UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

INSTITUTO DE BIOCIÊNCIAS DE SÃO VICENTE

PÓS-GRADUAÇÃO EM BIODIVERSIDADE AQUÁTICA

Fitoterápicos padronizados para o tratamento de doenças crônicas: *Rhizophora mangle*

Mestrando: Leonardo Mendes de Souza Mesquita

Prof. Dr. Wagner Vilegas

Orientador

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Ao meu avô João Mendes de Souza e padrinho João Carlos Lopes de Souza

(*in memorian*)

"Non c'è niente da capire non c'è niente da spartire

Il successo a volte toglie il privilegio di soffrire

Finisce sempre bene altrimenti non è finita"

Fedez

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Lista de abreviaturas, siglas e símbolos

λ	Comprimento de onda
ABTS	2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AERM	Extrato acetônico das cascas de *Rhizophora mangle*
ACN	Acetonitrila
ESI	Electrospray ionization (Ionização por electrospray)
EtOH	Etanol
FIA	Flow Injection Analysis (Análise por injeção em fluxo ou análise por inserção direta da amostra)
FIA-ESI-IT-MS	Flow Injection Analysis - Electrospray Ionization - Ion Trap - Mass Spectrometry (Espectrometria de Massas acoplada a um ion-trap com interface de Ionização por Electrospray e inserção direta da amostra)
HFD	Dieta hiper lipídica (High fat diet)
HPLC	High Performance Liquid Chromatography (Cromatografia Líquida de Alta Eficiência)
HPLC-PDA	High Performance Liquid Chromatography - Photodiode Array Detector (Cromatografia Líquida de Alta Eficiência acoplada com Detector de Arranjo de Fotodiodos)
HRF	Heterociclic Ring Fission (Clivagem Heterocíclica)
IT	Ion Trap
kiTT	Insulin tolerance test (Teste de tolerância a insulina)
$[M - H]^-$	Molécula desprotonada
m/z	Relação Massa/Carga
MALDI-TOF	Matrix-assisted laser desorption/ionization-Time of flight (Ionização e dessorção a laser assistida por matriz)
MeOH	Metanol
MS	Mass Spectrometry (Espectrometria de Massas)
NAFLD	Non-alcoholic fatty liver disease (Doença hepática gordurosa não alcoólica)
PAs	Proantocianidinas
PAD	Photodiode Array Detector (Detector com Arranjo de Fotodiodos)
QM	Quinone methide (quinona metídeo)
RDA	Retro-Diels-Alder
tr	Tempo de retenção
UV	Ultravioleta
RP18	Reversed Fase octadecylsilan (Fase reversa octadecilsilano)

Sumário

RESUMO

As espécies vegetais contendo substâncias bioativas são, cada vez mais, objeto de pesquisas, levando a alternativas para tratamentos terapêuticos ou revelando substâncias que posteriormente possam ser exploradas com o intuito de produzir fármacos. Estudos de plantas são de grande importância, em razão do vasto número de metabólitos secundários que podem ser encontrados. Eles têm cada vez mais atraído a atenção da sociedade, mostrando-se uma fonte alternativa aos altos custos dos medicamentos alopáticos. Mas, para que as plantas sejam usadas com eficácia e segurança, são necessários estudos multidisciplinares. A Agência Nacional de Vigilância Sanitária e o Ministério da Saúde aprovaram o uso de 71 plantas medicinais no tratamento contra diabetes, úlceras, inflamações e outras doenças crônicas. Contudo, essa é uma lista ainda limitada, pois várias espécies não são disponíveis o ano todo e/ou nas várias regiões do Brasil. Em contrapartida, *Rhizophora mangle* (Rhizophoraceae, popularmente conhecida como mangue vermelho) é uma espécie que ocorre no Brasil desde o Pará até Santa Catarina. É comumente utilizada pelas populações tradicionais costeiras, principalmente para o tratamento de diabetes, hemorroidas, analgesia e dores estomacais. Recentemente, nosso grupo de pesquisa verificou que o extrato acetônico das cascas de *R. mangle* possui atividades antioxidante e antiulcerogênica, e é eficaz no tratamento da colite experimental. Contudo, foi realizada apenas uma avaliação preliminar da composição química das cascas. Por isso, este trabalho abordou o estudo químico de *R. mangle*, enfatizando o estudo das cascas. Desenvolvemos estratégias analíticas baseadas em espectrometria de massas utilizando as técnicas de Electrospray-Ion Trap e Maldi-TOF, as quais permitiram a padronização qualitativa do extrato ativo. Como resultado, identificamos a presença de taninos condensados possuindo de 2 a 12 unidades de catequinas. Além disso, investigamos a atividade desse extrato no tratamento da obesidade e como anti-inflamatório, obtendo resultados promissores. Esses resultados fornecem subsídeos para o melhor entendimento das atividades farmacológicas previamente observadas.

Palavras chave: *Rhizophora mangle,* Diabetes, catequinas, analgesia

ABSTRACT

Plant species containing bioactive compounds are continuously being investigated, in order to search for alternative therapeutic treatments. These studies can reveal substances that can be explored in order to produce new drugs. Investigation of plants are of great importance, because of the large amounts of secondary metabolites that can be found. They have increasingly attracted the attention of the society, proving to be an alternative source to the high costs of allopathic medicines. However, multidisciplinary studies are necessary to ensure that plants are used with effectively and safety. The National Sanitary Surveillance Agency and the Ministry of Health have approved 71 medicinal plants to be used against diabetes, ulcers, inflammation and other chronic diseases. However, this list is still limited because several species are not available year-round and/or in the various regions of Brazil. In contrast, *Rhizophora mangle* (Rhizophoraceae, popularly known as red mangrove) is a Brazilian species that occurs from Pará to Santa Catarina. It is commonly used by traditional coastal populations, mainly for the treatment of diabetes, hemorrhoids, analgesia and stomach pains. Recently, our research group verified that the acetone extract of *R. mangle* barks has antioxidant and antiulcerogenic activities and it is also effective in the treatment of the experimental colitis. However, only a preliminary evaluation of the chemical composition of the barks was previously carried out. Thus, this work deals with the chemical study of *R. mangle*, emphasizing the study of barks. We developed analytical strategies based on Electrospray- Ion trap mass spectrometry as well as MALTI-TOF techniques that allowed the qualitative standardization of the active extract. As a result, we could identify a sequence of condensed tannins with polymerization degree from 2 to 12. Besides, we tested this extract to treat obesity and as anti-inflammatory, with promising results.These results allowed us to better understant the farmacological activities observed.

Keywords: *Rhizophora mangle,* Diabetes, catechins, analgesy

Identificação da proposta

A presente dissertação de mestrado, intitulada "Fitoterápicos padronizados para o tratamento de doenças crônicas: *Rhizophora mangle*", financiada pela Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP - 2014:23113-0) faz parte do Projeto BIOTA FAPESP (2009/52237-9), coordenado pelo Prof. Dr. Wagner Vilegas. Os resultados aqui apresentados concentram-se na caracterização química do extrato acetônico (70% v/v) de cascas de uma árvore advinda do ecossistema Manguezal, *Rhizophora mangle* L. (Rhizophoraceae). Tal extrato já havia sido investigado e observou-se que possui ação para o tratamento de injúrias gastro-intestinais, com patentes depositadas no Instituto Nacional da Propriedade Indústrial (INPI). Entretanto, não se sabia com precisão quais eram as substâncias responsáveis por tais atividades. Portanto, o objetivo principal deste trabalho foi responder à seguinte pergunta:

"Qual é a composição química das cascas de <u>Rhizophora mangle?</u>"

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CONSIDERAÇÕES INICIAIS

No contexto do panorama nacional e mundial, as doenças crônicas representam um sério problema no sistema público de saúde. Dentre elas estão aquelas relacionadas ao sistema gastrintestinal (úlceras, colites, diarréia e inflamações), diabetes, dores, problemas do sistema imunológico e câncer.

Além disso, importantes atividades farmacológicas foram encontradas em extratos acetônicos e hidroalcoólicos e/ou nas frações n-butanólica (n-BuOH) e em acetato de etila (EtOAc) das cascas de *R. mangle* para o tratamento de doenças crônicas, especialmente úlceras e colite. Os extratos atuaram no fortalecimento das linhas de defesa antioxidantes e na redução da concentração de citocinas pró-inflamatórias (de Faria et al., 2012a, 2012b). A elucidação completa dos mecanismos envolvidos nessas ações farmacológicas abre novas de possibilidades para outros estudos farmacológicos relacionados, como por exemplo, investigação pré-clinica e clínica em pacientes com DII. Além disso, o estudo de *R. mangle* é importante como estratégia para ampliar o número de plantas disponíveis para uso no sistema público de saúde, tendo em vista que ocorre desde o Pará até Santa Catarina. Adicionalmente, comunidades tradicionais costeiras usam *R. mangle* em suas enfermidades, mesmo não havendo respaldo e segurança quanto a sua constituição química.

A úlcera péptica é uma doença crônica dos tempos modernos, acelerada por fatores como vida atribulada (estresse), vícios comuns (cigarro e álcool) e dieta pobre e desequilibrada, sendo muitas vezes fatal, mesmo tendo altas chances de cura. Porém, sua reincidência é de quase de 100% após dois anos de interrupção do tratamento. Por isso, é um sério problema de saúde pública (Leong, 2009, Sone et al., 2008). Outra doença que tem elevada incidência e prevalência é a Doença Inflamatória Intestinal (DII) que engloba, fundamentalmente, duas doenças distintas: a Doença de Crohn (DC) e a Colite Ulcerativa (CU).

A colite ulcerativa é mais comum que a Doença de Crohn, sendo que a incidência e prevalência da DII variam amplamente devido às diferentes metodologias de estudo utilizadas. Estudos afirmam que ambas as doenças tornaram-se mais comuns no último século, especialmente devido à influência de fatores ambientais associados ao estilo de vida moderno e urbanizado (Crohn, 2000), já que fatores como estresse e dieta estão envolvidos na origem dessa doença. No Brasil, apesar da inexistência de dados epidemiológicos oficiais, a incidência de colite ulcerativa e da Doença de Crohn tem aumentado nos últimos anos segundo a Associação Brasileira de Colite Ulcerativa e Doença de Crohn, atingindo níveis similares aos de países desenvolvidos (Loftus, 2004).

Vários medicamentos têm sido usados para tratar essas doenças inflamatórias. No entanto, devido a sérios efeitos colaterais, é necessário encontrar novas alternativas (Duffy et al., 2014). As plantas de manguezais são fontes potenciais de compostos biologicamente ativos e possuem ampla aplicação em práticas etnofarmacêuticas. A padronização de fitoterápicos é pré-requisito para qualidade, além da constância dos efeitos terapêuticos e segurança do usuário.

Tendo em vista essas condiderações, na primeira etapa deste trabalho aplicamos técnicas de espectrometria de massas usando inserção direta do extrato em sistema Electrospray-Ion Trap para caracterizar as moléculas de baixo e médio peso molecular. Foi realizada uma análise *full-scan*, seguida pela fragmentação MS^n de cada íon. Usando essa abordagem, detectamos a presença de taninos condensados com unidades poliméricas de 2 a 4 unidades de catequinas. Paralelamente, tendo em vista que um dos problemas que mais tem atingido a população mundial é a obesidade, e que essa é uma doença multifatorial, a qual gera vários problemas associados, como diabetes mellitus tipo 2, pressão alta, problemas no fígado, depressão, etc. Decidimos testar a atividade desse extrato no tratamento de Diabetes mellitus tipo 2, e gerar um respaldo científico ao conhecimento tradicional. Observamos que o

extrato possui ação significante na resistência à insulina, bem como melhora nos problemas relacionados ao fígado. Esses resultados resultaram no manuscrito "Extract of the mangrove tree *Rhizophora mangle* L. induces antiradical effects and reverse insulin resistance in a model of diet-induced obesity", a ser submetido no *Journal of Ethnopharmacology*.

Tendo em vista que a técnica de FIA-ESI-IT-MS/MS não é adequada para o estudo de moléculas de alto peso molecular, na segunda etapa utilizamos a técnica MALDI-TOF para caracterizar os taninos de unidades poliméricas superiores. Como resultado, foram detectados taninos condensados com até 12 unidades de catequina, afzelequina e galocatequina. Além disso, avaliamos a atividade do extrato para ação analgésica, uma vez que o conhecimento popular sugere tal atividade. Esses ensaios mostraram que o extrato possui ação equivalente à da morfina. Essa parte do trabalho serviu de base para o manuscrito "Evaluation of antinociceptive activity of polyphenols contained in the standardized extract of *Rhizphora mangle*", a ser submetido para o *Evidence-based complementary and alternative medicine*.

CAPÍTULO I

Resistência à Insulina e comorbidades associadas a ser submetido no Journal of ethnopharmacology

Extract of the mangrove tree *Rhizophora mangle* L. induces antiradical effects and reverse insulin resistance in a model of diet-induced obesity.

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Glossary: ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); **AERM:** Acetonic extract of *Rhizophora mangle* barks; **HFD:** High fat diet; **HPLC-PDA:** High performance liquid chromatography coupled to Photodiode Array; **HRF:** Heterocyclic ring fission; **FIA-ESI-IT-MS:** Flow injection analysis electrospray-iontrap mass spectrometry; **kiTT:** Insulin tolerance test; ***m/z*:** mass/charge ratio; **NAFLD:** Non-alcoholic fatty liver disease; **PAs:** Proanthocyanidins; **RDA:** Retro Dies-Alder **QM:** Quinone-methide

Abstract

Ethnopharmacology relevance: An ethnopharmacological survey indicated that the *Rhizophora mangle* L. (Rhizophoraceae), a medicinal species commonly found in marine ecossystems in intertropical zone of the world, can be used as anti-diabetic therapeutics. Even though there is traditional knowledge, there is no scientific basis to prove the action of its phytochemicals and the mechanism of action of this activity.

