIA PLENA**, v. 8, n. 9, 2012.

- MERETO, E.; GHIA, M.; BRAMBILLA, G. Evaluation of the potential carcinogenic activity of Senna and Cascara glycosides for the rat colon. **Cancer Letters**, v. 101, n. 1, p. 79-83, 1996.
- MERTEN, C. Vibrational optical activity as probe for intermolecular interactions. **Physical Chemistry Chemical Physics**, 2017.
- MERTEN, C.; SMYRNIOTOPOULOS, V.; TASDEMIR, D. Assignment of absolute configurations of highly flexible linear diterpenes from the brown alga *Bifurcaria bifurcata* by VCD spectroscopy. **Chem Commun (Camb)**, v. 51, n. 90, p. 16217-20, 2015.
- MOREAU, J. P.; MOREAU, S.; SKINNER, S. Comparative physiological disposition of some anthraquinone glycosides and aglycones. **Biopharmaceutics & Drug Disposition**, v. 6, n. 3, p. 325-334, 1985.
- NAFIE, L. A. **Vibrational Optical Activity**. John Wiley & Sons Ltd, UK, 2011.
- NOMIZU, K. et al. Antioxidants from steamed used tea leaves and their reaction behavior. **Bioscience Biotechnology and Biochemistry**, v. 72, n. 7, p. 1682-1689, 2008.
- OTTAVIANI, J. I. et al. The stereochemical configuration of flavanols influences the level and metabolism of flavanols in humans and their biological activity in vivo. **Free Radical Biology and Medicine**, v. 50, n. 2, p. 237-244, 2011.
- PORTT, L. et al. Anti-apoptosis and cell survival: A review. **Biochimica Et Biophysica Acta-Molecular Cell Research**, v. 1813, n. 1, p. 238-259, 2011.
- PRADO, L. C. et al. The gastroprotective effects of *Eugenia dysenterica* (Myrtaceae) leaf extract: the possible role of condensed tannins. **Biological & Pharmaceutical Bulletin**, v. 37, n. 5, p. 722-30, 2014.
- RAUWALD, H. W.; LOHSE, K.; BATS, J. W. Establishment of configurations for the two diastereomeric C-glucosylanthrones Aloin A and Aloin B. **Angewandte Chemie International Edition in English**, v. 28, n. 11, p. 1528-1529, 1989.
- REGAZZONI, L. et al. A rapid profiling of gallotannins and flavonoids of the aqueous extract of *Rhus coriaria* L. by flow injection analysis with high-resolution mass spectrometry assisted with database searching. **J Pharm Biomed Anal**, v. 72, p. 202-7, 2013.
- ROERIG, J. L. et al. Laxative Abuse Epidemiology, Diagnosis and Management. **Drugs**, v. 70, n. 12, p. 1487-1503, 2010.

RUE, E. A.; RUSH, M. D.; VAN BREEMEN, R. B. Procyanidins: a comprehensive review encompassing structure elucidation via mass spectrometry. **Phytochemistry Reviews**, 2017.

SAEZ-AYALA, M. et al. Factors influencing the antifolate activity of synthetic tea-derived catechins. **Molecules**, v. 18, n. 7, p. 8319-41, 2013.

SAITO, M. et al. Antiulcer activity of grape seed extract and procyanidins. **Journal of Agricultural and Food Chemistry**, v. 46, n. 4, p. 1460-1464, 1998.

SCHLEEP, S.; FRIEDRICH, H.; KOLODZIEJ, H. The first natural procyanidin with a 3,4-cis configuration. **J. Chem. Soc., Chem. Commun**, n. 5, 1986.

SCHORKHUBER, M. et al. Effect of anthraquinone-laxatives on the proliferation and urokinase secretion of normal, premalignant and malignant colonic epithelial cells. **Eur J Cancer**, v. 34, n. 7, p. 1091-8, 1998.

SCHWARZENBERG, A. et al. Differentiation of isomeric dinitrotoluenes and aminodinitrotoluenes using electrospray high resolution mass spectrometry. **Journal of Mass Spectrometry**, v. 49, n. 12, p. 1330-1337, 2014.

SHAN, Y. et al. Oxidative tea polyphenols greatly inhibit the absorption of atenolol. **Front Pharmacol**, v. 7, p. 192, 2016.

SHIGEMATSU, M.; KOBAYASHI, T.; TANAHASHI, M. Solvent effect on the conformations of phenol, anisole and guaiacol simulated with MOPAC2000. **Journal of Computer Chemistry Japan**, v. 13, n. 3, p. 177-182, 2001.

SILVA, D. B. et al. Mass Spectrometry of Flavonoid Vicenin-2, Based Sunlight Barriers in *Lychnophora* species. **Scientific Reports**, v. 4, 2014.

SIRK, T. W. et al. Molecular binding of catechins to biomembranes: relationship to biological activity. **J Agric Food Chem**, v. 57, n. 15, p. 6720-8, 2009.

SONG, G. P. et al. Synthesis and bioactivity of novel xanthone and thioxanthone L-rhamnopyranosides. **Rsc Advances**, v. 5, n. 45, p. 36092-36103, 2015.

