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CARACTERIZAÇÃO DA ATIVIDADE ENTOMOTÓXICA INDUZIDA PELO EXTRATO METANÓLICO DE *ARAUCARIA ANGUSTIFOLIA* EM BARATAS DA ESPÉCIE *PHOETALIA PALLIDA*

DISSERTAÇÃO DE MESTRADO

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Orientador: Prof. Dr. Chárist on André Dal Belo

São Gabriel

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Caracterização da atividade entomotóxica induzida pelo extrato metanólico de *Araucaria angustifólia* em baratas da espécie *Phoetalia pallida*./ Thiago Carrazoni de Freitas.

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RESUMO

Neste trabalho foi demostrado, pela primeira vez, a atividade inseticida do extrato metanólico de *Arauacaria angustifolia* (Bert.) O. Kuntze 1898 (AAME) e seu metabólito secundário, a quercetina, em baratas da espécie *Phoetalia pallida*. A investigação fitoquímica preliminar, realizada através da Cromatografia em Camada Delgada (CCD), demonstrou a presença do flavonóide quercetina no AAME. A confirmação da presença da quercetina no extrato foi obtida por Cromatografia Líquida de Alta Eficiência (HPLC), com tempo de retenção em 10.6min. A determinação de fenólicos totais indicou uma alta concentração destes compostos (1,03%) no AAME. Os ensaios para a determinação da dose letal mínima (DLM) em baratas da espécie *P. pallida*, confirmaram a atividade inseticida do extrato em doses a partir de (800µg/g de animal). A mesma atividade foi demonstrada pela quercetina (80µg/g), porém, com maior potência comparada ao AAME. Ambos, AAME (200µg/g) e quercetina (40µg/g) foram eficazes em bloquear significativamente a atividade da enzima acetilcolinesterase no inseto. Os ensaios de atividade biológica demonstram uma atividade neurotóxica do AAME e da quercetina, tanto em nível central quanto periférico do inseto. Dessa forma, o AAME (200µg/g) e a quercetina (40µg/g) induziram um aumento no tempo total de *grooming* realizado pelo inseto, (138.11±5s /30min; $p<0.05$ e 230±5s/30min; $p<0.05$), respectivamente. A administração de Atropina (40µg/g), previamente à adição de quercetina (40µg/g) induziu um aumento significativo na atividade de *grooming* quando comparado à quercetina isoladamente (350±3s/30min; $p<0.05$). Quando o animal foi pré-tratado com Metoctramina (40µg/g), um inibidor seletivo de receptores colinérgicos M_2-M_3, observou-se o maior aumento no tempo de *grooming* induzido pela quercetina (40µg/g) (600±8s/30min; $p<0.01$ teste t). Por outro lado, o pré-tratamento com o SCH23390 (40µg/g), um inibidor seletivo de receptores DA-D_1, reduziu significativamente o *grooming* induzido pela quercetina (40µg/g) (90±6s/30min; $p<0.05$). A administração de Tiramina (40µg/g) e hidroxilamina (40µg/g), um inibidor da guanilato ciclase, aumentaram significativamente o tempo de grooming induzido pela quercetina (204±15s/30min; $p<0.05$) e (325±15s/30min; $p<0.01$), respectivamente. Quando ensaiados em preparação músculo coxal-adutor metatorácico de *P. pallida*, tanto o AAME (200µg/g de animal) quando a quercetina (40µg/g) induziram bloqueio neuromuscular irreversível em 120 min de registros (50±9%; $p<0.05$ e 100±7%; $p<0.05$), respectivamente. Este último resultado indica uma ação direta do extrato de *A. angustifolia* sobre o Sistema Nervoso Periférico da barata. Os resultados mostrados neste trabalho validam a atividade inseticida do AAME em *P. pallida*. Compostos fenólicos presentes no extrato provavelmente estão envolvidos na atividade inseticida, como demonstrado pela quercetina. O mecanismo de ação inseticida é complexo, e envolve tanto a neurotoxicidade sobre o Sistema Nervoso Central quanto sobre o Periférico. A ativação de uma cascata de eventos que se inicia com a ativação de autoreceptores muscarínicos no soma dopaminérgico e/ou em interneurônios colinérgicos, induziria um aumento da concentração do IP_3 citosólico bem como do Ca^{2+} favorecendo a liberação da dopamina no sistema nervoso central do inseto. Quanto à atividade bloqueadora neuromuscular, estudos mais detalhados usando-se moduladores farmacológicos de receptores de glutamato e do ácido-gama-aminobutírico (GABA) poderão elucidar esse efeito.

ABSTRACT

We have demonstrated, for the first time, the insecticidal activity of *Araucaria angustifolia* methanolic extract (AAME) and one of its chemical secondary metabolites, quercetin against *Phoetalia pallida*. Preliminary investigation of AAME phytochemical compounds by thin layer chromatography showed the presence of quercetin. The total phenolic contents measured by spectrophotometry showed high content of phenolic acids (1,03%). High Performance Liquid Cromatography (HPLC) proved the presence of quercetin that was eluted at 10.6min. In doses ranging from 800-1200µg/g (animal weight) AAME was effective to kill all cockroaches injected after 24 hours. Quercetin was more effective than AAME whole extract being lethal at 80µg/g (animal weight). Both AAME (200µg/g of animal weight) and quercetin (40µg/g of animal weight) were able to induce a significative blockage at insect acetylcholinesterase activity. these compounds also induced increase in the time of grooming activity (138.11±5s /30min; $p<0.05$) and (230±5s/30min; $p<0.05$), respectively. The injection of atropine (40µg/g) combined with quercetin (40µg/g of animal weight) significantly increased the grooming levels to over the control values of quercetin (350±3s/30min; $p<0.05$). When methoctramine (40µg/g), a selective inhibitor of M_2-M_3 cholinergic receptor was combined with quercetin (40µg/g of animal weight) there was the highest increase on the grooming pattern (600±8s/30min; $p<0.01$). When the SCH 23390 (40µg/g), a selective DA-D_1 receptor blocker was administrated 15min earlier the treatment with quercetin (40µg/g) there was a significative inhibition of the grooming levels (90±6s/30min; $p<0.05$). Treatments with Tyramine (40µg/g) and Hydroxylamine (40µg/g), a Guanylate Cyclase (GC) induced a significative increase in the quercetin-induced grooming activity (204±15s/30min; $p<0.05$) and (325±15s/30min; $p<0.01$), respectively. Both AAME and quercetin were able to complete inhibit cockroach neuromuscular transmission in 120 min recordings (50±9% n=6; $p<0.05$) and (100±7% n=10; $p<0.05$), respectively . The later result, point out to a direct action of the extract on insect peripheral nervous system. The results presented here validate the insecticide activity of *A. angustifolia* methanolic extract in *P. pallida*. Phenolic compounds presents in this extract era likely to be involved in the insecticide activity of AAME. The mechanism of insecticide activity is complex and involve both Central and Peripheral Nervous System. The activation of events that initiate with the activation of Muscarinic Auto-receptors and/or cholinergic interneurons, could lead to an increase of cytosolic IP_3 and Ca^{2+} concentration which could induce a dopamine release in the insect Nervous System. Concerning to the neuromuscular blockage, a further pharmacological investigation would be necessary to clarify this effect. However, one cannot disregard a direct action of quercetin on insect NMDA receptors.

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LISTA DE ABREVIATURAS

Extrato metanólico de *Arauacaria angustifolia* - AAME

N-metil-d-aspartato – NMDA

Dopamina – DA

Transportador de dopamina - DAT

Receptor dopaminérgico – $D_{(x)}$

Receptor muscarínico – $M_{(x)}$

Adenosina-5'-trifosfato – ATP

Monoamina oxidase - MAO

Cromatografia Líquida de Alta Eficiência – HPLC

Cromatografia em Camada Delgada - TLC

Ácido gama-aminobutírico – GABA

Fostatidil inositol 3-quinase – PI_3

Bloqueador de receptor D_1 – SCH23390

Equivalência de Ácido Gálico – GAE

Acetilcolina – ACh

Acetilcolinesterase - AChE

Adenilato Ciclase – AC

Proteina Kinase A – PKA

Receptores Muscarínicos de Acetilcolina – mAChRs

Fosfolipase C – PLC

Monofosfato Cíclico de 8-Bromoadenosia - 8-BrcAMP

Trifosfato de inusitol – IP3

Monofostato de Guanosina

Monofostato Cíclico de Guanosina – cGMP

Receptores de Tiramina – TyrRS

Monofosfato Cíclico de Adenosina - cAMP

Sumário

1. INTRODUÇÃO

1.1. Pesticidas

Os pesticidas podem ser definidos como sendo qualquer substância ou mistura de substâncias capaz de prevenir, destruir ou repelir pragas, podendo estas serem caracterizadas como: insetos, ervas daninhas, roedores ou ainda um conjunto de outros organismos indesejados (Ecobichon, 2001). Em uma situação ideal, os pesticidas deveriam atuar unicamente sobre o alvo desejado, o que não acontece na prática, pois a maioria dos pesticidas não é específica para uma única espécie, exercendo sua ação tóxica sobre espécies não-alvo, incluindo humanos (Rattan, 2010).

Atualmente, existem várias classes de pesticidas, com diferentes propósitos e mecanismos de ação. De acordo com Costa (2008), a classificação mais comum dos inseticidas se dá por suas espécies-alvo, sendo essa dividida principalmente em quatro grandes grupos: inseticidas, herbicidas, fungicidas e rodenticidas.

1.2. Inseticidas

Os inseticidas são substâncias capazes de atrair, repelir ou matar insetos alvo, sendo o seu uso descrito desde a antiguidade, quando extratos vegetais eram usados principalmente para manter os cereais livres de insetos-pragas, por meio de fumigação ou aplicação direta nos mesmos (Addor, 1994; Casida e Quistad, 1998; Thacker, 2002). A partir da segunda metade do século passado, devido à necessidade da agricultura em larga escala e a evolução dos processos de síntese química (Hapeman et al., 2003), agentes sintéticos foram substituindo os compostos naturais derivados de plantas, principalmente pelo seu amplo espectro de ação e poder residual.

Infelizmente, o que parecia uma vantagem, principalmente em relação à eficácia prolongada dos inseticidas químicos industriais, provou-se uma ameaça não apenas para os ecossistemas envolvidos (Grue et al., 1982; Pauli et al., 1999), mas também para os seres humanos. Nesse sentido, a literatura é farta em relação aos problemas de saúde pública envolvendo a exposição prolongada aos inseticidas químicos, que podem, por exemplo, induzir câncer (Tongeren et al., 2012), genotoxicidade (Zeljezic e Garaj-Vrhovac, 2001; Çakir e Sarikaya, 2005), doenças

neurodegenerativas (Sutedja et al., 2009; Kapka-Skrzypczak, 2011; Kamel et al., 2007), além de desordens relacionadas à memória, cognição e ao sistema visual (Çakir e Sarikaya, 2005).

