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O EFEITO FACILITATÓRIO DE FRAÇÕES DE *Casearia sylvestris* SW. SOBRE A FISIOLOGIA DO SISTEMA ESQUELÉTICO MOTOR DE MAMÍFERO E AVE

Sorocaba/SP
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Dissertação apresentada à Banca Examinadora do Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade de Sorocaba, como exigência parcial para obtenção do título de Mestre em Ciências Farmacêuticas.

Orientador: Profa. Dra. Yoko Oshima Franco.

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Dissertação aprovada como requisito parcial para obtenção do grau de Mestre no Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade de Sorocaba.

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Dedico,

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A coragem é a primeira das qualidades humanas
porque garante todas as outras.

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RESUMO

Inúmeras potencialidades medicinais foram relatadas com extratos oriundos de diversas partes da planta *Casearia sylvestris.* Estudos prévios demonstraram que extratos tanto aquoso quanto hidroalcoólico de *C. sylvestris* expressaram significativo efeito facilitatório em modelo nervo frênico-diafragma de camundongos. O benefício clínico na busca de novos agentes facilitadores é amplo. Dependendo da sua farmacodinâmica, sob o parâmetro da junção neuromuscular, pode-se pensar em aplicações nas doenças neuromusculares que causam paralisia, como adjuvantes no tratamento de envenenamentos causados por toxinas, na reversão de bloqueadores neuromusculares, entre outros. Os objetivos deste estudo foram: a) identificar farmacologicamente o efeito facilitatório em frações orgânicas de diferentes polaridades obtidas de folhas de *C. sylvestris*; b) Analisar músculos resultantes de tratamentos com frações neurofacilitadoras através de microscopia de luz; c) Determinar o efeito da fração facilitadora em relação à colinesterase e creatinoquinase (CK); e, d) investigar a farmacodinâmica da fração neurofacilitadora. As frações de *C. sylvestris* (hexano, diclorometano, acetato de etila e metanol) foram ensaiadas em preparações nervo frênico-diafragma (NFD) de camundongos na concentração de 200 µg/mL. Músculos diafragmas tratados com frações identificadas como neurofacilitadoras foram submetidos à microscopia de luz para verificar possível miotoxicidade; e bioquimicamente foi determinada a atividade de CK em preparações biventer cervicis (BC) de pintainho tratadas com a fração metanólica (FM) na concentração de 240 µg/mL.O estudo farmacodinâmico foi realizado utilizando-se ferramentas farmacológicas em preparações estimuladas direta ou indiretamente. Em alguns protocolos foram aplicados estímulos tetanizantes (40 Hz). Experimentos realizados com frações de diferentes polaridades da *C. sylvestris,* na concentração de 200 µg/mL, mostraram que as frações acetato de etila e metanólica foram as responsáveis pelo efeito de facilitação. A análise histológica comprovou a ausência de danos ao tecido muscular de preparações tratadas com essas frações, em relação ao controle Tyrode. A FM foi selecionada para os ensaios farmacodinâmicos em virtude do maior rendimento obtido. Do estudo farmacodinâmico, com fármacos de atuação em sítios específicos da junção neuromuscular, temos que: em preparações previamente curarizadas, a FM demonstrou atividade antagonista de diferente intensidade, assim como os fármacos neostigmina e 3,4 diaminopiridina (3,4 DAP). A aplicação de estímulo tetanizante (40 Hz) não apresentou a inibição de Wedensky, característica de anticolinesterásicos como a neostigmina. Em

preparações previamente tratadas com dantrolene, a FM não apresentou nenhum efeito, excluindo-se a possibilidade de competição com o dantrolene no receptor rianodina. Em preparações estimuladas diretamente, a adição de FM não produziu nenhum efeito sobre o sarcolema. Em preparação BC a FM não alterou as contraturas induzidas pela adição de acetilcolina exógena (ACh) e cloreto de potássio (KCl). Experimentos com nifedipina mostraram que o efeito facilitador da FM pode ser modulado por cálcio extracelular. A participação de receptores pré-juncionais foi excluído pelos resultados obtidos com atropina. A tetrodotoxina antagonizou o efeito facilitador da FM, sendo que o mesmo não ocorreu com 3,4 DAP e neostigmina. Os resultados apresentados mostram que o local de ação da FM ocorre à nível pré-sináptico através da ativação de canais de sódio neuronais, podendo a neurofacilitação ser modulada pelo cálcio extracelular.

Palavras-chave: *Casearia sylvestris*. Facilitação pré-sináptica. Junção neuromuscular.