SONG, R. et al. Metabolic analysis of rhubarb extract by rat intestinal bacteria using liquid chromatography-tandem mass spectrometry. **Biomedical Chromatography**, v. 25, n. 3, p. 417-426, 2011.

SPARTAN 14. Spartan 14, Wavefunction Inc., Irvine, CA, USA 2014.

SRINIVAS, G. et al. Molecular mechanism of emodin action: transition from laxative ingredient to an antitumor agent. **Medicinal Research Reviews**, v. 27, n. 5, p. 591-608, 2007.

SU, Y. T. et al. Emodin induces apoptosis in human lung adenocarcinoma cells through a reactive oxygen species-dependent mitochondrial signaling pathway. **Biochem Pharmacol**, v. 70, n. 2, p. 229-41, 2005.

SZABO, S. "Gastric cytoprotection" is still relevant. **Journal of Gastroenterology and Hepatology**, v. 29, p. 124-132, 2014.

SZABO, S. et al. Role of vascular factors, including angiogenesis, in the mechanisms of action of sucralfate. **American Journal of Medicine**, v. 91, p. S158-S160, 1991.

TAYLOR, P. W.; HAMILTON-MILLER, J. M.; STAPLETON, P. D. Antimicrobial properties of green tea catechins. **Food Sci Technol Bull**, v. 2, p. 71-81, 2005.

TOZZI, F. et al. Features associated with laxative abuse in individuals with eating disorders. **Psychosomatic Medicine**, v. 68, n. 3, p. 470-477, 2006.

TSUKIDA, K.; SUZUKI, N. Hydroxyanthrones in plants containing hydroxyanthraquinones .7. a new method for the microdetermination of 1,8-dihydroxyanthrone derivatives and the seasonal variance of their content in plants. **Yakugaku Zasshi-Journal of the Pharmaceutical Society of Japan**, v. 74, n. 10, p. 1092-1096, 1954.

WALKER, N. I.; BENNETT, R. E.; AXELSEN, R. A. Melanosis-coli - a consequence of anthraquinone-induced apoptosis of colonic epithelial-cells. **American Journal of Pathology**, v. 131, n. 3, p. 465-476, 1988.

WHO. Monographs on Selected Medicinal Plants - Volume 2. Geneva, 2016.

WU, X. F. et al. Simultaneous qualitative and quantitative determination of phenolic compounds in *Aloe barbadensis* Mill by liquid chromatography-mass spectrometry-ion trap-time-of-flight and high performance liquid chromatography-diode array detector. **Journal of Pharmaceutical and Biomedical Analysis**, v. 80, p. 94-106, 2013.

WU, Y. Y. et al. Aloe-emodin (AE) nanoparticles suppresses proliferation and induces apoptosis in human lung squamous carcinoma via ROS generation in vitro and in vivo. **Biochem Biophys Res Commun**, 2017.

YANG, H. et al. CFTR chloride channel as a molecular target of anthraquinone compounds in herbal laxatives. **Acta Pharmacologica Sinica**, v. 32, n. 6, p. 834-839, 2011.

YANG, Z. F. et al. Comparison of in vitro antiviral activity of tea polyphenols against influenza A and B viruses and structure-activity relationship analysis. **Fitoterapia**, v. 93, p. 47-53, 2014.

ZHANG, L. et al. Emodin targets mitochondrial cyclophilin D to induce apoptosis in HepG2 cells. **Biomed Pharmacother**, v. 90, p. 222-228, 2017.

APPENDIX

APPENDIX

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1. Sequential fragmentation (ESI-MS³) of cascarosides

Figure A1: Sequential fragmentation (ESI-MS³) of Cascarosides in positive mode.

Figure A2: Sequential fragmentation (ESI-MS³) of Cascarosides in negative mode.

2. 10-hydroxycascaroside B NMR spectra

Table S1: NMR data of 10-hydroxycascaroside B (Comparison with Cascaroside B (MeOD; 300MHz) (MANITTO et al.; 1993))