Segundo Costa (2008), todos os inseticidas utilizados atualmente são substâncias neurotóxicas, ou seja, atuam alterando a fisiologia do sistema nervoso do inseto. Apesar de possuírem relativa alta eficiência em controlar pragas, os compostos inseticidas utilizados no presente não são espécie-específicos, podendo exercer sua ação neurotóxica em espécies não-alvo, inclusive em mamíferos os quais podem ser altamente sensíveis a esta toxicidade. Agentes inseticidas em geral (seja natural, seja sintético) desempenham um importante papel no controle de insetos-praga, especialmente em países em desenvolvimento (Costa, 2008). Por outro lado, ultimamente, têm sido frequentes os relatos de falha no controle de pragas com a utilização de inseticidas sintéticos (Taylor, 1989; Collins et al., 2002), principalmente devido ao uso indiscriminado destes, o que acarretou no desenvolvimento gradativo de resistência à compostos sintéticos por artrópodes (Chagas, 2003). Por estes motivos, a busca por novos modelos de inseticidas com alta especificidade, baixo custo de produção e menor poder residual torna-se pertinente do ponto de vista econômico e de saúde pública.

1.3. **Atividade Inseticida de compostos vegetais**

Do ponto de vista biológico, a coevolução entre as plantas e os insetos é reconhecida (Ryan e Byrne, 1988), podendo explicar a seleção de um amplo espectro de metabólitos secundários usados como defesa (e.g. um ou mais compostos químicos usados como defesa contra insetos) (Bown et al., 2006). Atualmente, a maior motivação em se promover estudos, com enfoque na determinação da atividade inseticida de compostos botânicos, é o baixo impacto ao meio ambiente. Associado a esse último aspecto, existe a demanda crescente por produtos saudáveis e atóxicos para os humanos e outros mamíferos (Rattan, 2010).

Até o momento, vários exemplos de compostos vegetais que efetivamente demonstram atividade inseticida, podem ser encontrados na literatura. Por exemplo, Adler et al. (1986) utilizaram uma formulação a base de extratos de sementes de nim (*Azadirachta indica*), verificando a sua atividade repelente, inseticida e inibidora do

crescimento contra *Blattella germânica* e *Periplaneta americana*, duas espécies de baratas comuns em ambientes urbanos. El-Naggar et al. (1989) investigaram os efeitos de extratos de *Citrullus colocynthis* sobre diversos insetos-praga incluindo as espécies de baratas *B. germânica* e *P. americana*, verificando o seu potencial inseticida. Guardiola et al. (1990) estudaram os efeitos de compostos isolados de *Schinus mole*, identificando o timol como o composto com maior eficiência na atividade repelente e inseticida. Outras plantas como *Citrus sinensis*, *Laurus nobilis*, *Lonicera tatarica*, *Sorbus aucuparia*, *Lantana camara*, *Pteridieum aquilinum*, *Cestrum aurantiacum*, *Fagus sylvatica*, *Dryopteris filixmas*, *Quercus petrea* dentre outras, também já foram investigadas com essa finalidade e tiveram seus efeitos comprovados (Gallo et al., 1996). Prabhakaram e Kamble (1996) avaliaram a toxicidade da azadiractina para o controle de populações resistentes de *B. germânica* a inseticidas, comprovando a sua eficácia. Glicosinolatos foram isolados de *Crambea byssinica* e demonstram a sua eficácia no controle de *Musca domestica*, *Aedes aegypti*, *Tribolium castaneum*, *Oryzeaphylum surinamensis*, *Diabrotica virgifera virgifera* e *B. germânica* (Tsao et al., 1996).

Inseticidas botânicos podem atuar de diferentes formas sobre a fisiologia do inseto, geralmente como supressores alimentares. Como exemplo deste mecanismo temos a ação de *Artemisia judaica* contra *Spodoptera littoralis* (Abdelgaleil et al., 2008); *Nigella sativa* contra *Callosobruchus chinensis* (Chaubey, 2008) e *Litsea spp* contra *Trichoplusia ni* (Jiang et al., 2009). Se por um lado observa-se um grande número de trabalhos associados ao estudo da atividade inseticida de compostos vegetais, por outro, raramente se observa a indicação do mecanismo de ação responsável pela atividade inseticida desses compostos (Rattan, 2010).

1.4. ***Araucaria angustifolia***

Segundo levantamentos etnofarmacológicos realizados com populações rurais do Brasil (Arcego 2005), evidenciou-se um aumento no uso de plantas para obtenção de inseticidas naturais que possam ser utilizados no controle de insetos-praga, e dentre as diversas plantas que desempenham este papel, a espécie *Araucaria angustifolia* (Bert.) O. Kuntze destacou-se como potente inseticida e acaricida (Castro 2009). Entretanto testes laboratoriais envolvendo a atividade inseticida de *A. angustifolia* são

escassos e até o momento a bibliografia não apresenta nada relacionado ao mecanismo de ação do efeito inseticida desta planta.

A. angustifolia é uma gimnosperma de grande porte que ocorre na floresta ombrófila mista, um ecossistema único dentro do domínio Mata Atlântica (Almeida, 2003). Esta gimnosperma é conhecida pelos seguintes nomes populares: Pinheiro, pinheiro-brasileiro, pinheiro-do-paraná, pinho, pinho-brasileiro, pinheiro-das-missões ou araucária. Ela também é descrita em inglês como Parana-pine (Carvalho, 1994). Suas folhas recebem a denomição de acículas (Figura 1-a), são coreáceas, agudas, de 3 a 6cm de comprimento, alternadas e dispostas densamente (Gemtchújnicov, 1976). As flores são unissexuais diclinas, sendo o esporófilo reunido em forma de cone (Schultz, 1990). Estróbilos masculinos alongados, cujo eixo comporta numerosos microsporófilos, cada um sustentando oito ou mais microsporângios e os microspóros (pólem) não possuem câmaras de ar. Os estróbilos femininos são grandes e arredondados, e são chamados de pinhas, os macrosporos são numerosos, comportando cada um apenas um óvulo. Na parte superior do endosperma situam-se oito a 15 arquegônios. As sementes são grandes (pinhão), de cor marrom, contendo grande quantidade de reservas nutritivas (Gemtchújnicov, 1976).

A busca de novos agentes farmacologicamente ativos através da triagem de fontes naturais, como extratos de plantas, tem levado à descoberta de muitas moléculas protótipo que desempenham importante função no tratamento de doenças humanas, ou à descoberta de compostos com potencial biotecnológico, como atividade inseticida. Nesse contexto, a literatura relata a presença de metabólitos secundários como flavonóides e ácidos fenólicos nas folhas de *A. angustifolia* (Yamagushi, 2005, 2009), tais compostos podem estar associados a efeitos inseticidas (Viegas, 2003).

Nesse trabalho propomo-nos investigar o mecanismo de ação relacionado à atividade inseticida induzida pelo extrato metanólico de *Araucaria angustifolia*, promovendo a análise fitoquímica-bioguiada além de ensaios comportamentais e eletromiográficos em baratas da espécie *Phoetalia pallida*. Nesse sentido, foram demonstrados os principais sítios celulares envolvidos no mecanismo de toxicidade pelo extrato, bem como os aspectos de sinalização intracelular decorrentes dessa interação tóxica.

Figura 1-a. Acículas de *Araucaria angustifolia*. Para o trabalho em questão tais folhas foram coletadas de um exemplar da espécie localizado no município de São Gabriel, RS, Brasil. Fonte: **(**http://commons.wikimedia.org/wiki/File:Leaf_of_araucaria_angustifolia.jpg**)**

1.5. O **sistema nervoso de baratas como modelo para bioensaios de Toxinologia**

As baratas são insetos-praga primitivos nos quais a maioria dos sistemas fisiológicos é carente de especialização. Se por um lado, a falta de especialização pode ser um entrave para a comparação com alguns sistemas biológicos animais, do ponto de vista da neurotoxicologia torna-se um importante instrumento na investigação do mecanismo de ação de compostos químicos com atividade tóxica sobre o sistema nervoso (Fig 2-a) (Stankiewicz et al., 2012). Por exemplo, já foram identificados mais de 200 neurotransmissores e seus respectivos receptores no sistema nervoso de baratas, que são idênticos aos de outros insetos superiores e apresentam grande homologia em sua estrutura molecular aos de animais vertebrados, dentre eles os seres humanos.

Nesse aspecto, a junção neuromuscular da barata se vale do neurotransmissor glutamato, muito comum no sistema nervoso de mamíferos, para produzir contração muscular pela ativação dos receptores de N-Metil-D-Aspartato (NMDA). Por essa razão, as baratas são reconhecidas como modelos extremamente úteis em ensaios de neurobiologia (Huber et al., 1990). Uma grande vantagem do uso desses animais em experimentos, no campo da toxinologia, é a possibilidade de se investigar várias funções do sistema nervoso por meio de ensaios bioquímicos, como inibição da

enzima Acetilcolinesterase; ensaios comportamentais como, atividade de *grooming* (ato de limpeza dos órgãos sensoriais) e ensaios eletrofisiológicos, como preparação nervo-muscular, podendo estes serem realizados em modelos naturais *in vivo*, *in situ* ou mesmo *in vitro*. Além disso, no caso dos bioinseticidas, muitos deles têm como alvo principal o sistema nervoso, o que facilita, de certa forma, a descrição do seu mecanismo de ação, bem como a evidenciação do grau de seletividade (Stankiewicz et al., 2012). Como exemplo, podemos citar os piretróides naturais e sintéticos, que atuam por causar uma ativação persistente dos canais de sódio na junção neuromuscular, induzindo uma despolarização persistente das membranas, sendo esta última, letal para o inseto (Costa, 2008; Soderlund, 2012).

Outros agentes, como por exemplo, algumas toxinas de venenos animais, atam através da ligação ao sistema nervoso central do inseto, levando a um aumento na liberação do neurotransmissor dopamina, induzindo efeitos como letargia e diminuição da locomoção (Weisel-Eichler et al., 1999; Libersat, 2003). Além desses mecanismos celulares, também tem sido descritos aspectos bioquímicos na intoxicação induzida por agentes inseticidas naturais (Bullangpoti et al., 2006), por exemplo, a inibição da produção de ATP nas mitocôndrias (Yamamoto e Kurokawa, 1970; Storey, 1981) ou ainda, inibição da enzima acetilcolinesterase, causando acumulação de acetilcolina na fenda sináptica e, com isso, mantendo a membrana pós-sináptica em constante estado de estimulação, culminando na morte do inseto (Kostyukovsky et al., 2002; Mills et al., 2004; Shaaya e Rafaeli, 2007).