Aim of the study: Evaluate the effects of an acetonic extract of *Rhizophora mangle* barks (AERM) on type 2 diabetes and oxidative stress, as well as to try to relate these activities with the major phytochemicals in the extract.

Material and methods: The main chemical constituent of the extract were analyzed by high performance liquid chromatography (HPLC) and flow injection analysis electrospray-iontrap mass spectrometry (FIA-ESI-IT-MS).The antioxidant activity was evalueted by ABTS assay. High-fat diet fed mice was used as model of type 2 diabetes associated to obesity. Glucose homeostasis was evaluated, as well as, hepatic alterations induced by obesity.

Results and discussion: The phytochemical profile from AERM indicated the presence of catechins derivatives, which have the ability to polymerize and form proanthocyanidins. Derivatives of quercetin and quinic acid were also detected. These metabolites have nutritional importance as they are excellent natural antioxidants and inhibit the production of proinflammatory cytokinins. The AERM showed high antioxidant activity (608.8 μmol Trolox/g). Obese mice treated with *R.mangle* (50mg/kg) presented improvements in insulin resistance and hepatic steatosis probably associated to an important antioxidant activity.

Conclusions: *Rhizophora mangle* barks present mainly proanthocyanidins, which display intense antioxidant activity and reverse insulin resistance and hepatic steatosis associated to obesity, supporting previous claims in traditional knowledge.

Keywords**:** antioxidant activity, mass spectrometry, NAFLD, Polyphenols, type 2 diabetes.

1. Introduction

Despite approximately 40% of the world's population lives within 100 kilometers of coastline, oceans have not etnomedicinal history, and there is no certainty how many people depend on marine organisms for supply of medicines Narchi et al. (2015). Furthermore the expropriation of these traditional coastal communities, displaced this politically and economically disadvantaged populations from their traditional knowledge (Hoegh-Guldberg et al 2007; Doney et al., 2009). Besides, global climate change causes rising sea levels and ocean acidification, leading to critical chances in this environment.

In the Brazilian coast line, one of the most representative bioma is the mangrove. This ecosystem is characterized it is a transitional environment between terrestrial and aquatic ecosystems, and is much threatened by real estate speculation and harbor construction

(Cordeiro and Costa, 2010). Mangrove plants are potential sources of biologically active compounds and have wide application in etnofarmaceutic practices. The habitat of these species is under stressful environmental conditions (salinity, temperature, tidal fluctuations and anoxic soil). These plants are perfectly adapted to this inhospitable environment both morphologically and physiologically: exhibit pneumatophores, adventitious roots, salt excretion through foliar glands, and viviparity through propagules. Therefore, they might present many substances which protect them these environmental conditions (Nebula et al., 2013).

Rhizophora mangle L. (Rhizophoraceae) is one of the most proeminent species in mangrove ecossystems, and it occurs from Amazon until Santa Catarina. In the Latin America traditional medicine, *R. mangle* is commonly used to treat angina, asthma, pain, diarrhea, ulcers, inflammation, tumors, seizures and diabetes (Kandil et al., 2004; Nebula et al., 2013). The polyphenolic compounds such as proanthocyanidins (PAs - tannins) consisting of oligomers and polymers by deflavan-3-ol units are the most abundant compounds in *Rhizophora* genus. PAs are widely distributed in the plant kingdom and are the second-most frequent phenolic substances, after lignins (He et al., 2008).

Polyphenols are important secondary metabolites for the treatment of various chronic diseases, such as diabetes mellitus (Pandey and Rizvi, 2009). According to the American Diabetes Association (2009), diabetes mellitus is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Is a complex illness requiring multifactorial risk-reduction strategies beyond glycemic control (ADA, 2016). The global prevalence of type 2 diabetes, characterized by the ineffective use of insulin by body, is significantly increased associated to epidemic obesity (WHO, 2016). About 60% of Brazilian people are overweight or obese and 12 millions of people have type 2 diabetes (IBGE, 2015). In this study, we investigated the effect of standardized acetone extracts of *Rhizophora*

mangle barks (AERM) in improving glucose homeostasis in an experimental model diet-induced obesity in mice. The potential antioxidant of extract was also evaluated.

2. Materials and methods

2.1 Sample taxon

The barks of *Rhizophora mangle* L. (Rhizophoraceae) were collected from the estuarine system of ecological station of Juréia-Itatins (Peruíbe, São Paulo - Brazil - 24º25´40´´S – 47º05´20´´W). The collecting the material had prior authorization from the Brazilian authorities (IBAMA/MMA: 52497-1). The species was indentified by Dr. Paulo Sampaio. A voucher specimen (n° 11459) has been deposited at the Herbarium HUSC of the Santa Cecilia University (Santos, São Paulo - Brazil).

2.2 Chemical characterization

2.2.1 Preparation of plant extract

Fresh barks of *R. mangle* were washed, shade dried, powdered in a knife mill and sieved through a #60 mesh sieve. The powder (50 g) was extracted with 0.5 L acetone (70% v/v) and macerated for seven days at room temperature (24°C), protected from light. The macerate was filtered through Whatman N°.1 filter paper and concentrated in a rotary flash evaporator at a temperature not exceeding 35°C. The extract (7 g, 14 %) was lyophilized and stored in amber bottles and allocated in a freezer (-40 °C). In order to minimize the interference of very high order polymeric compounds, a solid-phase extraction (SPE) was made. An aliquot (10 mg) of the extract was submitted to the SPE using RP18 cartridge, eluted with H_2O/MeOH 8:2 (v/v) (5 mL). The eluate was filtered through the nylon membrane and directly analyzed by HPLC-PDA and ESI-IT-MS^n .

2.2.2 HPLC-PDA

The chemical composition of acetonic extract of *R. mangle* was investigated by High Performance Liquid Chromatography coupled to a Photodiode Array Detector (HPLC-PAD), using a Jasco (Tokyo, Japan) HPLC equipped with a PU-2089 quaternary solvent pump, a MD-2010 PAD and an AS-2055 autosampler. The analytical column maintained at room temperature (25 °C), was a Phenomenex Synergi Hydro RP18 (250 mm × 4.6 mm H × i.d.; 4 m) with a Phenomenex security guard column (4.0 mm × 2.0 mm H × i.d.). Separation of phenolic acids, flavonoids, flavan-3-ols and proanthocyanidins was established using the mobile phase of water (eluent A) and acetonitrile (eluent B), solvent A containing 0.1% formic acid, with the following gradient program: 5–50% B (30 min), 30-85% B (30-35 min), isocratic 85% B (45 min), 85–100% B (45-70 min), return to 5% B (2 min), and the column was reequilibrated with the initial conditions for 18 min before the next injection. The flow rate was 1.0 $mL.min^{-1}$, and the total run time was 70 min. EZChrom Elite Data System software (Chromatec, Idstein, Germany) was used for detection operation and data processing. The identification of the compounds was performed by retention time comparison and UV spectral analyses.

2.2.3 FIA-ESI-IT-MS^n

Flow injection analysis (FIA) was performed using a Thermo Fisher Scientific ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization source. The MS and MS/MS analysis in negative ion mode were selected after calibration infusing a standard solution of (+)-catechin (1 $\mu g.mL^{-1}$ in methanol) at a flow rate of 5 $\mu L \cdot min^{-1}$ and working under the following conditions: capillary voltage −31 V, spray voltage 5 kV, tube lens offset 75 V, capillary temperature 300 °C, sheath gas (N_2) flow rate 8 (arbitrary units). Negative ion mass spectra were recorded in the range *m/z* 100–2000 Da. The first event was a

full scan mass spectrum to acquire data on ions in the *m/z* range. The second scan event was an MS/MS experiment performed by using data-dependent scan that was carried out on deprotonated molecules from the compounds at collision energy of 25-30% and activation time of 30 ms. Data acquisition and processing were performed using the Xcalibur software.

2.3 Experimental model of diet-induced obesity

2.3.1 Animals

Six-week-old Swiss male mice, free of specific pathogens, were obtained from the Multidisciplinary Center for Biological Research (CEMIB; State University of Campinas, Campinas, SP, Brazil). Experiments were performed in accordance with the principles outlined by the National Council for the Control of Animal Experimentation (CONCEA, Brazil) and received approval from the Ethics Committee of São Francisco University, Bragança Paulista, SP, Brazil (Protocol 001.02.16). Animals were maintained on a 12:12 h artificial light–dark cycle with humidity and temperature controlled.

2.3.2 Diet-induced obesity and *R. mangle* treatment

After random selection, mice were introduced to control (15% energy from fat) or high-fat diets (HFD; 60% energy from fat) as previously described (De Oliveira et al., 2012). Body weights were assessed weekly. After 8 weeks, the control and HFD animals were randomly divided in groups (n=5-6 each). During the next four weeks, mice received orally with 5 or 50 mg/kg/day of AERM.

2.3.3 Blood glucose levels and insulin tolerance tests

In the last day of AERM treatment, mice were deprived of food for 6 h and a blood drop was collected from their tails. Glucose was measured using the glucose oxidase method.

Insulin (1.5 $U.kg^{-1}$) was administered by an i.p. injection, and blood samples were collected for blood glucose determination at 0, 10, 15, 20 and 30 min. The rate constant for glucose disappearance during an insulin tolerance test (kITT) was calculated using the formula 0.693/T1/2. The glucose T1/2 was calculated from the slope of the least square analysis of blood glucose concentrations during the linear decay phase.

2.3.4 Necropsy and sample collection

Mice were fasted for 6 h and anesthetized by xylasine/ketamine overdose (0.1 mL/30g body weight of 1:1 v/v of 2% xylasine and 10% ketamine). Adipose tissue depots (epididymal, subcutaneous, perirenal and mesenteric), liver and gastrocnemius muscle were carefully dissected, weighted and expressed as a percentage of body weight (b.w.). Liver samples were collected and stored at -80°C for further analyses.

2.3.5 Histological analyses of the liver

Hydrated 5.0 μm sections of paraformaldehyde-fixed, paraffin embedded liver specimens were stained using the hematoxylin–eosin method to evaluate the presence of liver steatosis. Steatosis quantification was performed by counting steatosis (macrovesicular and microvesicular) against a grid of 144 points. For the total lipid extractions, liver samples were homogenized in NaCl 0.9% and after a chloroform and methanol misture (2:1 v/v) was added (Folch et al., 1957). The chloroform layer was collected, dried under N_2 and reconstituted in PBS buffer. Triglycerides were measured using commercial kit (Laborlab, Brazil).

2.4 Antioxidant activity

2.4.1 Preparation of extract for antioxidant activity assays

A hydrophilic extract was prepared with 100 mL of 80% cold acetone by agitation with a magnetic homogenizer (Tecnal, Piracicaba, Brazil) for 15 min; the slurry was filtered, and the solids were washed twice with an additional 100 mL of 80% acetone and then concentrated in a rotary evaporator (T < 35 °C).

2.4.2 Chemical antioxidant assays

ABTS and potassium persulfate were dissolved in distilled water to a final concentration of 7 mM and 2.45 mM respectively. These two solutions were mixed and the mixture allowed to stand in the dark at room temperature for 16 h before use in order to produce ABTS radical ($ABTS^{\bullet+}$). For the study of phenolic compounds the ABTS radical solution was diluted with distilled water to an absorbance of 1.00 at 734 nm. Phenols (final concentrations 0.0001-0.01 mg/ml) or Trolox standards (final concentration 0-20 mM) were added to diluted ABTS•+ solution and the absorbance reading was taken 6 min after mixing using the spectrophotometer. Results are presented as the ability of phenols to scavenge of free radical $ABTS^{\bullet+}$ (Trolox equivalent antioxidant capacity).

2.5 Statistical analyses

The results were expressed as the means together with the corresponding standard errors of the mean (SEM). Statistically significant differences were determined using analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons using GraphPad Instat (GraphPad Software, Inc**.,** La Jolla, CA, USA). p values < 0.05 were considered to be significant.

3. Results and discussion

3.1 Chemical characterization of the extract

In order to obtain the most useful chemical information and best separation in the fingerprint chromatograms of AERM, the mobile phase compositions, gradient elution procedure and detection wavelength were optimised. With the aim of enhancing the resolution, glacial formic acid (FA) was added to the binary mixture of methanol–water. To acquire better selectivity and higher efficiency, different concentrations of FA (0.05%, 0.1% and 0.5%) in the aqueous phase were also investigate. At the end, the mobile phase consisting of water-0.1% FA solution was chosen for the determination of *R. mangle* acetonic extract with large number of peaks on the chromatogram within 70 min.

Using the optimised HPLC-PDA method we obtained baseline resolution. We could observe the presence of seven peaks in AERM, with maximum absorbance values around 232 nm and 278 nm, which covered more than 90% of the total area (Figure 1). Based on the UV spectrum of each chromatographic peak (Figure 2), the constituents of AERM could be classified catechin derivatives (λ_{max} 280 nm), which can polymerize, forming condensed tannins, known as proanthocyanidins (PAs) (Rohr et al., 2000). Another fact that corroborates the presence of condensed tannins in this extract is the presence of a low chromatographic resolution peak eluting between R_t 55-70 min. The presence of tannins is common in plants occurring in the mangrove ecosystem (Ravikumar et al., 2011). In fact, Oo et al. (2008) and Zhang et al. (2010) described the occurrence of such compounds in *R. apiculata* and *R. mangle* are catechins, epicatechins and epigalocatechins.