	¹ H-Experimental	¹ H-Literature	¹³ C - Experimental	¹³ C -Cascaroside B	DEPT- 135
1	-----	-----	160.8	162.0	-----
1a	-----	-----	118.5	120.2	-----
2	6.86 (s)	6.83 (s)	115.3	114.0	(CH)
3	-----	-----	150.7	151.0	-----
4	7.40 (s)	6.98 (s)	117.3	117.2	(CH)
4a	-----	-----	147.3	143.4	-----
5	7.26 (8.0 Hz; d)	7.27 (d; 7.4Hz)	121.7	124.6	(CH)
5a	-----	-----	146.7	145.4	-----
6	7.66 (8.0 Hz; 7.6Hz; dd)	7.53 (dd; 8.3Hz; 7.4Hz)	135.7	134.6	(CH)
7	7.33 (7.6 Hz; d)	7.36 (d; 8.3Hz)	115.5	116.7	(CH)
8	-----	-----	158.4	159.0	-----
8a	-----	-----	123.0	125.0	-----
9	-----	-----	190.9	192.1	-----
10	-----	4.59 (d; 1.9Hz)	76.8	46.1 (CH)	-----
11	4.62 (s)	4.62 (s)	64.6	64.5	(CH ₂)
1'	3.21 (d; 9.6 Hz)	3.31 (dd; 9.6 Hz; 1.9 Hz)	83.9	85.6	(CH)
2'	2.99 (9.6 Hz; 9.2 Hz; dd)	2.99 (dd; 9.6 Hz; 8.7 Hz)	70.8	71.7	(CH)
3'	3.27 (m)	3.26 (dd; 8.7 Hz)	79.0	79.8	(CH)
4'	2.84-2.93 (m)	2.84 – 2.93 (m)	73.1	72.0	(CH)
5'	2.84-2.93 (m)	2.85 – 2.93(m)	81.2	81.3	(CH)
6'	3.45-3.60 (m)	3.51 (dd; 11.9 Hz; 2.1 Hz); 3.36 (dd; 11.9 Hz; 4.9 Hz)	62.5	63.0	(CH ₂)
1''	5.02 (d; 8.0Hz)	4.95 (d; 7.7 Hz)	102.5	103.2	(CH)
2''	3.66 (m)	3.65 (dd; 7.7 Hz; 8.7 Hz)	74.4	74.7	(CH)
3''	3.45-3.60 (m)	3.41-3.47 (m)	77.5	77.8	(CH)
4''	3.45-3.60 (m)	3.41-3.47 (m)	70.8	71.2	(CH)
5''	3.45-3.60 (m)	3.41-3.47 (m)	77.9	78.5	(CH)
6''	3.89 (d; 12.2 Hz); 3.73 (dd; 12.2 Hz; 4.6 Hz)	3.93 (dd; 12.1 Hz; 1.8 Hz); 3.72 (dd; 12.1 Hz; 5.4 Hz)	62.1	62.6	(CH ₂)

Figure A3: ¹H NMR spectra of 10-hydroxycascaroside B (D₂O; 400MHz).

Figure A4: Expansion of ^1H NMR spectra (3.90 – 2.80 ppm) of 10-hydroxycascaroside B (D_2O ; 400MHz).

Figure A5: Expansion of ^1H NMR spectra (4.50 – 5.60 ppm) of 10-hydroxycascaroside B (D_2O ; 400MHz).

Figure A6: Expansion of ^1H NMR spectra (6.80 – 7.80 ppm) of 10-hydroxycascaroside B (D_2O ; 400MHz).

Figure A7: NOESY spectra of 10-hydroxycascaroside B (MeOD; 400MHz).

Figure A8: Expansion of 10-hydroxycascaroside B NOESY spectra (MeOD; 400MHz).

Figure A9: ¹³C NMR spectra of 10-hydroxycascaroside B (D₂O; 100MHz).

Figure A10: DEPT-135 NMR spectra of 10-hydroxycascaroside B (D_2O ; 100MHz).

3. 10-hydroxycascaroside A spectra

Figure

Table S2: NMR data of 10-hydroxycascaroside A (400 MHz) in comparison with Cascaroside A (MeOD; 300MHz) (MANITTO et al.; 1993).

	¹ H - Experimental	¹ H-Literature	¹³ C - Experimental	¹³ C -Cascaroside A	DEPT-135
1	-----	-----	161.9	161.9	-----
1a	-----	-----	118.7	120.5	-----
2	6.94 (s)	6.85 (s)	115.2	114.4	(CH)
3	-----	-----	150.5	150.3	-----
4	7.35 (s)	7.00 (s)	119.6	118.6	(CH)
4a	-----	-----	145.1	141.2	-----
5	7.45 (d; 8.0 Hz)	7.26 (dd; 7.7Hz; 1.0Hz)	121.0	123.6	(CH)
5a	-----	-----	149.5	147.2	-----
6	7.65 (t; 8.0Hz)	7.54 (dd; 8.3Hz; 7.7Hz)	135.9	135.7	(CH)
7	7.77 (d; 7.6 Hz)	7.37 (dd; 8.3Hz; 1.0Hz)	116.0	118.5	(CH)
8	-----	-----	158.7	159.1	-----
8a	-----	-----	123.8	125.0	-----
9	-----	-----	191.3	192.4	-----
10	-----	4.58 (d; 2.2Hz)	77.0	46.1 (CH)	-----
11	4.68 (AB syst.; 14.8Hz)	4.60 (AB syst.; 15.2Hz)	64.6	64.5	(CH ₂)
1'	3.26 (d; 8.8 Hz)	3.34 (dd; 9.6 Hz; 2.2 Hz)	83.7	85.0	(CH)
2'	2.96 (t; 9.0 Hz)	2.94 (dd; 9.6 Hz; 8.8 Hz)	71.6	71.7	(CH)
3'	3.20 (d; 9.2 Hz)	3.24 (dd; 8.8 Hz)	79.4	79.8	(CH)
4'	2.8 (t; 8.8 Hz)	2.79 – 2.90 (m)	73.0	72.0	(CH)
5'	2.88 (m)	2.79 – 2.90 (m)	81.6	81.4	(CH)
6'	3.41-3.55 (m)	3.50 (dd; 11.7 Hz; 2.4 Hz); 3.32 (dd; 11.7 Hz; 5.2 Hz)	63.2	63.5	(CH ₂)
1''	4.94 (d; 8.0Hz)	4.91 (d; 7.5 Hz)	105.1	104.9	(CH)
2''	3.61 (dd; 9.2 Hz; 7.6 Hz)	3.60 (dd; 7.5 Hz; 9.1 Hz)	75.0	75.0	(CH)
3''	3.41-3.55 (m)	3.51 (dd; 9.1 Hz)	77.2	77.2	(CH)
4''	3.41-3.55 (m)	3.37-3.47 (m)	71.2	71.2	(CH)
5''	3.41-3.55 (m)	3.37-3.47 (m)	78.7	78.6	(CH)
6''	3.91 (dd; 12.2 Hz; 2.4 Hz); 3.72 (dd; 12.2 Hz; 5.2 Hz)	3.89 (dd; 12.0 Hz; 2.2 Hz); 3.70 (dd; 12.0 Hz; 5.2 Hz)	62.5	62.5	(CH ₂)

