UNIVERSIDADE ESTADUAL PAULISTA
INSTITUTO DE BIOCIÊNCIAS
CÂMPUS DO LITORAL PAULISTA

Avaliação de compostos polifenólicos nos efeitos induzidos pela sPLA2 de veneno cortálico e botrópico

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SÃO VICENTE - SP
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Tese apresentada ao Instituto de Biociências - Câmpus do Litoral Paulista, UNESP, para obtenção do título de doutor no Programa de Pós-Graduação em Biodiversidade Aquática.

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TITULO DA TESE **Avaliação do sistema antioxidante enzimático e não enzimático sobre a ação farmacológica induzida pela sPLA2 de veneno**

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Sumário

RESUMO

A explosão respiratória está fortemente associada ao processo inflamatório, uma vez que diversos compostos antioxidantes estão envolvidos na potencialização ou neutralização deste processo. Diversos peróxidos inorgânicos e orgânicos são produzidos durante esse processo, como os hidroperóxidos de lipídio. Hidroperóxidos de lipídio são estáveis, extremamente reativos e podem induzir a apoptose celular. Tais lipídios modificados podem ser produzidos durante o processo inflamatório induzido pelas fosfolipases A2 (PLA2) que constituem o veneno de serpentes, que possui como consequência de sua atividade catalítica a produção de ácido araquidônico pela quebra de fosfolipídios de membrana, e estes então seriam oxidados por espécies reativas de oxigênio (ROS), formando os hidroperóxidos araquidônicos, que agravam o quadro inflamatório. Deste modo, nesse trabalho avaliamos o efeito protetor de compostos polifenólicos e extratos ricos em tais compostos de diferentes espécies vegetais no curso da inflamação e miotoxidade e no efeito inflamatório pró-oxidante induzido pela PLA2 purificada de veneno crotálico e botrópico. Nossos resultados mostraram que diversos compostos polifenólicos são capazes de diminuir os efeitos provocados pela PLA2, interagindo diretamente, inibindo a enzima e possivelmente atuando na captura de ROS.

Palavras-chave: tanino, quercetina, edema, metabólitos secundários, envenenamento.

ABSTRACT

Respiratory burst is strongly associated with inflammatory process, since several antioxidant compounds are involved in potentiation or neutralization of this process. Various inorganic and organic peroxides are produced during this process such as lipid hydroperoxides. Lipid hydroperoxides are stable, highly reactive and can induce cellular apoptosis. Such modified lipids can be produced during the inflammatory process induced by phospholipases A2 (PLA2) which are present in snake venom, and produces, because of its catalytic activity, arachidonic acid by the breakdown of membrane phospholipids, and these would then be oxidized by reactive oxygen species (ROS), forming the arachidonic hydroperoxides, which aggravates inflammation. Thus, in this work we evaluated the protective effect of polyphenolic compounds and extracts rich in such compounds of different plant species throughout the inflammation and myotoxicity and in pro-oxidant effect induced by PLA2 purified from bothrops and crotalus venom. Our results showed that polyphenolic compounds can decrease effects caused by PLA2, by directly interaction, inhibiting the enzyme and possible acting to capture ROS.

Key words edema**:** tannin, quercetin, edema, secondary metabolites, poisoning.

CAPÍTULO I
Introdução

1. Considerações gerais

Em âmbito mundial, o envenenamento ofídico é um problema de saúde pública importante em áreas rurais dos países tropicais e subtropicais, incluindo países da África, Ásia, Oceania e América Latina. No caso do Brasil, os acidentes ofídicos passaram a ser notificados, regularmente, ao Ministério da Saúde (MS) brasileiro somente a partir de 1986 (WHO, 2010). Tais acidentes são considerados como doença ocupacional que ocorre principalmente em períodos de intensa atividade humana no campo, acometendo diversos trabalhadores rurais, principalmente em países em desenvolvimento, onde o acesso ao soro antiofídico é precário ou até mesmo inexistente (WARRELL, 2010; LEMOS et al., 2009). Os principais sintomas são complicações sistêmicas com duração de horas ou até dias após o envenenamento, como insuficiência renal, necrose, hemorragias e problemas respiratórios (BOCHNER & STRUCHINER, 2003; AMARAL et al., 1991).

O Brasil é o país da América do Sul com maior índice de envenenamentos registrados, aproximadamente 25 mil casos de acidentes com cascavéis por ano (MINISTÉRIO DA SAÚDE, 2011), e por isso esses acidentes são considerados um grave problema de saúde pública não somente pela frequência em que ocorrem, mas também pela morbidade e mortalidade que ocasionam (WARRELL, 2010). Só no Brasil, no ano de 2002 foram registrados pela FUNASA (Fundação Nacional de Saúde – Ministério da Saúde) 158.495 casos de envenenamento por serpentes. Em estudos recentes de 2012, o Ministério da Saúde notificou 139.772 acidentes ofídicos e que são compatíveis aos de 2002. De modo geral, o gênero *Bothrops* (jararaca) é o que apresenta o maior número de acidentes, cerca de 75% dos casos registrados e com letalidade 0,31%. Em segundo lugar aparecem os acidentes com *Crotalus* (cascavel), sendo cerca de 6,5% dos casos e com letalidade de 1,87%, em terceiro o gênero *Lachesis* (surucucu) com 1,15% dos casos e letalidade de 0,95% e o gênero *Micrurus* (coral verdadeira) com 0,35% dos casos e com letalidade de 0,52% (FONSECA, 2012).

Contudo, esses números estão subdimensionados devido ao problema da subnotificação, devido ao fato de que muitos destes acidentes ocorrem entre os moradores de áreas rurais que têm dificuldade no acesso aos serviços de saúde (CALVETE et al., 2009). Neste contexto, como nova política global, em 2009 a Organização Mundial de Saúde (OMS) passou a considerar o acidente ofídico

como uma Doença Tropical Negligenciada (DTN) com o objetivo de garantir uma maior visibilidade e, consequentemente, estimular a elaboração de políticas públicas, visando diminuir os problemas decorrentes do envenenamento causado pelas serpentes.

Já foi demonstrado que as fosfolipases A2 secretórias (sPLA2) de veneno de serpentes crotálicas e botrópicas são potencialmente pró-inflamatórias e essa atividade envolve a produção de ácido araquidônico e biossíntese de eicosanoides, utilizando os mesmos mecanismos catalíticos e receptores de sPLA2 não pancreática de mamíferos (LÄTTIG et al., 2007; OLIVEIRA et al., 2005; TOYAMA et al., 2009). Estudos realizados por Valente e colaboradores (1998) mostraram que sPLA2 de *Crotalus durissus terrificus* podem modular a atividade metabólica de mitocôndrias isoladas, elevando o consumo de oxigênio e aumento da respiração celular, o que promove mudanças da estrutura das mitocôndrias isoladas (VALENTE et al., 1998). Adicionalmente, a ação pró-inflamatória induzida por sPLA2 de mamíferos são potentes indutores da atividade oxidativa na célula, aumentando o nível de espécies reativas de oxigênio (ROS). De acordo com estas observações, trabalhos realizados por Sampaio e colaboradores (2003) demonstraram que o veneno de *C. durissus terrificus* pode levar a um aumento da produção de óxido nítrico (NO•) e peróxido de hidrogênio (H_2O_2).

A soroterapia tradicional é capaz de neutralizar os efeitos tóxicos sistêmicos, impedindo muitas vezes a morte de pacientes, porém a ação do antiveneno não é imediata, não conseguindo neutralizar de forma rápida as proteínas do veneno de serpentes relativas aos danos teciduais, e o seu efeito é sentido após certo intervalo de tempo (PICOLO et al., 2002). Esta eficiência depende da administração imediata de antiveneno adequado, tempo de administração e da quantidade de anticorpos presentes (HIFUMI et al., 2015). Além disso, a soroterapia apresenta problemas, tais como induzir reações adversas como reações cutâneas, broncoespasmo, edema de glote, hipotensão (anafiláticos), febre, erupção cutânea e artropatia (reações tardias) e ineficácia na neutralização dos danos teciduais locais (GUTIERREZ et al., 2009; ALMEIDA et al., 2012). Alguns desses fatores podem ser pelo fato dos anticorpos não conseguirem neutralizar os efeitos fisiopatológicos decorrentes do aumento do estresse oxidativo celular induzido pelas toxinas do veneno de serpentes, além

das reações alérgicas contra o próprio soro. Ademais, estudos recentes demonstram que extratos de plantas encapsuladas em nano partículas podem reduzir a letalidade e toxicidade do veneno, sugerindo que o aumento do estresse oxidativo celular tem um papel crucial na evolução da patologia induzida pelo veneno (SAHA et al., 2015).

Durante a inflamação, que pode ser causada por ação conjunta das sPLA2 e serino proteases, mastócitos e leucócitos são recrutados para o local danificado (quimiotaxia) levando a uma “explosão respiratória” caracterizada pelo alto consumo de oxigênio e a acumulação de ROS como o radical ânion superóxido (O2-•) e peróxido de hidrogênio (H_2O_2), espécies que podem gerar o radical hidroxila (•OH) de forma direta ou indireta por meio de reações químicas, como as de Fenton e Harber Weiss (HALLIWELL & GUTTERIDGE, 1992). Os ácidos nucleicos, proteínas e lipídios são alvos importantes das ROS, e seu ataque pode levar a um aumento no risco de mutagênese devido à modificação no DNA. Neste contexto, os organismos necessitam de uma efetiva proteção contra tais moléculas, para tal, utiliza antioxidantes enzimáticos (superóxido-dismutase, glutationa-peroxidase, catalase e peroxirredoxinas) e não enzimáticos (glutationa, vitaminas C e D) (GOZAL & KHEIRANDISH-GOZAL, 2008).

As células do processo inflamatório sintetizam, por sua vez, mediadores solúveis, como metabólitos de ácido araquidônico, citocinas e quimiocinas, o que leva ao recrutamento de mais células envolvidas no processo inflamatório para o local lesionado, aumentando deste modo a produção de ROS, fechando um feedback pró-inflamatório. Além disso, esses mediadores-chave podem ativar cascatas de transdução de sinal, induzir mudanças nos fatores de transcrição como os fatores de transcrição nuclear K-β (NFK-β) e o transdutor de sinal/ativador de transcrição 3 (STAT 3), entre outros, que medeiam a resposta ao estresse celular. Ademais, com a indução da COX-2, relatou-se a síntese de óxido nítrico pela enzima óxido nítrico sintetase induzível (iNOS), grande expressão do fator de necrose tumoral (TNF), interleucina-1 (IL-1), interleucina-6 (IL-6) e alterações na expressão de micro-RNAs específicos. Este ambiente oxidativo/inflamatório prolongado danifica as células vizinhas saudáveis e, durante um longo período, pode levar a carcinogênese (REUTER et al., 2010). Cabe ressaltar que apesar do NO• gerado pela iNOS ser um importante sinalizador celular, em um ambiente com estresse oxidativo ele pode reagir com o O2-•,

gerando peroxinitrito (NOO-) o qual pode ser bastante danoso à célula (SZABÓ et al., 2007). De fato, alguns autores sugerem que impedir a formação do NOO- ou sua decomposição eficiente em processos inflamatórios pode resultar em uma nova frente terapêutica para o tratamento de processos inflamatórios (SZABÓ et al., 2007). O estresse oxidativo, além de seu papel na inflamação, também tem uma grande importância no processo mionecrótico sendo considerado um agente potencial para destruição das fibras musculares (TONON et al, 2012).

1.2. Compostos polifenólicos de plantas e o processo inflamatório

Substâncias de origem vegetal, pertencentes às mais diversas classes químicas, possuem atividade anti-inflamatória comprovada cientificamente, como os terpenos e terpenoides, taninos, alcaloides, ligninas, saponinas, cumarinas e flavonóides (NATÉRCIA, 2005). A descoberta de novos fármacos a partir de plantas conduziu ao isolamento de muitas substâncias que ainda hoje são utilizadas clinicamente ou então serviram como protótipos para a síntese de novos fármacos (KATIYAR et al., 2012). Estima-se que 40% dos medicamentos disponíveis na terapêutica atual foram desenvolvidos a partir de fontes naturais: 25% de plantas, 13% de microrganismos e 3% de animais. Estes produtos têm sido frequentemente utilizados pelos humanos para combater doenças causadas pelos mais diversos patógenos (CALIXTO, 2005).

Vários trabalhos mostram que compostos polifenólicos, podem auxiliar as defesas antioxidantes, a fim de neutralizar os efeitos dos ROS (SANDERS et al., 2001; COSKUN et al., 2005; ZHANG et al, 2011). Diversos estudos *in vitro* mostram que os flavonoides, uma dentre muitas classes de compostos fenólicos, podem atuar como anti-inflamatórios por vários mecanismos, incluindo o sequestro de ROS, inibição de enzimas relacionadas ao processo de geração de ácido araquidônico, regulação de células inflamatórias e modulação da produção e expressão gênica de moléculas pró-inflamatórias (NAVARRO-NUNEZ et al., 2008 e GARCIA-LAFUENTE et al., 2009). A quercetina é um dos flavonoides mais abundantes no reino vegetal (MARTÍNEZ-FLOREZ et al.,2002) e sua atividade anti-inflamatória e antioxidante já foi elucidada em outros estudos (COMALADA et al, 2005; ZHANG et al, 2011).

Até o presente momento, já se sabe que o poder antioxidantes de diversos compostos polifenólicos de plantas podem reagir com ROS e neutralizá-los; e diversos trabalhos de nosso grupo também mostram que compostos polifenólicos de plantas, mas especificamente os flavonoides também podem interagir com as sPLA2, diminuindo de forma significativa a atividade enzimática destas enzimas (XIMENES et al., 2012; TOYAMA et al., 2014a & b), isso também pode ser aplicável às fosfolipases A2 citosólicas (cPLA2), que possuem a mesma configuração de sítio ativo das sPLA2. Os nossos estudos mostram que determinados compostos polifenólicos de plantas podem também induzir uma significativa mudança em nível de estrutura secundária, afetando principalmente as estruturas em alfa-hélice (BELCHOR et al., 2017).

Algumas patologias incluindo doenças cardiovasculares, aterosclerose, distúrbios imunes, tumoral e mais recentemente as doenças neurodegenerativas tem como pano de fundo o processo inflamatório e as sPLA2 apresentam-se como um potencial agente indutor destas doenças (CUNNINGHAM et al., 2006, CHALIMONIUK, 2012 e CHEN et al., 2012) e ao interagirem com determinadas drogas anti-inflamatórias podem se desnovelar e expor regiões moleculares da sPLA2 que induzem a formação de agregados e precipitados proteicos. Estudos recentes mostram que em determinadas condições, alguns compostos podem induzir mudanças no comportamento cinético das sPLA2, seguido por uma mudança conformacional. Quando a concentração local de PLA2 é elevada, ocorre a formação de oligômeros de baixo peso molecular que com o tempo formam fibras amiloides e precipitados celulares que atrapalham o funcionamento celular correto prejudicando o tecido e o organismo (CODE et al., 2008).

Os flavonoides apresentam atividade antioxidante, uma vez que sequestram ROS e quelam íons metálicos. Esta propriedade atua sobre o radical hidroxil (•OH) e o ânion superóxido (O2-•), espécies reativas envolvidas na iniciação da peroxidação lipídica. São conhecidos cinco mecanismos que possibilitam a propriedade antioxidante dos flavonoides: opressão da formação de espécies reativas do oxigênio a partir da inibição de enzimática da ciclooxigenase, lipoxigenase ou xantina oxidase; quelação de íons metálicos que iniciam a produção de radicais hidroxila pela reação de Fenton ou Harber-Weis; sequestro de radicais livres; proteção das defesas antioxidantes por induzir a fase II de enzimas como a glutationa transferase, aumentando a excreção de espécies

oxidadas; indução de enzimas como a metalotioneína que age como quelante de metais (BEHLING et al., 2004).

A atividade antioxidante de flavonoides pode ser utilizada também como terapia complementar no tratamento de câncer, uma vez que estes compostos podem reduzir os efeitos colaterais da quimioterapia e radioterapia por reduzir sua toxicidade, além de auxiliar na redução do tamanho do tumor e no aumento da longevidade dos pacientes. Em relação à prevenção, os flavonoides são importantes pois podem inibir a carcinogênese química (geração de radicais livres endógenos, radiação ultravioleta e raio-X) e biológica (relativo à bactéria *Helicobacter pylori* que causa úlcera estomacal e está associada ao desenvolvimento de câncer no estômago). Entretanto, estas atividades encontradas nos flavonoides são provavelmente provenientes da ação sinérgica dos diversos compostos polifenólicos encontrados nos extratos das plantas (BEHLING et al., 2004; MACHADO et al.,2008)**.**

Em relação às doenças cardiovasculares, os flavonoides revelam uma proteção devido à diminuição da concentração sérica de LDL e inibição da agregação plaquetária. Além disso, a manutenção da integridade vascular é possivelmente dada pela inibição da peroxidação lipídica, atividades da lipoxigenase, da ciclooxigenase e da fosfolipase A2, bem como a inibição da oxidação da LDL, da agregação plaquetária e promoção da vasodilatação (BEHLING et al., 2004). Estudos revelam que flavonoides extraídos da berinjela (*Solanum melongena)* e extrato de tangerina apresentam diminuição nos níveis de colesterol total e triacilgliceróis no sangue (MACHADO et al.,2008). Além disso, flavonoides encontrados no vinho tinto e suco de uva tinto mostram uma diminuição na oxidação do colesterol LDL, redução da pressão arterial, assim como uma melhoria nos fatores de risco relacionados ao desenvolvimento da aterosclerose (GIEHL et al.,2007). A busca por novos compostos polifenólicos tangem principalmente novos compostos anti-inflamatórios e mais especificamente contra a atividade das sPLA2 e cPLA2 (LÄTTIG et al., 2007, MAHALKA e KINNUNEN, 2013, QUACH et al., 2014).

1.3 Relevância do trabalho

Neste contexto, esta tese pretende contribuir, buscando ampliar o conhecimento do mecanismo de ação das sPLA2 de veneno, proteína com proeminente ação tóxica durante o envenenamento. Além disso, mostrar as interações dos compostos naturais polifenólicos com a molécula da sPLA2 que podem não seguir um padrão semelhante já que a presença de glicosilações, metilações, entre outras podem aumentar significativamente a inibição da atividade enzimática da sPLA2 (XIMENES et al., 2012 e TOYAMA et al., 2014). Nos trabalhos a seguir mostramos a importância de compostos antioxidantes exógenos para a neutralização dos níveis de ROS, uma vez que em algumas patologias é observado que os sistemas antioxidantes endógeno pode ser suplantado pela produção de ROS.

2. Objetivos do trabalho

Analizar o papel de diferentes compostos polifenólicos de fontes naturais durante o curso da inflamação causada por sPLA2 de veneno botropico e cotrálico e suas interações com tal enzima.

2.1 Objetivos especificos

Verificar o potencial inibitório in vivo e in vitro da quercetina e de alguns derivados metilados na ação da sPLA2 de *Bothrops jararacussu*.

Verificar os feitos da apigenina 8-C-rhamnosil, flavonol isolado da *Peperomia obtusifolia* sob as enzimas fosfolipase A2 secretória, fosfolipase A2 citosólica e COX-2.

Verificar os feitos da casuaractina, tanino extraido da folha de *Laguncularia racemosa* sob fosfolipase A2 de *Crotalus durissus terrificus*.