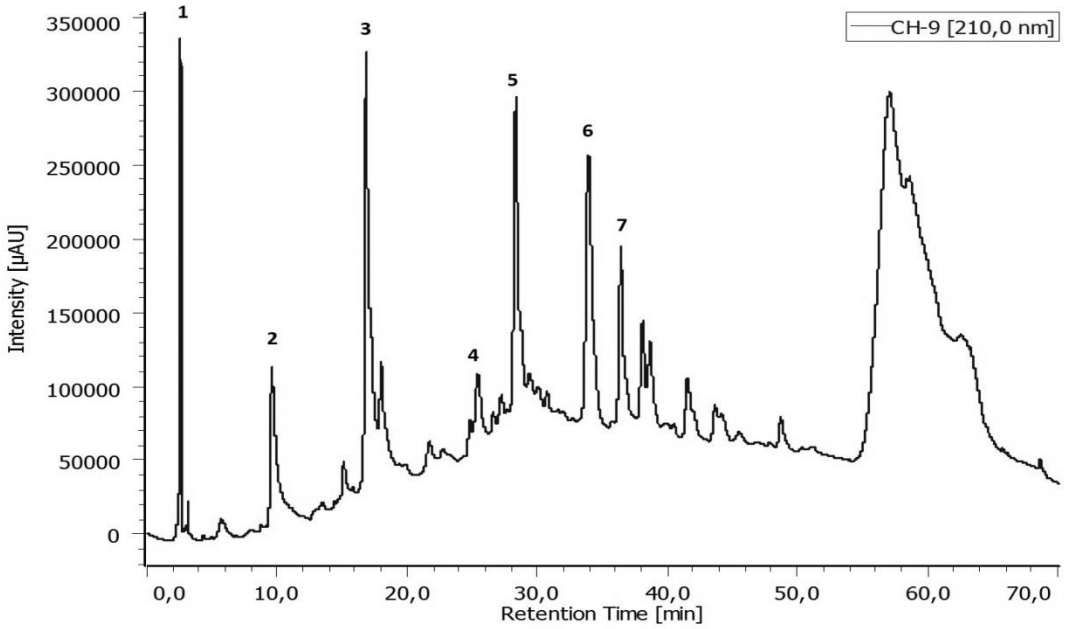

Figure 1. Analytical HPLC-PAD chromatogram recorded at 210 nm of the compounds identified on the acetone extract 70% of the barks of *R. mangle*.

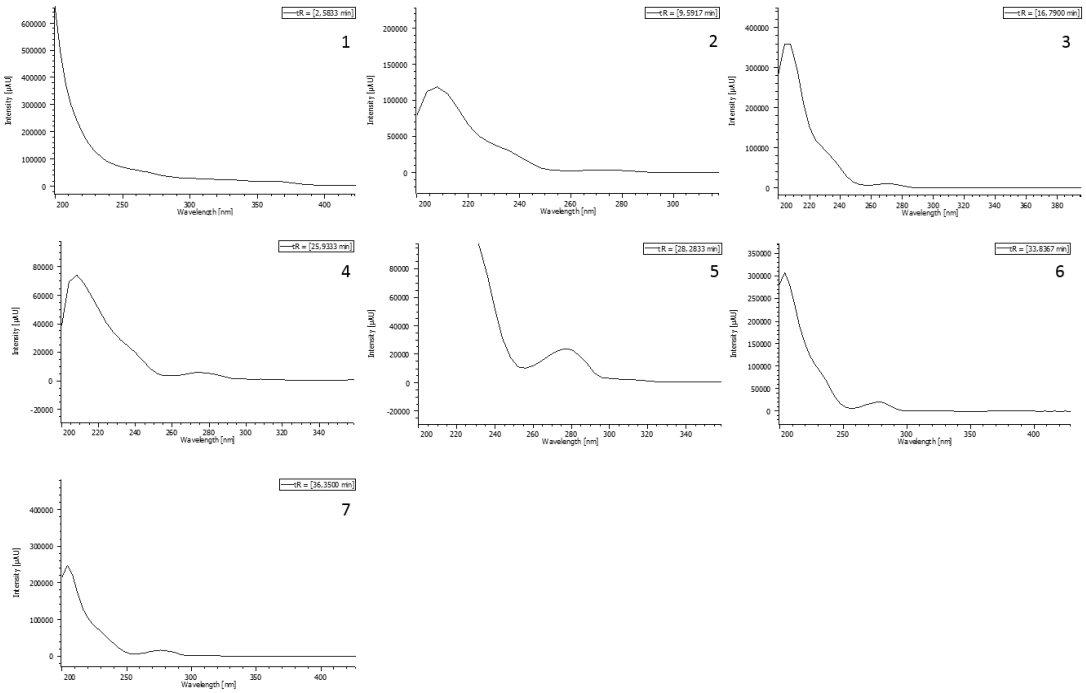

Figure. 2. Spectra in the UV region to the main eluted peak on the chromatogram of figure 1.

However, HPLC-DAD was not enough to fully characterize the concensed tannins in AERM. According to Li and Deinzer (2007), ESI-MS techniques have been used efficiently

for the characterization of several natural compounds, mainly polyphenolic compounds. MS/MS fragmentation of the precursor ion in an ion trap analyser can generate product ions that give additional information about the structure of these compounds. Direct flow injection – electrospray ionization – mass spectrometry analysis were already applyed to establish the polyphenol profile of complex matrices (Fulcrand et al., 2008). Thus, we decided to use this technique in order to obtain a preliminary qualitative metabolic fingerprint of the AERM, after a clean-up using SPE.

In order to obtain qualitative information on PAs in *R. mangle* extract, a sample rich of these compounds was prepared and directly injected into ESI source of the mass spectrometer. We tested both positive and negative ionization. The best results were obtained using negative mode. It was already reported that negative ionization is more sensitive and selective than the positive one (Maldini et al., 2009). Figure 3 and Table 1 show the ESI-MS fingerprint obtained using *full scan*, indicating the $[M-H]^-$ ions of the extract. After MS^n experiments with each peak observed in the full scan spectrum, for the identification and characterization of the PAs, three main fragmentation mechanisms were observed: Retro-Diels-Alder (RDA), Quinone Methide (QM) and Heterocyclic Ring Fission (HFR) (Rodrigues et al., 2007). The fragmentation patterns obtained evidenced the presence of two series of polymeric proanthocianidin. In the *full scan* experiment, the *m/z* 289 ion represents a unit of catechin. Besides, we also observed a first series of ions separated by 288 Da corresponding to ion peaks of dimeric (*m/z* 577) and trimeric (*m/z* 865) PAs. We detected a second series of PAs, based on catechins bounded to hexose moieties (*m/z* 451: Monomer; *m/z* 739: dimer). A third series of PAs is represented by catechins linked to deoxyhexose moieties (*m/z* 435, monomer; *m/z* 723, dimer). In order to check the possibilities, MS^n experiments were performed.

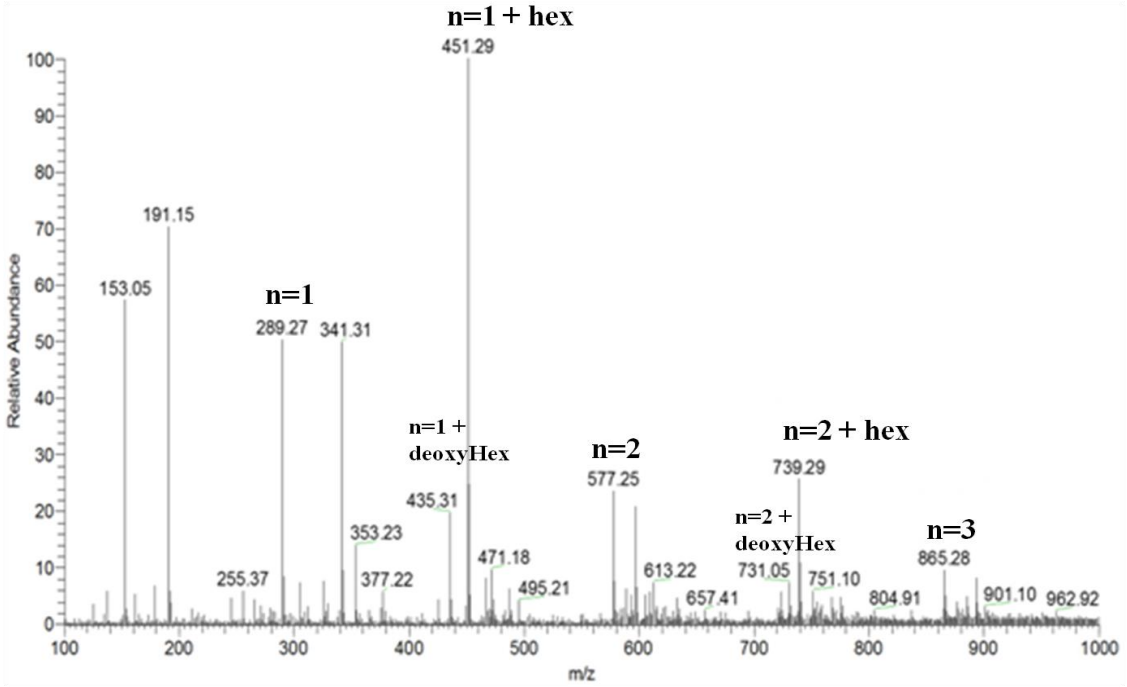

Figure. 3. Mass spectrum of first-order in full scan mode, from acetone extract of *R. mangle* evaluated in negative mode ionization. n= number of catechins units; hex = hexose unit; deoxyHex = deoxyhexose unit

Table 1. *m/z* $[M-H]^-$ ion, MS^n fragments of the compounds obtained by FIA-ESI-IT-MS^n of the acetonic extract from *R. mangle* bark's.

***m/z* [M-H]$^-$**	**MS^2**	**MS^3**	**Proposed name**
289	137 [M-152-H]$^-$		catechin
435	283 [M-152-H]$^-$	137 [M-152-146-H]$^-$	catechin + deoxyhexose
451	299 [M-152-H]$^-$	137 [M-152-162-H]$^-$	catechin + hexose
515	353 [M-162-H]$^-$	191 [M-162-162-H]$^-$	dicaffeoyl quinic acid
577	451 [M-126-H]$^-$		catechin dimer
	425 [M-152-H]$^-$		
	289 [M-288-H]$^-$		
609	463 [M-146-H]$^-$		rutin
	301 [M-308-H]$^-$		
723	571 [M-152-H]$^-$	419 [M-152-H]$^-$	catechin dimer + deoxyhexose
739	587 [M-152-H]$^-$	569 [M-18-H]$^-$	catechin dimer + hexose
		435 [M-152-H]$^-$	
865	577 [M-288-H]$^-$	451 [M-126-H]$^-$	catechin trimer

		425 [M-152-H]$^-$	
		289 [M-288-H]$^-$	
1153	865 [M-288-H]$^-$	847 [M-18-H]$^-$	catechin tetramer
		739 [M-126-H]$^-$	
		587 [M-278-H]$^-$	
		577 [M-288-H]$^-$	
		451 [M-414-H]$^-$	

The MS² spectrum of the ion at *m/z* 577 (Figure 4) showed major fragments at *m/z* 451, 425 and 289. The ion at *m/z* 451 it arises from the loss of 126 mass units, corresponding to the HRF fragmentation. The ion of *m/z* 425 derived from the loss of 152 Da was identified as arising from RDA fragmentation. The fragment at *m/z* 289 [M-288-H]$^-$ was assigned to a QM fragmentation. These fragmentation patterns were also described (Tala et al. 2013), and corroborate with the presence of PAs.

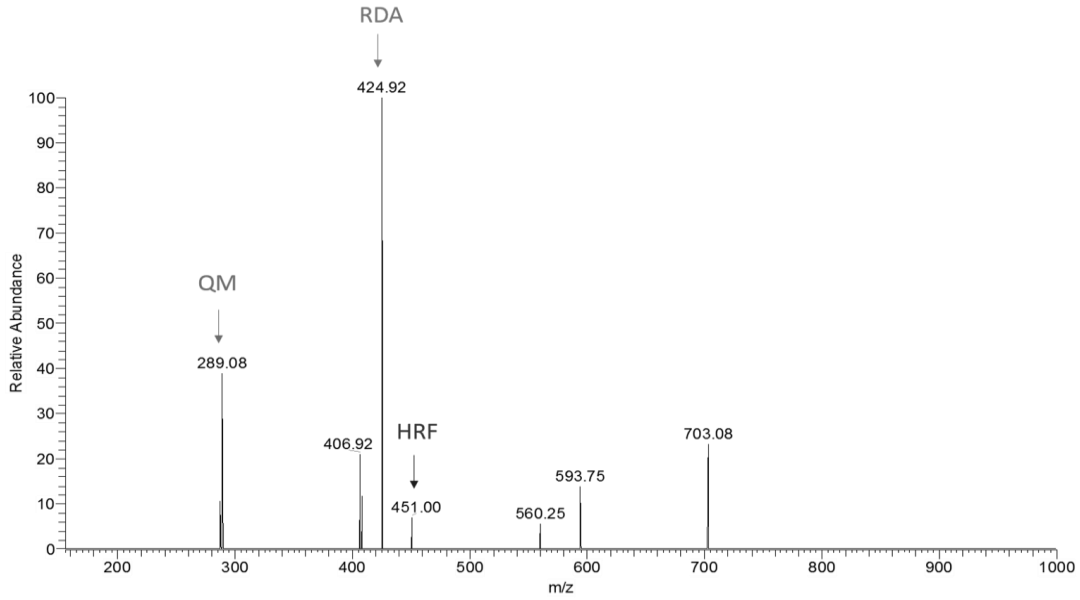

Figure 4. MS/MS spectrum of the ion at *m/z* 577 evidencing the main patterns of fragmentation of PAs . HRF = heterocyclic ring fission; RDA = Retro Dies-Alder; QM = quinone-methide.