Figure A11: ^1H NMR spectra of 10-hydroxycascaroside A (MeOD ; 400MHz).

Figure A12: Expansion of ^1H NMR spectra (2.80 – 4.00 ppm) of 10-hydroxycascaroside A (MeOD; 400MHz).

Figure A13: Expansion of ^1H NMR spectra (4.60 – 5.00 ppm) of 10-hydroxycascaroside A (MeOD; 400MHz).

Figure A14: Expansion of ^1H NMR spectra (6.70 – 7.80 ppm) of 10-hydroxycascaroside A (MeOD ; 400MHz).

Figure A15: NOESY spectra of 10-hydroxycascaroside A (MeOD; 400MHz).

Figure A16: Expansion of NOESY spectra of 10-hydroxycascaroside A (MeOD; 400MHz).

Figure A17: ¹³C NMR spectra of 10-hydroxycascaroside A (MeOD; 100MHz).

Figure A18: DEPT-135 NMR spectra of 10-hydroxycascaroside A (MeOD; 100MHz). (Reversed phase)

4. Cascaroside B spectra

Table S3: ^1H NMR data of Cascaroside B (MeOD; 400 MHz) in comparison with literature data (MeOD; 300MHz). (MANITTO et al.; 1993).

	^1H (Experimental)	^1H (Literature)
2	6.83 (s)	6.83 (s)
4	6.99 (s)	6.98 (s)
5	7.26 (s)	7.27 (d; 7.4Hz)
6	7.52 (sL)	7.53 (dd; 8.3Hz; 7.4Hz)
7	7.35 (s)	7.36 (d; 8.3Hz)
10	-----	4.59 (d; 1.9Hz)
11	4.61 (d; 8.8 Hz)	4.62 (s)
1'	3.29 – 3.34 (m)	3.31 (dd; 9.6; 1.9)
2'	2.99 (t; 9.2 Hz)	2.99 (dd; 9.6; 8.7)
3'	3.25 (d; 8.8 Hz)	3.26 (dd; 8.7)
4'	2.84-2.92 (m)	2.84 – 2.93 (m)
5'	2.84-2.92 (m)	2.85 – 2.93(m)
6'	3.38 (d; 4.8 Hz) 3.42-3.54 (m)	3.51 (dd; 11.9; 2.1); 3.36 (dd; 11.9; 4.9)
1''	----- (solvent)	4.95 (d; 7.7)
2''	3.65 (m)	3.65 (dd; 7.7; 8.7)
3''	3.42-3.54 (m)	3.41-3.47 (m)
4''	3.42-3.54 (m)	3.41-3.47 (m)
5''	3.42-3.54 (m)	3.41-3.47 (m)
6''	3.93 (d; 11Hz); 3.72 (dd; 12 Hz; 4.8Hz)	3.93 (dd; 12.1;1.8); 3.72 (dd; 12.1; 5.4)

Figure A19: ¹H NMR spectra of Cascaroside B (MeOD; 400MHz).

Figure A20: Expansion of ^1H NMR spectra (6.70 – 7.70 ppm) of Cascaroside B (MeOD ; 400MHz).

Figure A21: Expansion of ^1H NMR spectra (3.20 – 3.90 ppm) of Cascaroside B (MeOD ; 400MHz).

Figure A22: Expansion of ^1H NMR spectra (2.70 – 3.60 ppm) of Cascaroside B (MeOD; 400MHz).

Figure A23: NOESY spectra of Cascaroside B (MeOD; 400MHz).

Figure A24: Expansion of NOESY spectra of Cascaroside B (MeOD; 400MHz).

5. Cascaroside A NMR spectra

Table S4: NMR data (^1H ; ^{13}C e Dept-135) of Cascaroside A (MeOD; 400 MHz) in comparison with literature data (MeOD; 300MHz; MANITTO et al; 1993).