ABSTRACT

Several medicinal potentialities were mentioned to extracts from parts of *Casearia sylvestris* plant. Previous studies showed that either aqueous as hydoralcoholic extracts from *C. sylvestris* expressed significantly facilitatory effect on mice phrenic nerve-diaphragm model. The clinical benefits in the search of new facilitatory agents are enormous. Depending on of its mechanism of action, under neuromuscular junction, it is possible to apply in neuromuscular diseases that lead to paralysis, as adjuvant in envenomation treatments induced by toxins, in the neuromuscular blockers recovery, among others. The aims of this study were: a) to identify pharmacologically the facilitatory effect in organic fractions of different polarities obtained from *C. sylvestris* leaves; b) To evaluate muscles resulting from neurofacilitating fractions using light microscopy; c) To evaluate cholinesterase inhibition and creatine kinase activity of neurofacilitating fraction; and, d) to evaluate the mechanism of action of the neurofacilitating fraction. The *C. sylvestris* fractions (hexane, dichloromethane, ethyl acetate and methanol) were assayed using mice phrenic nerve-diaphragm (PND) preparations at 200 µg/mL. Diaphragm muscles resulting from pharmacological assays that expressed facilitatory effect were submitted to light microscopy in order to verify a possible myotoxicity, and were biochemically evaluated the CK activity from experiments carried out using chick biventer cervicis treated with methanol fraction at 240 µg/mL. The mechanism of action study was carried out using pharmacological tools in isolated preparations (in) directly stimulated. In some protocols, tetanic stimulation (40 Hz) was used. Experiments carried out with *C. sylvestris* fractions from different polarities at 200 µg/mL showed that ethyl acetate and methanol fractions were responsible by the facilitatory effect. Histological analysis showed the cell damage absence in preparations treated with these fractions, compared to Tyrode control. The methanol fraction was selected for further mechanism of action studies due its major outcome. From this study, using agents that act in specific places of neuromuscular junction, we have: MF act as antagonist of different intensity, in pre curarized preparations, as neostigmine and 3,4 diaminopyridine (3,4 DAP). PND preparation exposed to MF under tetanic stimuli (40 Hz) did not show the Wedensky inhibition, characteristic of cholinesterase inhibitors, as neostigmine. MF did not show any effect in pretreated preparations with dantrolene, excluding competition against dantrolene at ryanodine receptor. MF addition in preparations directly stimulated did not produce any effect on sarcolemmal membrane. In BC preparation, MF fraction did not change the contracture induced by exogenous acetylcholine (ACh) and potassium chloride (KCl). Experiments with nifedipine

showed that the facilitatory effect of MF can be modulated by extracellular calcium. The sharing of prejunctional receptors was excluded by obtained results with atropine. Tetrodotoxin antagonized the facilitatory effect of MF, in opposite to 3,4 DAP and neostigmine. The results showed that MF act presynaptically via neuronal sodium channel activation and the neurofaciltion can be modulated by extracellular calcium.

Key words: *Casearia sylvestris.* Presynaptic facilitation. Neuromuscular junction.

LISTA DE ABREVIATURAS E SIGLAS

NFD	Nervo frênico-diafragma
CK	Creatinoquinase
BC	Biventer cervicis
FM	Fração metanólica
3,4 DAP	3,4 diaminopiridina
Hz	Hertz
ACh	Acetilcolina
KCl	Cloreto de potássio
MeOH	Metanol
JNM	Junção neuromuscular
NAChRs	Receptores nicotínicos de acetilcolina
TTX	Tetrodotoxina
PEG 400	Polietilenoglicol 400
BNM	Bloqueadores neuromusculares
ChE	Colinesterase

SUMÁRIO

1 INTRODUÇÃO

A diversidade da flora brasileira é muito grande com mais de 43.020 espécies registradas e muitas ainda não descritas (LEWINSOHN; PRADO, 2005). O desconhecimento do potencial dessas plantas torna o Brasil frágil, quanto à descoberta de novos fármacos, ao patenteamento de processos biológicos e à realização de parcerias em pesquisas com instituições científicas internacionais, onde ambos possam compartilhar do conhecimento obtido (PEIXOTO; MORIM, 2003).

Estudos multidisciplinares mostram a abundância de metabólitos encontrados nos biomas brasileiros, onde muitas dessas substâncias apresentam atividades biológicas e farmacológicas, que podem contribuir para o desenvolvimento de fármacos, agroquímicos, cosméticos e suplementos alimentares (JOLY et al., 2011). A presença de diferentes fitoquímicos nas plantas é ampla: carotenóides (alfa-caroteno, betacaroteno e licopeno), fenólicos (ácidos fenólicos, flavonóides, cumarinas e taninos), alcalóides, compostos contendo nitrogênio e compostos orgânicos sulfurados, entre outros (LIU, 2004).

A planta *Casearia sylvestris* SW. possui grande valor medicinal, com atividade biológica diversa e presença de vários grupos fitoquímicos, distribuídos em extratos e frações de diferentes polaridades, obtidos de várias partes da espécie (FERREIRA, 2011). É relevante explorar sua atividade farmacológica utilizando-se estrutura dotada de rica variedade de receptores e moléculas, com múltiplos sítios de ação para substâncias (MARTYN, 2009) - a junção neuromuscular -, que do ponto de vista anatômico representa a sinapse nervo-músculo; do fisiológico, a contração muscular e do farmacológico, o estudo de novos fármacos (CINTRA-FRANCISCHINELLI et al., 2008a).

2 REVISÃO BIBLIOGRÁFICA

2.1 Junção neuromuscular

A junção neuromuscular (JNM) é formada por três importantes regiões, a pré-sináptica, que compreende o axônio e a terminação nervosa motora; a fenda sináptica, espaço onde o neurotransmissor é liberado; e a pós sináptica, que compreende uma região especializada para deflagrar o acoplamento excitação-contração. A informação é transmitida do nervo para o músculo através da liberação de acetilcolina (ACh), que por sua vez ativa receptores nicotínicos (nAChRs) (FAGERLUND; ERIKSSON, 2009).