The series of PAs containing hexose-catechins was also investigated using the same approach. The MS² spectrum of the ion of *m/z* 739 generated the product ion at *m/z* 587 [M-

152-H]$^-$, due to a RDA fragmentation (Figure 5A), followed by another RDA [M-152-152-H]$^-$ fragmentation, generating the ion at *m/z* 435 (Figure 5B). This fragmentation pattern allowed us to deduce the position of the sugar moiety either at ring A or D (Figure 5). Similar fragmentation patterns occur with the third series of Pas containing a deoxyhexose moiety (*m/z* 435 and 723).

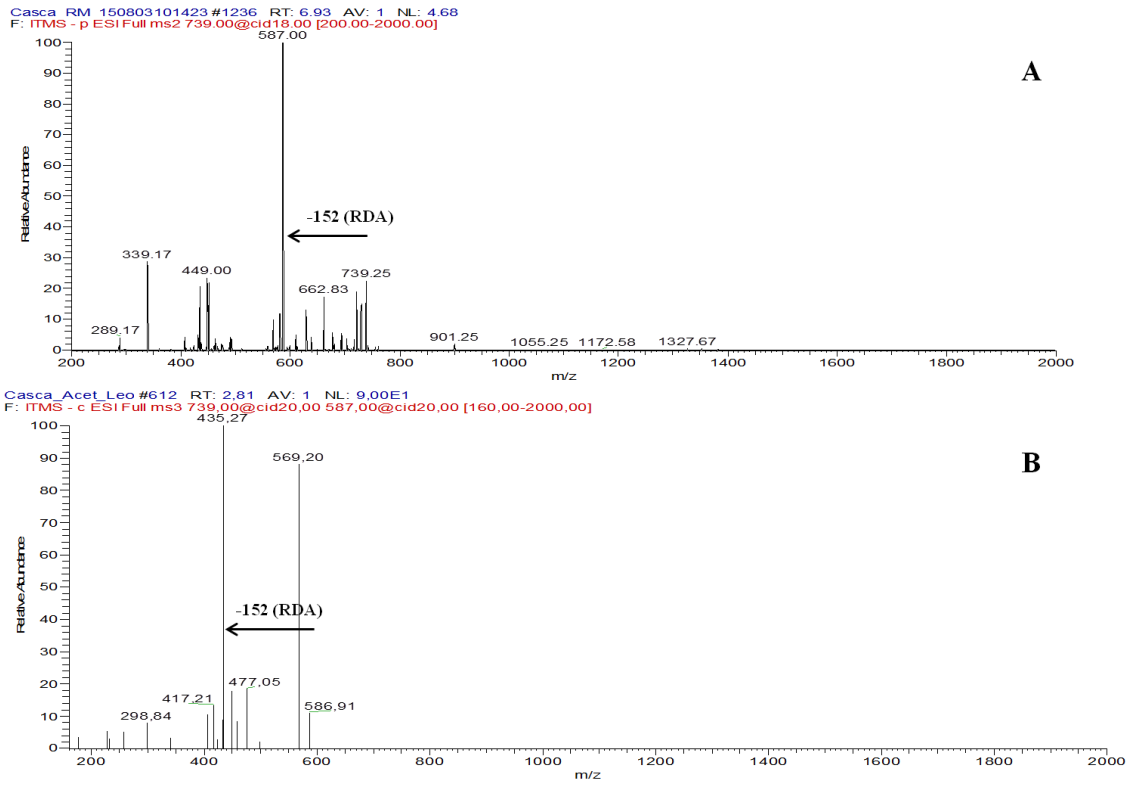

Figure 5. MS/MS spectrum of the ion at *m/z* 739, evidencing the main patterns of fragmentation of hexosyl PAs. RDA = retro Dies-Alder. (**A**): MS^2 spettra at ion *m/z* 739 [M-152-H]$^-$; (**B**): MS^3 spectrum at ion *m/z* 739 [M-152-152-H]$^-$.

However, we observed that these type of fragmentation pattern does not occur with higher molecular weight molecules, such as catechin tetramers (*m/z* 1153). The MS^2 spectrum of the precursor ion at *m/z* 1153 generated the product ion at *m/z* 865 [M-288-H]$^-$, corresponding to the quinone-methide (QM) fragmentation. The MS^3 spectrum of the precursor ion at *m/z* 1153 [M-288-H]$^-$ showed major product ions at *m/z* 847, *m/z* 739, *m/z* 587 and *m/z* 451. The product ion at *m/z* 847 was derived from loss of water [M-18-H]$^-$. The

product ion at *m/z* 739 was due to HRF [M-126-H]$^-$. HRF + RDA yielded the product ion at *m/z* 587 [M-278-H]$^-$; and the product ion at *m/z* 451 was generated from a QM + HRF fragmentation [M-414-H]$^-$. Figure 6 shows the fragmentation pathway proposed for the molecules of higher molecular weight, which have mixed fragmentations, not detected in smaller molecules.

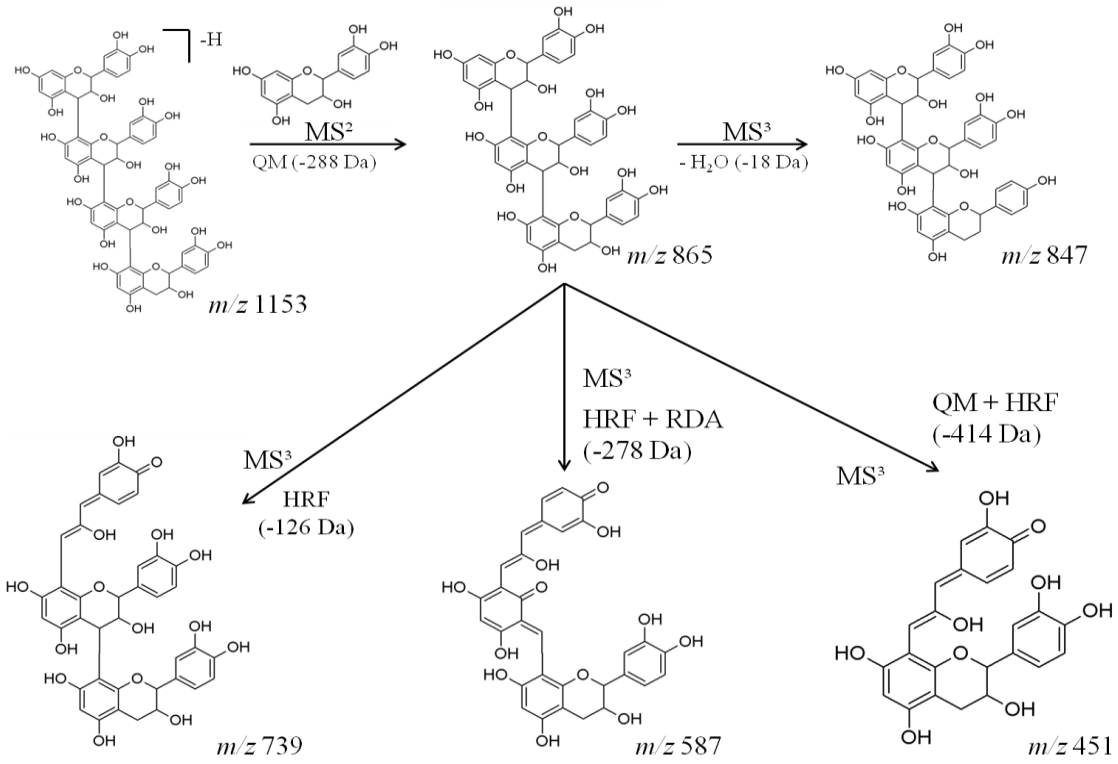

Figure 6. Fragmentation pathways of a possible tetrameric proanthocyanidin found in AERM. The main fragmentation mechanisms involved are: HRF, RDA and QM.

Although the acetone extract of *R. mangle* is composed mostly of catechin derivatives, other substances were found. Quinic acid derivatives (*m/z* 191) were detected in the negative mode. MS^2 fragmentation of the precursor ion at *m/z* 515 generated the product ion at *m/z* 353 [M-162-H]$^-$ (figure 7A, Table 1). The MS^3 fragmentation of the precursor ion of *m/z* 515 produced the product ion of *m/z* 191 [M-162-162-H]$^-$ (Figure 7B, Table 1), characteristic of dicaffeoyl-quinic acids (Gouveia and Castilho, 2012). Probably, these substances are produced due to biotic and abiotic stress conditions in which the plant is submitted in the ecosystem (Gouveia and Castilho, 2012). In addition, this class of

compounds has antioxidant, antiviral, anti-bactericidal, anti-inflammatory, cardiovascular risk reduction, diabetes type 2 and Alzheimer's disease beneficial role (Farah et al. 2008). This class of substances had not been reported in the literature for the genus *Rhizophora*.

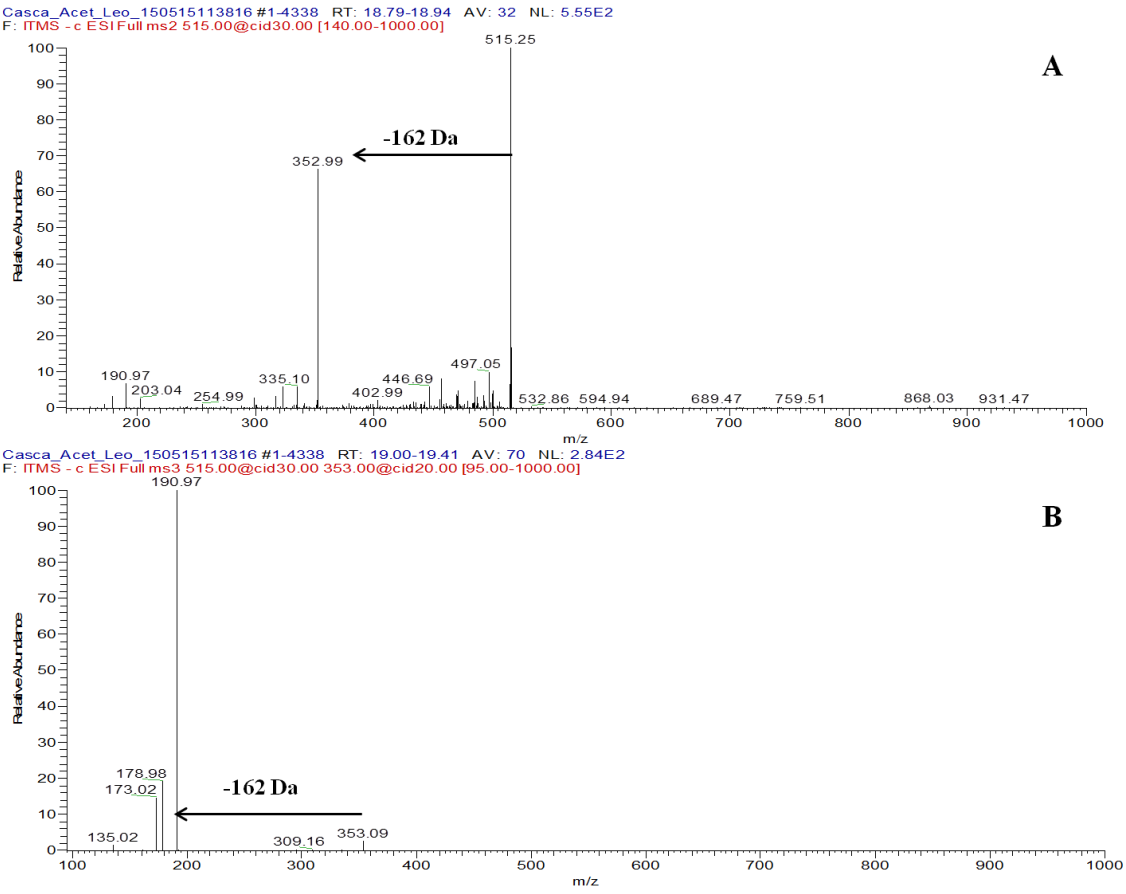

Figure 7. MS/MS spectrum of the ion at *m/z* 515, evidencing the main patterns of fragmentation of dicaffeoylquinic acids. (**A**): The MS^2 spectrum of the ion at 515 $[M-162-H]^-$; (**B**): The MS^3 spectrum of the ion at *m/z* 515 $[M-162-162-H]^-$.

Another substance detected by FIA-ESI-IT-MS presented *m/z* 609. The MS^2 fragmentation of the precursor ion *m/z* 609 produced two major fragments. The ion at *m/z* 463 $[M-146-H]^-$ (Figure 8) probably is due to the loss of a deoxyhexose moiety, whereas the ion at *m/z* 301 $[M-308-H]^-$ (figure 8) arises from the loss of an hexose moiety, which led us to propose the presence of rutin, a flavonoid commonly found in several plant families, which was already detected in extracts of *R. mangle* (Kandil et al., 2004).

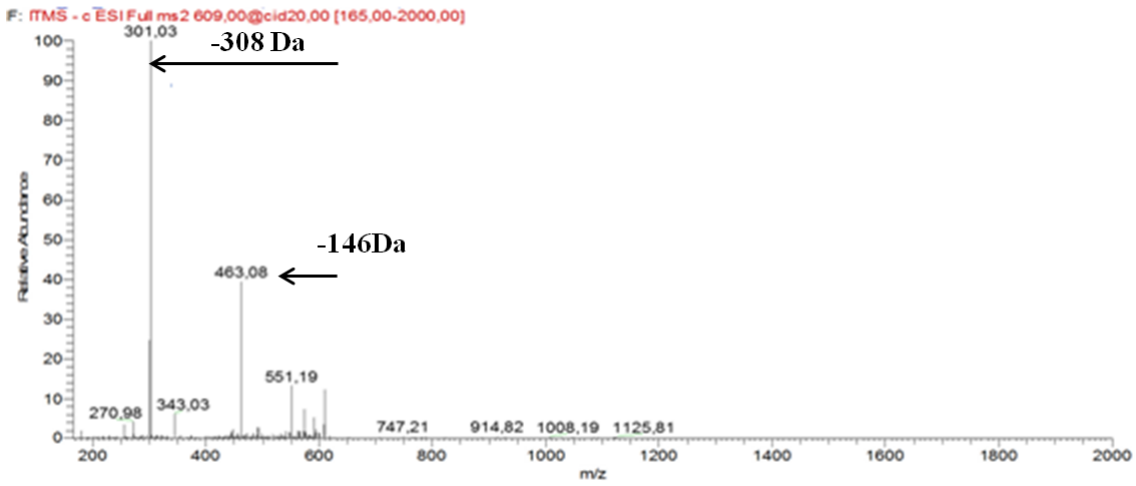

Figure 8. The MS^2 spectrum of the ion at *m/z* 609, evidencing the main patterns of fragmentation of rutin.