CAPÍTULO II
Artigos científicos

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Evaluation of Rhamnetin as an Inhibitor of the Pharmacological Effect of Secretory Phospholipase A2.

Novo Belchor M[1,2], Hessel Gaeta H[3], Fabri Bittencourt Rodrigues C[4], Ramos da Cruz Costa C[5], de Oliveira Toyama D[6], Domingues Passero LF[7], Dalastra Laurenti M[8], Hikari Toyama M[9].

Abstract

Rhamnetin (Rhm), 3-*O*-methylquercetin (3MQ), and Rhamnazin (Rhz) are methylated derivatives of quercetin commonly found in fruits and vegetables that possess antioxidant and anti-inflammatory properties. Phospholipase A2 (PLA2) displays several important roles during acute inflammation; therefore, this study aimed at investigating new compounds able to inhibit this enzyme, besides evaluating creatine kinase (CK) levels and citotoxicity. Methylated quercetins were compared with quercetin (Q) and were incubated with secretory PLA2 (sPLA2) from *Bothrops jararacussu* to determine their inhibitory activity. Cytotoxic studies were performed by using the J774 cell lineage incubated with quercertins. In vivo tests were performed with Swiss female mice to evaluate decreasing paw edema potential and compounds' CK levels. Structural modifications on sPLA2 were made with circular dichroism (CD). Despite Q and Rhz showing greater enzymatic inhibitory potential, high CK was observed. Rhm exhibited sPLA2 inhibitory potential, no toxicity and, remarkably, it decreased CK levels. The presence of 3OH on the C-ring of Rhm may contribute to both its anti-inflammatory and enzymatic inhibition of sPLA2, and the methylation of ring A may provide the increase in cell viability and low CK level induced by sPLA2. These results showed that Rhm can be a candidate as a natural compound for the development of new anti-inflammatory drugs.

KEYWORDS:

Bothrops jararacussu; anti-inflammatory; methylated quercetins; phospholipase A2; rhamnetin

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Article

Evaluation of Rhamnetin as an Inhibitor of the Pharmacological Effect of Secretory Phospholipase A2

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Abstract: Rhamnetin (Rhm), 3-*O*-methylquercetin (3MQ), and Rhamnazin (Rhz) are methylated derivatives of quercetin commonly found in fruits and vegetables that possess antioxidant and anti-inflammatory properties. Phospholipase A2 (PLA2) displays several important roles during acute inflammation; therefore, this study aimed at investigating new compounds able to inhibit this enzyme, besides evaluating creatine kinase (CK) levels and citotoxicity. Methylated quercetins were compared with quercetin (Q) and were incubated with secretory PLA2 (sPLA2) from *Bothrops jararacussu* to determine their inhibitory activity. Cytotoxic studies were performed by using the J774 cell lineage incubated with quercertins. In vivo tests were performed with Swiss female mice to evaluate decreasing paw edema potential and compounds' CK levels. Structural modifications on sPLA2 were made with circular dichroism (CD). Despite Q and Rhz showing greater enzymatic inhibitory potential, high CK was observed. Rhm exhibited sPLA2 inhibitory potential, no toxicity and, remarkably, it decreased CK levels. The presence of 3OH on the C-ring of Rhm may contribute to both its anti-inflammatory and enzymatic inhibition of sPLA2, and the methylation of ring A may provide the increase in cell viability and low CK level induced by sPLA2. These results showed that Rhm can be a candidate as a natural compound for the development of new anti-inflammatory drugs.

Keywords: rhamnetin; methylated quercetins; phospholipase A2; anti-inflammatory; *Bothrops jararacussu*

1. Introduction

Secretory phospholipases A2 (sPLA2) are proteins with a molecular mass of approximately 13 to 20 kDa. They are widely found in various animals, such as mammals, and in the venom of snakes, and they are almost exclusively calcium dependent. These enzymes have the same structural elements for enzymatic catalysis and have significant structural similarities among themselves [1–4]. sPLA2 and the calcium-dependent cytosolic PLA2 have the same enzyme triad, which allows these enzymes to hydrolyze membrane glycerophospholipids to produce arachidonic acid and lysophospholipids. Excessive hydrolysis of membrane phospholipids by activated sPLA2 may lead to altered membrane function, leading to functional membrane failure and cell death. In addition, free fatty acids and lysophospholipid hydrolysis products are precursors of bioactive pro-inflammatory mediators, such as eicosanoids and the platelet-activating factor (PAF) [4]. In particular, sPLA2 in snake venom is an

attractive therapeutic target due to its accessibility, ease of purification and, in the case of sPLA2 in humans, high levels of systemic enzymatic activity, which characterize and contribute to most of the inflammatory disorders, including neurodegenerative diseases. Therefore, the discovery of new inhibitors of sPLA2 is essential to produce new safe and effective drugs with fewer side effects compared to the current therapy [5,6].

Flavonoids are a class of special metabolites from plants, characterized by the flavan nucleus. These compounds are widely distributed in the leaves, seeds, bark, and flowers of plants; in addition, a recent estimate showed that human's intake ranges from 26 mg to 1 g per day [7]. Quercetin (Q) is a flavonol widely found in tea (*Camellia sinemensis*), wine, beer, fruits, and vegetables [8]. Additionally, this compound exhibits antioxidant and anti-inflammatory activities [9]. A methylated derivative of Q shows lipophilicity, which promotes the compound's access to the cell. *Achyrocline satureioides*, *Coriandrum sativum*, and *Rhamnus petiolaris* are species that show the presence of 3-*O*-methylquercetin (3MQ), rhamnetin (Rhm), and rhamnazin (Rhz), respectively, and have anti-inflammatory activities. In all of these cases, there is no evidence of the effect of methylated quercetins, such as Rhm, against inflammation mediated by sPLA2 and the consequent mobilization of arachidonic acid metabolism or the possible side effects, including cytotoxic activities [10–12]. Considering the Q activities, this work aimed at evaluating 3MQ, Rhm, Rhz, and Q regarding this anti-inflammatory function, besides analyzing their cytotoxic activities toward the J774 cell lineage and evaluating the creatine kinase (CK) levels. In addition, the methylation of free hydroxyl groups in flavones results in more metabolically-stable derivatives with superior membrane-penetrating properties and, thus, vastly improves bioavailability, which should improve their ability to act inside cells [13]. The anti-inflammatory effects of Rhm by modulation of kinases, oxidative stress, and suppression of pro-inflammatory mediators have already been observed [11], however, there are no studies that reveal sPLA2's inhibitory action of this compound and the relation of the structure and function with other quercetin derivatives. Thus, in this study, the inhibitory potential of Q and their methylated derivatives in vitro and in vivo were evaluated against sPLA2 from *Bothrops jararacussu*. To find safe and effective compounds, cytotoxic effects were evaluated using the cell lineage J774 as a model for cytotoxicity and CK levels were assayed as a model of acute toxicity in vivo.

2. Results

2.1. Purity of sPLA2

sPLA2 purity was evaluated by high-performance liquid chromatography (HPLC). Figure 1 shows the chromatographic profile of the eluted native sPLA2 from *Bothrops jararacussu* with a retention time of 22.71 min.

2.2. Enzymatic, Circular Dichroism, Edema, and Myotoxic and Cytotoxic Effects

2.2.1. Enzymatic Assay

All assays were conducted using sPLA2 from *Bothrops jararacussu* that was previously incubated with each compound (Figure 2A); under these conditions, 3MQ, Rhm, Rhz, and Q in the enzymatic assay revealed that, among the methylated quercetins, Rhz showed higher inhibition than 3MQ and Rhm. The inhibitory activity of Rhz was similar to Q. Despite the fact that Rhz showed methylation in the A ring and B ring, the presence of 3-OH in the C ring was likely a common group in Rhm, Rhz, and Q flavonols, leading to sPLA2 inhibition (Figure 1B). However, Rhz exhibits a higher inhibition than Rhm due the presence of the methylated group on the B ring. A greater interaction with sPLA2 was observed in Q, showing more inhibition among the quercetins studied. In addition, analysis of the results presented in Figure 2B,A showed that replacement of the OH group in the C ring by a methyl group almost completely abolished the inhibitory capacity of 3MQ.

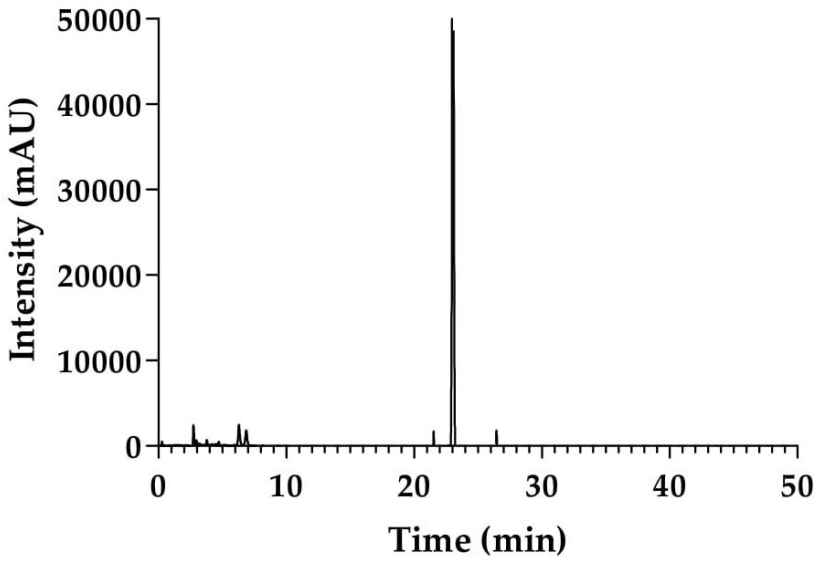

Figure 1. sPLA2 purity profile by high-performance liquid chromatography (HPLC). The enzyme purification was performed with a TSKgel SP-5PW (7.5 cm ID × 7.5 cm·L) using a non-linear gradient with buffer B (ammonium bicarbonate 1.0 M) at a constant flow rate of 1.0 mL/min. The active sPLA2 underwent a new chromatographic step on a reverse phase HPLC using a C5 semi-analytical column. In this step, the chromatographic column was pre-equilibrated with buffer A (0.1% TFA) for 30 min at a flow rate of 1 mL/min. Samples of sPLA2 (1 mg) were dissolved in 250 µL of buffer A and separated using the chromatographic column. Elution of the sPLA2 was performed with a continuous linear gradient of buffer B (66% acetonitrile in 0.1% TFA), and the monitoring of the chromatographic profile was at 280 nm.

2.2.2. Circular Dichroism

Modifications in sPLA2 were evaluated via circular dichroism, and the effects in the α-helix and β-sheet are observable in Table 1. Figure 2C shows the changes as a result of the interactions of quercetins with sPLA2, which occurred mainly in the region corresponding to the α-helices. These results reveal the changes in the secondary structure of this protein due to the interaction with 3MQ, Rhm, Rhz, and Q (Figure 2C).

Table 1. Variation of the α-helix and β-sheet percentages in sPLA2 and in the quercetins incubated with the protein.

	sPLA2	3MQ	Rhm	Rhz	Q
α-helix	45.30%	26.80%	27.00%	54.70%	23.40%
β-sheet	14.80%	18.30%	18.00%	13.40%	18.80%

2.2.3. Paw Edema Assay of Chemically-Treated sPLA2

All quercetins showed a significant inhibitor potential against sPLA2; thus, to evaluate the co-protection effect, the edema inhibition potential of the quercetins was evaluated. The compounds were administered 30 min before sPLA2 application. All flavonols studied for treatment of sPLA2 strongly decreased the maximum edema peak induced by native sPLA2 from *Bothrops jararacussu*. Moreover, Rhz and Q significantly diminished the edema induced by sPLA2 after 120 min. These results suggest that methylation substituents in the A and B ring and the presence of the 3-OH group in the C ring should play an effective role for the anti-inflammatory effect against edema induced by this native sPLA2 (Figure 2D).

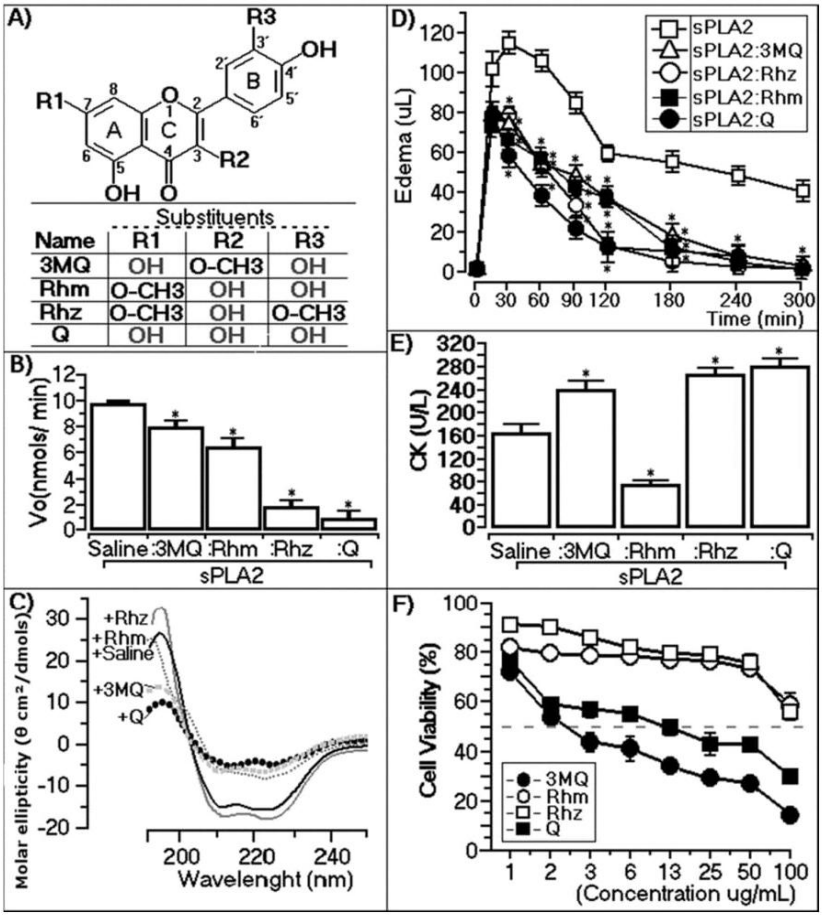

Figure 2. Structures of the quercetins assayed with the enzymatic, circular dichroism, edema, creatine kinase levels, and cytotoxic assays. (**A**) The structures of all flavonols assayed here and R1, R2, and R3 indicated the principal substituents found in all of the flavonols; (**B**) The effect of the previous treatment of the sPLA2 samples on the enzymatic activity after 30 min compared with native sPLA2. 3MQ, Rhm, Rhz, and Q. Each column represents the mean and SD of six replicates ($n = 6$), and the asterisk (*) means $p < 0.05$; (**C**) CD spectra of isolated sPLA2 (saline) and all quercetins incubated with the enzyme. Data over the range of 190–280 nm are shown and are expressed in theta machine units in molar ellipticity (θ cm^2/dmols), and each spectrum represents the analysis of three CD runs; (**D**) Graphic of the edema performed after 30 min of administration of the four quercetins evaluated (10 μg protein and 12.5 μg compound). The results are expressed as the mean ± standard deviation ($n = 5$), and the * means $p < 0.05$. We used the ANOVA and Bonferroni a posteriori tests; (**E**) Myotoxic activity of native and pretreated sPLA2 via measurement of the creatine kinase assay. Each column is shown as the mean and SD of $n = 5$ for each sample including saline, and ANOVA was used as the statistic assay with Dunnett as the a posteriori test. The * shows groups with significant differences with sPLA2 ($p < 0.05$); (**F**) To evaluate the cytotoxic potential of all compounds, MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was performed with the J774 macrophages. Cells were incubated previously with each compound, and each point in the cell viability graph represents the mean and SD of 12 experiments.

2.2.4. CK Levels of Chemically-Treated sPLA2

Named bothropstoxin II (BthTX-II), the PLA2 from *B. jararacussu* induced a rapid increase in plasma creatine kinase levels [14]. Thus, if compounds inhibit this enzyme, lower CK levels were observed, indicating less muscle damage. In this way, the CK levels were quantified to compare

the compounds' potential to decrease muscle injury. The results showed different activities for each compound. Q, 3MQ, and Rhz incubated with sPLA2 do not decrease the damage of this enzyme, and among these groups, CK levels were similar. By the other side, sPLA2 previously treated with Rhm potentially decreased CK release (Figure 2E). These data suggest that Rhm-treated sPLA2 neutralizes the muscle injury effect of native sPLA2 from *Bothrops jararacussu*. The structural analysis of all flavonols assayed here shows that the methylation in the A ring of Rhm is important for neutralization of the CK levels.

2.2.5. Cytotoxic Assay of Methylated Quercetins

To evaluate the cytotoxic potential of all compounds, MTT assay was performed with the J774 cell lineage. Figure 1F shows the different cell responses of J774 cell lineage to quercetins. 3MQ and Q revealed a higher toxicity with CC_{50} = 3566 and 10,371 μg, respectively. Otherwise, Rhm and Rhz showed no toxicity toward J774 cell lineage. The analyses of these results suggest that replacement of the 3-OH group with the O-CH3 group in the C ring is involved with cytotoxic effects of 3MQ and Q (Figure 2D).

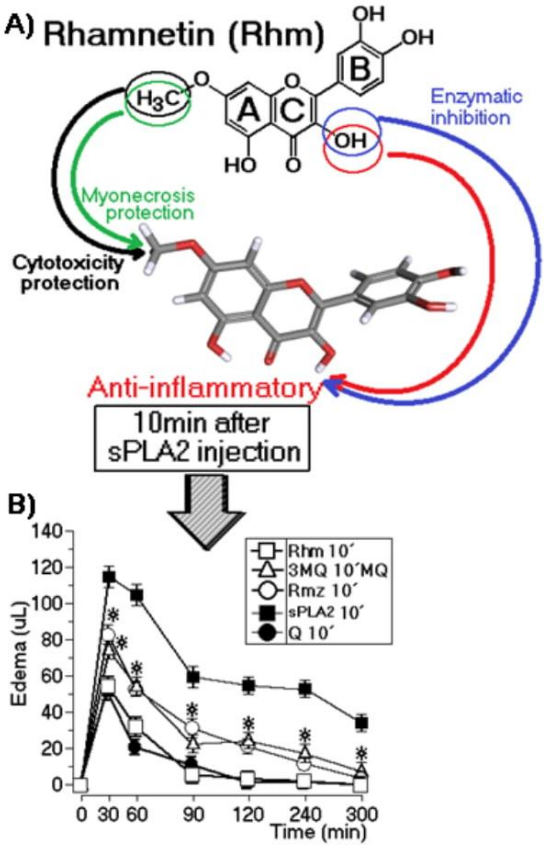

Figure 3. Structure-function relation between molecular regions of Rhm and its activities. (**A**) Molecular structure of Rhm in which the 3-OH in the C ring should lead to enzymatic and pharmacological inhibition and OCH_3 in the A ring should lead to cytotoxic and myotoxic protection; (**B**) Flavonol injection after 10 min of sPLA2 application (10 μg protein and 12.5 μg compound). The results are expressed as the mean ± standard deviation (n = 5), and the * means $p < 0.05$ using the ANOVA and Bonferroni a posteriori tests.