A ACh é sintetizada a partir da colina e da acetilcoenzima A e internalizada em vesículas na região pré-sináptica, algumas são mantidas próximas a locais de liberação imediata, conhecida como zona ativa (RIZZOLI; BETZ, 2005). Quando liberada na fenda sináptica se liga aos receptores nicotínicos na membrana pós-sináptica, ocorrendo a despolarização da membrana e contração muscular (MERIGGIOLI; SANDERS, 2009).

A resposta muscular pode variar dependendo do tipo de droga atuante. O efeito facilitatório é atribuído a substâncias que potencializam a contração muscular quando preparações neuromusculares são submetidas a estímulos maximais, isolados e indiretos, e aquelas denominadas anti-miastênicas e antagonistas do curare (RIKER; STANDAERT, 1966), como exemplos.

Os canais de sódio voltagem-dependentes são responsáveis pela despolarização que ocorre no terminal nervoso e se propaga através da junção neuromuscular (CATTERALL, 2000a). Existem várias substâncias com efeito sobre os canais de sódio, algumas como a tetrodotoxina (TTX) atuam sobre as subunidades α causando bloqueio do canal, outras como a batracotoxina e as plantas aconitina e veratridina, possuem afinidade pelo canal aberto, levando a ativação do canal e como consequência diminuindo a inativação (CESTELE; CATTERAL, 2000; HEMMINGS, 2009).

Quando o potencial de ação atinge o terminal nervoso faz com que o cálcio extracelular entre através dos canais de cálcio voltagem-dependentes e libere as vesículas contendo ACh. Entretanto, existe outro canal de cálcio dependente de AMP cíclico que permite a entrada de mais cálcio prolongando a resposta (CATTERALL, 2000b). O cálcio também está presente no retículo sarcoplasmático, e quando ocorre a despolarização dos túbulos transversais, o canal rianodina é ativado pelo potencial de ação ocorrendo a liberação do cálcio do retículo sarcoplasmático e promovendo a contração muscular (SANCHEZ; STEFANI, 1978;

ALMERS; PALADE, 1981). Tem-se como exemplo o polietilenoglicol 400 (PEG 400) que atua tanto na membrana do sarcolema como sobre o receptor rianodina, causando facilitação na contração muscular (OSHIMA et al., 2010).

A formação de sinapses requer uma coordenada localização de elementos pré e pós-sinápticos em futuros sítios de inervação. No músculo de mamíferos em desenvolvimento, axônios motores crescem junto com a região central do músculo e, em seguida, estendem-se em ramos individuais que terminam nas fibras musculares (BURDEN, 2002). Os receptores de acetilcolina agregados em justaposição com os terminais nervosos pré-sinápticos, resultam em uma faixa focal de junções neuromusculares. Padrões de inervação focal são mais prevalentes e melhor estudadas em mamíferos, entretanto inervação multi-focal, onde formam-se sinapses em vários pontos distribuídos ao longo do comprimento ou nas extremidades de fibras musculares, são comuns em animais vertebrados, tais como aves (COERS, 1967; KHANNA et al., 2003) e resultam na produção de contraturas. Assim músculos com inervação multi-focal podem ser estimulados por agonistas colinérgicos aplicados de forma exógena, e também pela estimulação de seu nervo-motor, permitindo que os efeitos pré-juncionais sejam distinguidos dos efeitos pós-juncionais (VATANPOUR, 2003).

2.2 A importância clínica de agentes que causam um efeito facilitador

2.2.1 Doenças neuromusculares

A miastenia gravis é uma patologia autoimune, onde grande parte dos pacientes apresentam anticorpos que se ligam a receptores nicotínicos presentes na região pós sináptica. Ocorre redução da ligação com a acetilcolina dificultando, assim, a transmissão neuromuscular (VINCENT; PALACE; HILTON-JONES, 2001; CARR et al.; YU et al., 2010). Como consequência, fadiga, fraqueza muscular generalizada, ptose, diplopia, dificuldade de mastigação e deglutição, falta de força nos membros e insuficiência respiratória, essa última condição é definida como crise miastênica e pode levar a óbito (THOMAS et al., 1997; PONSETI; ESPÍN; ARMENGOL, 2000; SCHERER; BEDLACK; SIMEL, 2005; SKEIE et al., 2006; CHAUDHURI; BEHAN, 2009).

O tratamento inicial da miastenia é realizado com medicamentos que buscam melhorar a transmissão neuromuscular. Esse objetivo é alcançado com os inibidores de acetilcolinesterase, sendo eles a neostigmina, o edrofônio (de curta ação) e a piridostigmina (TUNER, 2007; GILHUS et al., 2011). Com a evolução da doença são adicionados fármacos imunossupressores de curto prazo (corticosteroides) como a prednisona (DONALD; EVOLI, 2010) e de longo prazo como azatioprina, ciclosporina, ciclofosfamida, metotrexato, rituximab (SATHASIVAM, 2011).