3.2 Free radical scavenging activity

The relatively stable organic radical $ABTS^{\bullet+}$ has been widely used in the determination of antioxidant activity of different plant extracts (Sharma and Singh, 20113). Antioxidant substances are characterized in that, even at low concentrations, they prevent delay and repair oxidative stress (de oliveira et al., 2014). The reduction capacity of $ABTS^{\bullet+}$ was determined by the decrease in its absorbance at 734 nm, witch is reduced by antioxidants. The acetonic extract from *R. mangle* showed intense antioxidant activity (608.8 µmol Trolox/g). Zhang et al. (2010), reported intense antioxidant activity of *R. mangle* and *R. mucronata* ethanolic extracts by the DPPH assay, and attributed this effect to the large amount of condensed tannins present in the extract. Takara et al. (2008) evaluated the antioxidant activity of the *R. stylosa* species and showed that the sugar moietys present in the condensed tannins further increase the efficiency of free radical sequestration, which probably happens with our extract.

3.3. Hepatoprotective and insulin sensitizer activity

Mice fed during 8 weeks with high-fat diet becomes obese when compared with mice fed with standard diet, that can be observed by the increase in the final body weight and by the increase of the visceral and subcutaneous adipose tissue depots (Table 2). An increased of liver weight was also observed in the obese mice, suggesting the presence of hepatic alterations associated to obesity (Table 2). Four weeks treatment with AERM was not enough to statistically reduce the body weight gain and adiposity in HFD or control mice, but liver weight was significantly reduced in obese mice treated with *R. mangle* when compared to obese non-treated mice (Table 2).

Interestingly, glucose basal blood levels were also reduced in obese mice treated with AERM, as well as, these mice were tolerant to insulin, as we can observed by the kITT value (Table 2). Hyperglycemia and insulin resistance are routinely associated to obesity. The food ingestion was not different between obese groups, only higher dose of AERM employed in our study was able to reduce the amount of food ingestion in the control group (Table 2).

The improvements in glucose homeostasis could be related to the antioxidant activity of acetonic extract of *R. mangle*. The glucose- and fat-overloaded adipocytes secrets reactive oxygen species (ROS), free fatty acids and several pro-inflammatory adipokines (TNF-α, resistin, IL-1β, etc) to cause insulin resistance in muscles and liver (Han et al., 2010). ROS also can be generated by macrophages, which accumulate in adipose tissue in obesity and an increase in ROS production was described in visceral adipose tissue of genetically and diet induced obesity (Furukawa et al., 2004). Insulin resistance is associated to obesity and characterized by diminished capacity of the cells to respond to insulin. The insulin signaling occurs when insulin bind its receptor that results in autophosphorylation and enhanced tyrosine kinase activity. The phosphorylation of insulin substrate receptor (IRS) occurs subsequently and stimulate signaling cascade of glucose metabolism. ROS seems to be

involved in activation of jun-N-terminal kinase (JNK) that causes a defective phosphorylation in IRS disturbing insulin signaling (Manna and Jain, 2015).

In addition, oxidative stress also interferes with glucose metabolism through the regulation of the FoxO family of Forkhead transcription factor (FoxO1) that is a negative regulator of insulin sensitivity (Nakae et al., 2002). Oxidative stress in adipocytes plays a central role in insulin resistance and ROS secreted by adipocytes leads to insulin resistance in many tissues. The presence of phenolic compounds, such as flavonoids, phenolic acids and proanthocyanidins increase antioxidant defense mechanisms (de Oliveira et al., 2014). Ezuruike and Prieto (2014) evaluated the effect of catechins and quercetins present in green tea, and found that these compounds have been shown to protect pancreatic islet cells from oxidative stress as well as help in the regeneration of β-cells. The presence of these compounds as well as other potent antioxidants, such as those found in the AERM can contribute to the holistic management of diabetes which includes the prevention of diabetic complications.

It is notorious that a hyperlipidic diet generates obese individuals, and with this, associated comorbidities such as insulin resistance and type 2 diabetes mellitus (Kaur, 2014). Obesity is a multifactorial disease without a pharmacological cure, and so the best treatments are those related to lifestyle changes, such as periodic exercise and healthy eating. However, modern lifestyle can make these practices more difficult and exacerbate these symptoms, since the increase of obesity and related diseases has been a reality in recent years. Therefore, an excellent strategy for the prevention of these diseases is the supplementation with phenolic compounds, since they are nutraceuticals acting as complementary treatment (Bahandoran et al. 2013)

Hepatic dysfunction is routinely associated to obesity. The umbrella of hepatic alterations is collectively denominated nonalcoholic fatty liver disease (NAFLD) and includes

steatosis and steatohepatitis with possibility to progression to cirrhosis and hepatocellular carcinoma (Gual et al., 2016). As we can observe, obese mice present steatosis after 14 weeks of high-fat diet, but in mice treated with AERM at dose of 50 mg/kg, hepatic steatosis and triglycerides content is significantly reduced (Figure 9). Hepatic steatosis develops as a consequence of intrahepatic triglyceride accumulation from free fatty acids (FFAs) released from the adipose tissue (lipolysis), FFAs synthesized in the liver from excess carbohydrate (de novo lipogenesis) that are then esterified to storage triglycerides for future energy needs, or triglycerides coming from the diet (Bril and Cusi, 2016).

Regarding insulin resistance (secondary to obesity), adipose tissue lipolysis is increased determining an increase of FFAs into the circulation. The intrahepatic lipid accumulation (ectopic fat) can result in cellular dysfunction, reduced insulin suppression of hepatic glucose production, hyperinsulinemia (a strong promoter of de novo lipogenesis) and/or cell death. The metabolic disturbance in hepatocytes creates a positive feedback loop (ie, steatosis causes hyperglycemia/hyperinsulinemia, which then worsen steatosis) (Bril and Cusi, 2016). In addition, NAFLD pathogenesis is multifactorial, intestinal hormones by affecting food intake, body weight and insulin resistance as well as bacterial products that can affect the secretion of these hormones has also a role in the progression of disease (Koukias et al., 2016). ROS also participate directly in pathogenesis of NAFLD, because elevated fatty acid catabolism in hepatocytes causes excessive electron flux in the mitochondrial electron transport chain, impairing the oxidative capacity of the mitochondria and stimulates the peroxisomal and microsomal pathways of fat oxidation. This increase ROS production that causes oxidative stress and cell death also mediated by JNK dependent pathways (Rolo et al., 2012).

Murase et al. (2002) demonstrated that supplementation with catechins resulted in a significant reduction of high-fat-diet-induced body weight gain, visceral and liver fat

accumulation, because these compounds promote lipid metabolism in the liver. In spite of weight variation did not present a significant difference in any of the treatments, the decrease in the experimental liver steatosis is notorious with supplementation of acetonic extract at 50 $mg.Kg^{-1}$ (Figure 9E). Therefore, we propose that the improvement of the NAFLD, it is also through the mechanisms of antioxidant defense, such as reported by Luo et al. (2012), evaluating catechins derivatives and Martinez-Flores et al. (2005) and Crespo et al. (2008), evidencing that quercetin and rutin act in the improvement of NAFLD in response to the reduction of oxidative stress. NAFLD evaluated in this study is associated with the consumption of a HFD. One of the possible recommendations to avoid the progression of NAFLD is the consumption of healthy foods, with reduce calorie intake (Sullivan, 2010; Hernandez-Rodas et al., 2015). The dietary intake of the control group, supplemented with 50 $mg.kg^{-1}$, showed a significant decrease at food intake when compared to the control group without supplementation. This result demonstrates that the supplementation of the AERM may be a strategy for prevention and progression of NAFLD when linked to healthy diet.

High molecular weight molecules are more difficult to be absorbed by the organism, making it difficult to bioavailability (Monach et al., 2005). Prasain et al. (2009), reported that catechin trimeres, present in in grape seed extracts, are absorbed by the gut, and are detected in plasma and urine, suggesting they are bioavailable, and act as natural antioxidants. However, proanthocyanidins are able to form complexes with proteins, generating a physical defense in the gastrointestinal mucosa (Jesus et al., 2012). We suggest, therefore, in this study that this mechanism may serve as a barrier in the absorption of glucose, generating an improvement in the diabetogenic process reported in this study.

Table 2. Body weight, body composition and biochemical parameters of control mice, control mice treated with AERM extract 5 mg.kg^{-1} (Control5) or 50 mg.kg^{-1} (Control50), obese mice (HFD) and obese mice treated with *R. mangle* extract 5 mg.kg^{-1} (HFD5) or 50 mg.kg^{-1} (HFD50).

	Control	Control5	Control50	HFD	HFD5	HFD50
Body weight at 10th week (g)	42.2±1.4	41.7±1.2	43.0±1.8	55.0±1.2*	52.0±2.0	53.0±1.2
Final body weight (g)	44.7±1.8	45.2±1.6	43.5±1.3	61.2±2.4	56.0±2.7	55.2±1.3
Δ Body weight (%)	5.8±1.2	8.2±1.5	1.6±2.5	9.8±2.6	6.2±2.7	4.2±2.2
Food intake[a] (kcal/day)	24.8±1.8	23.0±0.6	21.0±0.7#	25.5±1.1	24.3±0.9	23.4±1.9
Epididimal fat (g)	1.8±0.3	1.5±0.2	1.5±0.1	2.7±0.1*	2.9±0.2	2.7±0.4
Epididimal fat (% body weight)	4.0±0.4	3.4±0.3	3.7±0.1	4.4±0.1	5.1±0.4	5.1±0.8
Subcutaneous fat (g)	0.7±0.1	0.5±0.1	0.6±0.1	1.3±0.1*	1.0±0.1	1.2±0.2
Subcutaneous fat (% body weight)	1.6±0.2	1.2±0.1	1.5±0.1	2.1±0.1*	1.8±0.3	2.2±0.2
Liver (g)	1.8±0.1	1.9±0.1	1.7±0.1	3.0±0.3*	2.1±0.1#	2.2±0.2#
Liver (% body weight)	4.2±0.2	4.2±0.1	4.0±0.1	4.9±0.3	3.7±0.3#	3.9±0.1#
Gastrocnemius muscle (g)	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
Gastrocnemius muscle (% body weight)	0.5±0.1	0.4±0.0	0.5±0.0	0.3±0.0	0.4±0.0	0.3±0.0
Basal blood glucose (mg/dL)	166±5	150±4	152±5	226±11*	201±22	197±6#
kITT	5.4±0.3	5.7±0.7	5.0±0.5	2.0±0.4*	2.6±0.8	3.2±0.3#

[a]Food intake was measured during the treatment with *R. mangle* extract. * $p<0.05$ when compared with control group and # $p<0.05$ when compared with non-treated group. (n=6-7)

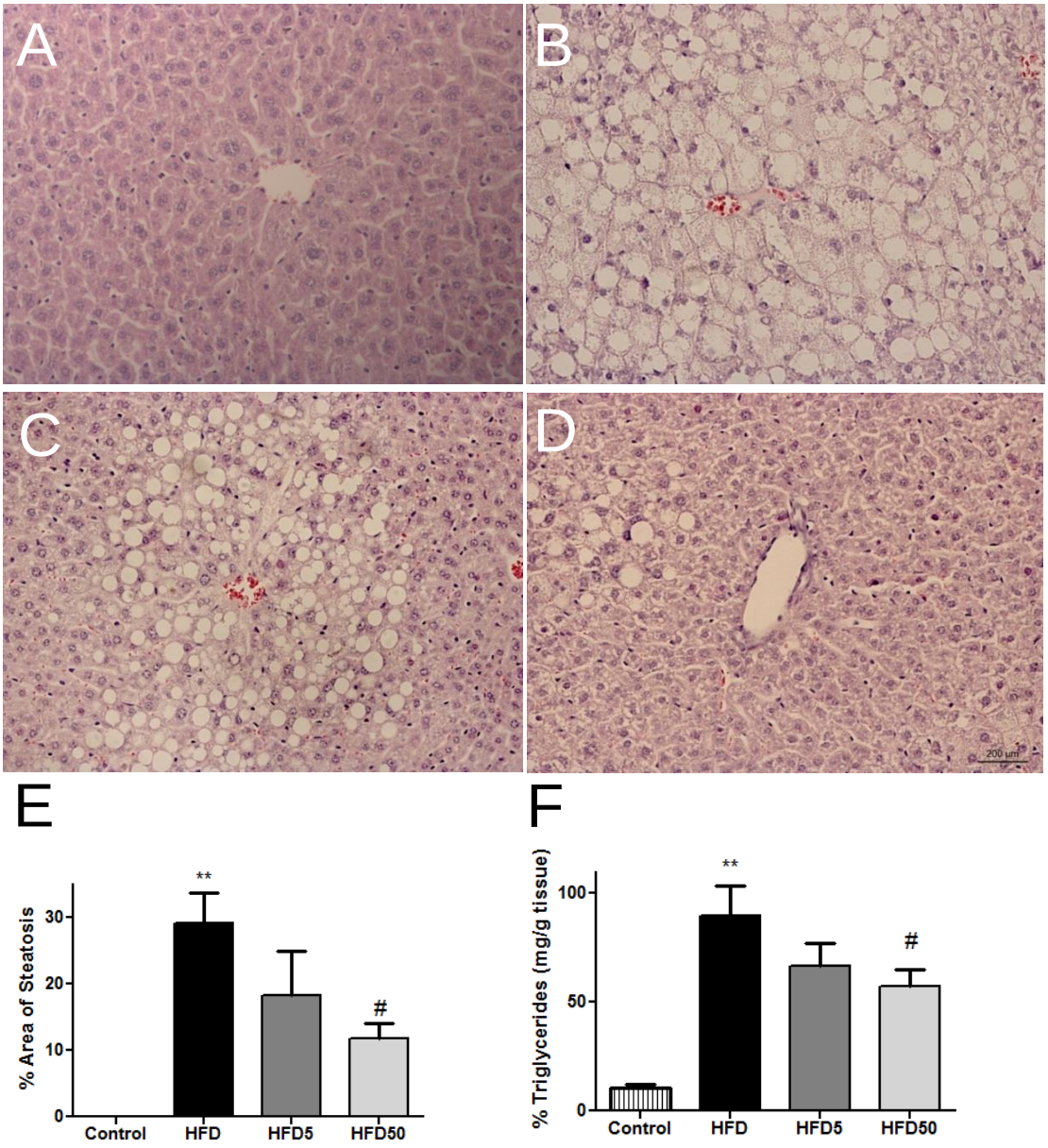

Figure 9. Liver histology of control mice (A), obese mice (B) and obese mice treated with AERM 5 mg.kg^{-1} (C) or 50 mg.kg^{-1} (D). Steatosis measurement (E) and triglycerides content (F). Hematoxylin–eosin staining of 5.0 µm liver sections. Magnification: 200×. Steatosis measurement in 5 random power field of 5 mice per group. ** $p<0.01$ when compared with control group and # $p<0.05$ when compared with HFD group. (n=5)