2.3. Edema Assay After Injection of sPLA2

In Figure 1, we summarize the effects of the chemical treatment of sPLA2 with 3MQ, Rhm, Rhz, and Q, and we concluded that Rhm has significant characteristics that include the presence of structural elements that specifically neutralize or strongly diminish the enzymatic activity of the pharmacological effects induced by sPLA2 from *Bothrops jararacussu* and do not induce significant cytotoxic activity. In the second edema assay, we injected each flavonol in the animals group 10 min after injection of sPLA2. Figure 3 shows that Rhm and Q essentially abolished edema induced by sPLA2, and both flavonols eliminated the edema 80 min after injection of sPLA2, whereas 3MQ and Rhz eliminated edema 4 h after injection of sPLA2.

3. Discussion

Plant extracts are characterized by the presence of active compounds and studies reveal the PLA2 inhibitory potential of extracts and isolated compounds. Crude aqueous extracts of *Casearia sylvestris*, *Piper umbellatum*, and *Piper peltatum* show this inhibitory activity [15–20]. Numerous studies have shown the anti-inflammatory and antioxidant activities of flavonoids and Q is widely distributed in vegetables and fruits [8,13]. Its potential and distribution highlight the search for new molecules and the need to understand their structures and functions. The methylation of polyphenolic compounds seems to be the second most significant conjugation reaction from a nutritional and metabolic point of view [21].

There are studies that have evaluated the effect of flavonoids and glycosylated flavonoids on the course of the enzymatic and pharmacological activity of secretory phospholipases A2 [5,6]. Moreover, these studies deserve further efforts to elucidate the mechanism of action of these types of flavonoids on sPLA2, including studies of the conformational changes of flavonoids that modify their antioxidant properties to pro-oxidants, and these mechanisms should be better understood. The chemical structure of polyphenols gives them the ability to act as free radical scavengers. The type of compound and the degree of methylation influence this capacity, and the number of hydroxyl groups is another parameter that determines the antioxidant activity [22]. Thus, within molecular logic, and with substantial data showing the antioxidant effects of various flavonoids, it would be assumed that any protective or neutralizing effect of flavonoids on sPLA2 could be associated with the presence of different numbers of OH moieties in the B ring of the flavonols. These characteristics may contribute to flavonoid's antioxidant activity as well as its toxicity [23,24]. Despite the relevant role of sPLA2 in the course of the inflammatory process, there are not methylated flavonoid studies on the enzymatic and pharmacological activity of sPLA2.

Results presented herein about the treatment of sPLA2 samples in the presence of Q and its derivatives clearly showed that it is not only the hydroxyls present in these flavonoids that are capable of neutralizing the pharmacological and enzymatic activities of sPLA2 from *Bothrops jararacussu*. The combination of methyl groups, the hydroxyl moiety present in the C-3 of the C ring and C-′4 of the B ring are shown to be essential to inhibit the enzymatic activity of sPLA2, as are the groups responsible for the neutralization of the edematogenic effects induced by this sPLA2 in this study. It can be accounted for because the C and B rings of some flavonoids may play a crucial role in the interaction of these compounds with the catalytic site of sPLA2 [5]; however, in the case of Rhm, the presence of the OH group in the C ring is crucial to decrease both the edematogenic and enzymatic activities of sPLA2. Besides that, the hydroxyl group present in the C-′4 also shows an enzymatic inhibitory role. In addition, our results showed that the methyl group present in the A ring of Rhm can be responsible to the absence of cytotoxicity and inhibition of high CK levels induced by sPLA2 from *B. jararacussu* venom. Once macrophages are essential in the primary response in inflammation [25], Rhm is revealed to be a compound that inhibits inflammation without impairing the viability of the body´s natural defence.

One important point is that the methylation of flavonoids may increase their hydrophobicity and, thus, increase the strength of the hydrophobic contacts between the protein and the flavonoid [26,27].

Studies with quercetrin reveal a high inhibition of sPLA2 from *Crotalus durissus terrificus*, possibly due the molecular interactions between the compound and the protein, as hydrogen bonding and hydrophobic interactions. The electrostatic interactions were observed between quercetrin and the amino acid residues Gly 30, Gly 32, His48, and Asp 49 and Ca^{2+} ion of sPLA2 leading to structural changes on the protein These interactions involves amino acids from de Ca^{2+}-binding loop (e.g., Gly 30) and catalytic site (e.g., Asp 49 and His 48) [5]. Thus, besides the essential role of 3-OH to inhibit sPLA2, 4'-OH of Rhm can interact with Gly-30 of the protein leading to a higher decrease of its activity. These data suggest an essential role of hydroxyls on the interactions between the compound and sPLA2 and its following inhibition.

The circular dichroism clearly shows that, in the case of sPLA2, both methylations and the presence of hydroxyls can change the CD spectrum of sPLA2. Q strongly decreased sPLA2 α-helix and shows the higher enzymatic inhibition. Despite Rhz exhibit also a potent inhibition of the enzyme, CD data revels that this compound do not leads strong modifications in sPLA2, conserving the structure. High structure modification is observed in 3MQ, however, this change leads a decrease in the inhibition activity. Rhm interact with the enzyme besides exhibit an inhibitory potential. In this study, the presence of OCH3 on the catechol group increase sPLA2 inhibition, without strongly changing the enzyme structure. Despite the presence of OCH3 on the B ring, leading to a higher inhibition activity than Rhm, this methylation leads to high toxicity and high CK. Thus, 3-OH presence is also associated with sPLA2 inhibition, and methylation on the A ring brings no toxicity and lower CK. Higher interaction with sPLA2 of compounds with an absence of 3-OH was observed in 3MQ in this study and in quercetin [5]. These data are robust enough to show that methylation in the A ring is important to essentially abolish the cytotoxic and muscle damage effects via a free radical neutralizing-dependent pathway.

All flavonoids tested in this work are methylated derivatives of Q, which occurs naturally and is found in several species of phytochemical interest. In the case of Rhm, there is considerable evidence showing that this particular flavonoid exerts its anti-inflammatory activity through two basic mechanisms: via envelope suppression of free radicals and by pro-inflammatory mediators [27]. These results show that the myonecrosis induced by several basic sPLA2s, such as sPLA2 from this work, would involve a strong increase in oxidative cellular stress, and the antioxidant capacity of Rhm would be very interesting for treatment of muscular degeneration induced by this protein. In vivo, all quercetins showed co-protection effects, revealing a decrease in paw edema at all times. The co-protection effect of Q was elucidated in another study [28,29], and its effect on chronic inflammation was higher than hesperidin (glycosylated and methylated quercetin). As it is known, there is a greater potential for aglycone flavonoids in terms of antioxidant activity in relation to the glycosylated forms [28,29]. This lowest potential is due to the hydroxyl substitution at the C-3 position in the C ring with a methylated or glycosylated group as a result of a decrease in OH and the changes in the molecular conformation of hydroxyl group. The flavonols with OH in the C-3 position of the C ring are planar; thus, compounds are able to conjugate and relocate their electrons, as well as increase the radical phenoxyl stability of the flavonoid. Therefore, 3-OH absence results in a twist on the B ring changing the antioxidant potential [24,28,29]. In this study, 3MQ reveals a loss of its sPLA2 inhibition activity; it also shows high cytotoxicity and CK. These data suggest the essential role of methylations in the A ring, the planar conformation's maintenance due to the presence of 3-OH and catechol group, besides the OH interactions between compound and protein to lead an inhibitory potential plus no toxicity against the immune system.

A decrease in paw edema was observed in *Cassia sophera* Linn, and this species has Rhm in its composition. In addition, *Coriandrum sativum* L. and *Prusmus cerasus* L. revealed the presence of Rhm, highlighting the potential of dietary species in phytochemical studies due to their benefits [11,12,30–34]. The suppression of the free radicals and pro-inflammatory mediators of Rhm would possibly inhibit of JNK and p38 MAPK activities by decreasing the COX2 pathway [11]. These pathways are important in the inflammation route, and our work shows that Rhm is beyond this activity; however, Rhm

can both modulate and modify the action of sPLA2, thereby decreasing inflammation not only by maintaining inflammatory stimuli, but by also neutralizing enzymatic and pharmacological effects of sPLA2. In addition, in this work, Rhm and Q abolished the inflammatory effect induced by sPLA2 within 10 min after its injection—i.e., even after the onset of inflammatory process—Rhm and Q were effective for edema neutralization (Figure 2). However, the cytotoxicity results showed that the best choice is Rhm, and the fact that this flavonoid has molecular regions involved in the inactivation of sPLA2 (Figure 2), reveals that this compound inhibits the action of sPLA2 by neutralizing the protein, itself, and protecting the cell from the effects of free radicals. Studies reveal a protection function of catechol moiety of Q, improving cellular uptake, metabolic stability, and lower toxicity of the methylation in Q. Additionally, structural modifications at the 5 or 7 position preserve most of the antioxidant capacity and this feature is observed in Rhm, once methylation occurs at C-7 [35].

Studies reveal that nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in inflammatory diseases. However, these drugs inhibit cyclooxygenase (COX) and COX-1 inhibition leads to gastrointestinal and renal side effects [36]. The enzymatic reaction involved in prostaglandin production by activation of COX is a two-step reaction: cyclooxygenase catalyses the formation of PG from arachidonic acid and a subsequent peroxidase reduces hydroperoxides (PGG2) and activates the cyclooxygenase and the peroxidase activity of COX can also generate reactive oxygen species. Thus, there is no total certainty of inhibiting COX-2 without inhibiting COX-1 and there is still a third group of COX (COX-3), which are inhibited by the same COX-1 inhibitory drugs [37]. It is also important to remember that all COX-2 drugs target the activity of converting arachidonic acid to prostaglandin, but forget that COX is comprised of bifunctional enzymes, where the reactions of bis oxygenase and peroxidase occur at distinct sites, which are structurally and functionally interconnected. NSAIDs bind specifically to the arachidonic acid binding site, but do not inhibit the peroxidase site that remains active, and COX-1 is found in most tissues and is related to the production of prostanoids involved in homeostasis processes in the body [38]. Thus, by looking at the entire spectrum of COX-2, COX-1, and COX-3, the search for an inhibitory drug with inflammatory activity as a function of the specific inhibition of sPLA2 is complex, since the inhibition is not complete and the production of prostanoids is physiological, and COX-1 can also be modulated.

In turn, in this study it is believed that the structure and molecular conformation of Rhm has anti-inflammatory activity and did not induce toxicity to cell lineages, nor to the experimental model; therefore, it can be considered a prototype anti-inflammatory drug.

4. Materials and Methods

4.1. Flavonoids and Reagents

To understand the effects of flavonoids on edema, muscle damage and the cytotoxic effects of 3MQ, Rhm, Rhz, and Q, each of these were obtained commercially from Sigma-Aldrich (St. Louis, MO, USA) All other chemicals, reagents, and kits were purchased from Sigma-Aldrich®, Bio-Rad (Hercules, CA, USA), Cayman Chemical (Ann Arbor, MI, USA), and BIOMOL International (Farmingdale, NY, USA).

4.2. Isolation, Enzymatic Kinetics, and Spectroscopic Evaluation of sPLA2

4.2.1. Purification of sPLA2 from *Bothrops jararacussu*

The dried venom (35 mg) was dissolved in 0.05 M of ammonium bicarbonate buffer (buffer A) followed by centrifugation (4500× *g* for 3 min). The supernatant was studied via an HPLC system Jasco system) coupled to the TSKgel SP-5PW (7.5 cm ID × 7.5 cm·L) Tosoh Bioscience column (Minato-Ku, Japan). sPLA2 was collected using a non-linear gradient with buffer B (ammonium bicarbonate 1.0 M) at a constant flow rate of 1.0 mL/min. The chromatography was monitored at 280 nm, and the fraction obtained was lyophilized and stored at −20 °C. Once the phospholipase A2 enzyme activity of the

corresponding fraction was confirmed, the active sPLA2 underwent a new chromatographic step on a reverse phase HPLC using a C5 semi-analytical column. In this chromatographic step, fractionation of sPLA2 was performed using C5 reverse phase chromatography, and the chromatographic column was pre-equilibrated with buffer A (0.1% TFA) for 30 min at a flow rate of 1 mL/min. Samples of sPLA2 (1 mg) were dissolved in 250 µL of buffer A and separated using the chromatographic column. Elution of the sPLA2 was performed with a continuous linear gradient of buffer B (66% Acetonitrile in 0.1% TFA), and the monitoring of the chromatographic profile was at 280 nm.

4.2.2. Enzymatic Characterization of sPLA2

The inhibitory potential of the quercetins against sPLA2 were observed. The enzyme was dissolved in a 0.9% NaCl saline solution at a final concentration of 1 mg/mL. sPLA2 samples were incubated with the quercetins in microplates for 20 min at room temperature in the presence of a chromogenic substrate for phospholipase (NOBA). The substrate was solubilized in acetonitrile at 1 mg/mL. To evaluate the inhibitory potential of the quercetins, sPLA2 was incubated with 0.25 mg/mL of the compounds for 30 min. Both were added to 0.02 M Tris-HCl, 0.15 M NaCl, and 1 mM $CaCl_2$ (pH 8) buffer for analysis via a SPECTRA MAX spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 405 nm. The measurements were performed throughout the incubation time (90 min) with five-minute intervals between readings. To calculate the percentage of inhibition, the rate of substrate consumption (slope of the line) was calculated using the formula

$$\frac{(Vo_{PLA2} - Vo_{incubated})}{Vo_{PLA2}} \times 100 \quad (1)$$

The data were expressed in percentage, and the error is in percentage points.

4.2.3. Circular Dichroism (CD)

J-815 CD Spectrometer (Jasco, São Paulo, Brazil) was used to evaluate the secondary structure of proteins (alpha helices, beta sheets, turns, and random secondary structures) in solution. It is characterized as a fast and economical method due to the small quantities of samples required. In this technique, polarized light is used in the distal ultraviolet (UV) range (from 180 to 260 nm). This technique allows the evaluation of the structural integrity of proteins, conformational changes, and processes of denaturation (unfolding) and renaturation (folding), making it possible to estimate the composition of the elements in the secondary structure of this macromolecule. The circular dichroism analysis was performed with 0.1 mg/mL of sPLA2, and the compounds were incubated at the same concentration (1:1). The isolated compounds were also used to evaluate the possible interference in the tests. The analysis was performed to eight convolutions, and data were treated by Spectra Manager.

4.3. Pharmacological Assay

4.3.1. Paw Edema Evaluation

In vivo experimental models were performed to evaluate the inhibition of acute inflammation caused by purified sPLA2 using randomly-chosen Swiss female mice (~25 g, $n = 5$). In vivo experiments were performed only after in vitro investigation of the inhibition or interaction activities of the compounds with sPLA2. Mice were injected with 50 µL (0.5 µg/g animal/12.5 µg per animal) of the compounds via peritoneal injection. After 30 min, via right posterior subplantar injection, 20 µL (10 µg) of PLA2 solution ($n = 5$) was inoculated. A saline solution (NaCl 0.9%) was used for negative control. Six groups were made: (1) Saline; (2) sPLA2; (3) sPLA2:3MQ; (4) sPLA2:Rhm; (5) sPLA2:Rhz; and (6) sPLA2:Q. The volume of the paws was monitored using the LE7500 Digital Plethysmometer (Panlab, Harvard Apparatus, Cornellà, Spain) for 4 h. After the tests, the mice were anaesthetized and sacrificed via cervical dislocation. In vivo experiments were performed

according to the institutional rules and they were approved by the ethics committee from UNESP, number 008-CEUA.

4.3.2. CK Level Measurement

In total, 20 μL (2.5 μg) of the compounds previously incubated for 30 min with sPLA2 (n = 5) were injected into the gastrocnemius muscle. After an average of 30 min, the animals' blood was harvested from the tail in a heparinized tube, which was centrifuged and frozen for further analysis. Control groups (n = 5) were submitted to the same procedure as the treated animals but were inoculated with 20 μL of 0.9% NaCl, 20 μL (2.5 μg) of the purified compounds or 20 μL (10 μg) of isolated sPLA2. After the tests, the mice were sacrificed via cervical dislocation. Seric creatine kinase (CK) levels were determine the according to the manufacturer of the kit (Bioliquid, Pinhais, Brazil).

4.4. Cytotoxic Effects

To evaluate the cytotoxic effects of the quercetins, 2×10^5 J774 cells were cultured in R10 medium with 3MQ, Rhm, Rhz, and Q (100.00 to 0.78 μg/mL). As a negative control, macrophages were cultivated in the medium and in DMSO as the vehicle solution (never exceeding 1% *v/v*). After 24 h, the cell viability was analysed via the MTT method. This cell lineage was employed in the present work as a model for cytotoxicity, as previously studied [39,40]. From a biological point of view, this cell lineage can be used as a prediction of the action of studied molecules on the immune system. Cytotoxic concentrations 50% (CC_{50}) was estimated using GraphPad Prism 5.0 software (La Jolla, CA, USA) for plotting and statistical analysis.

4.5. Statistical Analyses

Data are expressed as the means ± standard deviations. The results were analyzed by analysis of variance (ANOVA) of one or two routes followed by the Dunett or Bonferroni a posteriori test. Values of $p < 0.05$ were considered significant.

5. Conclusions

Flavonoid methylations may be extremely interesting for the generation of structural and functional variability. In the case of Rhm, the methylation position on the Q skeleton gave rise to a compound that may be extremely valuable from a therapeutic point of view. This study shows that the methylation position changes the compound´s function and, among the evaluated quercetins, Rhm is the best choice, exhibiting sPLA2 inhibitory activity, no cytotoxicity, and lower CK levels.

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Abbreviations

sPLA2	Secretory phospholipase A2
Q	Quercetin
3MQ	3-O-Methylquercetin
Rhm	Rhamnetin
Rhz	Rhamnazin

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Non-Clinical Studies for Evaluation of 8-C-Rhamnosyl Apigenin Purified from Peperomia obtusifolia against Acute Edema.

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Abstract

Compound 8-*C*-rhamnosyl apigenin (8CR) induced a moderate reduction in the enzymatic activity of secretory phospholipase A2 (sPLA2) from *Crotalus durissus terrificus* and cytosolic phospholipase A2 (cPLA2), but the compound also significantly inhibited the enzymatic activity of the enzyme cyclooxygenase. In vitro assays showed that the compound induced a slight change in the secondary structure of sPLA2 from *Crotalus durissus terrificus* snake venom. In vivo assays were divided into two steps. In the first step, the 8CR compound was administered by intraperitoneal injections 30 min prior to administration of sPLA2. In this condition, 8CR inhibited edema and myonecrosis induced by the sPLA2 activity of *Crotalus durissus terrificus* in a dose-dependent manner by decreasing interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), prostaglandin E2 (PGE2), and lipid peroxidation. This has been demonstrated by monitoring the levels of malondialdehyde (MDA) in rat paws after the course of edema induced by sPLA2. These results, for the first time, show that sPLA2 of *Crotalus durissus terrificus* venom induces massive muscle damage, as well as significant edema by mobilization of cyclooxygenase enzymes. Additionally, its pharmacological activity involves increased lipid peroxidation as well as TNF-α and IL-1β production. Previous administration by the peritoneal route has shown that dose-dependent 8CR significantly decreases the enzymatic activity of cyclooxygenase enzymes. This resulted in a decrease of the amount of bioactive lipids involved in inflammation; it also promoted a significant cellular protection against lipid peroxidation. In vivo experiments performed with 8CR at a concentration adjusted to 200 µg (8 mg/kg) of intraperitoneal injection 15 min after sPLA2 injection significantly reduced sPLA2 edema and the myotoxic effect induced by sPLA2 through the decrease in the enzymatic activity of cPLA2, cyclooxygenase, and a massive reduction of lipid peroxidation. These results clearly show that 8CR is a potent anti-inflammatory that inhibits cyclooxygenase-2 (COX-2), and it may modulate the enzymatic activity of sPLA2 and cPLA2. In addition, it was shown that *Crotalus durissus terrificus* sPLA2 increases cell oxidative stress during edema and myonecrosis, and the antioxidant properties of the polyphenolic compound may be significant in mitigating the pharmacological effect induced by sPLA2 and other snake venom toxins.