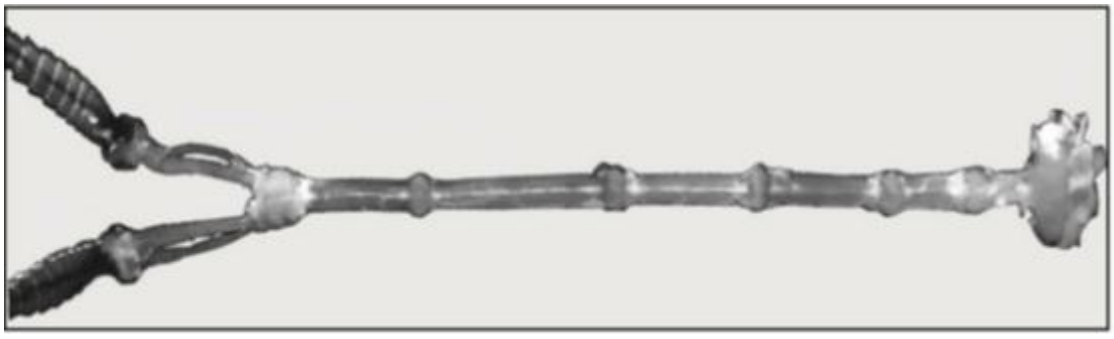

Figura 2-a. Cordão nervoso ventral de uma barata. O Sistema Nervoso de insetos é formado por um cordão nervoso ventral organizado em gânglios (Fonte: Stankiewicz et al., 2012) (Modificações: T.C. Freitas) .

2. OBJETIVOS

Este trabalho teve por objetivo validar o conhecimento popular sobre o potencial inseticida de *A. angustifólia* através de uma análise fitoquímica e de atividade biológica do extrato metanólico.

2.1. OBJETIVOS ESPECÍFICOS

1- Identificar os compostos bioativos presentes no extrato bruto de *A. angustifolia* por meio da técnica de cromatografia de camada delgada (CCD);

2- Identificar e quantificar os compostos bioativos presentes no extrato bruto de *A. angustifolia* por meio da Cromatografia Liquída de Alta Performance (HPLC);

3- Investigar o potencial bioinseticida do extrato bruto de *A. angustifolia* em modelo de *P. pallida*;

4- Determinar o mecanismo de ação bioinseticida do extrato de *A. angustifolia* sobre o sistema nervoso central usando modelos comportamentais em *P. pallida in vivo*;

5- Determinar o mecanismo de ação bioinseticida do extrato de *A. angustifolia* sobre o sistema nervoso periférico, usando a preparação neuromuscular de *P. pallida in vivo*.

3. RESULTADOS

Todos os resultados, bem como os itens Materiais e Métodos, Discussão e parte das Referências Bibliográficas que fazem parte desta dissertação estão apresentados sob a forma de manuscrito. O manuscrito está disposto na forma em que deverá ser submetido para a revista *Journal of Insect Physiology* (ISSN: 0022-1910).

4. MANUSCRITO

Insecticide Activity of *Araucaria angustifolia* (Bert.) O. Kuntze Methanolic Extract and Its Chemical Compound Quercetin in *Phoetalia pallida* Cokroaches

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Keywords: *Araucaria angustifolia*, *Phoetalia pallida*, insecticide activity, neurotoxicity, dopaminergic neurotransmission, glutamatergic neurotransmission, MAO, IP3.

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ABSTRACT

We have demonstrated for the first time the insecticidal activity of *Araucaria angustifolia* methanolic extract (AAME) and one of its chemical secondary metabolites, quercetin against *Phoetalia pallida*. Preliminary investigation of AAME phytochemical compounds by thin layer chromatography showed the presence of quercetin. The total phenolic contents measured by spectrophotometry showed high content of phenolic acids (1,03%). High Performance Liquid Cromatography (HPLC) proved the presence of quercetin that was eluted at 10.6min. In doses ranging from 800-1200µg/g (animal weight) AAME was effective to kill all cockroaches injected after 24 hours. Quercetin was more effective than AAME whole extract being lethal at 80µg/g. Both AAME (200µg/g of animal weight) and quercetin (40µg/g of animal weight) were able to induce a significative blockage at insect acetylcholinesterase activity, these compounds also induced increase in the time of grooming activity (138.11±5s /30min; $p<0.05$) and (230±5s/30min; $p<0.05$), respectively. The injection of atropine (40µg/g) combined with quercetin (40µg/g) significantly increased the

grooming levels to over the control values of quercetin (350±3s/30min; $p<0.05$). methoctramine (40µg/g), a selective inhibitor of M_2-M_3 cholinergic receptor was combined with quercetin (40µg/g) there was the highest increase on the grooming pattern (600±8s/30min; $p<0.05$). When the SCH 23390 (40µg/g), a selective DA-D_1 receptor blocker was administrated 15min earlier the treatment with quercetin (40µg/g) there was a significative inhibition of the grooming levels (90±6s/30min; $p<0.05$). Both hydroxylamine (40µg/g) and Tyramine (40µg/g) induced a significative increase in the quercetin-induced grooming activity indicating that nitric oxide donors and Catecholamines are key points on grooming activity-increase. Both AAME and quercetin were able to complete inhibit the cockroach twitch tension in 120 min recordings. The later results point out a direct action of the extract on insect peripheral nervous system. The results presented here validate the insecticide activity of *Araucaria angustifolia* methanolic extract. The mechanism of insecticide activity is complex and involve both central and peripheral nervous system. Mechanisms related to dopamine signaling and glutamate are likely to be involved but, IP3 signaling modulating Ca^{2+} cannot be disregarded. A direct effect of quercetin at cockroach neuromuscular junctions is a potential explanation for the neuromuscular impairment.

1. Introduction

In order to compete against the continuous threat provoked by different phytophagous insects, plants have developed a vast range of defense mechanisms that comprehend from morphological and structural characteristics to the synthesis of chemical compounds (Vandenborre et al., 2011).With the aim of repel or kill the aggressor, one of the most efficient mechanisms utilized by plants is the synthesis of low molecular weight compounds named secondary metabolites. The production of these chemical compounds can be both, passive or even wounded-induced (Bown et al., 2006). Therefore, most part of the insecticide effect induced by natural vegetal compounds is due to the selective interaction between two or more secondary metabolites with insect nervous system (Adeyemi et al., 2010). In this respect, analogues of secondary metabolites can interfere at insect nervous system, with cellular signaling systems or vital enzymes, which includes neurotransmitter synthesis, storage, release, binding, and re-uptake, receptor activation and function and enzymes

involved in signal transduction and blockage of metabolic pathways (Rattan et al., 2010).

Araucaria angustifolia (Bert.) O. Kuntze (Araucariaceae) known as "Parana pine" is an endemic conifer in southern Brazil (Handro, 1986). Its wood has economic importance as a raw material for paper and pulp production (Lorenzi, 1992). Due to the high content of phenolics, its knot powder has been used as a partial substitute for phenolic resins (Anderegg and Rowe, 1974; Campello and Fonseca, 1975). Previous ethnopharmacology research on this plant, showed that *A. angustifolia* needles are used traditionally as natural insecticide (Arcego, 2005) and acaricide (Castro, 2009) in rural communities of southern Brazil. However, to the best of our knowledge, the present study is likely to be the first demonstrating the chemical constitution associated to the mechanism of insecticide activity of *Araucaria angustifolia* extracts.

The aim of this work was to demonstrate the insecticide activity of *Araucaria angustifolia* needles methanolic extract, using *Phoetalia pallida in vivo* behavior models and neuromuscular preparation. The composition in total phenolic content and the presence of quercetin were also determined by UV spectrophotometric and HPLC methods.

2. Material and Methods

2.1. Reagents and solutions

All chemicals and reagents used were of the highest purity and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Methanol HPLC grade was purchased from Tedia (Fairfield, OH, USA). Tested solutions were prepared daily by dilution in insect saline immediately before use. We called insect saline a carbonate-buffered solution prepared essentially as described by Collins and Miller (1976) with the following composition in mM: NaCl 200.17, KCl 10.73, $MgSO_4$

0.996, $CaCl_2$ 3.40, $NaHCO_3$ 2.14, NaH_2PO_4 0.083 (pH 6.9 adjusted with NaOH 2N). All drugs were administered at the third abdominal hemocoel segment, at a final volume of 20µl, by means of a Hamilton syringe. Experiments were done at controlled room temperature (22-25°C).

2.2. Experimental Animals

All experiments were performed on adult male *Phoetalia pallida* cockroaches (3-4 month after adult molt). The animals were reared at laboratory conditions of controlled temperature (22-25°C) on a 12 h:12 h L:D cycle. All cockroaches were provided with water and dog chow *ad libitum.* Prior to the analysis of neurophysiological parameters, the minimum lethal dose of *A. angustifolia* methanolic extract (AAME) and its secondary methabolite quercetin were determined essentially as described by Kagabu et al. (2007).

2.3. Plant material

Araucaria angustifolia needles were collected in the rural area of São Gabriel, Rio Grande do Sul state, Brazil (30º20'18.63"S - 54º19'16.83"W). After appropriate identification by a plant taxonomist, a voucher specimen was deposited (register number: *HBEI 085*) at Bruno Edgar Irgang Herbarium (HBEI).

2.4. Extract preparation

One kilogram of *A. angustifolia* needles was collected, dried in a ventilated stove at 60^{o}C and powdered in a knife mill (Marconi, Model MA-680, Piracicaba, SP, Brazil). Dried and powdered material (500g) were extracted exhaustively with MetOH (4 x 500 ml) at room temperature. After filtration through a fine filter, the solvent was

removed by rotary evaporation (Model R-220, BÜCHI Labortechnik AG, Postfach, Switzerland) under reduced pressure at <45°C. The resulted semi-solid extract was then lyophilized and stored at -4°C until identification protocols.

2.5. Identification of extract secondary metabolites

Preliminary investigation on *A. angustifolia* methanolic extract (AAME) (15µl, 20µg) was performed by thin-layer chromatography (TLC) on silica sheets (60F254, aluminum backed, 200 µm layer thickness, 8.0 × 5.0 cm, Merck). The presence of flavonoids and phenolic acids was investigated using the adequate development systems and revealers (Pontual et al., 2012). After development, the sheets were air dried and sprayed with the indicators in a fume hood.