Em outros casos são encontrados anticorpos (anti-Musk) contra tirosina quinase músculo-específica, uma proteína da junção neuromuscular (HOCH; MCCONVILLE; HELMS, 2001). Esses pacientes também mostram fraqueza generalizada e peculiar envolvimento dos músculos faciais e/ou fraqueza dos músculos respiratórios (SCUDERI et al., 2002).

Na síndrome miastênica Lambert-Eaton são produzidos anticorpos contra canais de cálcio voltagem-dependente neuronais, ocorrendo a redução na liberação de acetilcolina e consequentemente da transmissão neuromuscular (MADDISON et al., 2001), resultando em fraqueza muscular, reflexos tendinosos, potenciação pós-tetânica e disfunção autonômica (LAMBERT; EATON; ROOKE, 1956; O'NEILL; MURRAY; NEWSOM-DAVIS, 1988; LINDQUIST; STANGEL, 2011).

O tratamento é realizado para amenizar os sintomas com fármacos como a piridostigmina (com ação anticolinesterásica) e guanidina (OH, 2009; WEIMER; WONG, 2009; LINDQUIST; STANGEL, 2011), podendo ser utilizado também imunossupressores como a prednisona, azatioprina (GUEVARA et al., 2006) e a ciclosporina (YUSTE ARA JR et al., 1996).

2.2.2 Insuficiências respiratórias provocadas por envenenamentos

A neurotoxina botulínica produzida pelo bacilo gram-positivo *Clostridium botulinum*, encontrado no solo, em água doce e salgada (HOROWITZ, 2005). Se dividem em 7 sorotipos (A, B, Ca, D, E, F e G) conhecidos e destes os responsáveis por 98,5% dos casos da patologia conhecida como botulismo são A, B e E (CAYA; AGNI; MILLER, 2004).

O mecanismo de ação da neurotoxina ocorre com a internalização e endocitose, seguida da mudança do pH com alteração conformacional da cadeia pesada e translocação da cadeia leve, resultando na clivagem, onde A e E clivam a SNAP-25; B, D, F e G clivam a VAMP/sinaptobrevina; C cliva a sintaxina e SNAP-25, impedindo o acoplamento da vesícula

contendo acetilcolina com a superfície interior da membrana celular, dessa forma não ocorre a fusão e liberação do neurotransmissor na fenda sináptica, inibindo a transmissão neuromuscular (JANKOVIC, 2004; DRESSLER; SABERI, 2005; DUTTON; FOWLER, 2007).

Os distúrbios apresentados pela toxina botulínica são paralisia, fraqueza generalizada, tontura, vertigem, visão turva, diplopia, boca seca, vômito, dor abdominal e desconforto ventilatório. Os sinais e sintomas podem apresentar uma variação dependendo da forma clínica do botulismo, que pode ser de origem alimentar, infantil, de feridas, oculto e inadvertido (CAYA; AGNI; MILLER, 2004).

O tratamento clínico do botulismo é realizado com avaliação frequente da equipe médica quanto aos distúrbios neurológicos, o estado respiratório e o surgimento de infecção. A administração de antitoxina botulínica deve ser realizada e tem atividade contra qualquer um dos sete sorotipos, mas não melhora a musculatura já paralisada (ZHANG; SUN; NIE, 2010). A 3,4 diaminopiridina pode auxiliar no antagonismo à paralisia muscular, causada pela toxina botulínica (ADLER et al., 1995).

A tetrodotoxina é uma neurotoxina presente nos ovários, fígado e em algumas espécies na pele de peixes baiacu e a sua ingestão pode causar envenenamento em humanos (GOTO et al., 1965). Sua ação é sobre os canais de Na^+ voltagem-dependentes, que são proteínas de membrana heteroméricas, formadas por uma subunidade principal, α (seis segmentos de α-hélices, quatro vezes repetidas), que se dobra formando poros seletivos de Na^+ através da membrana plasmática. A toxina se liga no sítio 1 da porção extracelular do canal e fecha o poro (SOONG; VENKATESH, 2006), bloqueando o potencial de ação, levando a morte por paralisia dos músculos respiratórios, por isso o paciente precisa de suporte ventilatório e monitoramento das condições vitais, não existindo antitoxina específica (SÃO PAULO, 2003).

2.2.3 Bloqueadores neuromusculares (BNM)

O bloqueio neuromuscular é compreendido como a capacidade de bloquear a transmissão neuromuscular através da interação de fármacos com receptores de ACh localizados na porção pós juncional da placa motora (BOWMAN, 2006).

O curare é um exemplo de substância que possui esse efeito bloqueador. Sua descoberta aconteceu por volta do século XVI quando exploradores observaram a sua

utilização, por índios da América do Sul, para paralisar animais (BOOIJ, 2000) e séculos depois experimentos envolvendo preparações neuromusculares levou ao conhecimento do local de ação do curare (BERNARD, 1850), resultando no inicio das pesquisas com a intenção de usar esse efeito bloqueador na terapêutica.

Em 1942, anestesistas canadenses realizaram a primeira cirurgia utilizando Intocostrin® (curare purificado) como relaxante muscular, desde então a técnica passou a ser usada na anestesia (GRIFFITH; JOHNSON, 1942).