KEYWORDS:

Peperomia obtusifolia; Piperaceae; edema; flavonoid; myonecrosis; snake venom phospholipase A2

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Non-Clinical Studies for Evaluation of 8-*C*-Rhamnosyl Apigenin Purified from *Peperomia obtusifolia* against Acute Edema

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Abstract: Compound 8-*C*-rhamnosyl apigenin (8CR) induced a moderate reduction in the enzymatic activity of secretory phospholipase A2 (sPLA2) from *Crotalus durissus terrificus* and cytosolic phospholipase A2 (cPLA2), but the compound also significantly inhibited the enzymatic activity of the enzyme cyclooxygenase. In vitro assays showed that the compound induced a slight change in the secondary structure of sPLA2 from *Crotalus durissus terrificus* snake venom. In vivo assays were divided into two steps. In the first step, the 8CR compound was administered by intraperitoneal injections 30 min prior to administration of sPLA2. In this condition, 8CR inhibited edema and myonecrosis induced by the sPLA2 activity of *Crotalus durissus terrificus* in a dose-dependent manner by decreasing interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), prostaglandin E2 (PGE2), and lipid peroxidation. This has been demonstrated by monitoring the levels of malondialdehyde (MDA) in rat paws after the course of edema induced by sPLA2. These results, for the first time, show that sPLA2 of *Crotalus durissus terrificus* venom induces massive muscle damage, as well as significant edema by mobilization of cyclooxygenase enzymes. Additionally, its pharmacological activity involves increased lipid peroxidation as well as TNF-α and IL-1β production. Previous administration by the peritoneal route has shown that dose-dependent 8CR significantly decreases the enzymatic activity of cyclooxygenase enzymes. This resulted in a decrease of the amount of bioactive lipids involved in inflammation; it also promoted a significant cellular protection against lipid peroxidation. In vivo experiments performed with 8CR at a concentration adjusted to 200 μg (8 mg/kg) of intraperitoneal injection 15 min after sPLA2 injection significantly reduced sPLA2 edema and the myotoxic effect induced by sPLA2 through the decrease in the enzymatic activity of cPLA2, cyclooxygenase, and a massive reduction of lipid peroxidation. These results clearly show that 8CR is a potent anti-inflammatory that inhibits cyclooxygenase-2 (COX-2), and it may modulate the enzymatic activity of sPLA2 and cPLA2. In addition, it was shown that *Crotalus durissus terrificus* sPLA2 increases cell oxidative stress during edema and myonecrosis, and the antioxidant properties of the polyphenolic compound may be significant in mitigating the pharmacological effect induced by sPLA2 and other snake venom toxins.

Keywords: *Peperomia obtusifolia*; Piperaceae; flavonoid; snake venom phospholipase A2; myonecrosis; edema

1. Introduction

More recently, plant extracts have been used as alternative anti-venom compounds for the management of snake bites and are used in conjunction with conventional antibody therapies [1,2]. In Brazil, *Crotalus durissus terrificus* venom is responsible for approximately 10% of snake bites and has a high mortality due to the toxic action of crotoxin exhibiting various pharmacological activities, including neurotoxicity, myotoxicity, nephrotoxicity, cardiotoxicity, and inflammation [3,4]. Recent studies have shown that snake venom secretory phospholipase A2 (sPLA2) has a mechanism of action highly like that of human sPLA2s, and the pharmacological activity of sPLA2 involves the generation of arachidonic acid. This may also involve the cross-talk between cytosolic phospholipases A2 (cPLA2) and other enzymes involved in arachidonic acid metabolism and associated with increase of cellular oxidative stress, such as pharmacological events induced by human secretory phospholipase A2 [5–7]. Recent studies show that both cyclooxygenase-2 (COX-2) and cytosolic phospholipase A2 (cPLA2), which are rigorously regulated by several mediators in several species, including several transcription factors activated during the inflammatory process, hydrolyze membrane phospholipids, which results in the release of arachidonic acid (AA), which is further converted by COX-2 and prostaglandin (PG) synthases to biologically-active PGs [8]. The genus *Peperomia* is used as an ornamental species, and others species have been used in folk medicine in some countries due to their anti-tumor, anti-inflammatory, antibacterial, and analgesic activities [9], and phytochemical studies in species of *Peperomia* showed the presence of a wide range of natural compounds, including lignans, polyketides, chromenes and chromanes, quinones, and flavonoids [10–12]. The 8-C-rhamnosyl apigenin (8CR) purified from *Peperomia obtusifolia* is an unpublished compound, and no pharmacological activity has been described for it. What is known about *Peperomia obtusifolia* is that it is a well-known ornamental foliage plant found in Mexico and parts of northern South America, and previous chemical research has demonstrated the presence of various polyphenolic compounds [13,14]. The aim of this study is to investigate the effect of 8-C-rhamnosil apigenin isolated from *Peperomia obtusifolia* on the toxic and pharmacological effects induced by purified secreted phospholipase A2 and on COX-2 and cPLA2.

2. Results

2.1. Structural and Biological Characterization of 8-C-Rhamnosyl Apigenin (8CR)

Peperomia species have been the source of various bioactive compounds, such as aromatic compounds and polyketides [12–17]. Previous studies with *P. obtusifolia* reported the isolation of chromanes, flavonoids, and lignans [18,19]. The n-BuOH phase from the MeOH extract of the aerial parts of *P. obtusifolia* was chromatographed on a Sephadex LH-20 column (Björkgatan 30, 751 84 Uppsala, Sweden), which afforded a phenolic compound characterized by analysis of its spectroscopic data. The UV spectrum revealed characteristic flavone absorptions at 269 and 331 nm. The 1H nuclear magnetic resonance (NMR) spectrum of the compound showed a singlet at δ 6.70 consistent with the H-3 of flavones, and this was supported by the observation of a carbon signal at δ 102.5 associated with the C-3 in its ^{13}C NMR spectrum. The B ring of the flavone was oxygenated only at C-4′ due to the two doublet signals at δ 8.10 (2H, d, J = 9.0 Hz) and 7.10 (2H, d, J = 9.0 Hz) assigned to H-2′/H-6′ and H-3′/H-5′, respectively. This substructure was confirmed through the signals at δ 124.9, 130.4, 115.8, and 164.7 obtained from the ^{13}C NMR spectrum and assigned to C-1′, C-2′/C-6′, C-3′/C-5′, and C-4′, respectively. An additional singlet at δ 6.52 bonded to a carbon atom at δ 96.7 suggested a penta-substituted A-ring. This signal exhibited a heteronuclear multiple-bond correlation (HMBC) with C5, C7, and C10, which confirmed the location of the aromatic proton at C6.

The presence of two additional doublets at δ 5.04 (1H, d, J = 9.8 Hz) and δ 0.62 (3H, d, J = 6.0 Hz) bonded to carbons at δ 72.2 and δ 18.2, respectively, suggested that the rhamnosyl moiety was C-attached to the flavone. The sugar linkage was determined to be C–C from the relatively upfield anomeric carbon resonances at δ 72.2, in contrast to the anomeric carbons of *O*-glycosides, which normally resonate at approximately δ 100.0 [20]. Thus, the rhamnose was determined to be bonded directly to the C-8 of the flavone. The sugar position linkage was confirmed by observation of the HMBCs between H-1″ and C-7, C-8, C-9, and C-3″. A comparison of the spectroscopic data with those reported in the literature allowed for the identification of the isolated flavonoid as a 5,7,4′-trihydroxyflavone 8-*C*-rhamnoside (8-*C*-rhamnosyl apigenin; 8CR), which is shown in Figure 1A. In Figure 1A, we also present a botanical illustration—*Peperomia obtusifolia*—to illustrate the plant; the figure was captured from the Internet.

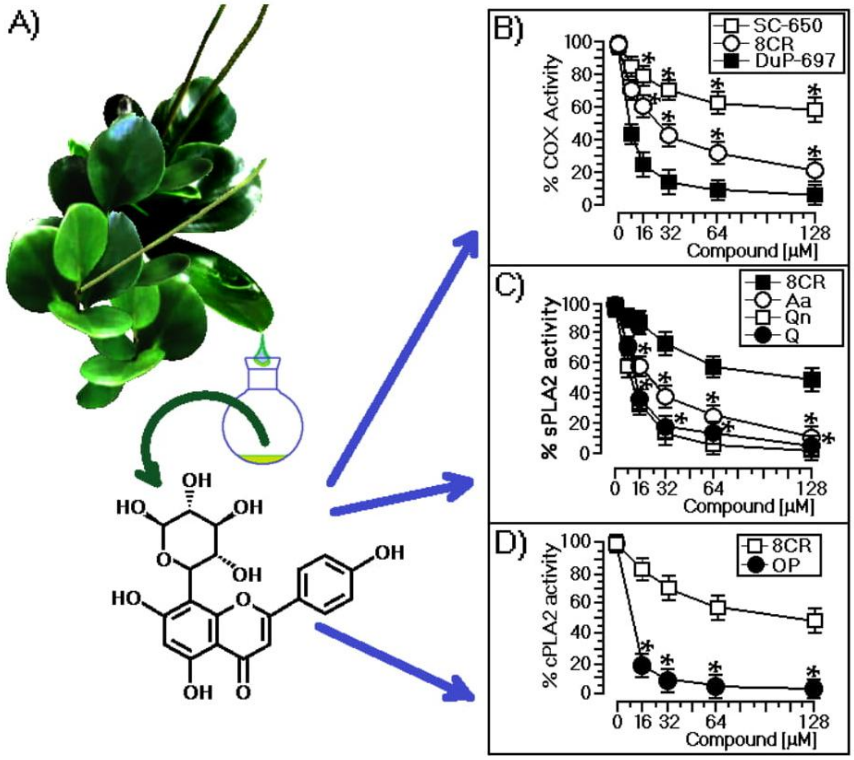

Figure 1. (**A**) Chemical structure of 8-*C*-rhamnosyl apigenin (8CR) isolated from *Peperomia obtusifolia* and the botanical illustration of the plant; (**B**) Comparative effects of 8CR, DuP-697, and SC-560 on cyclooxygenase-1 (COX-1) and COX-2 enzymatic activities. Each point represents the mean ± SEM of six animals in the percentage of remaining enzymatic activity of COX-1 or COX-2 without inhibitors; (**C**) Effects of 8CR, aristolochic acid (Aa), quercetin (Q), and quercitrin (Qn) on the enzymatic activity of sPLA2 isolated from *Crotalus durissus terrificus*; (**D**) The enzymatic effect of COX-2 in the presence of oleyloxyethyl phosphorylcholine (OP) and 8CR. All of the compounds used in this section were dissolved according to the supplier's information by adjusting the concentrations for 128, 64, 32, and 16 μM, and all assays were done with the material provided by the manufacturer. * indicates significant differences relative to a standard. All analyses were performed using analysis of variance (ANOVA, $p < 0.05$), and each bar represents $n = 5$.

In Figure 1B, we showed that 8CR significantly reduced the enzymatic activity of cyclooxygenase and presented an IC_{50} of 28.6 μM, but its activity was lower when compared to the effect of 5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl)-thiophene (DuP-697), which is a selective COX-2 inhibitor like other COX-2 inhibitors and showed an IC50 7.67 μM. In contrast, the 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560) compound

showed low inhibitory activity because it is a highly-selective compound for COX-1. In Figure 1C, 8CR induced only a marginal inhibitory effect on the enzymatic activity of sPLA2, exhibiting IC_{50} of 109.4 μM, whereas other flavonoids, such as quercetin (Q) and quercitrin (Qn), showed IC_{50} values of 13.5 and 9.2 μM, respectively. Commercial inhibitor compound sPLA2 (aristolochic acid, Aa) showed an IC_{50} value of 22.4 μM. The results presented in Figure 1C do not allow us to classify it as a specific inhibitor of sPLA2 compared with other flavonoids that were evaluated. In Figure 1D, we show the effects of 8CR and the oleyloxyethyl phosphorylcholine (OP) compound on the enzymatic activity of the cloned human cPLA2. Both enzymes were subjected to the same assay conditions. 8CR showed an IC_{50} value of 134 μM against the enzymatic activity of cPLA2, but this inhibitory effect induced by 8CR was insignificant when compared with the OP inhibitor.

2.2. Structural Shifts Induced by 8CR on the sPLA2 Molecule

The incubations of *C. d. terrificus* sPLA2 with purified 8CR (mol/mol) were performed according to the procedure described previously [21]. The results of reverse phase HPLC analysis showed that 8CR could form a stable complex with sPLA2, and that 8CR also changed the molecular structure of sPLA2, since the retention time of sPLA2 changed significantly in the presence of 8CR (Figure 2A). Compound 8CR, when incubated with sPLA2, also induced slight changes in the level of the secondary structure. Both sPLA2 and sPLA2:8CR were dissolved in 10 mmol/L sodium phosphate buffers, pH 7.4, and both proteins had their concentrations adjusted to 8.7 mmol/L. Both proteins were subjected to the same conditions of circular dichroism analysis using a J720 spectropolarimeter (Jasco Corp., Tokyo, Japan). Data collection was performed at room temperature with a scanning speed of 100 nm/min. Nine scans were obtained for each sample, and all spectra were corrected by subtracting buffer blanks (Figure 2B). The relative intrinsic fluorescence intensity of native sPLA2 or 8CR-treated sPLA2 was monitored with a Shimadzu spectrofluorimeter. Reaction mixtures of 2.0 mL in a 1-cm path length quartz cuvette consisting of 100 mmol/L Tris–HCl buffer (pH 7.4), sPLA2 (200 μg/mL), and 5 mmol/L calcium. Fluorescence was measured between 300 and 450 nm after excitation at 280 nm (Figure 2C).

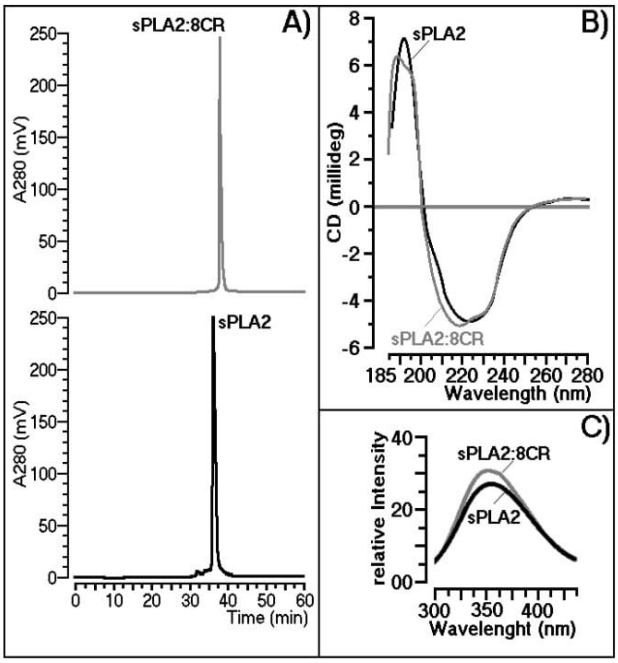

Figure 2. Comparison of the native secretory phospholipase A2 (sPLA2) and sPLA2 treated with 8CR in relation to: (**A**) chromatographic retention times; (**B**) circular dichroism curves and (**C**) fluorescence of the protein.

2.3. Protective Effect of 8CR against the Pharmacological Effects of sPLA2

The results presented in Figure 3A indicate that the edema induced from sPLA2 isolated from *Crotalus durissus terrificus* was strongly reduced in a dose-dependent manner by three different concentrations of 8CR. Acute edema induced by sPLA2 at times of 30 and 60 min were the most affected by a previous intraperitoneal (i.p.) injection of the compound. The sPLA2 isolated from *Crotalus durissus terrificus* showed maximum edema at 30 to 60 min, and exhibited edema values of 0.71 ± 0.018 and 0.77 ± 0.015 mL, respectively. Figure 3A presents edema values of 0.56 ± 0.021 and 0.62 ± 0.012 mL (n = 5) for animals that received 30 µg of i.p. doses of 8CR (1.2 mg/kg) at 30 and 60 min, respectively. Moreover, for animals that received 60 µg of i.p. doses of 8CR (2.4 mg/kg), the edema values at 30 and 60 min were 0.37 ± 0.023 and 0.45 ± 0.01 mL (n = 5, * $p < 0.05$), respectively. In addition, Figure 3A indicates that 180 µg of an i.p. dose of flavonoid (7.2 mg/kg) injected into the animals before the administration of sPLA2 exhibited edema values of 0.21 ± 0.021 mL (n = 5, * $p < 0.05$ at 30 min) and 0.26 ± 0.018 mL (n = 5, * $p < 0.05$ at 60 min). Edema induced by sPLA2 in the animals previously treated with indomethacin (positive control, 10 mg/kg) diminish edema values of 0.25 ± 0.018 mL (n = 5, * $p < 0.05$) at 30 min and 0.32 ± 0.022 mL (n = 5, * $p < 0.05$) at 60 min, respectively.

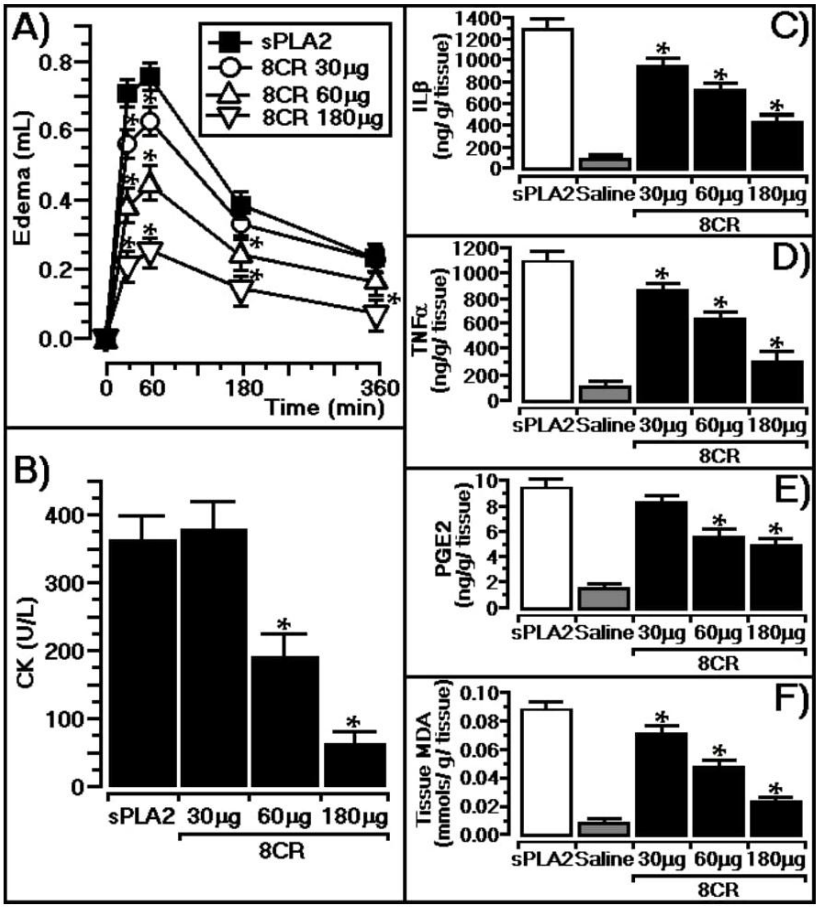

Figure 3. (**A**) Acute edema volumes at different times and administration doses of 8CR; (**B**) the protective effect of the flavonoid on the myotoxic activity induced by sPLA2; and in (**C**–**F**), all animals were treated with 30 µg (1.2 mg/kg), 60 µg (2.4 mg/kg), and 180 µg (7.2 mg/kg) 8CR prior to injection of sPLA2. After experiments, tissue homogenate from each animal was prepared for specific analysis following the procedures described in each protocol supplied by the manufacturers. * indicates significant differences relative to a standard. All analyses were performed using analysis of variance (ANOVA, $p < 0.05$), and each bar represents n = 5.