2.6. Total phenolic content

The total phenolic content was measured by spectrophotometry using the Folin-Ciocalteu method, with modifications (Nurmi et al 1996). Briefly, 1 mL of 1N Folin-Ciocalteu reagent was added to a 1 mL of sample, and this mixture was allowed to stand for 2-5 min before the addition of 2 mL of 20% Na_2CO_3. The solution was then allowed to stand for 10 minutes before reading at 750 nm in spectrophotometer using 1 cm quartz cells. The total polyphenol content was expressed as milligram gallic acid equivalent per milliliter (mg GAE/mL) of each extract. The samples were analyzed in triplicate.

2.7. High Performance Liquid Chromatography (HPLC)

The results obtained on TLC sheets were complemented by HPLC analysis, which was performed on a Prominence® Liquid Chromatograph Shimadzu instrument equipped with a LC-20AT pump, SIL-20A auto sampler, SPD-20AT PDA detector and CTO-20A column oven (Shimadzu Corporation, Kyoto, Japan). LC Solution V. 1.24 SP1 system software was used to control the equipment and to evaluate the data obtained. The assay was conducted using a reverse-phase technique. The analyses of quercetin and methanolic extracts were performed according to the method proposed by De Souza et al. (2002), which describes an isocratic elution protocol with a flow rate of 0.6 mL/min. The mobile phase was composed by a mixture of methanol and phosphoric acid 0.16M (53:47, v/v), being prepared daily, filtered through a 0.45μm membrane Millipore® filter (Milford, MA, USA) and sonicated before use. The wavelength of the DAD detector was set to 362 nm. An ODS-Hypersil Thermo Scientific C18 column (250 x 4.6 mm i.d., 5μm particle size) (Bellefonte, United States) was used. The HPLC system was operated at 25 °C. The injection volume was 20 μL.

For samples analysis, the extracts were diluted in a solution of ethanol in a proportion of 1:100 (v/v). The quercetin standard was prepared by dilution in ethanol at a concentration of 10 μg/mL. A co-injection was done by adding the quercetin solution to the extract sample solution. All solutions were filtered through a 0.45μm membrane filter from Millipore® before injection.

2.8. Assay for insect Cholinesterase Activity

The *in vitro* inhibition of AChE was evaluated according to the assays described by Ellman et al. (1961) modified by Franco et al. (2009). The whole amount

of protein was measure according to Bradford (1976). In brief, three cockroaches were injected with AAME (200 and 400µg/g animal weight), quercetin (40µg/g) and the well known anticholinesterasic trichlorfon (40µg/g), thirty minutes before the acetylcholinesterase analysis. The animals were previously anesthetized by chilling at -5ºC and their brains collected after cuticle removal. The material was mixed with 750µL of Kpi buffer pH 7.0 (500rpm/5min/4ºC) and 400µl of supernatant was collected. From this sample (50µl) was added to 50µl of 50mM DTNB, 500µl Kpi (pH 8.0) and 2.5µl acetylthiocoline. The reaction was measured during 60 seconds (s) at 412nm using a UV- Visible Spectrophotometer (model Evolution 60S, Thermoscientific, New Hampshire, USA) and analyzed by the software VISION lite (Thermoscientific).

2.9. Video-mounting apparatus for biological assays

For each specific biological assay, the activities were recorded during 30 min by using a video-camera (Panasonic coupled to a 50mm Karl-Zeiz lens) connected or not to an eyepiece of microscopy (Olympus, model SZ51, Germany). The camera had a frame-by-frame (60/s) and was connected to a PC (Infoway, ItauTec, Brazil). Video movies were latter analyzed using a HD Writer AE 2.6T system (Panasonic) with variable speed control.

2.10. Biological assays

2.10.1. Assay for insecticide activity

The insecticidal assay against adult *Phoetalia pallida*, was carried out essentially as described by Kagabu et al. (2007). Various concentrations of AAME and quercetin dissolved in 20µl insect saline, were injected between the third and the fourth abdominal segments of *P. pallida*. All the experiments were made in triplicate.

Five insects were used to test each dose and were kept at 22-25°C for 24 h after injection. The minimum dose at which three or more insects were considered killed was taken as the minimum lethal dose (MLD in µg). Paralyzed insects were also counted as having died.

2.10.2. Behavioral assays

For general behavioral study, animals were placed in a demarked open-field arena with a video camera mounted overhead.

2.10.2.1. Grooming activity

The grooming behavior of cockroaches was monitored in an opaque plastic box (29 cm x 18cm x 13 cm) with a clear plastic cover (Weisel-Eichler et al. 1999) and was recorded with a camera for later analysis of motion duration. The duration of continuous grooming in seconds was measured for a 30 min period immediately following treatment. Animals had never been in the testing box previously, and it was therefore a novel environment in all cases. The temperature in the testing room was maintained at 25–30 °C. Testing was performed 2–8 h after the beginning of the light cycle. Control cockroaches were injected with saline (Weisel-Eichler et al. 1999).

2.10.3 In vivo Cockroach Metathoracic Coxal-Adductor Nerve-Muscle Preparation

To analyze peripheral neurotoxicity, were used the *in vivo* cockroach metathoracic-coxal adductor muscle preparation (Oliveira et al., 2012). Animals were immobilized by chilling and mounted, ventral side up, in Lucite holder covered with 1cm soft rubber that restrained the body and provided a platform to which the metathoracic coxae could be firmly attached using entomologic needles. The left leg was then tied in the medial joint with a dentistry suture line connected to a 1g force

transducer (AVS Instruments, São Carlos, SP, Brazil). The transducer was mounted in a manipulator which allowed adjustment of muscle length. The exoskeleton was removed from over the appropriated thoracic ganglion. Nerve 5, which includes the motor axon to the muscle, was exposed and a bipolar electrode inserted to provide electrical stimulation. The nerve was stimulated at 0.5Hz/5ms, with twice the threshold during 120mim. The nerve was covered with mineral oil to prevent dryness. Twitch tension were recorded, digitalized and retrieved using a computer based software AQCAD (AVS Instruments, São Carlos, SP, Brazil). Data were further analyzed using the software ANCAD (AVS Instruments, São Carlos, SP, Brazil).

2.11. Data Statistical Analysis

The results were expressed as the mean ± SEM and were analyzed using analysis of variance (Two-Way ANOVA), followed Student "t" test as a *post hoc*. A p-value ≤ 0.05 indicated significance. Statistics and graphs were made using the Software OrigingPro 8.6 (OriginLab Corporation, MA, USA).

3. Results

3.1. Chemical investigation of Araucaria angustifolia methanolic extract compounds

Preliminary investigation of AAME phytochemical compounds by thin layer chromatography showed the presence of quercetin (data not shown). The presence of quercetin in the extracts from *A. angustifolia* was confirmed by evaluation of the chromatograms obtained by HPLC (Fig. 1). The chromatographic peak referred to quercetin was retained at 10.9 min, as can be observed by analysis of standard solution (Fig. 1). When the methanolic extract was submitted to chromatographic separation, a complex chemical profile was visualized, with detections of several substances in the

range of 7.5 – 20 min, including a peak at 10.695 min. A detailed comparison to standard reference allowed suggesting the presence of quercetin on plant material studied, even in low concentration.

3.1.1. Total phenolic content

In order to determine the total phenolic content in the studied plant material, the quantitative assay was applied according to the standardized protocol described for spectrophotometric determination. The results demonstrated a mean content of 23.27 mg GAE/mL of extract (RSD = 1.03%), illustrating a high content of phenolic in the methanolic extract.

3.2. Effect of sublethal doses of AAME and Quercetin on Brain AChE activity

The analysis of AChE activity of cockroach brain homogenates before and after injection of cockroaches with different sublethal concentrations of AAME (200 and 400µg/g animal weight), quercetin (40µg/g) and Trichlorfon (40µg/g) revealed a dose-dependent enzyme inhibition. Thus, the control values of AChE activity with saline was 185±3nmol TNB/min/mg protein. When AAME was incubated at (200 µg/g animal weight) the AChE activity decreased to 148.5±6nmol TNB/min/mg protein (n=3; $p>0.05$, Student "t" test). When AAME (400µg/g animal weight) was incubated there was a significant decrease of AChE activity (125.5±3nmol TNB/min/mg protein; n=3; $p<0.05$,Student *t* test) compared to control saline. On the other hand, a significative increase in AChE inhibition was also seen when quercetin (40µg/g) was incubated (73.5±5nmol TNB/min/mg protein; n=3; $p<0.05$, Student *t* test). Trichlorfon (40µg/g) administration, resulted in an AChE inhibition (24±6nmol TNB/min/mg protein; n=3; $p<0.05$, Student "t" test) (Fig.2).

3.3. Insecticide activity of AAME and Quercetin

To determine the insecticidal activity of *A. angustifolia*, four doses of the AAME were assayed (200, 400, 800 and 1600µg/g). For this, five cockroaches were injected at the third abdominal portion, and observed during 24h. After this period, the dose of (800µg/g) was considered as the minimum lethal dose (Fig. 3). Quercetin, a common phenolic compound in *A. angustifolia* seeds and barks (Cordenunsi et al., 2004; Seccon et al., 2010) was also assayed at 40, 80, 1200, and 1800µg/g, and showed to be lethal at 80µg/g and above. Once minimum lethal doses were identified, all the following biological protocols were carried out using sublethal concentrations.

3.4. Effect of sublethal doses of AAME and Quercetin on grooming activity

In saline injected cockroaches, the mean time of continuous grooming was 60± 8s/30min (n=20). We found that only the manipulation of the animal and the introduction of the rod of the syringe does not significantly interfere with the animal's normal behavior (67.5±12s/30min; n=10; $p>0.05$, Student "t" test).

All cockroaches treated with AAME showed a dose-dependent increase in grooming activity. Thus, AAME (200µg/g of animal weight) induced a significant increase in grooming activity (138.11±5s/30min; n=10; $p<0.05$, Student "t" test). With the highest dose assayed (400µg/g of animal weight) there was a further increase in the grooming parameters (185±8s/30min; n=10; $p<0.05$ Student "t" test) (Fig. 4). When quercetin (40µg/g) was assayed, there was also an increase in time spending grooming, that was higher than the all previously tested concentrations of AAME (230±5s/30min; n=10; $p<0.05$, Student "t" test) (Fig. 4).