Os bloqueadores neuromusculares foram alvo de intensa pesquisa com a descoberta de novos fármacos divididos em duas classes: os despolarizantes e os não despolarizantes.

Os fármacos despolarizantes necessitam de uma quantidade suficiente de moléculas para interagir com receptores nicotínicos pós juncionais e causar um bloqueio neuromuscular, resultando na despolarização da placa terminal (MOORE; HUNTER, 2001). O decametônio e o suxametônio tem um efeito semelhante àquele que é produzido por um excesso de ACh na junção neuromuscular (THESLEFF, 1955), sendo o segundo utilizado atualmente na clínica, apesar de seus efeitos secundários indesejáveis e a chance de desencadear hipertermia maligna (APPIAH-ANKAM; HUNTER, 2004).

Os fármacos que competem com a ACh para impedir a sua ação nos receptores nicotínicos são conhecidas como não despolarizantes (MOORE; HUNTER, 2001) e estão divididas em dois grupos: os benzilisoquinolínicos (atracúrio, mivacúrio, doxacurio, cisatracúrio e tubocurarina) e os aminoesteróides (pancurônio, vecurônio, pipecurônio e rocurônio) (APPIAH-ANKAM; HUNTER, 2004). Os fumaratos (gantacurio e CW 002) constituem um novo grupo de adespolarizantes em estudo, onde a pretensão é um rápido início de ação com um intervalo de tempo adequado, melhorando a fraqueza residual e os efeitos adversos (LIEN, 2011).

Milhões de pessoas utilizam BNM, anualmente, em procedimentos como cirurgias, no auxílio a ventilação mecânica ou na intubação traqueal (EIKERMANN et al., 2002; MENCKE et al., 2003; PAPAZIAN et al., 2010). Esse bloqueio neuromuscular instaurado não deve persistir, pois a permanência nesse estado pode causar fraqueza muscular e prejudicar a respiração, a permeabilidade das vias aéreas superiores, a deglutição e a tosse, tornando o paciente vulnerável a complicações graves no pós operatório (SUNDMAN et al., 2000; EIKERMANN et al., 2005; MURPHY, 2006; MURPHY et al., 2008; BUTTERLY et al., 2010).

A recuperação da função neuromuscular pode ocorrer através da administração de um anticolinesterásico, que resultará na inibição da acetilcolinesterase e no aumentando da

acetilcolina na fenda sináptica, deslocando o bloqueador neuromuscular adespolarizante (NAIR; HUNTER, 2004). A procura por novos fármacos com ação de reversão do bloqueio neuromuscular residual é relevante, visto a gravidade que esse estado clínico representa para o paciente.

2.3 *Casearia sylvestris* Sw.

A planta *Casearia sylvestris* Swartz (Figura 1), da família Salicaceae, é conhecida por vários nomes populares como erva-de-lagarto, guaçatonga, guaçatunga, vassitonga, chá-de-bugre e bugre-branco (TESKE; TRENTINI, 2001).

A guaçatonga pode ser lenhosa, arbustiva ou arbórea, com folhas inteiras, em ordem alternada, normalmente dística (WERLE et al., 2009). Proveniente da América tropical ocorre desde o México até a Argentina.

No Brasil, a espécie vegeta em abundância, sendo facilmente encontrada no Estado de São Paulo. A crença popular diz que o lagarto só enfrenta uma serpente após certificar-se da presença de um exemplar de erva-de-lagarto por perto (TESKE; TRENTINI, 2001).

Figura 1. Guaçatonga localizada no canteiro da Uniso.

Fonte: Elaboração própria.

As folhas de *Casearia sylvestris* Sw. tem como principais características ser simples, peciolada, coriácea, oblonga ou *elíptico-lanceolada*, estreitamente acuminada no vértice, de base simétrica ou levemente inequilateral. Em análise microscópica observa-se epiderme glabra, formada de células poligonais, de paredes retas na face superior e levemente curvas na inferior, que é revestida de estomas envolvidos por células não diferenciadas. O mesofilo é heterogênio e assimétrico, formado na parte superior por várias camadas de células paliçádicas e na inferior por um parênquima de células ovais ou arredondadas, onde se observam, também, grandes bolsas secretoras. O sistema libero-lenhoso é representado por um cordão lenhoso, arqueado, recoberto sobre a face inferior por um líber mole que contém vários canais secretores e por um periciclo fibroso. A face superior do cordão é igualmente recoberta por um arco de periciclo mais ou menos lenhificado. O parênquima fundamental das nervuras encerra, também, numerosas bolsas secretoras (SILVA, 1929).

As cascas e especialmente folhas da *C. sylvestris* SW são mencionadas em vários estudos como antidiarréicas, antipiréticas, anti-inflamatórias, antireumáticas e diuréticas. É indicado seu uso externo em afecções da pele pela sua ação cicatrizante (ALICE; SIQUEIRA; MENTZ, 1995).