4. Conclusions

In summary, the data obtained evidenced the presence of three series of PAs from 1 up to 4 catechin moieties, besides phenolic acids and flavonoids, which displayed intense antioxidant activity, besides nutraceutical action, improving in the inflammatory process caused by obesity. In addition, the extract experiment with mice showed that the acetonic extract from the barks of R. mangle displayed promising results for the treatment and prevention of NAFLD's. Taken together, these results suport the traditional knowledge about the use of *R. mangle* for the treatment of type II diabetes.

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CAPÍTULO II

Atividade Antinonceptíva

A ser submetido no evidence-based complementary and alternative medicine

Evaluation of antinociceptive activity of polyphenols contained in the standardized extract of *Rhizphora mangle*

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Abstract

Rhizophora mangle L. (Rhizophoraceae) species has been used as a traditional medicine. The present study was designed to characterize the chemical composition of the acetone extract (70% v/v) of *R. mangle* and evaluate the nociceptive effects. Mass spectrometry analysis, LC-MS/MS and MALDI-TOF, were performed in order to recognize the high molecular weight secondary metabolites present in the extract. Antinociceptive activity was tested through animalmodels in formalin, and tail flick assays. The results showed the presence of an extract rich in phenolic compounds derived from flavan-3-ol units. The polymers are composed of units of catechins, gallocatechins and afzelechins, as well as their monoglucosylated forms. Since *R. mangle* has already been used for the treatment of gastrointestinal injuries, which cause hard pains we investigated the anti nociceptive activity of the acetone extract from the barks of this species. The results evidenced in the Formalin and Tail flick biological assays revealed that the extract has activity similar to the positive control, morphine. The use of opioid medicines to treat pain is common but it causes several side effects. Therefore, the acetonic extract from the barks of *R. mangle* is an alternative form for the reduction of the use of opioids, thus giving support to the use of this plant in traditional medicine.

***Keywords*:** catechins, mass spectrometry, nociceptive effects, Polyphenols.

1. Introduction

The study of medicinal plants is characterized by a multidisciplinary approach, where various fields of chemistry and biology merge to unravel its therapeutic potential. The use of many plants in folk medicine is supported on a long history of application and empirical observation of their toxic and medicinal effects [1]. *Rhizophora mangle* L. (Rhizophoraceae) is a pioneer plant that dwelleth the estuarine ecosystem, it is considered a true mangrove specie. Mangrove plants are potential sources of biologically active compounds and have wide application in ethnopharmacology practices. The habitat of these species is under stressful environmental conditions (salinity, temperature, tidal fluctuations and anoxic soil). These plants are perfectly adapted to this harsh environment and have many substances that protect them from these conditions [2]

In traditional medicine, *R. mangle* is commonly used to treat angina, asthma, diarrhea, ulcers, inflammation, tumors, diabetes and pains [2,3]. Pain is the main reason for seeking medical consultations and represents high medical and economic costs to the community [4]. The continuous use of opioids can generate dependence and side effects, such as gastrointestinal problems and respiratory depression, and therefore the search for new analgesics, especially those of natural origin, being a challenge for medicinal chemistry [5].

The polyphenolic compounds such as proanthocyanidins (PAs - tannins) consisting of oligomers and polymers by flavan-3-ol units are most abundant in *Rhizophora* [6]. PAs are widely distributed in the plant kingdom and are the second-most frequent phenolic substances, after lignins [7]. Due its variable degree of polymerization (DP), the isolation and identification of the pas require the use of complex steps of separation and purification. Additionally, PAs are highly unstable compounds with complex molecular structures, thermolabile and photosensitive. Therefore, mass spectrometry (MS) techniques based on matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-

MS) and liquid chromatography coupled to the tandem mass pectrometry (LC-MS) has been attracting attention because of their ability to reliable analysis. In this context, the purpose of the present study was to characterize the chemical composition of the acetone extract of *R. mangle* bark's (AERM) and evaluate the analgesic activity.

2. Material and Methods

2. 1 Plant material

Fresh barks of *R. mangle* were washed, shade dried, powdered in a knife mill and sieved through a #60 mesh sieve. The powder (50 g) was extracted with 0.5 L acetone (70% v/v) and macerated for seven days without solvent chagne, at room temperature (24°C), protected from light. The macerate was filtered through Whatman N°.1 filter paper and concentrated in a rotary flash evaporator at a temperature not exceeding 35°C. The extract (7 g, 14 %) was lyophilized and stored in amber bottles and allocated in a freezer (-40 °C). In order to minimize the interference of very high order polymeric compounds, a solid-phase extraction (SPE) was made. An aliquot (10 mg) of the extract was submitted to the SPE using RP18 cartridge, eluted with H_2O/MeOH 8:2 (v/v) (5 mL). The eluate was filtered through the nylon membrane and directly analyzed by LC-MS/MS.

2.2 Preparation of plant extract (AERM)

Fresh barks of *R. mangle* were washed, shade dried, powdered in a knife mill and sieved through a #60 mesh sieve. The powder (50 g) was extracted with 0.5 L acetone (70% v/v) and macerated for seven days at room temperature (24°C), protected from light. The macerate was filtered through Whatman N°.1 filter paper and concentrated in a rotary flash evaporator at a temperature not exceeding 35°C. The extract (7 g, 14 %) was lyophilized and stored in amber bottles and stored in a freezer (-40°C).

2.3 Phytochemical evaluation

2.3.1 LC-ESI-MS/MS analysis

Mass spectrometry analyses were carried out in a Thermo Finnigan (San Jose, CA, USA) LCQ mass spectrometer equipped with an electro-spray ionization source, ion-trap analyzer and Xcalibur software for data processing. All analyzes were operated in negative mode. For UPLC-MS analysis: column Phenomenex, 300 $\mu L.min^{-1}$, followed by chromatographic using MeOH and 0.002% formic acid in water were added using a linear gradient from 0% to 100% MeOH over 5 min. The mass spectrometer was operating in the negative ion mode under the following conditions: flow 5 $\mu L\ min^{-1}$; the capillary temperature 270°C; 80 arbitrary units of nitrogen; gas assist with 5 arbitrary units.

2.3.2 MALDI-TOF

For the analysis using MALDI-TOF, 1.0 mg of the powdered vegetable material was diluted in 500 μL of MeOH containing 2,5-dihydroxybenzoic acid (DHB – 125 nm) and sodium iodide (1 nM) with addition of 1% trifluoroacetic acid (v/v). This solution was placed for 1 min by vortexing. After this, 1 μL of the solution suspension has been deposited on MALDI plates, made of stainless steel and allocated to air dry prior to analysis. The analysis of MALDI-MS were carried out a Voyager DE-RP coupled to the spectrometer flight time mass (TOF), equipped with a nitrogen laser (λ = 337 nm, pulse width 3rd). The intensity of the laser beam was experimentally attenuated slightly above the threshold level for forming ions from the tannin components. The ions generated by laser desorption were introduced in the flight tube with a 20 kV accelerating voltage in positive linear mode. The delay time is 100 ns. All mass spectra were acquired by averaging 100 individual laser shots [8].

2.4 Analgesic activity

2.4.1 Animals

This study used female Swiss mice (n = 6) at 8-12 weeks (25–30 g) were obtained from Campinas University (Campinas, São Paulo, Brazil). The animals were kept in temperature (22-26◦C) and light control (12 h light cycle) and were fed *ad libitum*. This study was conducted according to the standard ethical guidelines and approved by the Ethics Committee on Animal Use (019 CEUA/CLP - UNESP-São Paulo State University/Coastal Campus of São Vicente).

2.4.2 Formalin assay

Formalin test was done according to the modified method of Dubuisson and Dennis, (1977). Each animal was placed inside a box (30 × 30 × 30 cm, length × width × height) after formalin injection in right foot plantar area. Two responses were analyzed: acute-phase (0 to 5 min) and later-phase (15 to 30 min) [9, 10]. Thus, Swiss female mice were fasted for a period of 8 hours, and divided into three groups (n = 6), Control (saline), Morphyne (10 mg kg^{-1}, i.p., 30 min before formalin) and Bark treated with *R. mangle* extracts (10 mg kg^{-1}) injected peritonially. After one hour of each treatment, animals were given an intraplantar formalin injection (20 µ; 2%). The time (s) the animal licks the paw where the substance has been applied ("licking time") was clocked in two stages (acute-phase and late-phase) zero time is considered the time immediately after administration of formalin.

2.4.3 Tail flick assay

Tail-Flick test was evaluated Tail flick assay for evaluation of central analgeseic activity [11]. The tail (latency period) is used as a determination index of nociception. Data collected from mice with latency lower than two seconds were not used. Animals were

divided into three groups (n = 6), Control, Morphyne and Bark (Intraperitoneal injection of AERM) and an analgesymeter (Insight EFF 300L), with the tail docked in apparatus with light thermal source was used. The latency of tail withdrawal response is determined 0, 30, 60, 120 e 180 min before the AERM administration, inject peritoneally, with 10 $mg.kg^{-1}$ concentration. As positive control was used morphine (10 $mg.kg^{-1}$, i.p.).

2.5 Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M.). The statistical significance was determined using a One-way Analysis of Variance (ANOVA), followed by Tukey post hoc test $p < 0.05$ was considered as an indication of a significant difference.

3. Results and discussion

Previous studies carried out with species of the genus *Rhizophora* and Rhizophoracea family have demonstrated that these specimens are important sources of polyphenolic compounds (tannins) [3,6,12]. MS experiments were performed using LC–MS system equipped with an ESI source and an Ion Trap analyzer were made in order to investigate the presence of different compounds with the same molecular weight and then to perform a qualitative analysis on the PAs constituents occurring in the AREM extract.

In order to recognize the main class of compounds we performed LC-ESI-MS/MS analysis. The chromatographic separation was optimized in order to obtain an excellent separation and resolution. The LC-ESI-MS/MS obtained has satisfatory chromatographic resolution (Figure 1), with 11 separate compounds and their structure proposed (Table 1).

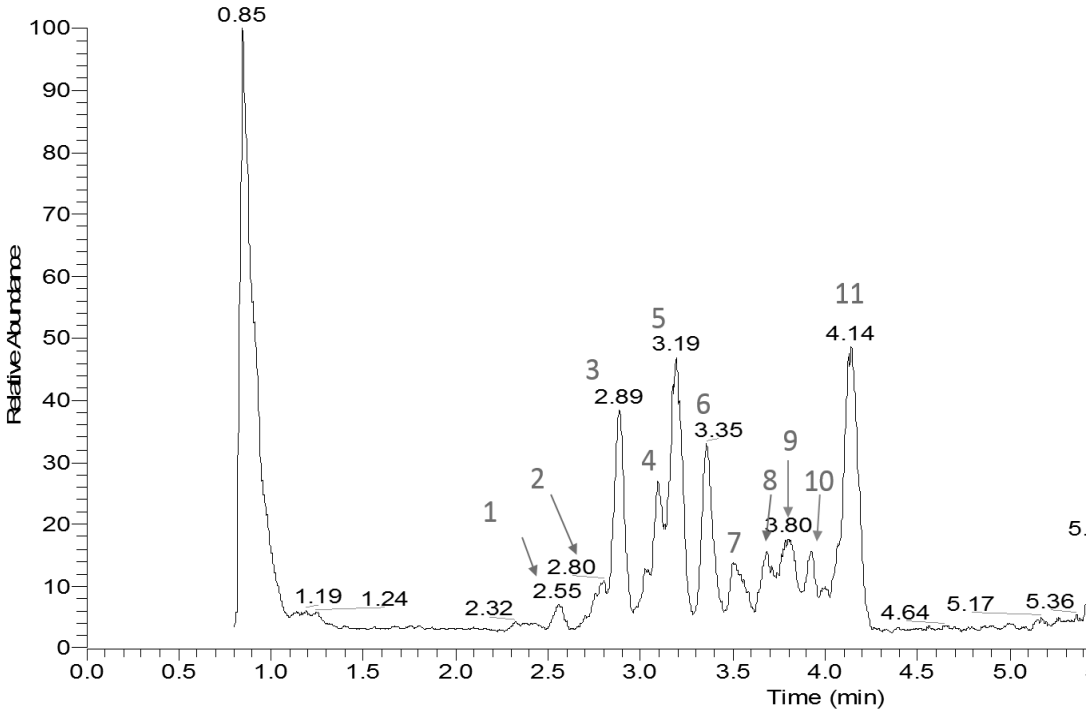

Figure 1. Total ion chromatogram in negative ion UPLC-MS analysis of proanthocyanidins present in the AREM.