	^1H - experimental	^1H -Literature	^{13}C - experimental	^{13}C – Literature	DEPT-135
1	-----	-----	161.9	161.9	-----
1a	-----	-----	120.6	120.5	-----
2	6.88 (sL)	6.85 (s)	114.4	114.4	(CH)
3	-----	-----	150.3	150.3	-----
4	7.07 (sL)	7.00 (s)	118.7	118.6	(CH)
4a	-----	-----	141.3	141.2	-----
5	7.28 (d; 8.0 Hz)	7.26 (dd; 7.7Hz; 1.0Hz)	123.7	123.6	(CH)
5a	-----	-----	147.3	147.2	-----
6	7.58 (t; 8.4Hz; 7.2 Hz)	7.54 (dd; 8.3Hz; 7.7Hz)	135.9	135.7	(CH)
7	7.40 (d; 8.4 Hz)	7.37 (dd; 8.3Hz; 1.0Hz)	118.4	118.5	(CH)
8	-----	-----	159.1	159.1	-----
8a	-----	-----	125.0	125.0	-----
9	-----	-----	192.4	192.4	-----
10	4.59 (s)	4.58 (d; 2.2Hz)	46.2	46.1	(CH)
11	4.63 (m)	4.60 (AB syst.; 15.2Hz)	64.5	64.5	(CH ₂)
1'	3.24-3.29 (m)	3.34 (dd; 9.6 Hz; 2.2 Hz)	85.0	85.0	(CH)
2'	2.96 (t; 8.8 Hz; 9.6 Hz)	2.94 (dd; 9.6 Hz; 8.8 Hz)	71.8	71.7	(CH)
3'	3.24-3.29 (m)	3.24 (dd; 8.8 Hz)	79.8	79.8	(CH)
4'	2.85 – 2.88 (m)	2.79 – 2.90 (m)	72.0	72.0	(CH)
5'	2.85 – 2.88 (m)	2.79 – 2.90 (m)	81.5	81.4	(CH)
6'	3.34-3.56 (m)	3.50 (dd; 11.7 Hz; 2.4 Hz); 3.32 (dd; 11.7 Hz; 5.2 Hz)	63.3	63.5	(CH ₂)
1''	4.95 (d; 7.6 Hz)	4.91 (d; 7.5 Hz)	104.7	104.9	(CH)
2''	3.63 (m)	3.60 (dd; 7.5 Hz; 9.1 Hz)	75.0	75.0	(CH)
3''	3.34-3.56 (m)	3.51 (dd; 9.1 Hz)	77.2	77.2	(CH)
4''	3.34-3.56 (m)	3.37-3.47 (m)	71.2	71.2	(CH)
5''	3.34-3.56 (m)	3.37-3.47 (m)	78.6	78.6	(CH)
6''	3.92 (dd; 12.0 Hz; 2.0 Hz); 3.73 (dd; 12.0 Hz; 6.2 Hz)	3.89 (dd; 12.0 Hz; 2.2 Hz); 3.70 (dd; 12.0 Hz; 5.2 Hz)	62.5	62.5	(CH ₂)

Figure A25: ¹H NMR spectra of Cascaroside A (MeOD; 400MHz).

Figure A26: Expansion of ^1H NMR spectra (2.80 – 3.20 ppm) of Cascaroside A (MeOD ; 400MHz).

Figure A27: Expansion of ^1H NMR spectra (3.20 – 3.66 ppm) of Cascaroside A (MeOD; 400MHz).

Figure A28: Expansion of ^1H NMR spectra (3.50 – 4.04 ppm) of Cascaroside A (MeOD; 400MHz).

Figure A29: Expansion of ^1H NMR spectra (6.88 – 7.64 ppm) of Cascaroside A (MeOD; 400MHz).

Figure A30: NOESY spectra of Cascaroside A (MeOD; 400MHz).

Figure A31: Expansion of NOESY spectra of Cascaroside A (MeOD; 400MHz).

Figure A32: ¹³C NMR spectra of Cascaroside A (MeOD; 100MHz).

Figure A33: DEPT-135 NMR spectra of Cascaroside A (MeOD; 100MHz).

Cascaroside D

	¹ H	¹ H-Literatura
2	6.66 (s)	6.64 (s)
4	6.83 (s)	6.82 (s)
5	7.27 (d; 7.6 Hz)	7.25 (d; 7.6 Hz)
6	7.53 (t; 8.4 Hz; 7.6 Hz)	7.51 (dd; 8.2 Hz; 7.6 Hz)
7	7.36 (d; 8.8 Hz)	7.35 (d; 8.2 Hz)
10	4.56 (s)	4.55 (d; 1.9Hz)
11	2.35 (s)	2.34 (s)
1'	3.30-3.34 (m)	3.30 (solvente)
2'	2.99 (t; 9.6 Hz; 8.8 Hz)	2.98 (dd; 9.6 Hz; 8.7 Hz)
3'	3.25 (d; 8.8 Hz)	3.26 (dd; 8.7 Hz)
4'	2.86-2.90 (m)	2.83 – 2.92 (m)
5'	2.86-2.90 (m)	2.83 – 2.92 (m)
6'	3.30 – 3.55 (m)	3.51 (dd; 11.9 Hz; 2.0 Hz); 3.36 (dd; 11.9 Hz; 4.9 Hz)
1''	4.94 (d; 8Hz)	4.92 (d; 7.7 Hz)
2''	3.65 (t; 8.4 Hz; 8 Hz)	3.64 (dd; 7.7 Hz; 8.9 Hz)
3''	3.40 - 3.55 (m)	3.41-3.48 (m)
4''	3.40 - 3.55 (m)	3.41-3.48 (m)
5''	3.40 - 3.55 (m)	3.41-3.48 (m)
6''	3.93 (m) 3.72 (dd; 12 Hz; 5.6 Hz)	3.92 (dd; 12.1 Hz; 1.7 Hz); 3.72 (dd; 12.1 Hz; 5.1 Hz)

Figure A34: ¹H NMR spectra of Cascaroside D (MeOD; 400MHz).