Em abordagem fitoquímica os grupos observados foram taninos, flavonóides, saponinas e terpenos (LUZ et al., 1998). O teor de óleos essenciais para droga seca foi de 0,12% (POSSOLO; FERREIRA, 1949), enquanto que pelo processo de hidrodestilação das folhas frescas foi obtido 0,4%, com concentração elevada de terpenos (77,78%) (WERLE et al., 2009).

A composição do óleo essencial da *C. sylvestris* SW é influenciada pelo fotoperíodo (dia e noite), ocorrendo inicialmente a biossíntese de sesquiterpenos monocíclicos, que ao longo do período sofreriam rearranjos na cadeia carbônica originando os outros sesquiterpenos (TININIS et al., 2006).

A planta *C. sylvestris* SW integra a Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS), a Resolução – RDC Nº 10, DE 9 DE MARÇO DE 2010 e o Formulário de Fitoterápicos, e a indicação quanto ao seu uso é destinado ao tratamento de dispepsia, gastrite e halitose, além do uso como antisséptico e cicatrizante em lesões tópicas (RENISUS, 2009; BRASIL, 2010; BRASIL, 2011).

A atividade antitumoral da planta *C. sylvestris* SW pode ser associada à ação citotóxica de sesquiterpenos como β-cariofileno e α-humuleno e a outros terpenóides (SILVA et al., 2008). A ação quimiopreventiva do extrato etanólico de *C. sylvestris* e da casearina X (um

diterpeno) foi constatada em estudo envolvendo partículas da queima da cana de açúcar, onde foi capaz de proteger o DNA contra danos reparáveis e não reparáveis (PIETRO et al., 2012).

O extrato de *Casearia sylvestris* Sw. apresenta atividade antiofídica, pois foi capaz de inibir a fosfolipase A_2 aumentando o tempo de sobrevida de camundongos que receberam doses letais de venenos de serpentes (BORGES et al., 2000).

Nesta linha, mas utilizando outro aparato experimental, Oshima-Franco et al. (2005) verificaram que extrato hidroalcoólico de guaçatonga era capaz de neutralizar a ação neurobloqueadora da bothropstoxina-I, uma miotoxina constituinte do veneno de *Bothrops jararacussu*.

A partir do estudo acima referido, os autores prosseguiram à extração das folhas de guaçatonga utilizando solventes orgânicos de diferentes polaridades (hexano, acetato de etila, diclorometano e metanol) verificando que a propriedade antibotrópica da planta era restrita à fração metanólica, indicativa de que os constituintes presentes seriam responsáveis pela ação anti neurobloqueadora, tanto da miotoxina como do veneno total (CINTRA-FRANCISCHINELLI et al., 2008b).

A premissa de trabalho, entretanto, vem das curvas doses-respostas de extratos tanto aquoso como hidroalcoólico, de folhas de *Casearia sylvestris*, que demonstraram um aumento da resposta contrátil (que aqui denominamos efeito facilitatório) em preparações nervo frênico-diafragma de camundongos, dependendo da concentração utilizada (OSHIMA-FRANCO et al., 2005).

3 OBJETIVOS

3.1. OBJETIVOS GERAIS

Identificar as frações de diferentes polaridades (hexano, diclorometano, acetato de etila e metanol) obtidas de extrato hidroalcoólico de *C. sylvestris*, responsáveis pelo efeito facilitatório (visualizado pelo aumento da amplitude da resposta contrátil), em preparações nervo frênico-diafragma de camundongos e biventer cervicis de pintainho; realizar um estudo farmacodinâmico das frações neurofacilitadoras, utilizando-se ferramentas farmacológicas clássicas validadas cientificamente, em modelo experimental de junção neuromuscular.

3.2. OBJETIVOS ESPECÍFICOS

Caracterizar farmacologicamente as frações em preparação nervo frênico-diafragma de camundongos e selecionar as frações neurofacilitadoras.

Analisar histologicamente os músculos de NFD.

Realizar *screening* farmacológico das frações neurofacilitadoras em preparação nervo frênico-diafragma de camundongo e em preparações biventer cervicis de pintainho para verificar a natureza sináptica (pré/pós).

Determinar a atividade da colinesterase (ChE) e da creatinoquinase (CK).

Realizar protocolos experimentais utilizando ferramentas farmacológicas clássicas, que atuam na junção neuromuscular, para o estudo farmacodinâmico.

4. ARTIGO: The facilitatory effect of *Casearia sylvestris* Sw. fractions on the function of mammalian and avian skeletomotor apparatus.

The facilitatory effect of *Casearia sylvestris* Sw. fractions on the function of mammalian and avian skeletomotor apparatus

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Running title: Neurofacilitation induced by methanol fraction of guaçatonga

