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SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO**

DANIEL PECORARO DEMARQUE

**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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PhD student: Daniel Pecoraro Demarque

Supervisor: Prof. Dr. Norberto Peporine Lopes

Co-supervisor: Dr. Christian Merten

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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
CasMWP	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

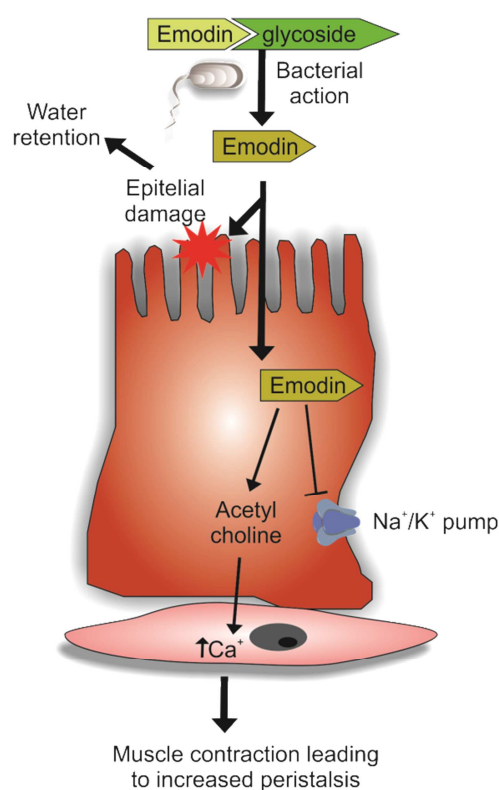


Figure 1: Emodin laxative action (Adapted from Srinivas et al. 2006).

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-melanosus coli, is a pigment present in macrophages from colonic mucosal cells in lamina propria. The melanosus 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 melanosus 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.

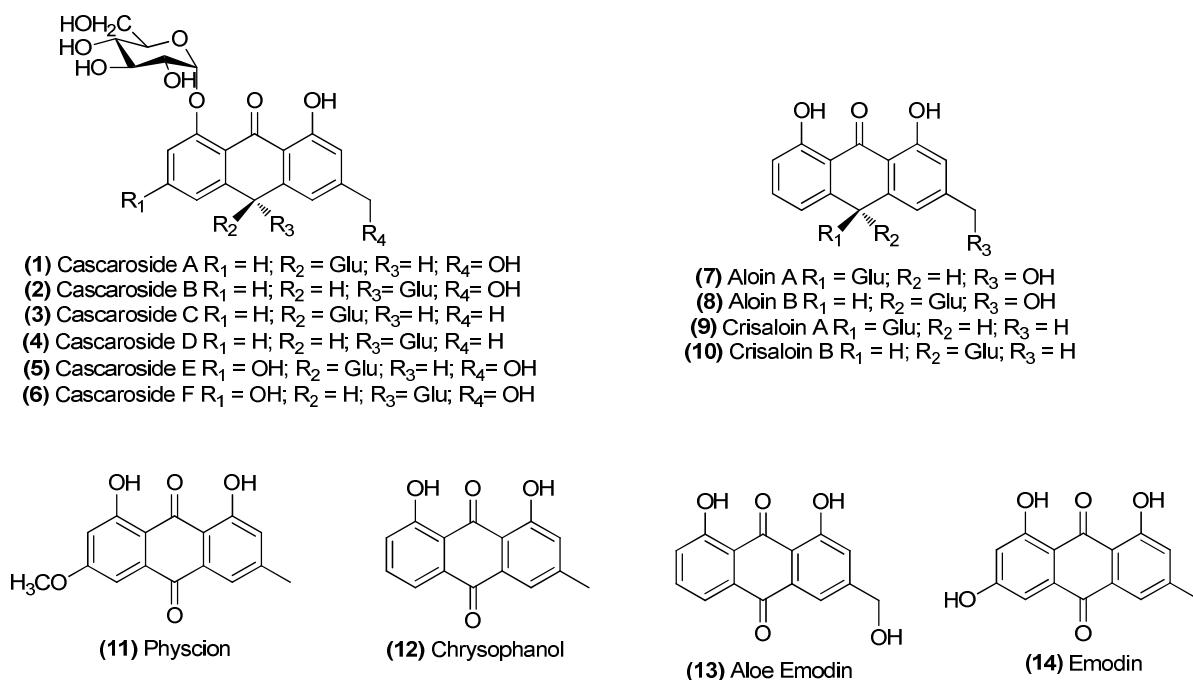


Figure 2: Anthraquinones found in *R. purshiana*.

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)**.