3.5. Effect of cholinergic modulators on Quercetin-induced grooming activity

When atropine (40µg/g), a non-selective muscarinic cholinergic receptor inhibitor, was assayed alone (76±2s/30min) there was no alteration compared to control grooming levels (75±14s/30min). The injection of atropine (40µg/g) combined with quercetin (40µg/g) significantly increased the grooming levels to over the control values of quercetin (350±3s/30min; n=10; $p<0.05$, Student "t" test) (Fig.5). When methoctramine (40µg/g), a selective inhibitor of M_2-M_3 cholinergic receptor was combined with quercetin (40µg/g) there was the highest increase on the grooming pattern (600±8s/30min; n=10; $p<0.05$, Student "t" test) (Fig.5). However, pirenzepine (40µg/g), a selective M_1-cholinergic blocker, added 15min earlier to quercetin (40µg/g) induced no significative alteration in the grooming levels of quercetin alone (230±5s/30min; n=10; $p>0.05$, Student "t" test) (Fig. 5).

3.6. Effect of dopamine modulators, Hydroxilamine and Tyramine on Quercetin-induced grooming activity

Since dopamine (DA) activity is mediated by dopamine receptors at pre- and postsynaptic neuronal membrane (e.g D_2 and D_1 receptors families), the protocols described below aimed to verify the influence of dopaminergic modulators on quercetin-induced grooming activity. Thus, metoclopramide (40µg/g), a DA-D_2 receptor antagonist, injected 15min before quercetin (40µg/g) inhibited significantly the quercetin-induced grooming activity (100±12s/30min; n=10; $p<0.05$, Student "t" test) (Fig. 6). When the SCH 23390 (40µg/g), a selective DA-D_1 receptor blocker was administrated 15min earlier to quercetin (40µg/g) there was also a significative inhibition of grooming levels, even below the metoclopramide treateament values (90±6s/30min; n=10; $p<0.05$, Student "t" test) (Fig. 6). Dopamine release depends on calcium entrance at nerve terminals in order to induce exocytosis (Rozov et al. 2001).

Nitric oxide is thought to be involved in Dopamine release by increasing the opening probability of L-type calcium channels (Büyükuysal, 1997). We used the NO donor hydroxylamine, in order to verify the influence of nitric oxide cascade in the quercetin-induced grooming increase. Thus, hydroxilamine (40µg/g) alone, induced a slight increase of grooming activity compared to control saline values (75±8s/30min). When hydroxilamine (40µg/g) was applied 15min prior quercetin (40µg/g) there was a significant increase in grooming activity compared to quercetin alone (325±15s/30min; n=10; $p<0.05$, Student "t" test) (Fig 5). In order to verify the influence of cathecolamines in the quercetin-induced increase of grooming behavior we used tyramine, an agonist of tyramine receptors at insect nerve terminals. Tyramine (40µg/g) alone, induced a high increase of grooming activity compared to control saline values (182±8s/30min, n=10; $p<0.05$, Student "t" test). When tyramine (40µg/g) was applied 15min prior quercetin (40µg/g) there was an increase in grooming activity compared to quercetin alone (204±15s/30min; n=10; $p<0.05$, Student "t" test) (Fig 6).

3.7. Neuromuscular blockade induced by AAME and Quercetin at in vivo cockroach nerve-muscle preparation

To further analyze the effect of AAME and quercetin on cockroach peripheral nervous system, we used the i*n vivo* metathoracic coxal-adductor nerve-muscle preparation. The administration of insect saline alone did not interfere with neuromuscular responses during 120min recordings (n=6) (Fig. 7). The injection of AAME (200 and 400 µg/g of animal weight) induced a dose and time-dependent neuromuscular blockade in 120 min recordings. When the minimum dose of AAME (200µg/g) was assayed there was 50±9% blockade of twitch tension in 82min (n=6, $p<0.05$, ANOVA) and 68±6% in 120min recordings (Fig 7). The injection of the highest concentration of AAME (400µg/g) induced 50±5% blockade in 45min and

95±3% inhibition of the twitches in 120min recordings (n=6, $p<0.05$, ANOVA) (Fig.7).

When quercetin was assayed (10µg/g and 40µg/g), the same dose and time-dependent effect was observed at neuromuscular parameters. Thus, for the lowest concentration assayed, there was only 45 ±15% neuromuscular blockade in 120 min recordings (n=6, $p<0.05$ compared to control saline) (Fig 7). When the highest concentration was assayed, there was 50 ±13% inhibition of the twitch tension at 25 min and complete inhibition of the muscle strength at 120 min (n=6, $p<0.05$, ANOVA) (Fig 7). In all preparations, the increase of the frequency of electrical stimulation was unable to recovery the neuromuscular function.

4. Discussion

In this work we have demonstrated for the first time the insecticide activity of *Araucaria angustifolia* needles extract, and one of its constituents quercetin. The toxic activity induced by the AAME is related to a neurotoxic action probably by the inhibition of insect acetylcholinesterase (AChE). In this regard, a number of plants have been ascribed as having insecticide activity, to target mainly at insect nervous system (Richards and Cutkomp, 1945, Soderlund, 1995; Bloonquist, 1996), but few have demonstrated a direct correlation between inhibition of insect AChE and toxicity (Hieu et al., 2012). Acetylcholinesterase plays role in cholinergic synapses that is essential for insects and higher animals (Fournier and Mutero, 1994). Inhibition of AChE causes accumulation of acetylcholine at the synapses, so that the post-synaptic membrane is in a state of permanent stimulation, which results in ataxia i.e. general lack of co-ordination in the neuromuscular system, and eventual death (Singh and Singh, 2000; Aygun et al., 2002). It has been also reported that the essential oils act by

a reversible competitive inhibition of AChE enzymes isolated from electric eel, heads of house fly, cockroaches, horse serum and bovine erythrocytes (Rattan, 2010). To date, monotherpenoids, present at plant essential oils, are among the compounds named to induce neurotoxicity by inhibition of insect AChE (Coats et al., 1991, Hieu et al., 2012). We also investigated the composition of secondary metabolites in the AAME. We found a great amount of phenolic compounds and the presence of quercetin in the extract. Concerning the chemical constituents and the classes of metabolites, the phenolic compounds are involved on essential functions and interfere in several biological systems, mainly due their known antioxidant potential with hydrogen-donating radical scavenger properties (Rice-Evans et al 1996; Urquiaga and Leighton 2000). In addition, the presence of the flavonoid quercetin was identified in the whole extract by comparison with the standard reference. In our experimental conditions, quercetin has also demonstrated inhibitory actions on cockroach brain AChE, corroborating the previous literature for mammals (Jung and Park, 2007) and insects (Pontual et al., 2012). This observation reinforces the relevance of quercetin in the insecticide activity of *Araucaria angustifolia*. Therefore, to the best of our knowledge, it is the first time that the flavonoid quercetin was assayed for insecticide activity in cockroaches. This may improve the biotechnological applications of the AAME, especially because quercetin is an important antioxidant compound of humans dietary (Cartea et al., 2011).

AAME showed to be lethal to cockroaches at relatively moderated concentrations, confirming the traditional knowledge (Arcego, 2005). A number of plant extracts have demonstrated insecticidal activity (Rattan, 2010) and their mode of action and site of effect for the insecticide activities have been studied by various authors (Hiort et al., 1999; Michaels et al., 2010; Vandemborre et al., 2011). In this

respect, insecticides exert a broad range of effects on insects and other arthropods, e.g. neuroexcitation resulting in hyperactivity, tremor and rigid paralysis due to energy depletion and neuromuscular fatigue, while neuroinhibition results in immobility and paralysis because of possible oxygen deprivation and/or reduced respiratory capacity that ultimately leads to mortality (Scharf et al., 2003). It is important to investigate behavioral patterns in insects to elucidate the mode of action of novel and conventional insecticides, and their response in the environment by minimizing their contact with the toxic material (von Keyserlingk et al., 1985).

The effects induced by AAME and quercetin on grooming activity showed to be similar. Grooming in insects serve the function of cleaning the outer body surface and may have other functions as well, such as courtship behavior, social signaling, displacement activity and de-arousal (Spruijt et al. 1992). In insects, a neural center involved in grooming behavior is not well identified but, it was demonstrated, that dopamine (DA) may act as the main neurotransmitter associated with this response (Weisler-Eicheler et al. 1999). Signaling by extracellular DA is regulated by several mechanisms such as diffusion from the synapse, enzymatic degradation and reuptake of DA by the DA transporter (DAT). In addition to that, a muscarinic (M) cholinergic mechanism underlies activation of the central pattern generator in insects, probably by activation of serotoninergic/dopaminergic interneurons (Buhl et al., 2008). Indeed, in our experimental conditions, the previous treatment with atropine, an unspecific blocker of muscarinic receptors, followed by quercetin treatment increased the quercetin-induced grooming behavior. Therefore, at sites in the insect brain where muscarine stimulation is effective, behavioral changes are evoked by forskolin, an activator of adenylate cyclase (AC); 8-BrcAMP-activating protein kinase A (PKA); and 3-isobuty-1-methylxanthine, leading to the accumulation of endogenously

generated cAMP through inhibition of phosphodiesterases. This suggests that the mAChRs mediated-excitation occurs by stimulating the AC/cAMP/PKA pathway. Because the inhibition of AC, PKA, or PLC by various individually applied substances entirely suppressed muscarine-evoked behaviour parameters in insects, activation of both pathways, AC/cAMP/PKA and PLC/IP3/diacylglycerine, appeared to be necessary to mediate the excitatory effects of mAChRs (Wenzel et al., 2002). Furthermore, activation of insect mAChRs by muscarinic agonists demonstrated to increase cytosolic IP3 and Ca^{2+} concentrations, showing a selectivity for antagonists that is similar to vertebrate M1 and M3 subtypes but distinct from M2 AChRs (Millar et al., 1995, Honda et al., 2007). Our data are in agreement with the literature, since pirenzepine counteract the quercetin-induced increase in grooming activity. In addition, at nerve terminals, phosphatidylinositiol (PI) 3-kinase is involved in DA transporter activation and the degradation of (IP3) (Carvelli et al., 2002). Quercetin is also thought to inhibit phosphatidylinositiol 3-kinase (Nanua et al., 2006), reinforcing that the AAME modulation of insect behavior is likely to involve the increase of IP3 signaling, following activation of mAChRs. We also investigated the influence of tyramine and hydroxylamine on quercetin-induced increase of cockroach grooming behavior. Tyramine activates tyramine receptors (TyrRs) at insect central nervous system, modulating insect behavior by increasing citosolyc Ca^{2+} by the IP3 synaling (Farooqui, 2007). However, in our experimental conditions, tyramine was unable to increase quercetin-induced increase of grooming behavior, suggesting that TyrRs are probably not involved in this effect. Further experiments using TyrRs specific antagonists should improve this knowledge. Regarding to hydroxylamine, it is a nitric oxide regenerator (Atoine et al, 1996). Nitric oxide (NO) is a membrane-permeant messenger molecule generated from the amino acid L-arginine. NO can activate

soluble guanylyl cyclase leading to the formation of cyclic GMP (cGMP) in target cells (Bicker, 2001). NO is an atypical neurotransmitter since it is not packaged in synaptic vesicles but rather diffuses from its site of production and moves readily through cell membranes. The principal function of NO appears to be as an activator of the heterodimeric heme protein soluble guanylyl cyclase (sGC). Activation of guanylyl cyclase in insect neurons is related to excitatory process of neurotransmission by modulation of intracellular Ca^{2+} stores (Bicker, 2001). We suggest that hydroxilamine potententiate the quercetin-induced increase in grooming behavior in cockroaches by a synergism in Ca^{2+} signaling pathway at dopaminergic nerve terminals.