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Abstract

The facilitatory effect of apolar to polar fractions of *Casearia sylvestris* Sw. on the function of mouse phrenic nerve diaphragm (PND) and chick biventer cervicis (BC) skeletomotor apparatus was investigated, using the forces elicited by indirect (3V) *versus* direct (30V) nerve muscle stimulation and antagonists as pharmacological tools. Methanol (MeOH) and ethyl acetate fractions, but neither hexane nor dichloromethane, exhibited a facilitatory effect on PND (3V). MeOH fraction was chosen for further assays addressed to: 1) presynaptic sites (axons or nervous terminal); 2) post synaptic sites (cholinergic receptor, sarcolemma or T-tubule); and 3) synaptic cleft (acetylcholinesterase enzyme). In curarized (d-tubocurarine) preparations, MeOH fraction did not exhibit facilitation (30V). It was also unable to reverse the dantrolene-blockade (3V). A dual response was obtained when MeOH fraction was added into curarized preparations mimicking neostigmine or failing completely (3V). Using 3,4-diaminopyridine (3,4 DAP) the MeOH fraction decreased the twitches amplitude, whereas under high-frequency tetanus (40 Hz), it increased the tetanic tension. Using BC preparation the MeOH fraction did not induce changes on contractures of exogenous acetylcholine and potassium chloride addition. Atropine excluded the modulation by prejunctional receptors. Nifedipine showed that the neurofacilitation is modulated by extracellular calcium. Tetrodotoxin did not avoid the facilitatory effect of 3,4 DAP, neither neostigmine, but it did antagonize the MeOH fraction one. We can suggest that the site of action of MeOH fraction is at the presynaptic level by an activation of neuronal sodium channels. Extracellular calcium can also modulate the neurofacilitation.

Keywords: *Casearia sylvestris*; Chick biventer cervicis; *guaçatonga*; Mouse phrenic nerve-diaphragm preparation; neuromuscular junction.

Introduction

The medicinal use of parts of *Casearia sylvestris* Sw. (Salicaceae) plant has been described as antiseptic, anti-febrile, blood depurative, and in the syphilis treatment (leaves, barks and roots); asthmatic bronchitis (leaves infusion); anti-diarrheal properties (barks) and anti-snake bite (leaves and barks) [1]. The essential oil has gastric antiulcer and anti-inflammatory activities [2]; the hydroalcoholic extract has antinociceptive action [3], anti-inflammatory and antioxidant properties [4]; aqueous and methanolic extract from their leaves have antibothropic activity [5, 6]; and methanolic extract also showed a cardiovascular protective action [7]. More properties of *C. sylvestris* were well described by elsewhere, including the innumerous isolated bioactive compounds [8, 9].

These widespread medicinal properties of *C. sylvestris* in opposite to no genotoxic effects and not modified effect inducing DNA damage by alkylating agents cyclophosphamide and methyl methane sulfonate in Hepatoma Tissue Culture (HTC cells) of Ratus norvegicus and Chinese hamster V79 cells, respectively [10], or low acute toxicity, confirmed by sub chronic daily testing [11], lead researchers to exploit them.

In this context, an unexplained facilitatory effect (visualized by an augment in the twitches amplitude) of aqueous, hydroalcoholic and methanolic extract from leaves of *C. sylvestris* was described using mouse phrenic nerve-diaphragm preparation [6, 12]. From the pharmacological point of view, neurofacilitation is important against muscle fatigue or paralysis resulting from pathological conditions such as neuromuscular diseases, some envenoming, or even against residual neuromuscular blockade in the postoperative period. Therefore, it is mandatory to clear the mechanism of action for drugs candidates which to that, aiming to the future therapeutic application.

The muscle fatigue can occur by a failure in the motoneurons excitation, or in the neural signal transmission, or in the muscle response face to neural excitation, and it could have central as peripheral origin [13, 14]. At the central level, anticholinesterasics have importance by the acetylcholine content increase, one of the multiple pathogenic factors in Alzheimer's disease [15]. At peripheral level, innumerous diseases are located at pre- and postsynaptic sites, since a failure of action potential up to failure of excitation-contraction coupling. These mechanisms (involving branch point vs. presynaptic vs. postsynaptic) of fatigue at the neuromuscular junction were very well described by Sieck and Prakash [16].

The intentional muscle paralysis occurs by using neuromuscular blocking agents (NMBA) in clinical anesthesia, resulting in profound muscle relaxation to permit tracheal intubation and ensure immobility.

However, in the postoperative period the muscle recovery is influenced by a marked variation according to the patient sensitivity to NMBA [17]. Hence, neurofacilitating agents can help to reduce the incidence of morbity and mortality associated with residual paralysis.

For this study *Casearia sylvestris* was selected, which is a plant able to induce a facilitatory effect and with high medicinal potential and low toxicity, for obtaining apolar to polar fractions from *C. sylvestris* hydroalcoholic extract. The objectives were to identify the fractions responsible by this effect, as well as, to clear the facilitatory mechanism using mammalian and avian neuromuscular apparatus.

Materials and Methods

Plant material

The leaves of *C. sylvestris* were collected on 11/08/2007 by T. M. Camargo and M. C. Ferraz from adult plants of an orchard at the University of Sorocaba (UNISO, SP, Brazil) (23°30′05.90″S, 47°23′40.84″W). A voucher specimen was deposited in the UNISO herbarium (HUS046 number) after identification by E. A. Lopes from the Instituto de Botânica at São Paulo City (SP, Brazil).