Figure A35: Expansion of ^1H NMR spectra (2.30 – 4.00 ppm) of Cascaroside D (MeOD ; 400MHz).

Figure A36: Expansion of ^1H NMR spectra (4.44 – 5.16 ppm) of Cascaroside D (MeOD; 400MHz).

Figure A37: Expansion of ^1H NMR spectra (6.60 – 7.60 ppm) of Cascaroside D (MeOD; 400MHz).

7. Cascaroside C spectra

Table S6: NMR data (¹H) of Cascaroside C (MeOD; 400 MHz) in comparison with literature data (MeOD; 300 MHz; MANITTO et al.; 1993)

	H - Experimental	H-Literature
2	6.60 (s)	6.69 (s)
4	6.80 (d; 1.6 Hz)	6.89 (d; 1.6 Hz)
5	7.19 (d; 7.6 Hz)	7.28 (dd; 7.7 Hz; 1.1 Hz)
6	7.57 (t; 8.4 Hz)	7.57 (dd; 8.3 Hz; 7.7 Hz)
7	7.31 (d; 8.0 Hz)	7.40 (dd; 8.3 Hz; 1.1 Hz)
10	4.46 (s)	4.56 (d; 2.2 Hz)
11	2.27 (s)	2.36 (s)
1'	3.33 (d; 8.8 Hz)	3.34 (dd; 9.6 Hz; 2.2 Hz)
2'	2.89 (t; 8.8 Hz; 9.6 Hz)	2.98 (dd; 9.6 Hz; 8.9 Hz)
3'	3.15 – 3.26 (m)	3.26 (dd; 8.9 Hz)
4'	2.77 (m)	2.82 – 2.93 (m)
5'	2.77 (m)	2.82 – 2.93 (m)
6'	3.76-3.85 (m) 3.37 (m)	3.52 (dd; 11.8 Hz; 2.3 Hz); 3.35 (dd; 11.8 Hz; 5.1 Hz)
1''	4.84 (d; 8.4 Hz)	4.93 (d; 7.5 Hz)
2''	3.52 (dd; 7.6 Hz; 9.2 Hz)	3.61 (dd; 7.5 Hz; 9.2 Hz)
3''	3.36 – 3.48 (m)	3.53 (dd; 9.2 Hz)
4''	3.36 – 3.48 (m)	3.40-3.48 (m)
5''	3.36 – 3.48 (m)	3.40-3.48 (m)
6''	3.82 (dd; 12.0 Hz; 2 Hz) 3.63 (dd; 12.4 Hz; 5.6 Hz)	3.92 (dd; 12.0 Hz; 2.1 Hz); 3.72 (dd; 12.0 Hz; 5.3 Hz)

Figure A38: ^1H NMR spectra of Cascaroside C (MeOD ; 400MHz).

Figure A39: Expansion of ^1H NMR spectra (2.20 – 2.96 ppm) of Cascaroside C (MeOD ; 400MHz).

Figure A40: Expansion of ^1H NMR spectra (3.12 – 3.86 ppm) of Cascaroside C (MeOD; 400MHz).

Figure A41: Expansion of ^1H NMR spectra (4.00 – 5.30 ppm) of Cascaroside C (MeOD; 400MHz).

Figure A42: Expansion of ^1H NMR spectra (6.40 – 7.70 ppm) of Cascaroside C (MeOD; 400MHz).

Figure A43: NOESY spectra of Cascaroside C (MeOD; 400MHz).

Figure A44: Expansion of NOESY spectra of Cascaroside C (MeOD; 400MHz).

8. Gallocatechol spectra

Figure A45: ^1H NMR spectra of Gallocatechol (MeOD ; 400MHz).

Figure A46: Expansion of ^1H NMR spectra (2.30 – 4.60 ppm) of Gallocatechol (MeOD ; 400MHz)

Figure A47: ¹³C NMR spectra of Gallocatechol (MeOD; 100MHz).

Figure A48: DEPT-135 NMR spectra of Galocatechol (MeOD; 100MHz).

9. Robinetinidol spectra

Figure A49: ^1H NMR spectra of Robinetinidol (MeOD; 400MHz).

Figure A50: Expansion of ^1H NMR spectra (6.00 – 7.00 ppm) of Robinetinidol (MeOD; 400MHz)

Figure A51: Expansion of ^1H NMR spectra (2.40 – 4.70 ppm) of Robinetinidol (MeOD ; 400MHz).

Figure A52: ¹³C NMR spectra of Robinetinidol (MeOD; 100MHz).

Figure A53: DEPT-135 NMR spectra of Robinetinidol (MeOD; 100MHz).

10. Epigallocatechin spectra

Figure A54: ^1H NMR spectra of Epigallocatechin (MeOD; 400MHz).

Figure A55: Expansion of ^1H NMR spectra (2.60 – 4.30 ppm) of Epigallocatechin (MeOD ; 400MHz)

Figure A56: ¹³C NMR spectra of Epigallocatechin (MeOD; 100MHz).