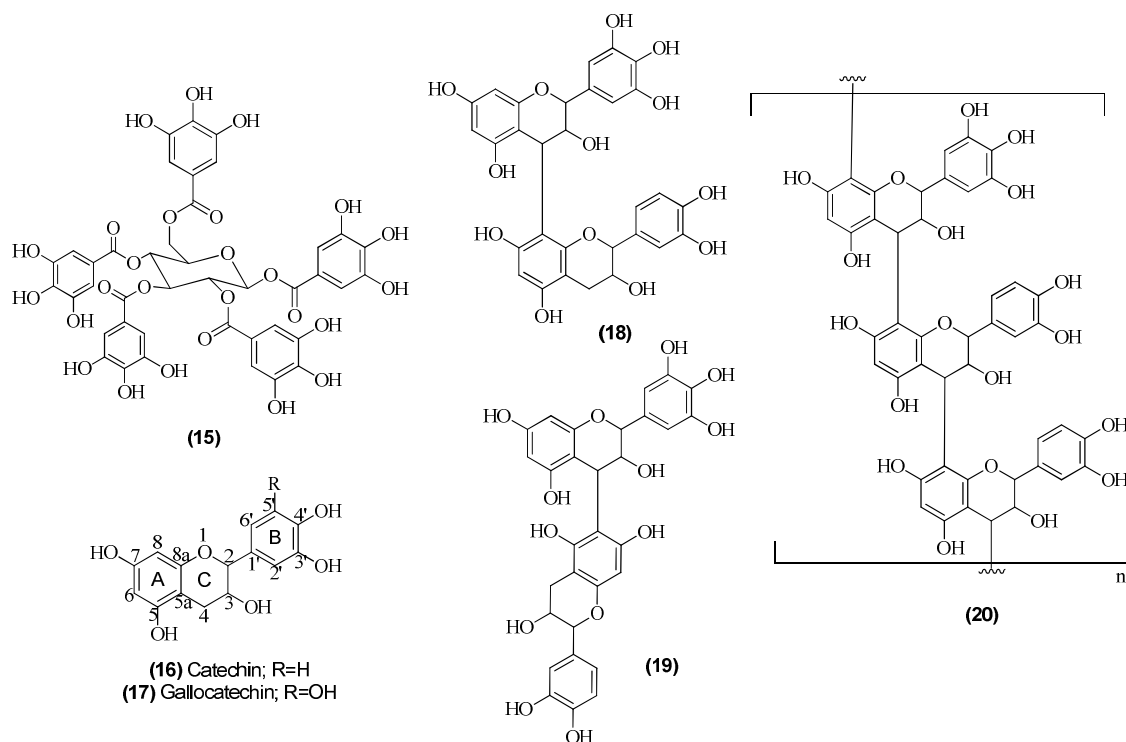


Figure 4: Examples of hydrolysable tannin (15), catechin (16), gallocatechin (17), procyanidin dimer linked C4-C8 (18), procyanidin dimer linked C4-C6 (19) and condensed tannin (20).

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.

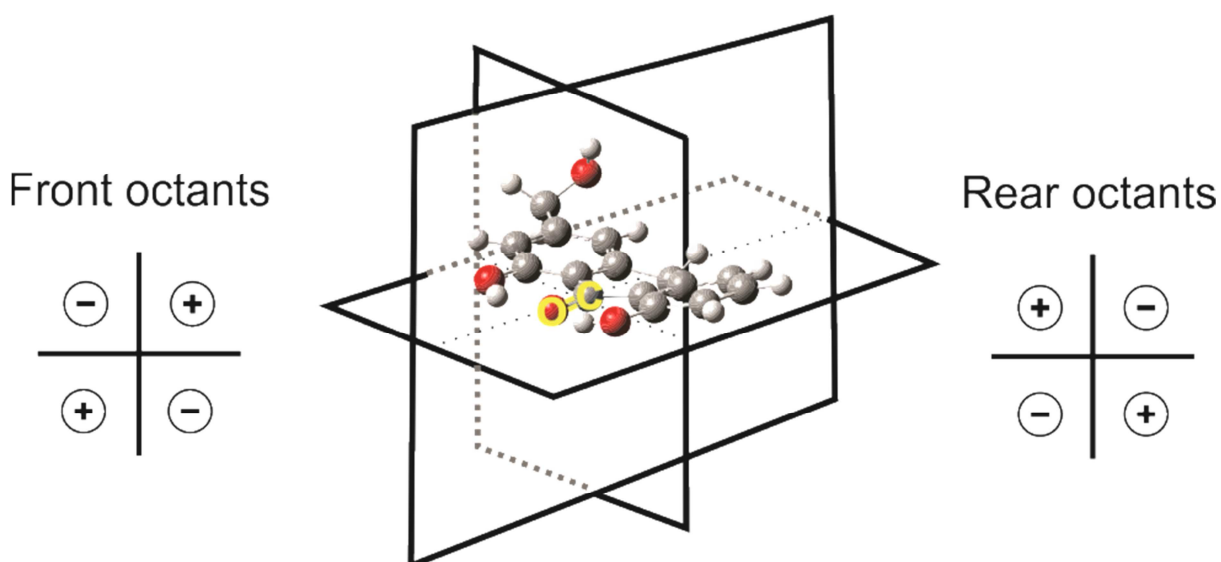


Figure 5: The octant rule: diagram to establish the octant signal.

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.

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.

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