Figure 3B presents the protective effect of the flavonoid against myotoxic activity induced by sPLA2. The native phospholipase A2 induced an increase in the plasma creatine kinase (CK) values of 368 ± 63 (n = 6), whereas the administration of 8CR at concentrations of 60 µg (2.4 mg/kg) and 180 µg (7.2 mg/kg) effectively decreased the myonecrosis activity induced by the native sPLA2 (CK values of 182 ± 34 and 52 ± 16 U/L, respectively; n = 5, * $p \leq 0.05$; Figure 3B). Indomethacin (10 mg/kg) significantly diminish myonecrosis induced by sPLA2 at 325 ± 23 U/L, respectively; n = 5, * $p \leq 0.05$. In addition, we observed that sPLA2 also induced a significant mobilization of interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and prostaglandin E2 (PGE-2), and significantly increased lipid peroxidation. IL-1β is recognized as a key mediator of inflammation, is an inducible cytokine, and is not generally expressed in healthy cells or tissue. TNFα is a powerful pro-inflammatory agent that regulates many facets of macrophage function. In particular, some studies demonstrated that PGE-2 is an important prostaglandin produced by acute inflammation and enhanced lipid peroxidation, which occurs during oxidative stress and results in the generation of lipid peroxidation end products, such as malondialdehyde (MDA). The results presented in Figure 3C–F show that the previous administration of 8CR in a dose-dependent manner strongly decreases IL-1β, TNFα, PGE-2, and lipid peroxidation, and better results were observed for 8CR concentrations adjusted at 180 µg (7.2 mg/kg) for each animal. Thus, i.p. application of 8CR in different doses induced a dose-response decrease in IL-1β, TNFα, PGE-2, and tissue MDA levels in the paws (n = 5, $p < 0.05$).

2.4. Antiophidian Activity Effect of 8CR against the Pharmacological Effects of sPLA2

The results presented in Figure 4A show that 8CR (8 mg/kg) administered 15 min after injection of sPLA2 reduced edema induced by sPLA2 at 60, 180, and 360 min from 0.81 ± 0.012, 0.47 ± 002 and 0.22 ± 0.01 mL to 0.47 ± 0.021, 0.23 ± 0.018 and 0.21 ± 0.01 mL, respectively (n = 5, * $p \leq 0.05$). 8CR's inhibition of edema was more effective than the anti-venom principally after 30 and 60 min, and indomethacin at all times observed. Figure 4B shows that the administration of 8CR at concentrations of 200 µg (8 mg/kg) decreased the myonecrosis induced by the native sPLA2 (CK values of 0.396 ± 27 U/L) to 0.238 ± 32 U/L (n = 5, * $p \leq 0.05$; Figure 4B).

As shown in Figure 4B, indomethacin (the positive control for edema) showed a marginal effect against myonecrosis induced by native sPLA2, and commercial snake anti-venom did not show this difference with 8CR. After three hours post-injection of sPLA2 and inoculation of 8CR, a group of five animals were sacrificed and swollen hind paw tissue homogenate was subjected to biochemical analysis for determination of COX-2, MDA, PGE-2, and cPLA2 analysis. These biochemical analyses showed that edema and myonecrosis induced by sPLA2 from *Crotalus durissus terrificus* involve prostaglandin overproduction, such as PGE-2, and an increase of oxidative stress. The results presented in Figure 4C–F show that the administration of 8CR 15 min after injection of sPLA2 strongly decreases PGE-2, lipid peroxidation, and COX-2, and significantly inhibited enzymatic activity of cPLA2.

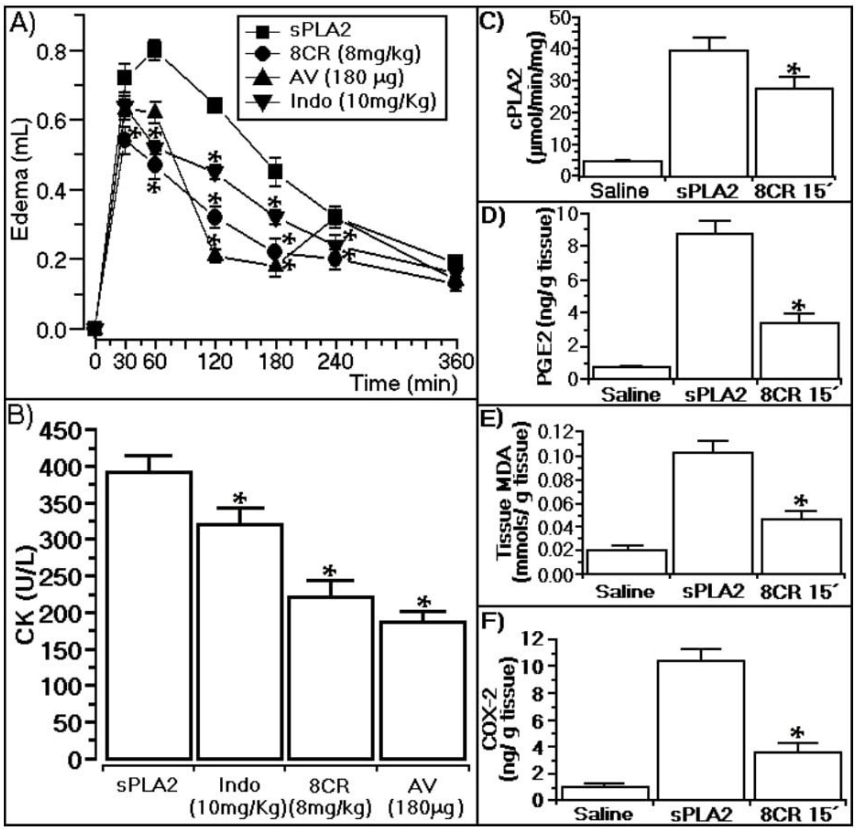

Figure 4. Effects of 8-*C*-rhamnosyl apigenin injected 15 min after the injection of sPLA2 from *Crotalus durissus terrificus*. In (**A**) we observe the anti-inflammatory effect of 8CR that neutralizes the edema induced by sPLA2; In (**B**), we see the protective effect of the flavonoid on the myotoxic activity induced by sPLA2; (**C**–**F**) show the hind paw tissue homogenate quantification of cPLA2, PGE-2, MDA, and COX-2. All biochemical determinations were established by swollen hind paw tissue homogenate analysis of five animals. In (**C**–**E**), each column of biochemical determination represents the mean ± SEM of five animals and * statistically significant differences ($n = 5$, $p < 0.05$). All animals were treated with 200 µg (8 mg/kg) 8CR prior to injection of sPLA2, and after the experiments swollen hind paw tissue homogenate from each animal was prepared for specific analysis following the procedures described in each protocol supplied by the manufacturers. AV (crotalic and bothropic commercial veterinary propose antivenom, Lema-Injex, São Paulo, Brasil). Indo (Indomethacin, Sigma-Aldrich, St. Louis, MO, USA).

3. Discussion

3.1. Structural and Biological Characterization of 8CR

No previous study has described the importance of COX-2 enzymatic activity in the action of sPLA2 from the venom *Crotalus durissus terrificus*. Studies performed by Moreira et al. [22] using animals after intraperitoneal injection of *Bothrops asper* sPLA2 and *Crotalus durissus terrificus* demonstrated that only *Bothrops asper* sPLA2 induced an increase in cytosolic expression, and COX-2 levels by leukocytes. Our results using sPLA2 from *Crotalus durissus terrificus* demonstrated the role and importance of COX-2 during the edema and myonecrosis induced by this protein. In addition, the pharmacological activities of sPLA2 were strongly dose-dependently reduced by 8-C-rhamnosyl apigenin by reducing enzymatic activity and COX-2 expression. Consequently, the ability of COX-2 to synthesize prostaglandins has been shown to be an important factor for edematogenic and myotoxic activity. The results of 8CR treatments demonstrated cross-talk between *Crotalus durissus terrificus* and

COX-2 sPLA2 in both pharmacological activities in edema and myonecrosis. The results presented here showed that 8CR inhibits the COX-2 enzyme more specifically than sPLA2 or cPLA2, but these results did not rule out the possibility that the higher concentrations of 8CR inhibited the enzymatic systems cPLA2 and sPLA2. Thus, 8CR appears to be a good candidate for the development of a new class of drugs that moderately reduces the enzymatic activity of two key molecules, sPLA2 and cPLA2, which may decrease the production of arachidonic acid. In addition, 8CR significantly decreases the production of bioactive prostanoids by COX-2 inhibition.

3.2. Structural Shifts Induced by 8CR on the sPLA2 Molecule

Drugs fail in the clinic for two main reasons: the first is that they do not work, and the second is that they are not safe and a great amount of money was involved. Thus, the pharmaceutical industry and, more recently, some academic centers, have streamlined a number of early processes to identify molecules that possess suitable characteristics to make acceptable drugs, including choice and specific analysis against molecular targets, such as protein [23,24]. In recent years, our groups have focused several efforts to use sPLA2 from *Crotalus durissus terrificus* as a molecular target for the evaluation of the potential application of natural products against acute edema. These investigations involve spectroscopic, chromatographic, enzymatic, and other studies for monitoring the effects of these compounds on the structure and function of sPLA2. Our investigation showed that, in the case of sPLA2 from *Crotalus durissus terrificus* venom, 8CR did not induce significant structural modification, and in the case of sPLA2, the smaller shifts observed did not affect the structure of sPLA2.

3.3. In Vivo Protective Effect of 8CR against the Pharmacological Effects of sPLA2

In Figure 3, all the results clearly show that edema and myonecrosis induced by sPLA2 involve the mobilization of several pro-inflammatory molecules, an increase in the enzymatic activity of cyclooxygenase by stimulation of COX-2 expression, and oxidative stress, which appear to be important elements for sPLA2-induced cellular damage. PGE-2 is one of several products generated by the overexpression of COX2, and has been widely characterized as an important eicosanoid involved in many inflammatory conditions, and may stimulate the generation of hydroxyl radicals and increase lipid peroxidation and cellular apoptosis [25,26]. The quantification of MDA was used to estimate the oxidative damage of cells that could lead to an oxidative attack on polyunsaturated lipids. During acute inflammation, tumor necrosis factor α (TNF-α) and IL-1β are responsible for a wide range of signaling events in cells, which lead to necrosis or apoptosis by increasing cellular oxidative stress [27–29]. Our results clearly showed that i.p. previous to 8CR significantly decreased the mobilization of pro-inflammatory cytokines induced by sPLA2, and significantly decreased the levels of MDA in a dose-dependent manner. The results also suggest that 8CR can inhibit lipid peroxidation and, consequently, the cytotoxic effects induced by sPLA2 from snake venom by decreasing free radical levels in cells and the COX-2 metabolism should be involved in oxidative stress [30].

3.4. Anti-Ophidian Effect of 8CR against the Pharmacological Effects of sPLA2

The results presented in Figure 4 show that 8CR administered intraperitoneally 15 min after sPLA2 injection of *Crotalus durissus terrificus* was able to abolish edema, as well as the myotoxic effect resulting from administration of the toxin. This protective or antiophidic effect of compounds at the dose of 200 μg (8 mg/kg) per animal virtually abolished the COX-2 enzyme activity; this inhibition probably should have induced a lower production of PGE-2, and probably of other pro-inflammatory interleukins. In addition, 8CR was also effective in neutralizing lipid peroxidation, which was indirectly measured by the biochemical quantification of MDA from swollen hind paw tissue homogenate aliquots. On the other hand, these results were obtained with the compound being administered at a dose of 200 μg (8 mg/kg) of 8CR, which also inhibited the activity of cPLA2. Thus, 8CR neutralized the effect of *Crotalus durissus terrificus* sPLA2 by inhibiting the major enzymes involved in arachidonic acid metabolism and by the ability to neutralize the toxic effects of lipid peroxidation. In addition,

our results show that 8CR showed much better therapeutic qualities than commercial anti-venom, mainly in the completion of edema or myotoxic activity induced by *Crotalus durissus terrificus* sPLA2.

4. Materials and Methods

4.1. Materials and General Experimental Procedures

Crotalus durissus terrificus whole dried venom was purchased from Bio-Agents serpentary (Faz Boa Esperanca, S/N, Zona Rural, Batatais, SP, CEP 14300-000, Brazil). The solvents, chemicals, and reagents used for protein purification and characterization (high-performance liquid chromatography (HPLC)-grade or higher) were acquired from Sigma-Aldrich Chemicals (St. Louis, MO, USA), Merck (Kenilworth, NJ, USA), and Bio-Rad (Hercules, CA, USA). Male Swiss mice (25 g) were obtained from the Multidisciplinary Center for Biological Research (CEMIB) of the State University of Campinas (UNICAMP, São Paulo, Brazil). The animals were maintained under standard conditions (22 $\pm$ 2 °C; 12 h light/dark cycle) with food and water available ad libitum. All animal experiments were performed in accordance with Brazilian Laws for the Care and Use of Laboratory Animals, and the protocols were approved by the Protocol No. 014-CEUA (23 August 2016) and Protocol No. 019-CEUA (23 August 2016). Briefly, 1H and 13C NMR spectra were recorded at 300 and 75 MHz, respectively, on a Bruker (Billerica, MA, USA) DPX-300 spectrometer. HMBCs were recorded on a Bruker Avance DRX-500 spectrometer. CD3OD (Tédia, Aparecida de Goiânia, Brazil) was used as the solvent, and TMS (Sigma-Aldrich) was used as the internal standard. Chemical shifts are reported in δ (units: ppm) and coupling constants (J) in Hz.

4.2. Extraction and Purification of 8CR

Aerial parts of *Peperomia obtusifolia* (L.) A. Dietr. (Piperaceae) were collected in Petrópolis, Rio de Janeiro, RJ, in February 2008. The identification of the plant was performed by Prof. Elsie Franklin Guimarães, Botanical Garden of Rio de Janeiro. A voucher specimen (RB: 393491) was deposited in the Herbarium of Rio de Janeiro Botanical Garden, Rio de Janeiro, Brazil. Fresh aerial parts (1000 g) were cut and extracted with methanol (MeOH) at room temperature. The crude methanolic extract (18.58 g) was suspended in MeOH:H_2O (1:2 v/v) and successively partitioned into hexane, CH_2Cl_2, EtOAc, and n-BuOH. Part of the n-BuOH phase (325 mg) was subjected to column chromatography (CC) over a Sephadex LH-20 column, and eluted with MeOH to yield seven groups (N1–N7). The N4 fraction (43.0 mg), which is composed of a pure compound, was identified through 1H and 13C nuclear magnetic resonance (NMR) spectroscopy as a rhamnosyl apigenin derivative. The correct position of the sugar was assigned by HMBCs, and the compound was characterized as 8-*C*-rhamnosyl apigenin (8CR; Figure 1) in accordance with data in the literature [31,32].

4.3. Purification of Secretory Phospholipase A2

Fractionation of phospholipase A2 from the total venom of *Crotalus durissus terrificus* was purified by two steps. In the first step, the total venom was injected into a molecular exclusion HPLC column (Superdex 75, 1 $\times$ 60 cm, Pharmacia, London, UK), and the chromatographic run was performed with a flow rate of 0.2 mL/min for the elution of crotoxin. After confirming the enzymatic activity and biochemical analyzes, as described in [9], it was subjected to reverse-phase chromatography using a µ-Bondapack C18 column (0.39 $\times$ 30 cm) with a flow rate of 1 mL/min for fraction elution of sPLA2 and crotapotin. The fractions of sPLA2 were then submitted to enzymatic assays and analyzed in electrophoresis in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer, as previously described [32].

4.4. Cytosolic PLA2, sPLA2, and COX-1 and -2

For the cytosolic PLA2 assay, we used a cPLA2 Assay Kit (catalog No. 765021), purchased from Cayman Chemical (Ann Arbor, MI, USA), and procedures were conducted according to the manufacturer's instructions. For the test, cytosolic phospholipase A2 (Holzer diagnostika, Hölzel Diagnostika Handels GmbH, Hohenzollernring 38, 50672 Cologne, Germany) was prepared following the manufacturer's recommendation. Moreover, inhibitor stock solutions of 8CR and oleyloxyethyl phosphorylcholine, a potent inhibitor of PLA2 (Santa Cruz Biotechnology, Dallas, TX, USA), were adjusted to prepare a final concentration of 1 μmol/L that was dissolved in 100 μL of DMSO and added to 10 mL of cPLA2 assay buffer (stock solution). After the preparation of each stock solution, we adjusted the specific volume of each inhibitor concentration, and mixed and stored them at room temperature in amber glass bottles until use. In this work, we performed this assay in sextuplicate, at 0, 16, 32, 64, and 128 μM of 8CR or OP. After preparation of recombinant cPLA2 under the same conditions indicated for the cPLA2 assay kit, we performed the enzymatic assay as described for the cPLA2 assay kit.

For sPLA2 inhibition, sPLA2 activity was measured by following a previously-described protocol [32] for a 96-well plate assay using 4-nitro-3-octanoyloxybenzoic acid (4N3OBA or NOBA, Enzo Life Sciences, Inc. Farmingdale, NY, USA) as the substrate. 8CR and aristolochic acid (Aa), quercetin (Q), and quercetrin (Qn) were adjusted to prepare a final concentration of 1 mmol/mL that was dissolved in 100 μL of DMSO and added to 10 mL of Tris-HCl 50 mmol/L buffer pH 8.0 (stock solution). After the preparation of each stock solution, we adjusted the specific volume of each inhibitor concentration, then mixed and stored them at room temperature in amber glass bottles until use. In this work, we made this evaluation in sextuplicate, at 0, 16, 32, 64, and 128 nmol/L for all compounds, including 8CR, Aa, Q, and Qn. These inhibitors were pre-incubated with native sPLA2 from *Crotalus durissus terrificus* (20 μL, 2 mg/mL) in reaction buffer (Tris-HCl 50 mmol/L buffer pH 8.0, 2 mmol/L of calcium) for one minute prior to the addition of substrate at 37 °C, and two min prior to the addition of sPLA2 substrate (4 μmol/L). Enzymatic reactions were carried out at 37 °C for 30 min and absorbance at 405 nm was recorded at regular intervals of 10 min. In the graph, each point represents the mean and SD of six replicates, and X indicates compounds used and its respective concentration.