Figure A57: DEPT-135 NMR spectra of Epigallocatechin (MeOD; 100MHz).

11. Analytical curves and statistical parameters

Analyte Name: 289.0 / 125.1 (Catechin)
Internal Standard: N/A

Data File	2017_08_2 BTM.wiff	Result Table	Barbatimão curva 01 03_08_2017
Acquisition Date	8/2/2017 12:52:37 PM	Algorithm Used	MQ4
Acquisition Method	Método Barbatimao.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = -0.02664 (\ln(x))^2 + 1.37087 \ln(x) + 3.07820$ ($r = 0.99928$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
20.00	5 of 6	2.075e1	103.7	2.22	10.7
50.00	6 of 6	4.776e1	95.5	2.46	5.1
100.00	6 of 6	9.407e1	94.1	4.41	4.7
125.00	6 of 6	1.343e2	107.4	10.57	7.9
250.00	6 of 6	2.551e2	102.1	19.01	7.5
375.00	6 of 6	3.777e2	100.7	5.46	1.5
500.00	6 of 6	4.977e2	99.5	19.87	4.0
1000.00	6 of 6	1.008e3	100.8	39.88	4.0
2000.00	6 of 6	1.974e3	98.7	51.11	2.6

Analyte Name: 305.0 / 167.1 (Gallocatechin)
Internal Standard: 595.0 / 269.0

Data File	2017_08_2 BTM.wiff	Result Table	Barbatimão curva 01 03_08_2017
Acquisition Date	8/2/2017 12:52:37 PM	Algorithm Used	MQ4
Acquisition Method	Método Barbatimão.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = 0.05452 (\ln(x))^2 + 0.45712 \ln(x) + -5.98583$ ($r = 0.99278$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
20.00	0 of 6	N/A	N/A	N/A	N/A
50.00	6 of 6	4.762e1	95.2	4.82	10.1
100.00	6 of 6	1.086e2	108.7	14.33	13.2
125.00	6 of 6	1.203e2	96.3	10.63	8.8
250.00	6 of 6	2.691e2	107.7	22.59	8.4
375.00	6 of 6	3.888e2	103.7	32.32	8.3
500.00	6 of 6	4.813e2	96.3	34.99	7.3
1000.00	6 of 6	8.936e2	89.4	49.13	5.5
2000.00	5 of 6	2.161e3	108.1	106.40	4.9

Analyte Name: 169.0 / 106.8 (Gallic acid)
Internal Standard: N/A

Data File	2017_08_2 BTM.wiff	Result Table	Barbatimão curva 01 03_08_2017
Acquisition Date	8/2/2017 12:52:37 PM	Algorithm Used	MQ4
Acquisition Method	Método Barbatimao.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = -0.05050 (\ln(x))^2 + 1.72231 \ln(x) + 0.24239$ ($r = 0.99793$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
20.00	6 of 6	2.385e1	119.3	9.89	41.5
50.00	6 of 6	4.259e1	85.2	10.09	23.7
100.00	6 of 6	9.974e1	99.7	17.92	18.0
125.00	6 of 6	1.290e2	103.2	24.67	19.1
250.00	6 of 6	2.969e2	118.8	30.41	10.2
375.00	6 of 6	3.908e2	104.2	26.18	6.7
500.00	6 of 6	4.636e2	92.7	27.61	6.0
1000.00	6 of 6	1.006e3	100.6	69.83	6.9
2000.00	6 of 6	1.943e3	97.2	46.34	2.4

Analyte Name: 579.0 / 297.0 (Cascaroside A)
Internal Standard: 457.0 / 169.0

Data File	2016_10_07 15ul.wiff	Result Table	Curva CAS CERTO met 01 15ul
Acquisition Date	10/8/2016 10:44:06 PM	Algorithm Used	MQ4
Acquisition Method	Método Cascara teste.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = -0.01395 (\ln(x))^2 + 1.10099 \ln(x) + -9.05172$ ($r = 0.99828$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
10.00	6 of 6	1.011e1	101.1	0.55	5.5
20.00	6 of 6	2.019e1	101.0	1.36	6.7
50.00	6 of 6	4.824e1	96.5	1.88	3.9
100.00	5 of 5	9.961e1	99.6	7.04	7.1
125.00	5 of 5	1.226e2	98.0	5.53	4.5
250.00	6 of 6	2.631e2	105.2	9.37	3.6
375.00	6 of 6	3.768e2	100.5	22.47	6.0
500.00	6 of 6	5.093e2	101.9	27.10	5.3
1000.00	6 of 6	9.721e2	97.2	36.27	3.7