Table 1. Events MS/MS^n, fragmentations performed during the chromatographic analysis

Peak	Tr (min)	[M-H]$^-$ (*m/z*)	MS^n events	Proposed ID
1	2.55	577	MS^2: 425 MS^3: 407, 272, 298, 228	catechin dimer
2	2.80	723	MS^2: 571, 577, 269	catechin dimer + deoxyhexose
3	2.89	577	MS^2: 425	catechin dimer
4	3.10	435	MS^2: 289, 136	catechin + deoxyhexose
5	3.19	739	MS^2: 577 MS^3: 559, 451, 289, 269	catechin dimer + hexose
6	3.35	451	MS^2: 341, 289	catechin + hexose
7	3.52	577	MS^2: 425	catechin dimer
8	3.60	577	MS^2: 425	catechin dimer
9	3.80	451	MS^2: 341	catechin + hexose
10	3.95	451	MS^2: 341	catechin + hexose
11	4.14	451	MS^2: 341	catechin + hexose

Compounds **1**, **3**, **7** and **8** were identified as catechin dimers based on their $[M-H]^-$ precursor ion at *m/z* 577 $[M-H]^-$, as well as after MS^2 fragmentation, which resulted in the formation of the products ions at *m/z* 425, *m/z* 407, *m/z* 272, *m/z* 298 and *m/z* 228, consistent with the data reported in the literature for catechin dimmers [13]. Compound **2 (***m/z* 723, $[M-H]^-$) was assigned as a catechin dimer with one deoxyhexose moiety; MS^2 fragmentation of this precursor ion led to the products ions at *m/z* 577 $[M-146-H]^-$, *m/z* 571 and *m/z* 269 [14]. Fragmentation of the precursor ion of *m/z* 435 $[M-H]^-$ (Compound **4**) led to the product ions at *m/z* 289 $[M-146-H]^-$ and *m/z* 136, thus suggestings a catechin core bounded to a deoxyhexose unit. Fragmentation of the compound **5 (***m/z* 739) led to the fragments at *m/z* 577 $[M-162-H]^-$, *m/z* 559, *m/z* 451, *m/z* 289 and *m/z* 269 [14], corresponding to a catechin core bounded to an hexose moiety. Compounds **6**, **9**, **10** and **11** were also identified as hexosyl-catechins, with *m/z* 451 $[M-H]^-$ and MS^2 product ions at *m/z* 341 $[M-162-H]^-$ [14].

There are two geometric isomers of catechins: catechin (CA) and epicatechin (EC), where CA is in a *trans* form and EC is in a *cis* form, in respect to the substituents at C2 and C3 [15]. Their structure consists of two benzene rings (A and B rings) connected through a pyran ring C. The subtle difference in molecular structure may result in drastic differences in pharmacological processes and therapeutic efficacy [16]. LC-MS/MS analyses does not allow to easily differenciate these isomers. Moreover, there are also the possibility of enantiomers of CA and EC. Since we detected peaks **1**, **3**, **7**, **8** (*m/z* 577) and peaks **6**, **9**, **10** and **11** (*m/z* 451) at different retention times and same *m/z,* we suggest the presence of isomers.

Catechins are classified as flavan-3-ol monomers and have the capacity to polymerize, forming oligomeric compounds with high molecular weight [17]. The polymeric character is evidenced by the chromatogram, however, the characterization of condensed tannins was limited with ESI-IT-MS/MS experiments. To solve this bottleneck, analysis using

MALDI-TOF mass spectrometry were performed to establish the degree (DP) of polymerization to the proanthocyanidins and its glucosylated

The spectra obtained by MALDI-TOF analyzes of the AREM presented two homologous series: one with 288 Da increments and another one with 288+162 Da increments, corroborating with those found using FIA-ESI-IT-MS technique.

The series containing 288 Da increments were attributed to the polymeric catechins, according to formula 290 + 288 (n-1) + 23, where n is the degree of polymerization (DP). Sodium adducts with degree of polymerization of up to 12 DP (3481.673 Da) were found, even with the decrease in the intensity of the intercepted ions (Figure 2A). In addition to this first serie of flavan-3-ol, each DP had a subset of masses with variation of -16 Da and +16 Da (Figure 2A). These subset can be explained by heteropolymers of repeating flavan-3-ol units containing an additional hydroxyl group (Δ +16 Da), characterizing a homologous series of gallocatechins (m/z 905.145 ~ 3497Da) (Figure 2A). The other series consists of heteropolymers of repeating flavan-3-ol units without a hydroxyl group (Δ -16 Da), that is, an homolougous series of afzelechins (m/z 873 ~ 3465) (Figure 2A).

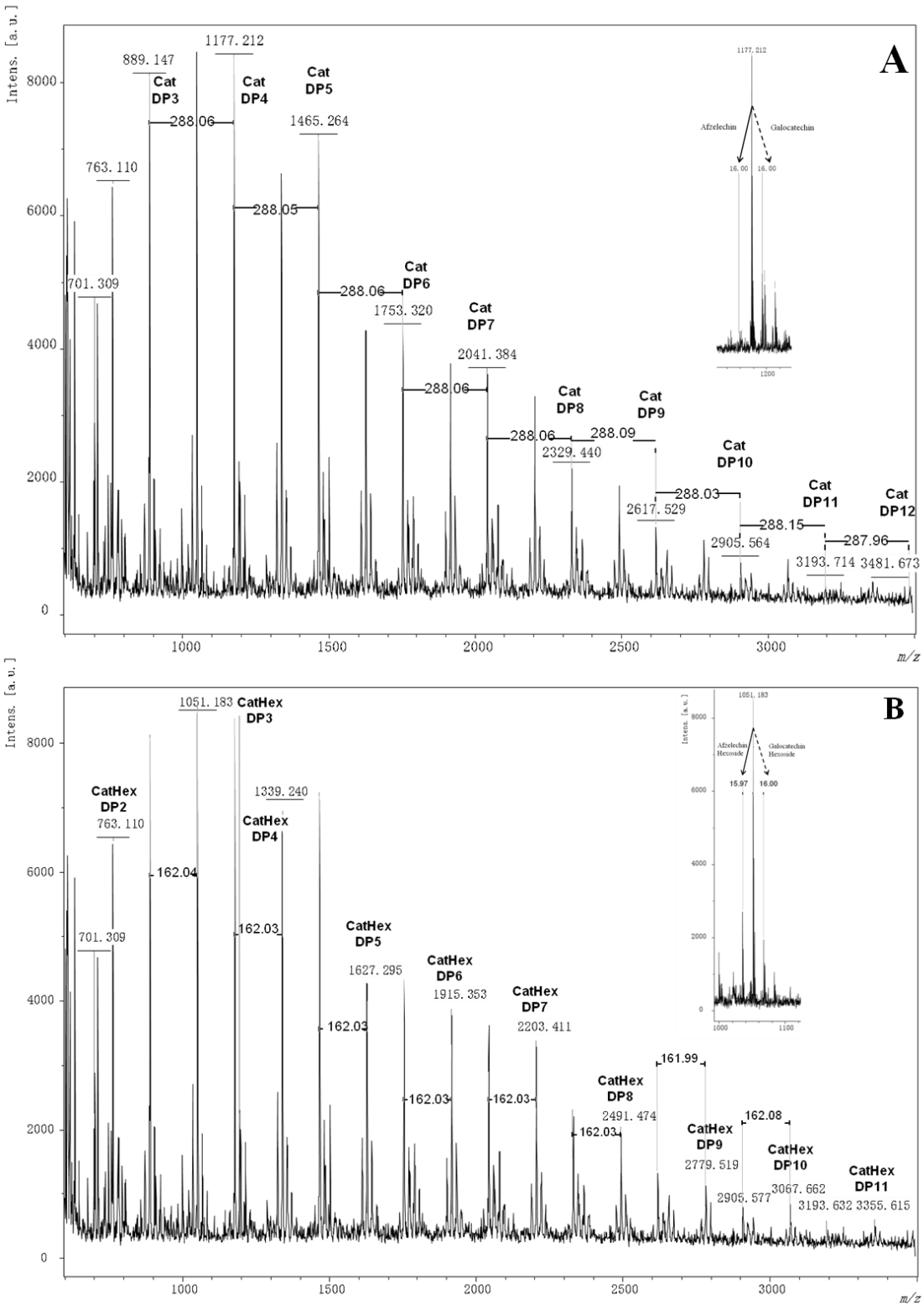

Figure 2. MALDI-TOF positive reflectron mode mass spectra of the condensed tannins from *R. mangle*. (**A**) catechins series with varying hydroxylation patterns, (**B)** catechins series with hexose group and varying hydroxylation patterns.

MALDI-TOF mass spectrometry experiments indicated the occurrence of a mixture of proanthocyanidins and its monohexoside derivatives which presented a variable DP (from 2 up to 12). Each precursor ion of the condensed tannins from AERM was always followed by mass signals at a distance of 162 Da, thus suggesting the presence of a series of monohexoside anthocyanidins, that can be explained with the formula 290 + 288 (n-1) + 162 + 23 (Figure 2B). Condensed tannins with an hexose group with DP 2 to 11 (*m/z* 763.110 ~ 3355.615) were detected in AERM (Figure 2B). Species with degree of polymerization up to DP 11 CatHex (m/z 763.110 ~ 3355.615 Da) could be observed (Figure 2B).

The same pattern with homologous series of gallocatechins and afazelequins could also be observed for monohexoside series, thus evidencing that the extract also possesses a series of monohexoside gallocatechins (*m/z* 779 ~ 3371) as well as a series of monohexoside afzelechins (*m/z* 747 ~ 3339). No compound with more than one hexoside group were detected in any homologous series evaluated. The peaks with the highest intensities in MALDI-TOF MS of the AREM were summarized in Table 2.

Table 2. Summary of peaks with the highest intensities in MALDI-TOF MS of the condensed tannins from AREM.

Polymer	**N1 Catechins**	**N2 Afzelechin**	**N3 Gallocatechins**	**N4 Glycoside**	**MW + Na**
DP 2	2	0	0	0	763.110
DP3	3	0	0	0	889.147
	3	0	0	1	1051.18
	2	1	0	0	747.133
	2	0	1	0	905.145
	1	2	0	0	-
	1	0	2	0	-
DP4	4	0	0	0	1177.212
	4	0	0	1	1339.240
	4	1	0	0	-
	3	0	1	0	905.145
	3	1	0	1	1035.211
	3	0	1	1	1067.178
	2	2	0	0	-
	2	0	2	0	
	2	2	0	1	
	2	0	2	1	

	1	3	0	0	
	1	0	3	0	
DP5	5	0	0	0	1465.264
	5	0	0	1	1627.295
	4	1	0	0	-
	4	0	1	0	1193.210
	4	1	0	1	1323.266
	4	0	1	1	1355.230
	3	2	0	0	-
	3	0	2	0	-
	3	2	0	1	-
	3	0	2	1	1084.189
	2	3	0	0	-
	2	0	3	0	-
	1	4	0	0	-
	1	0	4	0	-
DP6	6	0	0	0	1753.320
	6	0	0	1	1915.353
	5	1	0	0	-
	5	0	1	0	1481.252
	5	1	0	1	1611.313
	5	0	1	1	1643.291
	4	2	0	0	-
	4	0	2	0	-
	4	2	0	1	-
	4	0	2	1	1372.240
	3	3	0	0	-
	3	0	3	0	-
	2	4	0	0	699.293
	2	0	4	0	-
	1	5	0	0	-
	1	0	5	0	-
DP7	7	0	0	0	2041.384
	7	0	0	1	1769.318
	6	1	0	0	1738.328
	6	0	1	0	-
	5	2	0	0	-
	5	0	2	0	2203.411
	4	3	0	0	-
	4	0	3	0	-
	3	4	0	0	-
	3	0	4	0	-
DP8	8	0	0	0	2329.440
	8	0	0	1	2491.474
	7	1	0	0	2025.402
	7	0	1	0	2058.386
	6	2	0	0	-
	6	0	2	0	-
DP9	9	0	0	0	2617.529
	9	0	0	1	2779.519
	8	1	0	0	-

	8	0	1	0	2345.434
	8	1	0	1	2475.799
	8	0	1	1	2507.576
DP10	10	0	0	0	2905.564
	10	0	0	1	3067.662
	9	1	0	0	2602.470
	9	0	1	0	-
	8	2	0	1	-
	8	0	2	1	2523.489
DP11	11	0	0	0	3193.713
	11	0	0	1	3355.615
	10	1	0	0	-
	10	0	1	0	-
DP12	12	0	0	0	3481.673
	12	0	0	1	-
	11	1	0	0	-
	11	0	1	0	-

Rhizophora species are traditionally used for the treatment of pain [18], but no scientific support corroborates this action, especially for *R. mangle*. There are already works evidencing the positive therapeutic effect of *R. mangle* in the treatment of gastro intestinal diseases [13,20], which cause a high sensation of pain. Banerjee et al., [18] reported a significant reduction in pain severity and frequency of painful episodes, in patients who underwent alternative treatment with extracts rich in grape proanthocyanidins, reported a significant reduction in use of narcotic analgesics [20]. The mechanism of action by which tannins act in the treatment of pain is still poorly explained, but it is a reality [21,22]. In ordet to to evaluate the effect of AREM in the treatment of pain, we performed experimental tests with formalin and tail-flick assays.