In addition, we attempted to investigate if the insecticide effect of AAME was to a direct action on cockroach peripheral nervous system. We confirmed a significative blockage of neuromuscular twitches by AAME and Quercetin using *in vivo* cockroach nerve-muscle preparations. At insect neuromuscular junctions, glutamate is the main excitatory neurotransmitter and $GABA_{(A,B)}$ is the main inhibitory one (Osborne, 1996). The increase of neuromuscular blockade induced by both AAME and quercetin resemble drugs which act direct on receptor ion channels. At insect neuromuscular junctions there are mainly two types of ionotropic receptors. The first is the NMDA receptor which is activated by the excitatory neurotransmitter glutamate and the second is $GABA_A$ receptor which is activated by the inhibitory neurotransmitter gamma-aminobutyric –acid (Osborne, 1996). We did not attempt to investigate in which receptor AAME and quercetin is preferable targeting, but blockage of NMDA and/or activation or $GABA_A$ receptors cannot be disregarded. Some plant natural compounds are able to modulate both NMDA (Karangwa et al., 2007) as well as GABA receptors (Wang et al., 2006). In murines, quercetin and its

derivatives can indeed induce selective inhibitory actions on NMDA receptors (Mehdizadeh et al., 2009).

Conclusions

In conclusion, in this paper we demonstrated the insecticidal activity of *A. angustifolia* methanolic extract, using *P. pallida* as experimental model. This result confirms the traditional knowledge for *A. angustifolia,* as a natural insecticide. The insecticidal activity of AAME is mainly related to its inner anticholinesterase activity in insects, but also at direct action on insect neuromuscular junctions. The anticholinesterase activity of the AAME is probably devoid to the presence of the flavonoid quercetin in its constitution. The anti AChE activity initiates a cascade of events that probably begins with the activation of insect muscarinic receptors. This process causes a modulation of insect behavior by changing the dopaminergic pathways at insect central nervous system. The inhibitory actions of AAME and quercetin at insect neuromuscular junction should be exploited in a future detailed investigation, but inhibition of NMDA receptors is suggested.

5. Acknowledgements

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Figures and Legends

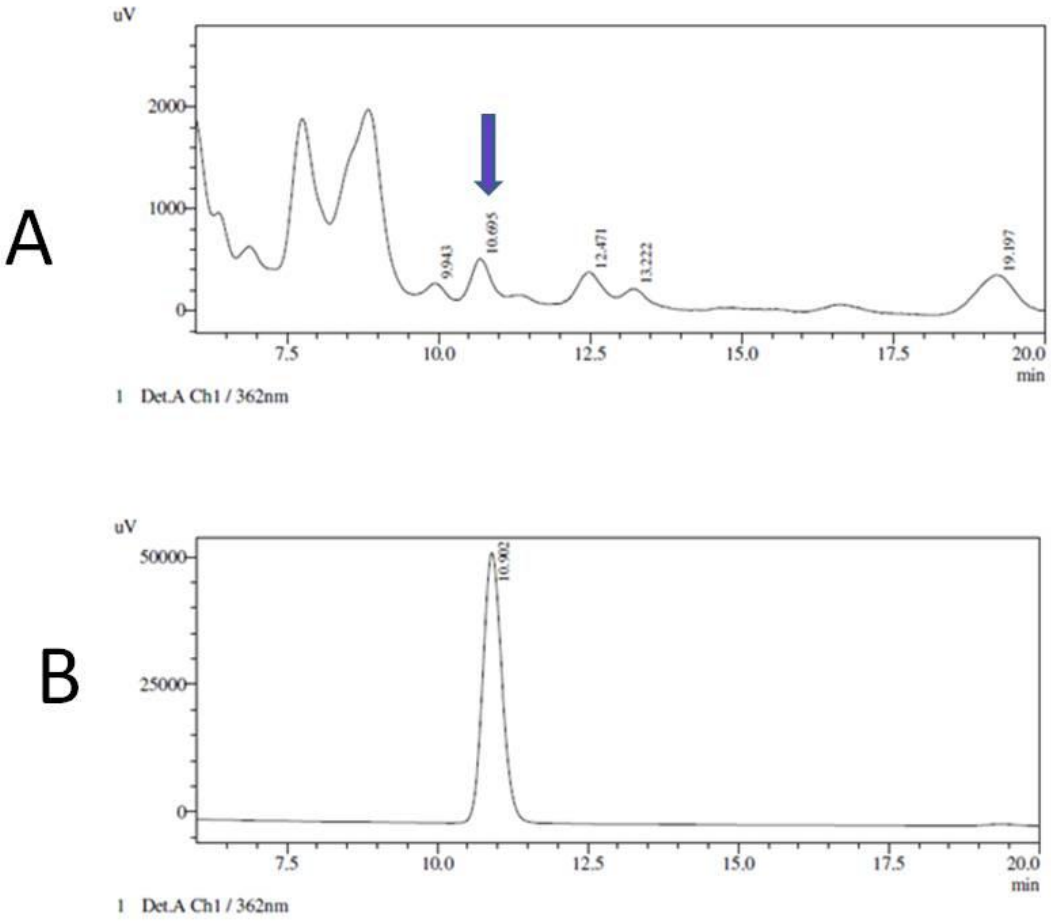

Fig. 1. High Performance Liquid Chromatography (HPLC) of *A. angustifolia* methanolic extract **(A)** and quercetin standard **(B)**. Note that on (A) there is an evident peak at 10.695 min. of retention time. When quercetin standard was eluted there was a higher peak almost at the same time, at 10.9 min. of retention time. This observation suggests the presence of quercetin on *A. angustifolia* methanolic extract located at 10.695 min. of retention time.

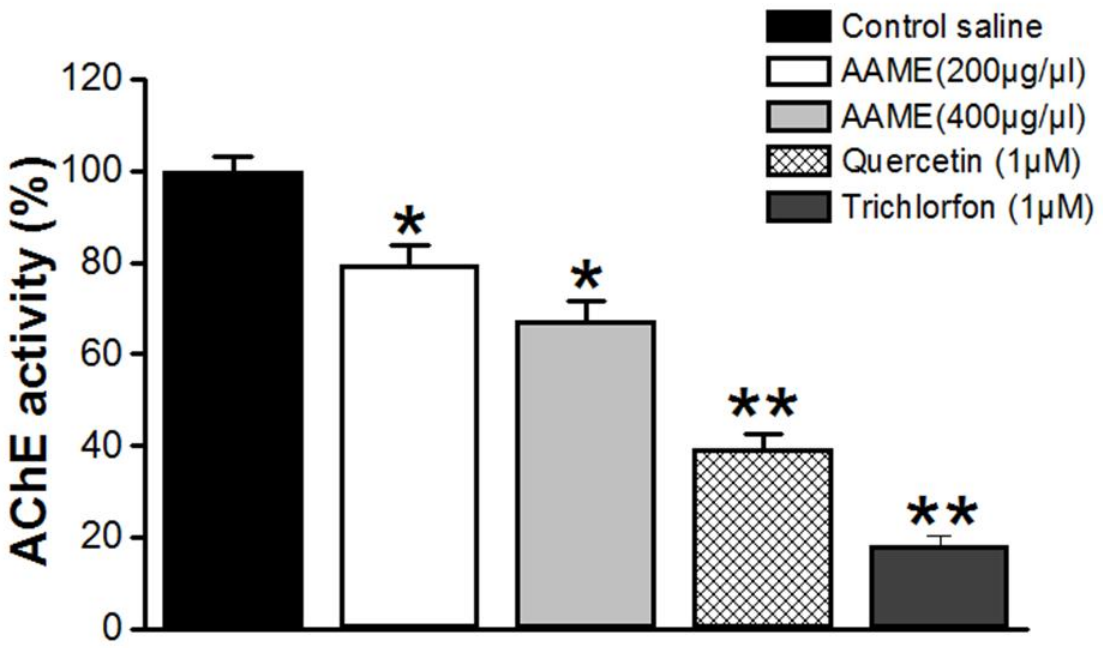

Fig. 2. Inhibition of acetylcholinesterase (AChE) activity by *Araucaria angustifolia* methanolic extracts (AAME) and quercetin. The graph shows the dose-response inhibition of AChE by AAME, compared to the flavonoid quercetin and the organophosphate trichlorfon. Data were expressed as nmol TNB/min/mg protein. The assay for AChE inhibition was carried out using different concentrations of AAME (200 and 400µg/µL), quercetin (1µM) and trichlorfon (1µM). Experiments were done in triplicate. (**significance at $p<0.01$ with Student "t" test).