Analyte Name: 579.0 / 297.0 (Cascaroside B)
Internal Standard: 457.0 / 169.0

Data File	2016_10_07 15ul.wiff	Result Table	Curva CAS CERTO met 02 15ul
Acquisition Date	10/8/2016 10:44:06 PM	Algorithm Used	MQ4
Acquisition Method	Método Cascara teste.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = 0.00902 (\ln(x))^2 + 0.90646 \ln(x) - 9.85116$ ($r = 0.99806$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
10.00	6 of 6	9.886e0	98.9	1.35	13.6
20.00	6 of 6	2.079e1	103.9	1.98	9.5
50.00	6 of 6	4.876e1	97.5	3.06	6.3
100.00	5 of 5	1.009e2	100.9	4.20	4.2
125.00	5 of 5	1.277e2	102.1	2.77	2.2
250.00	6 of 6	2.552e2	102.1	8.93	3.5
375.00	6 of 6	3.659e2	97.6	30.05	8.2
500.00	6 of 6	4.856e2	97.1	28.23	5.8
1000.00	6 of 6	1.025e3	102.5	34.38	3.4

Analyte Name: 563.0 / 281.0 (Cascaroside C)
Internal Standard: 457.0 / 169.0

Data File	2016_10_07 15ul.wiff	Result Table	Curva CAS CERTO met 01 15ul
Acquisition Date	10/8/2016 10:44:06 PM	Algorithm Used	MQ4
Acquisition Method	Método Cascara teste.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = -0.00543 (\ln(x))^2 + 1.03221 \ln(x) + -8.77707$ ($r = 0.99874$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
10.00	6 of 6	9.739e0	97.4	0.54	5.6
20.00	6 of 6	2.088e1	104.4	1.19	5.7
50.00	6 of 6	4.998e1	100.0	1.43	2.9
100.00	5 of 5	9.966e1	99.7	4.45	4.5
125.00	5 of 5	1.248e2	99.8	7.78	6.2
250.00	6 of 6	2.549e2	102.0	11.36	4.5
375.00	6 of 6	3.591e2	95.8	17.01	4.7
500.00	6 of 6	4.998e2	100.0	28.08	5.6
1000.00	6 of 6	1.021e3	102.1	27.41	2.7

Analyte Name: 563.0 / 281.0 (Cascaroside D)
Internal Standard: 457.0 / 169.0

Data File	2016_10_07 15ul.wiff	Result Table	Curva CAS CERTO met 02 15ul
Acquisition Date	10/8/2016 10:44:06 PM	Algorithm Used	MQ4
Acquisition Method	Método Cascara teste.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = 0.00419 (\ln(x))^2 + 0.95057 \ln(x) - 9.26598$ ($r = 0.99841$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
10.00	6 of 6	9.685e0	96.9	0.63	6.5
20.00	6 of 6	2.087e1	104.3	1.67	8.0
50.00	6 of 6	5.113e1	102.3	1.66	3.2
100.00	5 of 5	9.997e1	100.0	3.53	3.5
125.00	5 of 5	1.230e2	98.4	5.99	4.9
250.00	6 of 6	2.522e2	100.9	9.39	3.7
375.00	6 of 6	3.595e2	95.9	17.76	4.9
500.00	6 of 6	4.980e2	99.6	23.90	4.8
1000.00	6 of 6	1.028e3	102.8	38.33	3.7

Analyte Name: 595.0 / 269.0 (Cascaroside 10H-A)
Internal Standard: 457.0 / 169.0

Data File	2016_10_07 15ul.wiff	Result Table	Curva CAS CERTO met 01 15ul
Acquisition Date	10/8/2016 10:44:06 PM	Algorithm Used	MQ4
Acquisition Method	Método Cascara teste.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = -0.02961 (\ln(x))^2 + 1.20319 \ln(x) + -8.40329$ ($r = 0.99851$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
10.00	6 of 6	9.838e0	98.4	0.35	3.5
20.00	6 of 6	2.061e1	103.1	1.12	5.4
50.00	6 of 6	4.990e1	99.8	2.21	4.4
100.00	5 of 5	9.984e1	99.8	2.67	2.7
125.00	5 of 5	1.223e2	97.8	6.97	5.7
250.00	6 of 6	2.607e2	104.3	11.60	4.5
375.00	6 of 6	3.657e2	97.5	19.12	5.2
500.00	6 of 6	4.903e2	98.1	21.67	4.4
1000.00	6 of 6	1.019e3	101.9	47.23	4.6

Analyte Name: 595.0 / 269.0 (Cascaroside 10H-B)
Internal Standard: 457.0 / 169.0

Data File	2016_10_07 15ul.wiff	Result Table	Curva CAS CERTO met 02 15ul
Acquisition Date	10/8/2016 10:44:06 PM	Algorithm Used	MQ4
Acquisition Method	Método Cascara teste.dam	Instrument Name	API 3200
Project	Daniel Barbatimão\2016_05_11		

Regression Equation: $\ln(y) = -0.01337 (\ln(x))^2 + 1.11556 \ln(x) - 9.76651$ ($r = 0.99800$) (weighting: None)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
10.00	6 of 6	9.617e0	96.2	0.62	6.4
20.00	5 of 6	2.187e1	109.3	0.55	2.5
50.00	6 of 6	4.980e1	99.6	2.34	4.7
100.00	5 of 5	9.538e1	95.4	6.18	6.5
125.00	5 of 5	1.245e2	99.6	6.72	5.4
250.00	4 of 6	2.541e2	101.7	12.32	4.9
375.00	6 of 6	3.720e2	99.2	20.33	5.5
500.00	6 of 6	5.085e2	101.7	21.73	4.3
1000.00	0 of 6	N/A	N/A	N/A	N/A