In the COX-1 and COX-2 cyclooxygenase inhibition assay, we used the COX activity assay kit (Cayman Chemical, item number 760151) to investigate the effect of 8CR on the enzymatic activity of COX enzymes and compare them with another COX inhibitor, DuP-697 (Cayman Chemical, item number 70645), and SC-560 (Cayman Chemical, item number 70340). DuP-697 is a member of the diaryl heterocyclic group of selective COX-2 inhibitors and SC-560 is a member of the diaryl heterocyclic class of cyclooxygenase (COX) inhibitors, which includes celecoxib (Celebrex™) and rofecoxib (Vioxx™). Each commercial inhibitor was dissolved following the manual instructions provided by the manufacturer, and 8CR was dissolved in DMSO. All of the concentrations of inhibitors were prepared and diluted in COX assay buffer and the concentrations were adjusted. These inhibitors were pre-incubated with the enzyme in reaction buffer for one minute prior to the addition of arachidonic acid (AA). Assays were performed using 100 units of ovine COX-1 or ovine COX-2 (one unit of enzyme consumes one nanomole of oxygen per minute at 37 °C) in 0.1 M/L Tris-HCl buffer, pH 8.0, containing 20 μM AA, 5 mmol/L EDTA, 2 mmol/L phenol. Assays were initiated by the addition of 20 μM AA; the colorimetric COX assay was measured by monitoring the appearance of colorimetric oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm, and the COX activity was measured according to the manufacturer's instructions.

4.5. Pharmacological Assay and Biochemical Assays

4.5.1. Paw Edema

A paw edema assay was performed using previously described protocol [32]. Male Swiss mice (25 g) were anesthetized by inhaling halothane. Posterior paw edema was induced by a single subplantar injection of sPLA2. Paw volumes were measured immediately before the injection of the samples and at selected time intervals thereafter (0, 30, 60, 180, and 360 min) using a hydroplethysmometer (model 7150, Ugo Basile, Monvalle, Italy). The results are expressed as the increase in paw volume (mL) calculated by subtracting the initial volume. Each treatment was conducted for $n = 5$.

4.5.2. Evaluation of Myonecrosis

The myotoxic activity was evaluated by the plasma creatine kinase (CK) measurement released from damaged muscle cells. For this, we used a commercial CK-NAc kit (Laborlab, London, UK), as described in [32]. The right gastrocnemius muscle was injected with 50 µL of 0.5 mg/mL sPLA2 sample, while the control mice received only an equal volume of 0.15 M NaCl. After 3 h, the animals were anesthetized and samples were collected from the abdominal cavity into tubes containing heparin as an anticoagulant. The plasma was stored at −10 °C for a maximum of 12 h before the assay. The level of CK was then determined with 40 µL of plasma, which was incubated for 3 min at 37 °C with 1.0 mL of the reagent according to the protocol kit. The resulting activity was expressed in U/L. Each treatment was conducted for $n = 5$.

4.5.3. Determination of IL-1β, TNF-α, Prostaglandin E2 (PGE-2), and Malondialdehyde (MDA) Levels in Mice Paws

IL-1β, TNF-α, PGE-2, MDA, and COX-2 levels in mouse paw tissues were determined by colorimetric assay using tissue samples from paw tissue homogenization. Two hours after the injection of sPLA2, and two hours after the injection of cPLA2, five mice of each group were sacrificed, and the tissue samples were collected and weighed, snap frozen in liquid nitrogen, and stored at −80 °C to be processed for preparation of homogenates. Paw tissues (10% w/v) were homogenized in sodium phosphate buffer (0.1 M PBS, pH = 7.4) using a Disruptor Genie Cell Disruptor/Homogenizer (Scientific Industries, Inc.; 80 Orville Drive, Suite 102 Bohemia, New York 11716, USA). The homogenates were centrifuged at 9000× g for 20 min at 4 °C. The supernatants were collected, and IL-1β, TNF-α, PGE-2, and MDA levels were measured by respective enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, Waltham, MA, USA) according to the kit instructions. IL-1β, TNF-α, PGE-2, and MDA were determined using the following kits: IL-1β mouse ELISA kit (ab100704), mouse TNF-α ELISA kit (ab100747), prostaglandin E2 ELISA kit (ab133021) and lipid peroxidation (MDA) assay kit (colorimetric/fluorometric) (ab118970), mouse COX2 SimpleStep ELISA® kit (ab210574), cytosolic phospholipase A2 assay kit (ab133090), and secretory phospholipase A2 assay kit (ab133089), respectively, and the protocol used for each biochemical analysis followed the manufacturer's instructions (Abcam, Cambridge, MA, USA).

4.6. Statistical Analysis

The results are reported as the means ± SEM of the replicated experiments. The significance of the differences between the means was assessed by an analysis of variance followed by an analysis of variance (ANOVA) when several experimental groups were compared with the control group. The confidence limit for significance was 5%.

5. Conclusions

In conclusion, 8CR exhibited a generalized beneficial effect in terms of neutralizing the toxic effects of *Crotalus durissus terrificus* and sPLA2 snake venom, which included COX-2 neutralization in

a dose-dependent manner. The inhibition of the mobilization of cytokines and other pro-inflammatory mediators generated by an increase in arachidonic acid metabolism contributed significantly to the decrease of free radical levels in cells. Thus, the systemic effects induced by the sPLA2 toxin from *Crotalus durissus terrificus* may be dependent on increased oxidative stress and the generation of reactive oxygen species. These effects are not completely neutralized by the action of commercial anti-venom, even by inhibiting the pro-inflammatory action of sPLA2 over the course of 60 min. In addition to inhibiting COX-2 activity, the compound 8CR also inhibited the phospholipase A2 activity and showed a strong antioxidant activity, and this set of qualities was essential for the 8CR compound to be more effective than the anti-venom or indomethacin against edema induced by sPLA2 from *Crotalus durissus terrificus*. In addition, the protein–protein interaction performed with sPLA2 as a molecular tool to evaluate the effect of 8CR treatment on the structure and function of sPLA2 strongly suggests that the compound did not substantially affect the protein structure as demonstrated by chromatographic data, as well as by the CD spectroscopy studies and tryptophan intrinsic fluorescence. The analysis of all of the results strongly supports the possible complementary anti-venom therapy for the treatment of snake bites, and a new class of polyvalent anti-inflammatory drugs.

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Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

Abbreviations

SC-560	5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole)
8CR	8-*C*-Rhamnosyl apigenin
cPLA2	Citossolic phospholipase A2
sPLA2	Secretory phospholipase A2
DuP-697	5-Bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl)-thiophene)
Aa	Aristolochic acid
CK	Ceratine kinase
Cdt	*Crotalus durissus terrificus*
CD	Circular dichroism
IL-1β	Interleukin 1β
PG	Prostaglandins
COX	Cyclooxygenase
AA	Arachidonic acid
OP	Oleyloxyethyl phosphorylcholine
SEM	Standard Error of Mean

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3. Manuscrito a ser submetido à revista:
Marine drugs

Type of the Paper (Article)

Anti-inflammatory and anti-phospholipase A2 actions from Casuarictin a tannin from *Laguncularia racemosa* extracts

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Abstract: Anti-inflammatory drugs are widely used by humans. However, recent researches suggest their periodic use leads to severe renal and hepatic complications, especially when associated with some related disorders, such as constant use of alcoholic beverages. About 30% of drugs are derived from natural products and plant-based compounds are potential candidates to become an efficient bioactive. Mangrove plants are subject to high sunstroke, salinity and tide variation, leading unique chemical components which support their survival. Therefore, *Laguncularia racemosa*, one of the most abundant mangrove species, was used to investigate its anti-inflammatory and phospholipase A2 inhibitory potential. Leaves of *L. racemosa* were treated with different solvents to extract and separate the largest variety of compounds. These extracts were tested against PLA2 from *Crotalus durissus terrificus* venom, which initiates inflammatory process and leads an acute inflammation. To investigate the extract potential, PLA2 inhibition enzymatic assay and paw edema were performed. A great diversity of flavonoids glycosides were found, such as quercetin and myricetin derivatives in ethyl acetate and butanol extracts. Major component in butanol extract was identified as casuarictin also inhibited edema, both two glycosylated quercetins tested. Therefore, the methanol extract of *L. racemosa* could be effective as anti-inflammatory, highlighting mangrove study preservation.

Keywords: sPLA2, *Crotalus durissus terrificus*, inflammation, plant extract, natural compounds

1. Introduction

About 30% of all drugs evaluated as therapeutic agents come from natural products derived from plants. These compounds have often been used to combat diseases caused by most diverse pathogens in humans [1]. Many pharmacological tests allow characterizing the bioactivities of components obtained from plant extracts. These extracts and their purified compounds may become targets for biotechnological innovations [2]. Several protocols to search new drugs were developed such as the compound investigation that modulate key proteins to metabolic events, such as phospholipase A2 (PLA2) and cascade inflammation [3].

Secretory PLA2 (sPLA2) are involved in arachidonic acid release from cellular phospholipids for eicosanoid biosynthesis, especially during acute inflammation. However, the exact mechanism of sPLA2 action for arachidonic acid production or other pro-lipid-inflammatory still requires clarification of points not yet understood and involve since enzymatic activity of these proteins on membrane phospholipid, there presence of some specific receptor for these proteins that allowed specific interactions of some sPLA2 and modulate other enzymes such as cPLA2, PLC and increasing intracellular production of arachidonic metabolism and in some case this sPLA2 also modulate metabolic equilibrium of cell [4-6, 7]. Substantial increasing of intracellular arachidonic acid in cells leads increase cellular oxidative stress, increase of free radical and cellular damage [8,9]. Recent studies have demonstrated that snake venom sPLA2s have a mechanism of action that is highly similar to that of human sPLA2s [10], and some of the sPLA2s purified from humans can induce pharmacological events similar to those of snake venom phospholipase A2 [11]. sPLA2, an enzyme extracted from several snake venoms, exerts inflammatory and myotoxic effects resulting from membrane phospholipids cleavage in pro-inflammatory eicosanoids such as arachidonic acid. This activity can be inhibited by natural products such as flavonoids, preventing formation of arachidonic acid and, consequently, its inflammatory and myotoxicity actions [7]. During inflammation, modulated and intermediated by sPLA2 in pathological condition or during snake venom envenoming, we observed evident increase of free radicals. Excessive reactive species can deplete the endogenous antioxidant system, giving rise to a condition known as oxidative stress. During the condition of oxidative stress, the generation of free radicals can have several consequences. Reactive oxygen species can react with and cause damage to DNA, lipids and proteins. [7, 8,9]. Thus plant extract may be important source of exogenous antioxidant that increase cellular defenses against oxidative stress increasing and by other hand, we observed that natural products also modulate the enzymatic and pharmacological effects of sPLA2 and other important enzymes involved in the arachidonic acid [12,13]

Mangrove plants are submitted to a large diversity of biotic and abiotic pressures. The specimens contained in this ecosystem have to withstand herbivorism, microorganisms action, soil anoxity and high salinity [14].

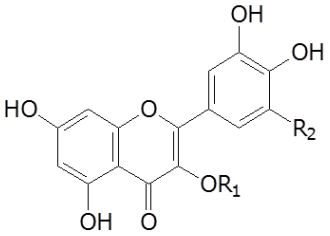
Consequently, the production of compounds is one of the adaptations adopted by plants that survive in these locations. Therefore, terpenoids and phenolic compounds such as flavonols, tannins, etc, are natural products frequently isolated from plants of mangrove habitats [15]. Among these compounds, various pharmacological activities are described in the literature [10] constituting a main source of new drug discovery [16]. However, this source of biologically active compounds is still poorly studied [17], and needs to be valued, since such an ecosystem is a powerful producer of organic matter and nutrients, providing habitat for numerous species, and its destruction has brought disastrous ecological and economic loss [18].

Laguncularia racemosa (Combretaceae), known as a white mangrove, is a perennial plant found in America, Africa [18-20] and South Asia. This plant together with *Avicennia* sp. and *Rhizophora mangle* represent a triple of the most characteristic species of mangrove [20]. Thus, the aim of this work is to evaluate the anti-inflammatory effects of extracts and some compounds obtained from *Laguncularia racemosa* leaves on the inflammatory model caused by sPLA2 purified from *Crotalus durissus terrificus* snake venoms.

2. Results

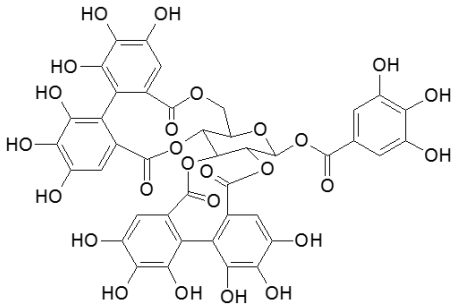
2.1. Fractionation of extract and chemical identification of compounds

The best results in enzymatic and paw edema assays were observed in n-buthanol (n-BuOH) and ethyl acetate (EtOAc) phases of methanolic extract, respectively, which led us to focus the identification analyzes in these two phases. EtOAc phase was analyzed by HPLC-UV-DAD showing 11 peaks, which 10 belonged to flavonoid group, due the presence of two characteristic absorption bands in the regions of 355-357 nm and 260-265 nm, suggesting the presence of flavonols skeleton [20]. The remaining peak, which had the shortest retention time (Rt = 3.7 min) and maximum absorption at 275 nm, was reported as belonging to tannin class. Due its greater abundance in n-BuOH phase this component was separated and identified through NMR, which will be discussed below (Figure 1(b)). The HPLC-UV-DAD-MS-MS analysis of EtOAc phase allowed the identification of ten flavonoids pertaining to quercetin and myricetin aglycones, which were elucidated as: myricetin-3-O-(galloyl)-glucoside (1), myricetin-3-O-glucoside (2), myricetin-3-O-xyloside (3), myricetin-3-O-arabinoside (4), myricetin-3-O-rhamnoside (5), quercetin-3-O-(galloyl)-glucoside (6), quercetin-3-O-glucoside (isoquercitrin; 7), quercetin-3-O-xyloside (reinoutrin; 8), quercetin-3-O-arabinoside (9) and quercetin-3-O-rhamnoside (quercitrin; 10) showed in Figure 1(a). Mass data used for identification of flavonoids are shown in Table 1.



Compound	Name	R_1	R_2
1	Myricetin-3-O-(galloyl)-glucoside	Gly-Gal	OH
2	Myricetin-3-O-glucoside	Gly	OH
3	Myricetin-3-O-xyloside	Xyl	OH
4	Myricetin-3-O-arabinoside	Ara	OH
5	Myricetin-3-O-rhamnoside	Rha	OH
6	Quercetin-3-O-(galloyl)-glucoside	Gly-Gal	H
7	Quercetin-3-O-glucoside	Gly	H
8	Quercetin-3-O-xyloside	Xyl	H
9	Quercetin-3-O-arabinoside	Ara	H
10	Quercetin-3-O-rhamnoside	Rha	H

(**a**)



Casuarictin (11)
[1-O-galloyl-2,3:4,6-di-O-(4,4',5,5',6,6'-hexahydroxydiphenoyl)-glucopyranose

(**b**)

Figure 1. Compounds identified from polar extracts of leaves from *Laguncularia racemosa*. (**a**) allow to characterize the bioactivities of components (**b**) Casuarictin, a tannin which is the major compound present in n-BuOH phase.

Table 1. Identification of compounds from *L. racemos*a leaf extracts and mass data from LC-MS-MS analysis.

N°	Compound	Negative Ionization	Positive ionization
1	Myricetin-3-O-(galloyl)-glucoside	631→479(bp) [M-H$^+$-Gal], 316, 271	-
2	Myricetin-3-O-glucoside	479 [M-H$^+$]→317(bp) [M-H$^+$-Gly], 287, 179	503 [M+Na$^+$], 481(pb) [M+H$^+$], 319, 74 481 [M+H$^+$]→319(pb) [M+H$^+$-Glc]
3	Myricetin-3-O-xyloside	449 [M-H$^+$]→317(bp) [M-H$^+$-Xyl], 179	-
4	Myricetin-3-O-arabinoside	449 [M-H$^+$]→327, 317(bp) [M-H$^+$-Ara], 179	451 [M+H$^+$], 319 [M+H$^+$-Ara], 99; 451→319(bp)
5	Myricetin-3-O-rhamnoside	-	465 [M+H$^+$], 319 [M+H$^+$-

			Rha], 99; 465→319(bp) 319→301, 290, 273, 245, 195(bp), 166, 153, 133
6	Quercetin-3-O-(galloyl)-glucoside	615 [M-H$^+$]→463(bp) [M-H$^+$-Gal], 449, 301 [Q-H$^+$]	-
7	Quercetin-3-O-glucoside	463 [M-H$^+$], 329; 463→301(bp) [M-H$^+$-Gly], 257	487 [M+Na$^+$], 465 [M+H$^+$], 384, 303 [M+H$^+$-Gly], 99; 465→303(bp), 243
8	Quercetin-3-O-xyloside	433 [M-H$^+$]→301(bp) [M-H$^+$-Xyl]	457 [M+Na$^+$], 435 [M+H$^+$], 303 [M+H$^+$-Xyl], 99; 435→303(bp), 204
9	Quercetin-3-O-arabinoside	433 [M-H$^+$]→301(bp) [M-H$^+$-Ara]	457 [M+Na$^+$], 435 [M+H$^+$], 303 [M+H$^+$-Ara], 99; 435→303(bp)
10	Quercetin-3-O-rhamnoside	447 [M-H$^+$]→301(bp) [M-H$^+$-Rha], 179	-

bp: base peak (100% relative intensity); Q: Quercetin; Gal: Galloyl; Gly: Glucose; Xyl: Xylose; Ara: Arabinose; Rha: Rhamnose.

n-BuOH extract of *L. racemosa* was subjected to Sephadex LH-20 column to separation of components yielding four groups. Group L1 revealed a major component with retention time at 3.7 minutes and maximum absorption at 275 nm, suggesting the presence of a tannin. The ^{1}H NMR spectra showed a singlet at δ 7.18 (2H) characteristic of a galloyl group, and four other singlets at δ 6.69, δ 6.57, δ 6.45 and δ 6.38 which were assignable to two hexahydroxydiphenoyl (HHDP) groups. Additionally, sugar moiety was assigned by the four doublets at δ 6.22 (*J*= 3.8 Hz), δ 5.62 (*J*= 3.8 Hz), δ 5.52 (*J*= 2.9 Hz) and δ 5.26 (*J*= 2.9 Hz), a multiplet at δ 4.80 and a double-doublet at δ 4.01 (*J*= 9.0, 2.0 Hz), indicating the presence of glucopyranose. The ^{13}C NMR spectra confirmed the presence of a galloyl and two HHDP groups esterifying the glucose moiety. HMBC correlations confirmed the galloyl unit at C-1 and two HHDP groups at C-2,C-3 and C-4,C-6. Therefore, the compound was identified as casuarictin (11) [1-O-galloyl-2,3:4,6-di-O-(4,4',5,5',6,6'-hexahydroxydiphenoyl)-glucopyranose; Figure 1(b)] by comparison of literature data [21].

Remaining groups (L2 to L4) of n-BuOH phase were composed by flavonols, which were analyzed by HPLC-UV-DAD-MS-MS. Compounds were identified as myricetin-3-O-xyloside (3), myricetin-3-O-rhamnoside (5), quercetin-3-O-(galloyl)-glucoside (6), quercetin-3-O-xyloside (reinoutrin; 8) and quercetin-3-O-rhamnoside (quercitrin; 10) showed in Figure 1(a). The respective mass data are shown in Table 1.