The Formalin test was performed to contribute to the tail-flick, whilst the former is better known to evaluate pain threshold or reflex through acute cutaneous stimulation, formalin injection induces a state that better approximates to clinical conditions, therefore evaluating tonic pain. Thus, in the first phase of the test, formalin predominantly evokes activity in C fibers, substance P and bradykinin, whilst histamine, serotonin, prostaglandins and bradykinin are involved in the second phase [23]. Therefore, the formalin test allows the

evaluation of analgesic effect in peripheral inflammatory processes, that is evoked especially in the second phase of the test. The stimulus provided by subcutaneous injection of formalin induced a behavioral response in animals treated with formic acid, in contrast to animals treated with morphine and AREM compound, that had almost no behavioral response to formalin injection in both phases of formalin test. Therefore, the formalin test showed that there is no difference between the analgesic effect of morphine and bark compounds, even in a test that provides a long-lasting pain stimulus such as the formalin test (Figure 3).

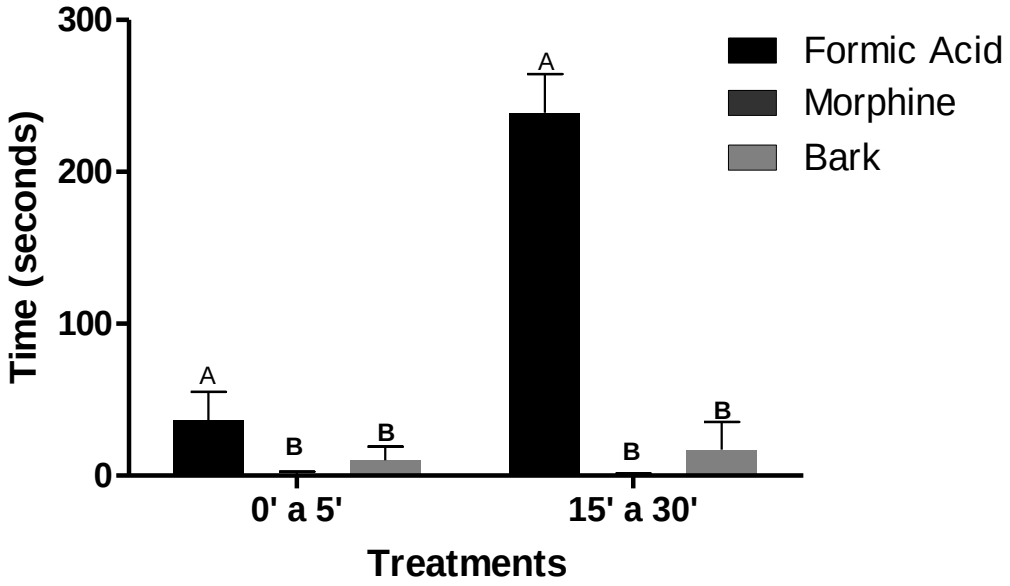

Figure 3. Formalin test. Significant difference was found between animals treated with formalin and those treated with the compound or morphine. There was no difference between the extracts and morphine. (Two-way ANOVA, Bonferroni post test ($p < 0.01$, $F = 229.7$, $df = 4$) $N = 5$. First column Time 0 'to 5' second '5' 30 ').

Tests with more phasic stimuli, such as the tail-flick test, provides an evaluation of antinociceptive effect in acute pain state. In this scenario, the response to thermal stimuli was evaluated using the tail-flick test. As it was shown in Figure 4, both the effect of morphine as well as the bark compounds, were substantially greater than in control (saline-treated animals), except for 60 minutes. This result indicates that the bark compounds had a spinal

effect similar to morphine, though the exact mechanism is still unknown. The reason behind this morphine-like effect presented by the AREM, may be due the action of catechins, as it was shown previously that these substances acts like blockers for sodium channels in neuronal cells [24]. Hence, this morphine-like effect observed for the bark compound has a significant clinical importance, as it could be used as a potential substitute for the morphine. Considering all the morphine's adverse effects (e.g. constipation, myoclonus, sedation, nausea and pruritus), the use of the AREM could improve the treatment of pain, specially chronically [25].

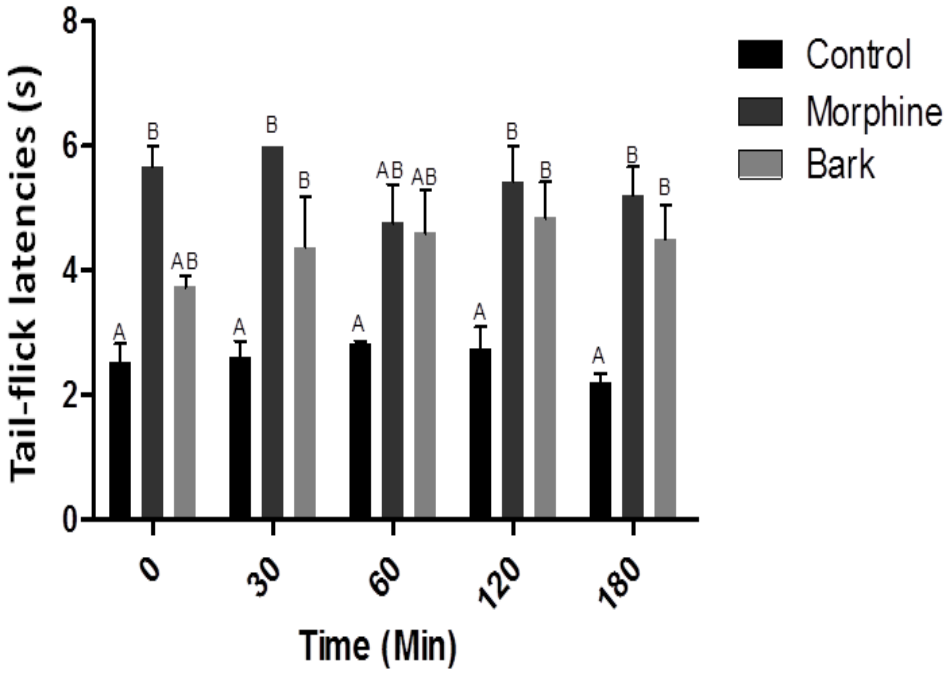

Figure 5. Tail-flick test. Morphine and bark application significantly increased tail-flick latencies while administration of saline (control) did not affect tail-flick latencies, except for 60 min. (Two-way ANOVA, Bonferroni post test, F=46,27, p<0,01, gl=4).

A scientific basis underpinned by the compounds of *Rhizophora mangle* may be helpful to explain its antinociceptive effects. Thus, *R. mangle* is rich in flavonoids, substances that have been investigated and found to possess pharmacological potentials such as antiviral, antimicrobial [26], anti-pyretic, analgesic, anti-inflammatory [27], and are accordingly to the findings above. Furthermore, *Pinus pinaster* is also rich in polyphenolic compounds, such as

afzelechins and galocatechins, same as found in *Rhizophora mangle*, and has been shown to have similar analgesic effects as *R. mangle* [28]. *Pinus pinaster* cause inhibition of Cyclo-oxygenase (COX-1 and COX-2) activity in serum samples of human volunteers. Cyclo-oxygenases enzymes are the major targets for the treatment of inflammation, fever and pain, due to its function, that is synthesis of mediators, such as prostaglandins, prostacyclin, and thromboxane. Which suggests, that the Cyclo-oxygenase blockage may also play a role in the analgesic effect of *Rhizophora mangle* due to polyphenolic compounds activity, nonetheless this hypothesis requires more investigation.

4. Conclusions

The research showed the potential of UPLC coupled to ion trap tandem mass spectrometry and MALDI-TOF analysis for the rapid and sensitive detection of phenolic compounds in the acetone extract from the barks of *R. mangle.* This technique allowed to detect the presence of a series of anthocyanidins in less than 5 min of analysis. MALDI-TOF mass spectrometry allowed to characterize all the polymeric substances present in the extract, revealing the presence of a series of condensed tannins based on catechins, another based on gallocatechins and a third one based on afzelechins. Besides, all of these series were acopanied by their respective glucosilated derivatives. The identification of these compounds has provided relevant data for fully charachterize the secondary metabolits present in this complex vegetal extracts. The chemical characterization of the extract also helped to understand the results observed in the Formalin and Tail Flick anti-inflammatory assays using experimental models, which demonstrated the antinoceptive effect of the acetonic extract from the barks of *Rhizophora mangle*. In conclusion, the present data indicate that the compounds presented in *R. mangle* acetone extract, based on anthocyanidins, contribute to the analgesic activity observed, giving support to the the use of this plant in traditional medicine.

Acknowledgments

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CAPÍTULO III

Considerações finais

Rhizophora mangle e sua utilização no tratamento de doenças crônicas

A presença de taninos é comum em plantas do ecossistema manguezal e conhecido para espécies do gênero *Rhizophora*. Atribui-se o efeito anti ulcerativo e anti colite de *R.*

mangle à presença desses compostos fenólicos. Há dois mecanismos de ação principais para a proteção de doenças gastro-intestinais: (i) químico, com ativação de defesas antioxidantes (Bonacorsi et al., 2012), e (ii) físico, com formação de uma camada tanino/proteína, que protege a mucosa gástrica promovendo maior resistência a lesões químicas, mecânicas e irritações (Jesus et al. 2012).

Dryden et al. (2006) estudou por meio de injeção intraperitoneal em ratos o efeito do extrato rico em polifenóis do chá verde em doenças gastrointestinais; constatando que houve diminuição da inflamação do cólon, fator de necrose tumoral (TNF-α) e aumento da expressão de hemeoxygenase-1 (HO-1), evidenciando um papel importante das defesas antioxidantes. Lee et al. (2004), avaliou o efeito de um dos componentes isolados do chá verde, epicatequina galato, confirmando que este polifenol pode melhorar os danos celulares da mucosa causado por contaminação de *Helicobacter pylori*; por meio de proteção do DNA, inibição da morte celular apoptótica induzida, repressão do fator de transcrição NF-κB e inibição de 5-lipoxigenase. Entretanto, todos esses mecanismos são iniciados com a glicosilação de TLR-4 (tall like 4), e o tratamento com epicatequina galato inibe efetivamente a ativação deste receptor.

Estudos mostram que muitos taninos possuem a capacidade de sequestrar radicais livres, atuando positivamente em muitas doenças, como câncer, esclerose, aterosclerose, anginas e outras doenças crônicas (Jesus et al. 2012). Muitas das drogas gastroprotetivas agem nos fatores de neutralização de secreção, como antiácidos, receptorers de H_2 e inibidores de bomba de prótons, assim como o medicamento omeoprazol, comumente utilizado. Berenguer et al. (2005), avaliou o efeito protetor de *R. mangle* em úlceras gástricas induzidas por anti-inflamatórios não esteroidais utilizando Dicoflenaco como modelo e concluiu que a melhora do quadro ulcerativo é devido as propriedades antioxidantes, as quais aumentam a atividade de enzimas GHH-Px e SOD, e por outro lado, diminuem a peroxidação lipídica.

Cheng et al. (2011) relatam o importante papel das catequinas na proteção da mucosa gástrica de camundongos. Possivelmente, esses metabólitos previnem lesões nas células do epitélio inestinal e induzem a expressão de HO-1 via modulação da translocação protéica de Nrf2. Esses resultados suportam o possível uso de catequinas como uma dieta preventiva contra injúrias gástro-intestinais causadas pelo estresse oxidativo. Portanto, o extrato acetônico de *R. mangle*, caracterizado nesse trabalho é uma excelente fonte de catequinas e compostos polifenólicos, responsáveis pelo efeito observado por nosso grupo de pesquisa e que foi objeto de patente (INPI: BR10201202881).

Apesar do AERM ser composto em sua maioria por taninos, outras substâncias foram encontradas, derivados de ácido químico e de quercetina. Essas substâncias de menor peso molecular, além de também atuarem nos mecanismos de defesa antioxidante, possuem atividade reportada no tratamento das doenças crônicas avaliadas no atual projeto. Neste trabalho, realizamos a caracterização qualitativa desses constituintes, tendo-se observado a presença de proantocianidinas, as quais podem ser correlacionadas com as atividades farmacológicas observadas. Provavelmente atuam no mecanismo de defesa antioxidante, na inativação da cascata de inflamação TL-4, no aumento da expressão enzimática de HO-1 e na formação de um complexo de ligação Tanino/Proteína, prevenindo lesões químicas, mecânicas e irritações. Além disso, derivados de catequinas já vem sendo empregados para o tratamento de injúrias gastro intestinais, o que corrobora com os resultados observados pelo nosso grupo de pesquisa.

Os taninos de *R. mangle* são uma mistura complexa e de alta polaridade. Ao que tudo indica, os extratos de *R. mangle* são futuros potenciais fitoterápicos de qualidade, pois apresentam sua composição química totalmente caracterizada, com ação farmacológica comprovada. A caracterização química dos metabólitos secundários majoritários permitiu testar esse extrato contra outras doenças crônicas: analgesia e diabetes, oriundas de um

processo inflamatório crônico/agudo e que exibe melhoras após a suplementação/aplicação destas substâncias.

Estes estudos não terminam aqui. Na próxima etapa deste trabalho pretende-se desenvolver formulações farmacêuticas que viabilizem o uso do extrato de *Rhizophora mangle*, de forma a avançar no aproveitamento racional da biodiversidade brasileira. Além disso, avançar nos estudos de biodisponibilidade de taninos, apesar do elevado peso molecular, apresentam vários benefícios medicinais e nutricionais, mas o mecanismo de ação ainda é pouco compreendido.

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