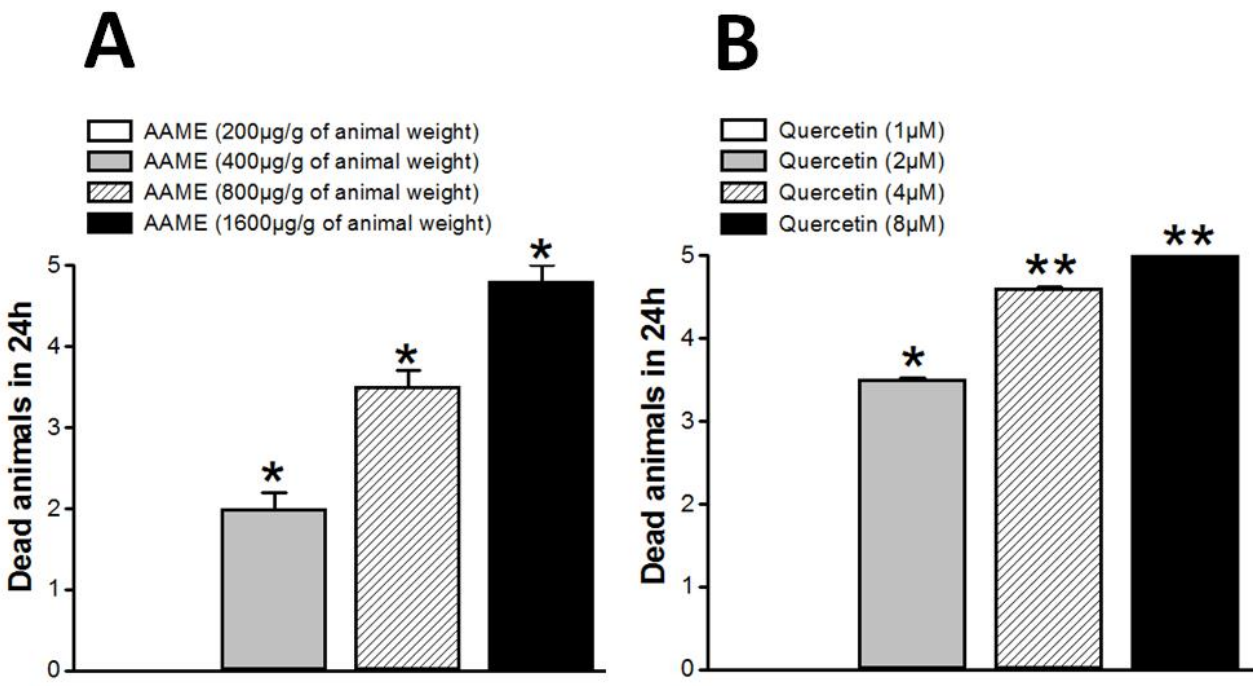

Fig. 3. Minimum Lethal Dose (MLD) of *Araucaria angustifolia* methanolic extract (AAME) **(A)** and quercetin **(B).** All treatments were done in triplicate (n=5) and the dose able to kill 3 or more insects was considered as the MLD (n=3). (**significance at $p<0.01$ with Student "t" test).

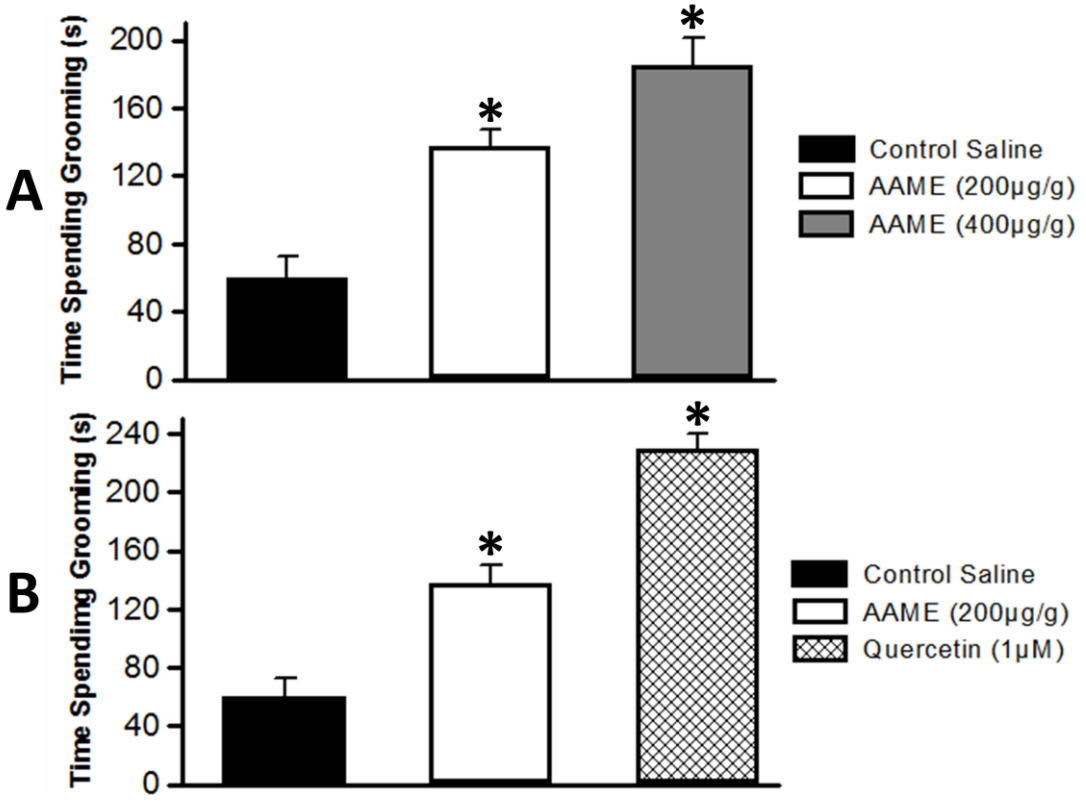

Fig. 4. Increase of grooming behavior by different sublethal doses of *Araucaria angustifolia* methanolic extract (AAME) **(A)** and quercetin **(B)**. The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. (*p<0.05 with Student "t" test)

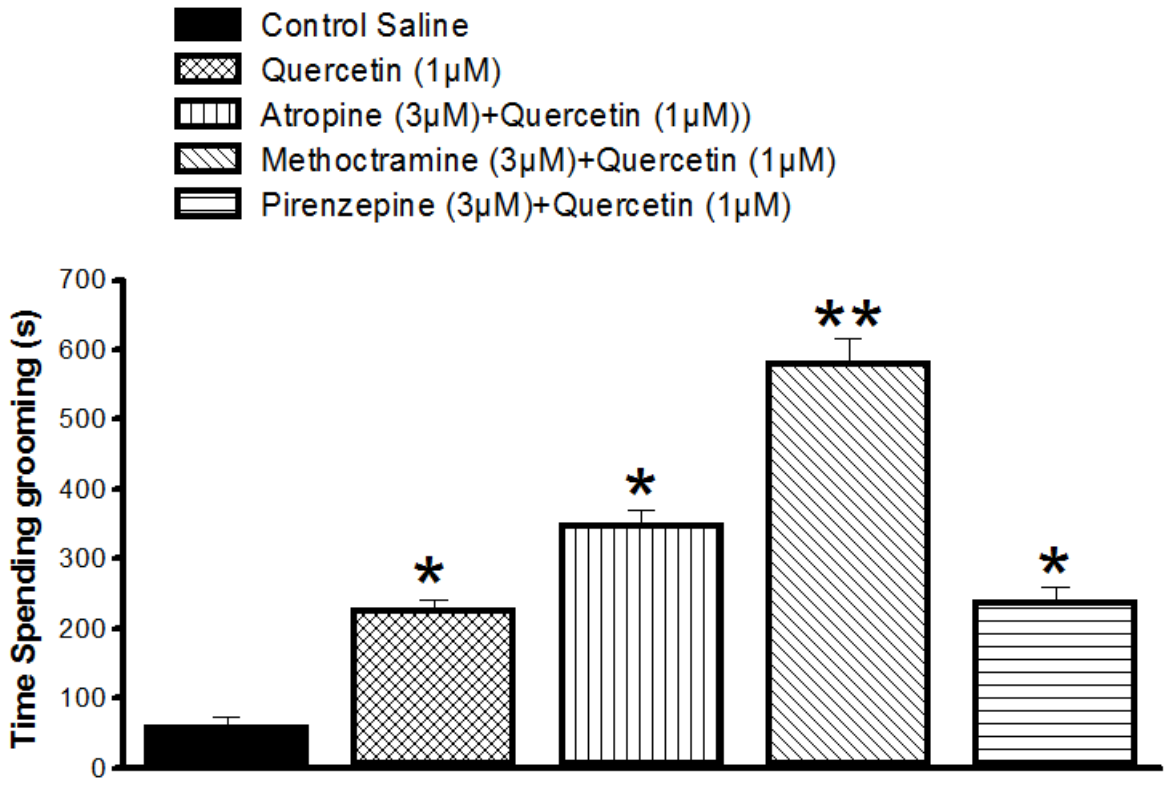

Fig. 5. Effect of different cholinergic modulators on Quercetin-induced grooming increase in cockroaches. Drugs were injected in the third abdominal segment 5 min before querectin (1µM). The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. (**p<0.01 with Student "t" test).

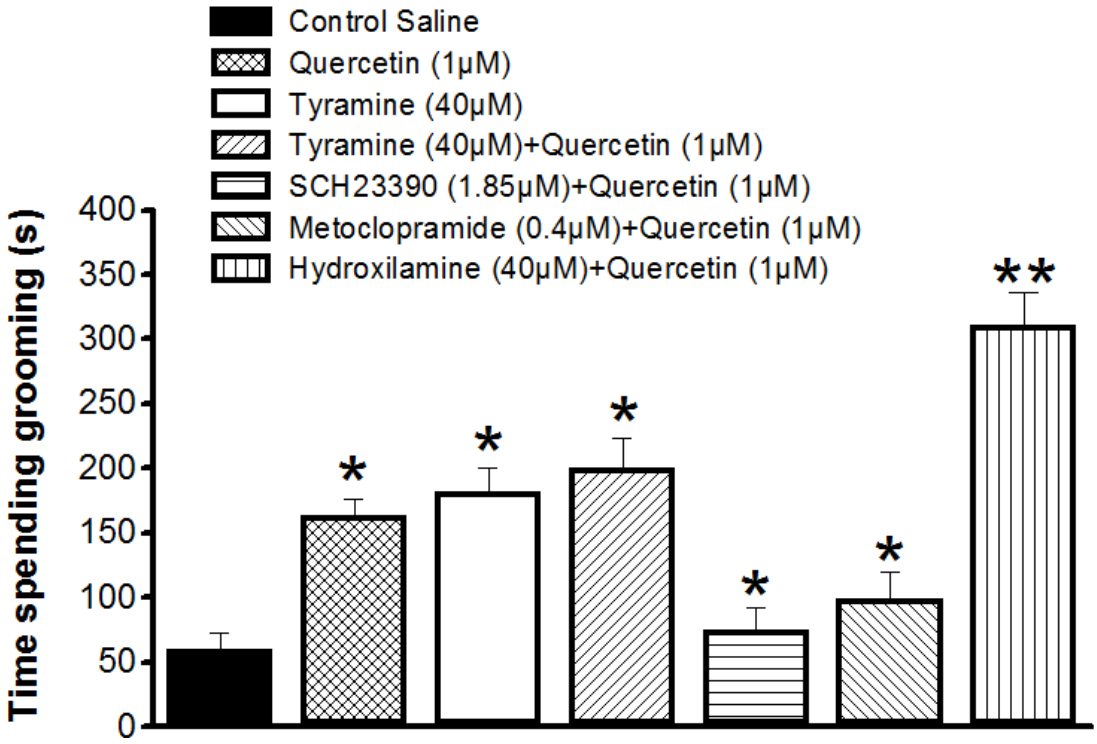

Fig. 6. Modulation of quercetin-induced increase in grooming activity by dopaminergic drugs. Drugs were injected in the third abdominal segment 5 min before quercetin (1µM). The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. (**p<0.01 with Student "t" test).