2.2. Polar phases assays

Polar phases were assayed for enzymatic inhibition with sPLA2 obtained from Crotalus durissus terrificus venom. There was a prominent decrease in sPLA2 activity was observed primarily in the butanolic phase, which showed inhibition of 64.49% ± 1.59 (Figure 2 a). The CK test quantifies the creatine kinase enzyme released by muscle tissue, in which sPLA2 is injected. The enzyme cleaves membrane phospholipids leading a lysis in muscle cells, and a release of cytoplasmic content, rich in creatine kinase [22]. The assays showed that both phases do not exert a myotoxic effect, because they do not significantly increase a creatine kinase release when compared to sPLA2 and saline injection. However, the extracts, when incubated with sPLA2 resulted in a decrease in myotoxicity in ethyl acetate phase and an increase in butanolic phase, confirmed by one-way analysis of variance ($p < 0.05$, $f = 47.49$ on Figure 2 b). Edematogenic inhibition activity of extracts was tested when injected 30 minutes before sPLA2, resulting in a prominent inhibition of partitions. In both extracts there was inhibition of phospholipase-induced edema ($p < 0.05$ EtOAc Cdt: $f = 11.71$, BuOH Cdt: 16,61), but EtOAc phase had no significant difference with PLA2 treatment of C. d. terrificus isolated in 180 minutes and 240 minutes (Figure 2 c). BuOH phase, on the other hand, was apparently more efficient in inhibiting edema, since it presented difference in all treatments (Figure 2 d).

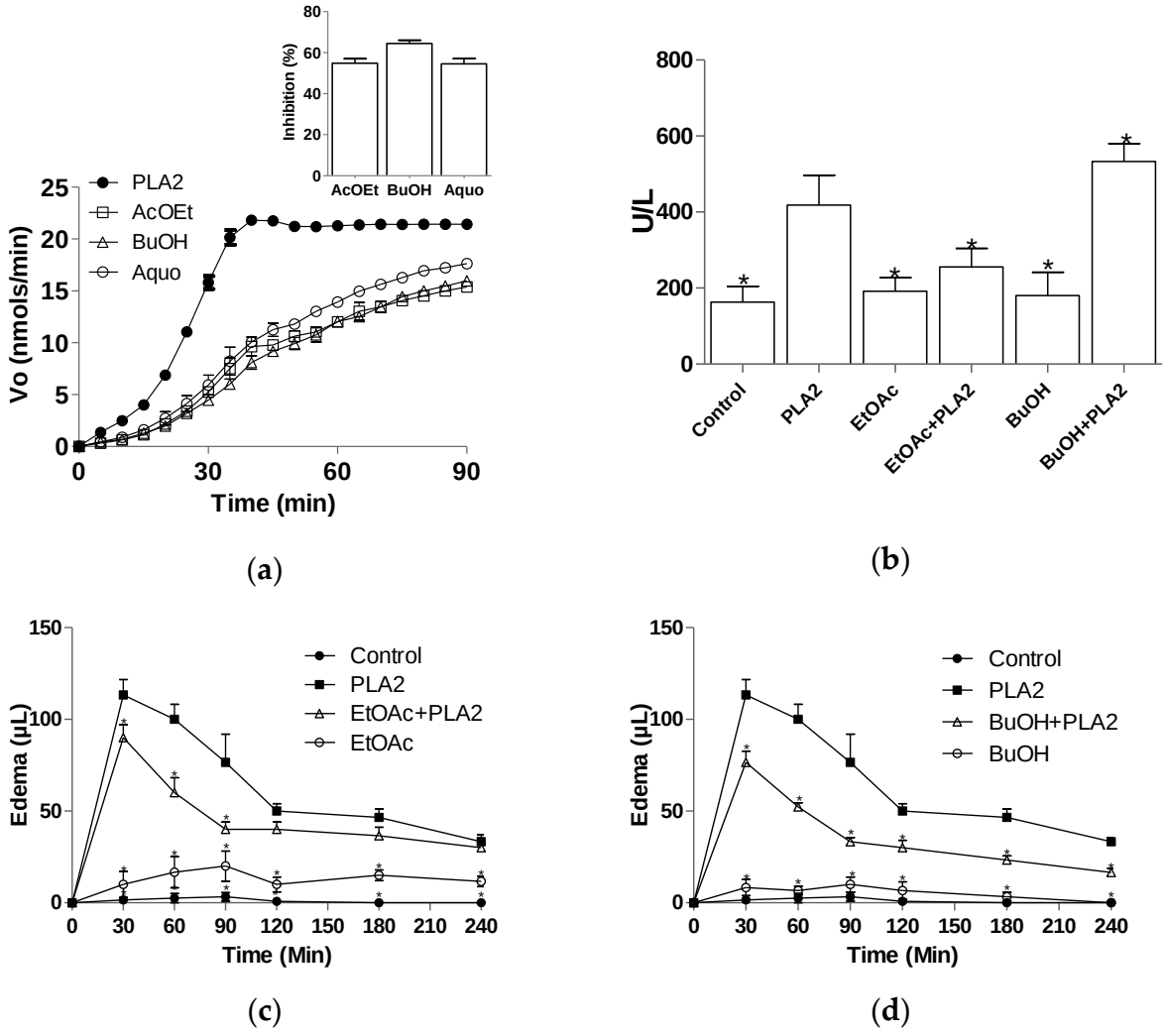


Figure 2. Enzyme activity assay (lines) and their respective inhibition percentages (bar chart) using secretory PLA2 (PLA2 at 0.1 µg/µL, black circles) of *C. d. terrificus* (**a**) and phases (0.1 µg/µl) ethyl acetate (EtOAc - cast square on **a**), butanolic (BuOH - cast triangle on **a**), aqueous (Aquo - cast circles on **a**) from methanolic extract of *L. racemosa*, all incubated with sPLA2 for 30 min, using NOB substrate (0.2 µg/µL) and monitored in a spectrophotometer at 405 nm for 90 min (n = 6). Creatine kinase break for indication of myotoxicity of PLA2 of *C. durissus terrificus* (PLA2, 1µg/g on **b**) compared to control (saline 0.9%) and ethyl acetate phases (EtOAc, 1µg/g on **b**) and buthanolic (BuOH 1µg/g on **b**) alone and incubated 1:1 (EtOAc+PLA2, BuOH+PLA2, 1µg/g on **b**) with PLA2 (1µg/g); ANOVA followed by the Bonferroni test, (*) indicate statistical difference p<0,05, **b**: f = 47. Paw volume of sPLA2 (1µg/g) isolated from venom of *C. durissus terrificus* in Swiss female mice previously treated with Saline 0.9% (Control, Black circle on **c** and **d**), with phase with ethyl acetate (EtOAc+PLA2, at 2µg/g cast triangles, on graph **c**) and butanol (BuOH+PLA2, at 2µg/g cast triangles in graph **d**) from the methanolic extract of *L. racemosa*, and only ethyl acetate phases (EtOAc cast circle 2µg/g on graph

c) and butanol (BuOH cast circle 2µg/g on graph **d**) injected alone into paw; Two-way ANOVA followed by Bonferroni test, (*) indicate statistical difference p <0.05, EtOAc: f = 11.71, BuOH: f = 16.61 (n = 6).

2.3. Isolated casuarictin assays

From n-BuOH phase the main compound casuarictin were isolated and were assayed for enzymatic inhibition with sPLA2 from *C. durissus terrificus* venom (Figure 3 a). In SEC interaction by casuarictin with PLA2 ware observed as specter of PLA2 decreased when incubated with casuarictin (Figure 3 b). After the in vitro effects were confirmed, CK was performed and inhibition of PLA2 ware confirmed (p <0.05, f = 25.58, Figure 3 c), verified by edematogenic activity in which casuarictin inhibited edema on 30 and 60 minutes (p<0.05, f = 8.391, Figure 3 d).

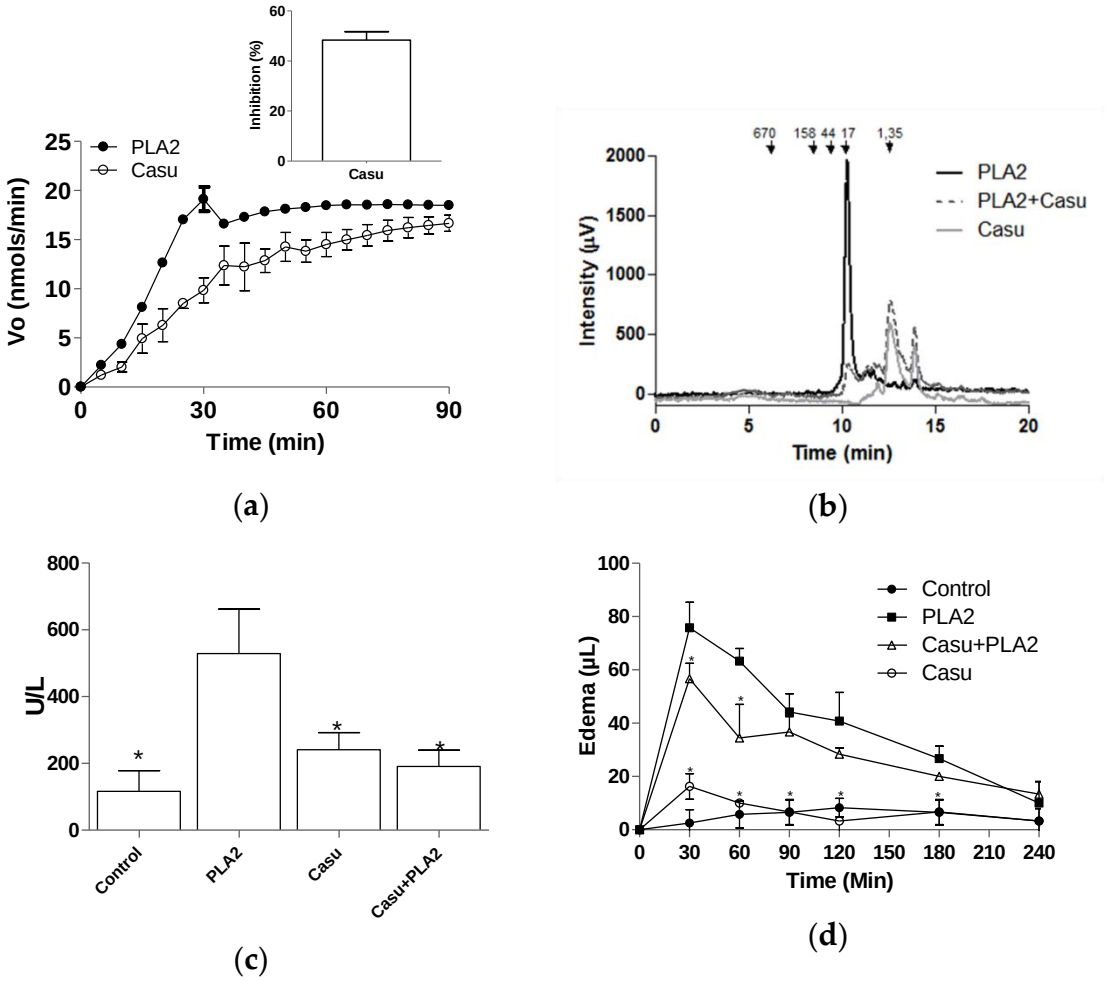


Figure 3. Enzyme activity assay (lines) and inhibition percentages (bar chart) using secretory PLA2 (PLA2 at 0.1 µg/µL, black circles) of *C. d. terrificus* (**a**) and casuarictin isolated from n-BuOH phase (Casu - cast circles on **a**) incubated with sPLA2 for 30 min, using NOB substrate (0.2 µg/µL) and monitored in a spectrophotometer at 405 nm for 90 min (n = 6). Size exclusion chromatography in Tris 0.1M and NaCl 0.1M of casuarictin, PLA2 and both previous incubated for 30 minutes (**b**). Creatine kinase break for indication of

myotoxicity of PLA2 of *C. durissus terrificus* (PLA2, 1μg/g) compared to control (saline 0.9%) and casuarictin (Casu, at 1μg/g on graph **c**) alone and incubated 1:1 (Casu+PLA2, 1μg/g on graph **c**) with sPLA2 (1μg/g) (n = 6). Paw volume of sPLA2 (1μg/g) isolated from venom of *C. durissus terrificus* in Swiss female mice previously treated with Saline 0.9% (Control, Black circle on graph **d**), with casuarictin (Casu+PLA2, at 2μg/g cast triangle on **d**) isolated from leafs extracts of *L. racemosa*, and casuarictin (Casu, at 2μg/g cast circle on graph **d**) injected alone into paw; Two-way ANOVA followed by Bonferroni test, (*) indicate statistical difference $p < 0.05$, $f = 8.391$ (n = 6).

3. Discussion

The great diversity of compounds and functions present in plants, and especially in mangrove plants, leads multitude of effects, such as antioxidants, antiviral, antibacterial, antifungal and anti-cancer, resulting in great interest in such compounds for industries of drugs and cosmetics [23]. Soil salinity is the major abiotic stress affecting plant productivity worldwide. Many crop species, which countless people rely for survival, are negatively affected by high salinity and in case of mangrove plants we found several physiological and metabolic adaptation that leads its growth in the grows in coastal saline or brackish water. Among this adaptation including differentiated natural products [24,25].

Among many components produced by plants, flavonoids can be distinguished because they have several therapeutic effects, such as anti-inflammatory, antioxidant, antimicrobial, etc. Due to this broad therapeutic spectrum, these classes possess particular interest to pharmacology [24]. Pharmacological actions have been demonstrated by plant extracts present in mangroves, such as antioxidant [26-28], healing [29], antibacterial [30,31], anti-inflammatory, antinociceptive and antitumor [16,32]. The present study shows evidence of bioactive compounds with significant anti-inflammatory activity present in *L. racemosa* leaf extract, a fact observed by enzymatic inhibition and paw edema assays.

Flavonoids have antioxidant and anti-inflammatory activities very well established by several studies [33-38]. The presence of flavonoids in EtOAc phase from methanolic extract of *L. racemosa* partly explaining the observed effects. However, the origin of antiedematogenic effects exerted by BuOH phase from methanolic extract may be related to major chemical constituent, the casuarictin, which showed an inhibition in tests mostly at beginning of edema, suggesting that the compound presence and its synergy with other phenolic components in BuOH is related to edema inhibition activity.

Casuarictin is a ellagitanin present in many plants such as strawberry [39](FUMAGALLI) and have anti-cancer [40](ISMAIL) and anti-inflammatory properties by IL-8 and NF-κβ pathway inhibition [39](FUMAGALLI), also shows gastric protection, furthermore acute edema induced by carragenan was inhibited by extracts from *Syzygium jambos* which also contain this tannin in its composition

[41](HOSSAIN). Especially the last mentioned activity corroborates to our anti-edematogenic results.

Enzymatic assay revealed an inhibition of sPLA2 activity when incubated with EtOAc and BuOH phases. Extracts and compounds isolated from plants already showed inhibition of sPLA2, such as *Althaea officinalis* (Malvaceae), *Abuta* sp. (Menispermaceae), *Astrocaryum volgare* (Arecaceae), *Baccharis microdonta and B. oxyodonta* (Asteraceae) [9,42], *Bactris gasipaes* (Arecaceae), *Bowdichia major* (Fabaceae), *Cassia hirsuta* (Fabaceae) [43], *Mandevilla illustris* (Apocynaceae) [44], among others [44-47]. Such inhibition may be related to affinity of extracts for sPLA2, and regulation of arachidonic acid synthesis [48]. Biondo et al. [44] analyzed aqueous extract of *Mandevilla velutina*, which showed a high inhibition of enzymatic and pharmacological action, with a greater specificity for sPLA2 of *Crotalus* sp. venom.

Methanolic extracts and their partitions of *Serjania erecta* also showed inhibition of *Bothrops* sp. PLA2 as well as edema and myotoxicity [49]. The edematogenic and myotoxic activity test was performed only with EtOAc and BuOH phases, due to their greater inhibition of sPLA2 activity *in vitro* assays. In this study, EtOAc and BuOH phases of *Laguncularia racemosa* effectively inhibited edema induced by phospholipase A2 of *C. durissus terrificus*. This fact is also shown by other studies with plant extracts, such as the methanolic extract of *Cordia verbenacea*, whose rosmarinic acid compound was isolated and inhibited edema and myotoxicity induced by total venom of *B. jararacussu*, which has in its composition a high concentration of sPLA2 [50].

Ethanolic extracts from *Trichomanes elegans*, *Dracotium croati*, *Bixa orellana*, *Brownea rosadenonte*, *Struthanthus orbicularis* and *Gonzalagunia panamensis* inhibited edema induced by *Bothrops* sp. [48]. Other plant extracts also showed an inhibition of phospholipase A2-induced edema [51]. The first plant to present inhibition of sPLA2 toxicity was *W. somnifera* [52]. The mechanisms of action of phospholipase A2 inhibitors and consequent decrease in arachidonic acid production are diverse and not fully understood, and may include links to specific domains, denaturation, modification of specific amino acid residues and others [53].

CK assay revealed no satisfactory results with BuOH phase, which did not inhibit creatine kinase release when compared to the injection of pure sPLA2 from *C. durissus terrificus* increasing myotoxic activity. According to Lättig et al. [13], these results allow us to infer that phase inhibition in EtOAc may be correlated with the presence of flavonoids, whereas the BuOH phase of methanolic extract has a low concentration of flavonoids in comparison to EtOAc phase. By associating these biological activities with the results from LC-MS-MS analysis of EtOAc phase of methanolic extract, in which ten O-glycosylated flavonoids pertaining to aglycones myricetin and quercetin were identified, there is evidence that these constituents inhibiting sPLA2.

Other studies showed a sPLA2 inhibition by quercetin aglycone [54] and quercetrin [55], corroborating what was found in this study. Our results showed a high protection by myotoxic activity by pure compounds compared with total extracts and low protection on edema, suggesting that the action of edema co-protection is related to the molecules synergism, and high affinity of the quercetins for the sPLA2 when in muscle.

Quercetin had already been found in *L. racemosa* collected in Nigeria [56]. In Combretaceae, quercetin has been found in vegetables such as *Guiera senegalensis* and *Pteleopsis suberosa* [57,58]. Casuarictin also has been found at Combretaceae like *Combretum glutinosum* [59] (jossang), *Terminalia arborea*, among others [60] (yoshida). In addition, species of Combretaceae have also shown to have compounds with pharmacological potential, so they can act as antioxidants, anti-inflammatory, anti-leishmania, cytotoxic, antiplatelet, bactericidal and fungicidal, such as phenolic compounds, terpenes and their derivatives [61-65]. However, until now, works with Combretaceae and even with *Laguncularia racemosa* are scarce. It has been isolated from *L. racemosa*, phenolic compounds with antioxidant and inhibitory activities of protein kinase [61], as well as polysaccharides, lipids and a sulfated nor-sesquiterpene [62], which suggests that its extract has potential action over other biological activities.

Thus, this study with extracts and partitions of *Laguncularia racemosa* shows the potential of these compounds in altering the enzymatic activity of PLA2 of *Crotalus sp*., evidenced by *in vivo* assays (paw edema). Such evidence ratifies the pharmacological potential of plants in less studied ecosystem, primarily in anti-inflammatory activities. Glycosylation is a key decoration in natural products from plants, resulting in chemical complexity and diversity of compounds. It influences their chemical properties and bioactivities [66]. Glycosylation enhances water solubility. It provides stability through the protection of reactive nucleophilic groups of the natural products and facilitates their storage and accumulation in plant cells [67,68]. Glycosylation is also known as one of the major factors determining natural product's bioactivity and bioavailability. Thus, we showed that mangrove plant clearly has antioxidant, anti-inflammatory and diminish myonecrosis induced by pro-inflammatory key protein (sPLA2), by other side, in case of Brazil littoral coast we found a very particular environment that can influenciate glycosylation of several compound including polyphenolic, that should involve in pharmacological beneficently of these extract from mangrove plants.

4. Materials and Methods

4.1 General experimental procedures

Crotalus durissus terrificus venom were supplied by Butantan Institute. Reagents for experimental trials were purchased from Promega, Sigma-Aldrich,

Fisher, Across, BioRad, GE Health Care Life Science. Chromatographic columns were purchased from Phenomenex or Sigma-Aldrich (Supelco). Sephadex LH-20 (GE Health Care) was used for column chromatographic separation, while silica gel 60 PF254 (Merck) was used for analytical TLC (0.25 mm).

^{1}H NMR and ^{13}C spectra were recorded, respectively, at 300 and 75 MHz in a Bruker DPX-300 spectrometer. CD_3OD (Aldrich) was used as solvent and TMS (Aldrich) as internal standard. Chemical shifts are reported in δ units (ppm) and coupling constants (*J*) in Hz. Analytical HPLC analysis were performed using a Dionex chromatograph model P680 with UV-Vis detector (model UVD 340U), and C-18 column (3.5 μm, 150 x 5 mm), with a flow rate of 0.6 $mL.min^{-1}$ using H_2O:HOAc 2% and MeOH as eluent. Mass spectra were obtained on a Bruker Daltonics MicroTOF mass spectrometer (ESI-MS) and Bruker Daltonics Ultro-TOF mass spectrometer (ESI-MS/MS).

Animals used *in vivo* tests were Swiss female mices (20-30 g), from Central Animal House of State University of Campinas, with appropriate certification from animal ethics committee of UNICAMP (413141), as well as the ethics committee of University São Paulo State, Biosciences Institute of the São Paulo Coast (UNESP IB-CLP) CEUA 002/2014, where tests were carried out. The animals were kept in polysulfone cages with high temperature resistance, transparent and with insulation filters, at room temperature 22-24ºC with light / dark cycles every 12 hours with food and water *ad libitum*.

4.2. Plant material, extraction, isolation and identification of compounds

Leaves of *Laguncularia racemosa* were collected in a mangrove of Praia Grande city (23° 59' 15"S, 46° 24' 18"W). The voucher specimen has been deposited at Herbarium of University of Santa Cecilia under number HUSC8256. In laboratory, leaves were washed in distilled water, dried and lyophilized. Powdered leaves of *L. racemosa* (39.4g) were exhaustively defatted with hexane (at room temperature). Sequentially, plant material was exhaustively extracted with MeOH affording 29.4 g of a syrupy green extract, after solvent removal under reduced pressure. The MeOH extract was resuspended in MeOH:H_2O (1:2, v/v) and then extracted successively with hexanes, CH_2Cl_2, EtOAc and n-BuOH. For subsequent use of extracts, they were solubilized in saline and 5% DMSO (dimethylsulfoxide). Extracts with higher enzymatic activity (EtOAc and n-BuOH) were analyzed for identification of compounds. EtOAc extract was analyzed through HPLC-UV-DAD-MS-MS resulting in the identification of flavonoids (**1-10**) and tannin (**11**). BuOH extract were subjected to CC over Sephadex LH-20 with MeOH as eluent to yield 4 groups. Groups L2 to L4 were analyzed by HPLC-UV-DAD-MS-MS resulting in the identification of flavonols (**3**, **5-6**, **8**, **10**). Group L1 was analyzed by NMR resulting in the identification of casuarictin (**11**) by comparison of literature data [20].

4.3. Purification of sPLA2 from snake venoms

Purification was performed following protocol developed by Toyama et al., (2000) [69]. The total venom of *Crotalus durissus terrificus* was fractionated on a TSK SP5PW ion exchange column (8mm x 80mm), previously equilibrated with a solution of 0.1 M ammonium bicarbonate, pH 7.8 (buffer A) for 30 minutes. Approximately 10 mg of sample was dissolved with 250 μL of buffer A (0.05 M ammonium bicarbonate, pH 7.8), after centrifugation and filtration sample was applied to the column. Samples were eluted through a continuous linear gradient of buffer B (ammonium bicarbonate 1.0, pH 7.9) and fractionation monitoring was performed at 280 nm absorbance. Subsequently, peaks containing phospholipase fraction were subjected to reverse phase chromatography with a C5 semianalytic column previously equilibrated with buffer A (0.1% TFA) for 20 minutes at a flow rate of 1 mL / min. Samples of sPLA2 (1 mg) were dissolved in 250 μL of buffer A and duly clarified and applied to the chromatographic column. sPLA2 elution was performed with a continuous linear gradient of buffer B (66% Acetonitrile in 0.1% TFA) and the chromatographic profile monitoring was at 280 nm. Samples were submitted to enzymatic kinetics to ascertain the activity of fractions.

4.4. PLA2 inhibition assay

Extracts potential inhibition on the enzymatic activity of sPLA2 was performed according to protocol described by Petrovic et al. (2001) [70]. The enzyme (sPLA2, previously purified) was solubilized with 0.9% NaCl saline at a concentration of 0.1 μg/μl and incubated with the extracts (0.1 μg/μl) for 20 minutes. The chromogenic substrate derived from ϱ-nitroanilide, 4-nitro-3-octanoyloxy benzoic acid (NOB) (Enzo Life Sciences), was solubilized in Acetonitrile PA 0.2 μg / μl. Both were added to 0.02M Tris-HCl, 0.15M NaCl and 1mM $CaCl_2$ (pH 8) buffer to be analyzed by spectrophotometer reading SPECTRA MAX (Molecular Devices, 28 CA), with wavelength at 405 nm. Readings occurred for 90 minutes at 5-minute intervals between readings. Absorbances were transformed in substrate reaction rate, in unit of (mmol / min). To calculate the inhibition percentage, the rate of substrate consumption (slope of the line) was calculated using the formula ((VoPLA2 - Vo Incubated extracts) / VoPLA2) *100. The data were then expressed in percentage and its error in percentage points.

4.5. Paw edema

To evaluate systemic effect of extracts under edema caused by sPLA2, Swiss female mice (24.2 ± 2.4 g) were randomly selected (n=6) in groups: Concentration of 2 μg / g was selected in sequel for sPLA2 tests from *Crotalus durissus terrificus*. The phases were injected into the peritoneum and after 30 minutes, 20 μL of sPLA2 (1 μg / g animal) was inoculated through right posterior subplantar injection (n = 6). For the negative control, saline solution (NaCl 0.9%) and extracts alone injected into the right hind paw were used. The volume monitoring of

edema occurred through a hydroplethysmometer (model 7150, Ugo Basile, Italy.). After tests, mice were anesthetized, and cervical dislocations were performed.

4.6. Inhibition of the myotoxic effect induced by sPLA2

EtOAc and n-BuOH phases (1μg/g) previously incubated for 30 minutes with sPLA2 (1 μg/g) were injected into right *gastrocnemius* muscle. After 30 minutes, animal's blood was collected from tail in a heparinized tube, which was centrifuged, and plasma separated and frozen for further testing. Control animals were submitted to same procedure as treated animals, only inoculated with 25μL of 0.9% NaCl, 25μL EtOAc phase and 25μl of the n-BuOH phase (n = 6). Serum CK levels were tested using CK-Nac Bioliquid kit. After addition of reagents to the serum, samples were placed in a water bath at 37°C for 3 minutes. Readings (at 340 nm) were performed at 1-minute intervals. The activity is expressed as unit per liter (U / L).

4.7. Statistical analysis

Data were expressed as mean ± standard deviation of mean for n animals (n=6). The results were analyzed by analysis of variance (ANOVA) of one or two ways to compare differences between treated positive and negative controls followed by the Dunett or Bonferroni *a posteriori* test. Values of $p < 0.05$ were considered significant.

5. Conclusions

Laguncularia racemosa extracts exhibited a great diversity of flavonols in EtOAc and BuOH phases from methanolic extract. In addition, compounds revealed a synergistic action in sPLA2 inhibition in paw edema and enzymatic tests, but pure compounds were more effective in protection of myonecrosis. These data highlight the mangrove potential for study of new extracts with anti-inflammatory properties and a great pharmacological potential for plant-based compounds, also emphasize the relevance of preserving this ecosystem by addition of evident economic importance to preserve these plants.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/link.

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contributed reagents/materials/analysis tools; Henrique Hessel Gaeta, Caroline Fabri Bittencourt Rodrigues, Mariana Novo Belchor, Marcelo José Pena Ferreira and Marcos Hikari Toyama wrote the paper."

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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CAPÍTULO III
Considerações finais

1. Considerações finais

Este trabalho contribuiu para ampliar o conhecimento do mecanismo de ação das sPLA2 de veneno, proteína com proeminente ação tóxica no envenenamento. Os flavonoides em particular teriam dois modos de ação, uma como elementos antioxidantes exógenos que estariam auxiliando os sistemas antioxidantes celulares na neutralização de ROS (BELCHOR et al., 2017; RODRIGUES et al., 2018). Além disso, o efeito inibitório de flavonoides sobre PLA2 secretória (sPLA2) mostraram que a inibição seletiva das sPLA2 de diferentes fontes quando incubada com vários flavonoides (LINDAHL & TAGESSON, 1997). Contudo são necessários estudos mais aprofondados quanto as proteínas que participam da remoção e neutralização das ROS durante o processo inflamatório. Além da forma que os compostos polifenólicos estariam de fato atuando no processo edematogenico causado pelo envenenamento.

Ao longo do desenvolvimento do trabalho durante a disciplina de estágio docência nos deparamos com alguns problemas sérios na educação que afeta também o nível superior, a evasão escolar e falta de interesse dos alunos em disciplinas com grande quantidade de conteúdo. Na tentativa de tornar a fixação de conteúdos e o processo de avalição mais interativos produzimos duas ferramentas para o ensino de bioquímica que podem ser encontradas em anexo a esse documento (TOYAMA et al., 2017; GAETA et al., 2017). Além disso tive a oportunidade de participar na experimentação e produção de uma patente do grupo de pesquisa relacionado ao processo de obtenção do extrato de *Rizophora mangle* e seu uso (RODRIGUES et al., 2017), que auxiliaram na experiencia e consolidação desse trabalho.

2. Conclusão

Nossos resultados mostraram que determinados compostos polifenólicos antioxidantes são capazes de diminuir os efeitos provocados pela PLA2, inibindo diretamente a enzima, também atuando na captura de ROS.

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Outras produções científicas no período

1. Patente: PROCESSO DE OBTENÇÃO DO EXTRATO DE *RIZOPHORA MANGLE* E SEU USO. - Auxílio na experimentação e produção do texto

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 014544 1

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE ESTADUAL PAULISTA JULIO DE MESQUITA FILHO
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 48031918000124
Nacionalidade: Brasileira
Qualificação Jurídica: Instituição de Ensino e Pesquisa
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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Reitoria

TERMO DE CESSÃO DE DIREITOS SOBRE PROPRIEDADE INTELECTUAL

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Cessionária UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" - UNESP, autarquia estadual de regime especial, criada pela Lei nº 952 de 30.01.1976, devidamente inscrita no CNPJ/MF sob o nº 48.031.918/0001-24, com sede na Rua Quirino de Andrade, 215, Centro, São Paulo (SP), CEP 01.049-010, neste ato representada nos termos da procuração anexada.

Pelo presente instrumento, nesta e na melhor forma de direito, os Cedentes autorizam os Cessionários a depositarem o pedido de patente intitulado **"PROCESSO DE OBTENÇÃO DO EXTRATO DE *RHIZOPHORA MANGLE* E SEU USO"** junto ao Instituto Nacional da Propriedade Industrial, cedendo todos os direitos patrimoniais a ele relativos na forma e para os fins do disposto na Lei 9.279 de 14.05.1996 e Lei 8.666 de 21.06.1993, Artigo 111, a título gratuito, sem qualquer restrição quanto à forma, tempo ou lugar, desde já ficando autorizadas quaisquer alterações que venham a ser consubstanciadas em futuras atualizações, modificações ou derivações tecnológicas.

Por ser a expressão da verdade, este documento é firmado na presença de duas testemunhas que também o assinam.

São Vicente 24 de Janeiro de 2017.

Cedentes:

LEONARDO MENDES DE SOUZA MESQUITA

CAROLINE FABRI BITTENCOURT RODRIGUES

WAGNER VILEGAS

MARCOS HIKARI TOYAMA

CLÁUDIA QUINTINO DA ROCHA

MARIANA NOVO BELCHOR

HENRIQUE HESSEL GAETA

2. Coautoria no trabalho: Evaluation of Potential Thrombin Inhibitors from the White Mangrove (*Laguncularia racemosa* (L.) C.F. Gaertn.).

Mar Drugs. 2015 Jul 21;13(7):4505-19. doi: 10.3390/md13074505.

Evaluation of Potential Thrombin Inhibitors from the White Mangrove (Laguncularia racemosa (L.) C.F. Gaertn.).

Rodrigues CF[1], Gaeta HH[2], Belchor MN[3], Ferreira MJ[4], Pinho MV[5], Toyama Dde O[6], Toyama MH[7].

Abstract

The aim of this work was to verify the effects of methanol (MeOH) and hydroalcoholic (HA) extracts and their respective partition phases obtained from white mangrove (Laguncularia racemosa (L.) C.F. Gaertn.) leaves on human thrombin activity. Among the extracts and phases tested, only the ethyl acetate and butanolic partitions significantly inhibited human thrombin activity and the coagulation of plasma in the presence of this enzyme. Chromatographic analyses of the thrombin samples incubated with these phases revealed that different compounds were able to interact with thrombin. The butanolic phase of the MeOH extract had the most potent inhibitory effects, reducing enzymatic activity and thrombin-induced plasma coagulation. Two glycosylated flavonoids in this partition were identified as the most potent inhibitors of human thrombin activity, namely quercetin-3-O-arabinoside (QAra) and quercetin-3-O-rhamnoside (Qn). Chromatographic analyses of thrombin samples incubated with these flavonoids demonstrated the chemical modification of this enzyme, suggesting that the MeOH extract contained other compounds that both induced structural changes in thrombin and diminished its activity. In this article, we show that despite the near absence of the medical use of mangrove compounds, this plant contains natural compounds with potential therapeutic applications.

KEYWORDS:

Laguncularia racemosa; flavonoids; plasma coagulation; thrombin

PMID: 26197325 PMCID: PMC4515630 DOI:10.3390/md13074505

3. Trabalhos acadêmicos de divulgação científica e método de ensino:

3.1. "METABOLIC RIDE" a conceptual evaluation tool for metabolic biochemistry teaching for graduate and postgraduate students in biological sciences and related areas

Journal of Biochemistry Education
Revista de Ensino de Bioquímica
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"METABOLIC RIDE" a conceptual evaluation tool for metabolic biochemistry teaching for graduate and postgraduate students in biological sciences and related areas

"METABOLIC RIDE" uma ferramenta de avaliação conceitual para o ensino de bioquímica metabólica para os alunos de graduação em ciências biológicas e áreas corelatas

Henrique Hessel Gaeta[1]; Bruna Doimi Ortolan[1]; Caroline Fabri Bittencourt Rodrigues[1]; Caroline Ramos da Cruz Costa[1]; Mariana Novo Belchor[1], Daniela de Oliveira Toyama[2]; Marcos Hikari Toyama[1].*

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Abstract

Biochemistry as a discipline have a high degree of difficulty. Otherwise, application of creative games as teaching methodology has spread in various disciplines. "METABOLIC RIDE" board game is a conceptual and perceptual evaluation tool for biochemistry teaching, aiming to review concepts transmitted in classroom, promoting a competitive challenge to students without denying tools that are at their disposal, stimulating their skills such as their creativity. Further, it makes possible to correlate metabolic routes and their interconnections to establish that metabolic pathways are not separated, such as a railway map. In addition, this game proved to be an excellent tool for student's complementary evaluation, which allowed to analyze the student's perception and thus realize that when properly stimulated some groups could show a great productive and creative capacity. However, this game demonstrated to students new ways to approach complex subjects in biochemistry using creativity.

Keywords: *Metabolic map; didactic game; metabolic integration*

Resumo

A disciplina de bioquímica no geral é considerada de alto grau de dificuldade. Contudo, a aplicação de jogos lúdicos como metodologia de ensino vem se disseminando em várias disciplinas. "METABOLIC RIDE" é uma ferramenta de avaliação conceitual e de percepção para o ensino de bioquímica, visando rever conceitos difundidos em sala de aula, promovendo um desafio competitivo aos estudantes sem negar as ferramentas que estão a sua disposição, estimulando diversas aptidões dos mesmos, como a sua criatividade. Ainda, possibilita correlacionar a importância das rotas metabólicas e suas interligações a fim de sedimentar que os caminhos metabólicos não estão separados, como um mapa ferroviário. Ademais, este jogo se mostrou uma excelente ferramenta de avaliação complementar, mostrando que quando devidamente estimulados alguns grupos foram capazes de mostrar uma capacidade produtiva e criativa. Além disso, mostrou-se novas formas de abordar temas complexos em bioquímica com práticas lúdicas.

Palavras-chave: Mapa metabólico; jogo didático; integração metabólica.

3.2. "Biotecnological war" conceptual evaluation tool for the biotechnology and protein chemistry teaching for undergraduate and postgraduate students in biological sciences

Journal of Biochemistry Education
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"Biotecnological war" conceptual evaluation tool for the biotechnology and protein chemistry teaching for undergraduate and postgraduate students in biological sciences

"Biotecnological war" uma ferramenta de avaliação conceitual e de percepção para o ensino de biotecnologia e química de proteínas para os alunos de graduação em ciências biológicas

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Abstract

Biochemistry in general is practically unanimous as a discipline with a high degree of difficulty, complex and "boring". So, practical and creative play games as teaching methodology has been disseminated in several disciplines of biological sciences. "Biotecnological war" board game is a proposal that was initially conceived as an alternative complementary tool for biochemistry teaching of proteins and peptides, challenging students, aiming to review concepts transmitted in classroom, stimulating student's abilities, such as their creativity, competitiveness, resource management and making possible to correlate biochemistry importance of proteins and peptides as new products. This game proved to be an excellent tool for complementary evaluation of students, which besides stimulating teamwork, also stimulated "a strong competitive spirit" within the classroom, which allowed to analyze students' perception in relation to the theme and mainly articulated group work.

Keywords: *Didactic game; protein chemistry; biotechnology.*

Resumo

A bioquímica no geral é taxada como uma disciplina com alto grau de dificuldade, complexa e "chata". Por outro lado, a aplicação de jogos lúdicos práticos e criativos como metodologia de ensino vem se disseminando em várias disciplinas em ciências biológicas. O jogo de tabuleiro "*Biotecnological war*" é uma proposta pensada como uma ferramenta complementar para o ensino de bioquímica de proteínas e peptídeos, desafiando os discentes, visando rever conceitos transmitidos em sala de aula, e estimulando aptidões dos estudantes, como sua criatividade, competitividade e gestão de recursos. Possibilitando ainda correlacionar a importância da bioquímica de proteínas e peptídeos com o desenvolvimento de produtos. Este jogo se mostrou uma excelente ferramenta de avaliação complementar dos alunos, e além de estimular o trabalho em equipe, também estimulou "um forte espírito competitivo", o que permitiu analisar a percepção dos alunos em relação ao tema e principalmente o trabalho em grupo.

Palavras-chave: : Jogo didático; química de proteínas; biotecnologia.