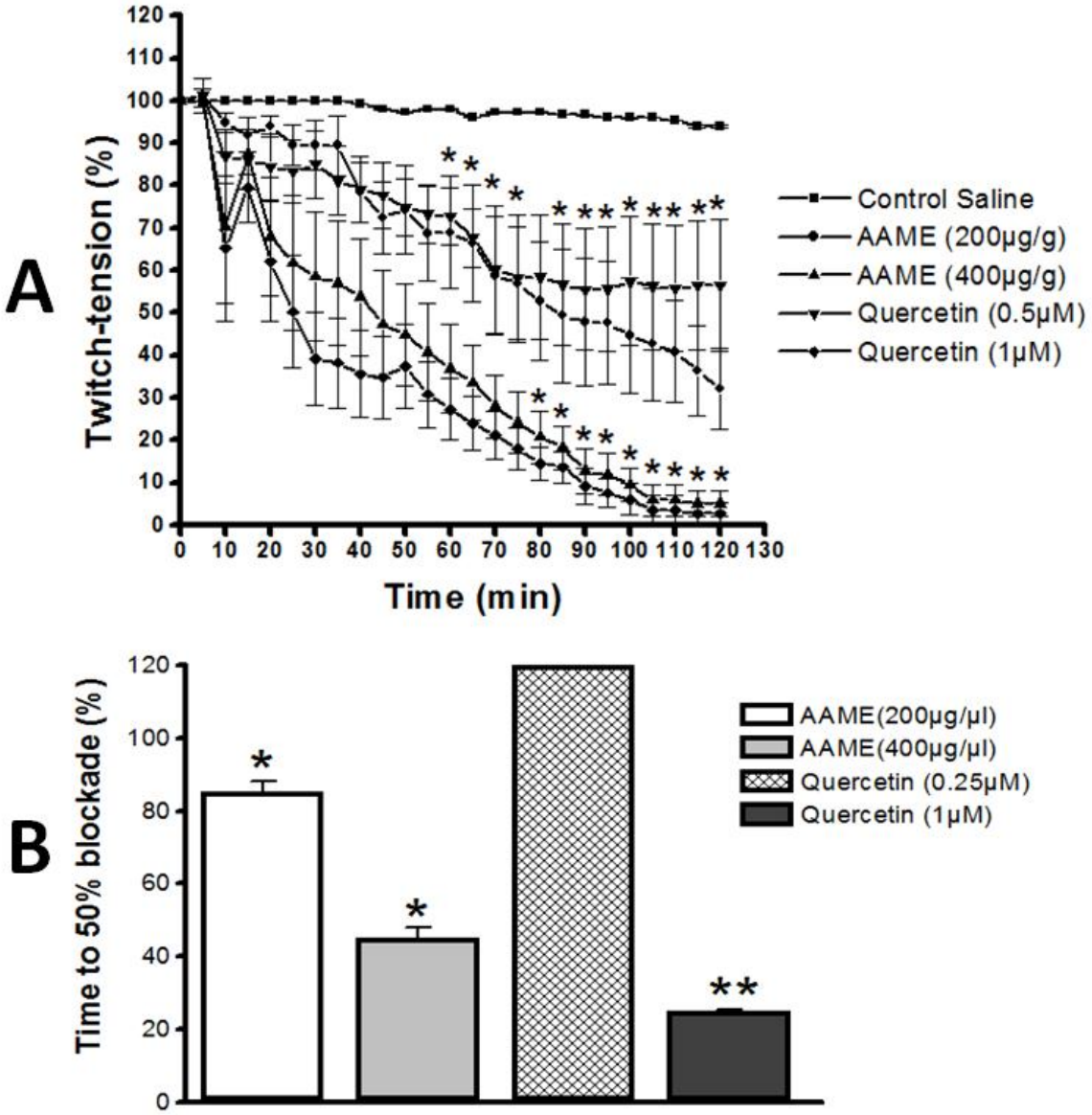

Fig. 7. Neuromuscular blockade induced by *Araucaria angustifolia methanolic extract* (AAME) and quercetin at *in vivo* cockroach coxal-adductor methatoracic nerve-muscle preparation. Panel (**A)** shows the dose-dependent effect induced by AAME (200 and 400µg/g of animal weight) and quercetin (1µM). On **B)**. the graph of the time required to 50% blockade. Note the dose-dependent inhibition of cockroach twitches (**p<0.01 with ANOVA two way or Student "t" test)

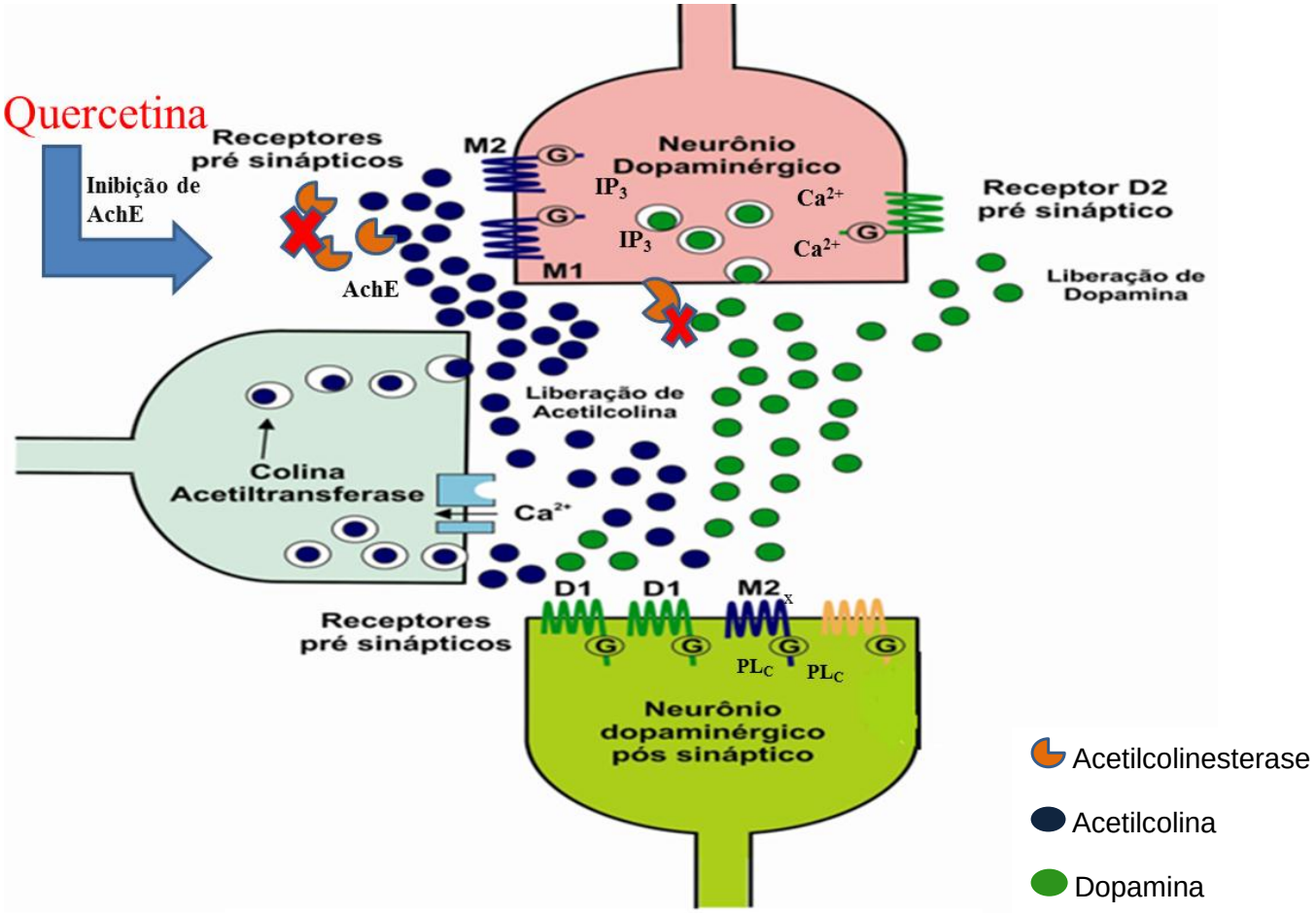

Fig. 8. Proposta de mecanismo de atividade inseticida induzida pelo extrato metanólico de *Araucaria angustifolia* em *Phoetalia pallida* sobre o sistema nervoso central**.** Nesse modelo, o extrato bruto, pela ação da quercetina, induz a inibição da atividade da enzima acetilcolinesterase. A inibição dessa enzima, em neurônios colinérgicos da barata, levaria ao aumento da concentração do neurotransmissor acetilcolina (ACh) nos terminais nervosos colinérgicos. A acetilcolina possivelmente ativaria receptores muscarínicos colinérgicos na região pré-sinaptica de neurônios dopaminérgicos (autoreceptores), levando a geração do segundo mensageiro trifosfofato de inositol (IP3), aumentando a concentração do Ca^{2+} intracelular, favorecendo a liberação do neurotransmissor dopamina. Em paralelo, receptores muscarínicos pós-sinapticos presentes em neurônios dopaminérgicos, induziriam a ativação de uma fosfolipase C (PLC), favorecendo a despolarização da membrana pós-sinaptica pela dopamina e/ou aumentando a excitabilidade desses neurônios à dopamina. (Fonte: G.D. Stürmer/ Modificações: T.C. Freitas).

7. CONCLUSÕES

Em nossas condições experimentais, os ensaios com o extrato metanólico de *Araucaria angustifolia*, validaram o seu potencial como inseticida natural, nesse contexto é apresentada uma proposta do mecanismo de ação da atividade inseticida deste extrato sobre *Phoetalia pallida* (Fig 8). Além disso, a análise fitoquímica detalhada do extrato bem como os ensaios de atividade biológica permitiu concluir:

-Existe uma quantidade importante de compostos fenólicos dentre os metabólitos secundários presentes no extrato;

- O flavonóide quercetina está presente nas amostras utilizadas do extrato metanólico de *Araucaria angustifolia.*

- A Atividade inseticida deve-se, em parte, provavelmente pela inibição da enzima acetilcolinesterase do inseto, provavelmente pela atividade da quercetina.

- A atividade anticolinesterasica induz alterações comportamentais no inseto, as quais estão relacionadas à modulação da neurotransmissão colinérgica e dopaminérgica, no sistema nervoso central.

-Um efeito direto sobre a junção neuromuscular do inseto foi observado e deve contribuir para a atividade inseticida do extrato.

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