Extraction and partition

The air-dried leaf powder (after drying by using a Marconi forced air circulation apparatus, and grinding to 10 mesh using a Wiley type Marconi®, MA 340 model, macromill) of *C. sylvestris* (3.110 kg) was extracted with 70% ethanol (EtOH) at room temperature for 2 hours, and the solution was evaporated *in vacuum* to give a hydroalcoholic extract (522.9 g). Part of this extract (373.2 g) was dissolved in a 80:20 methanol-water mixture and partitioned successively with the corresponding solvents to give a hexane (Synth®, Brazil); dichloromethane, CH_2Cl_2 (Synth®, Brazil); ethyl acetate, EtOAc (Synth®, Brazil); and methanol, MeOH (Ecibra®, Brazil) residues [18]. The solvents were evaporated to dryness, lyophilized (Thermo Electron Corporation® - ModuleD Freezer Dryer), and the yields corresponding to the obtained fractions were calculated, being 50.11 g, 47.24 g, 36.03 g, and 11 g, respectively. The dried fractions were then protected from light and humidity at room temperature until assayed.

Fractions solubilization for using in the pharmacological assays

Hexane, CH_2Cl_2, EtOAc and MeOH residues were solubilized in polyethylene glycol (PEG 400, 15 µL, Synth®), according to Cintra-Francischinelli et al. [19].

Cholinesterase (ChE) Inhibition Assay

The ChE inhibitory activity of the facilitatory fraction was determined by using colorimetric method (Labtest Diagnóstica S.A.®, Brazil), which calibration was made using a human calibrator (Calibra H). Normal (Qualitrol 1H) (Labtest Diagnostica S.A.®, Brazil) was used as negative control, according to the manufacturer. ChE activity was determined spectrophotometrically (Shimadzu®, model multispec-1501) at 405 nm wavelenght. The percentage of enzyme inhibition was calculated by comparing the enzymatic activity of 1, 0.5, and 0.25 mg/mL of facilitatory fraction with the same concentration of neostigmine (Sigma®), as a cholinesterase inhibitor. The experiment was run in triplicate.

Animals

Male Swiss white mice (26-32 g) were supplied by Anilab, Animais de Laboratório (Paulínia, S.P., Brazil) and HY-line W36 male chicks (4–8 days old) were supplied by Avicultura Santa Bárbara (Sorocaba, SP, Brazil). The animals were housed at 25 ± 3 °C on a 12 h light/dark cycle and they had access to food and water *ad libitum*. This project (protocol n^{o} 40/2012) was approved by the institutional Committee for Ethics in Research of Universidade Federal de São Carlos (UFSCAR), and the experiments were carried out according to the guidelines established by the Brazilian Society for Laboratory Animal Science (SBCAL).

Mouse phrenic nerve-diaphragm preparation (PND)

The diaphragm and its phrenic nerve branch were obtained from mice anesthetized with halothane inhalation (Cristália®, Campinas, Brazil) and killed by exsanguination. The diaphragm was removed according to the method described by Bülbring [20] modified for mice, and mounted under a tension of 5 g in a 5 mL organ bath containing Tyrode solution with the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, and glucose 11.1. After equilibration with a carbogen aeration mixture of 95% O_2/5% CO_2, the pH of this solution was set at 7.0. The preparations were stimulated indirectly (3V) with supramaximal stimuli (4X threshold, 0.06 Hz, 0.2 ms) delivered from a stimulator (Model ESF-15D, Ribeirão Preto, SP, Brazil) to the phrenic nerve through bipolar electrodes. Some curarized (d-tubocurarine, d-Tc) preparations were directly stimulated (30 V). Isometric twitch tension was recorded with a force-displacement transducer (Cat.7003, Ugo Basile®, Italy) coupled to a 2-channel recorder Gemini physiograph device (Cat. 7070, Ugo Basile®) via a Basic Preamplifier (Cat. 7080, Ugo Basile®). The PND myographic

record was performed according to Melo et al. [21]. The preparations were allowed to stabilize for at least 20 min before treatments with each 200 µg/mL organic fraction: hexane (Hex, n=7), dichloromethane (DCM, n=6), ethyl acetate (EtOAc, n=10), methanol (MeOH, n=10), or with Tyrode solution alone (control, n=6). The facilitatory effect was measured by an increase of twitches amplitude. A set of experiments was carried out aiming to investigate whether the facilitatory fraction acts at pre-, post- or synaptic cleft level using substances actives on mammalian neuromuscular junction, as a pharmacological tool (see used agents [22], all from Sigma®, and its concentrations on the Table 1). Some experiments were carried out using tetanic stimulation (40 Hz, during 5 seconds every 15 min), or direct stimuli (30 V).

Quantitative Histological Study

Preparations with facilitatory effect resulting from pharmacological assays were analyzed by quantitative morphometry aiming to observe some cell damage. Thus, at the end of the experiments (after 60 min), the selected preparations (n=3, each) were fixed in Bouin solution and processed by routine morphological techniques. Cross-sections (5 µm thick) of diaphragm muscle were stained with 0.5% (w/v) hematoxylin-eosin for microscopy examination. Any tissue damage like edema, myonecrosis characterized by atrophy of the muscle fibers, hyaline aspect, sarcolemmal disruption and lysis of the myofibrils was expressed in myotoxicity index (MI), *i.e.*, the percentage of damaged muscle cells number divided by the total number of cells in three non-overlapping, non-adjacent areas of each preparation [23].

Chick biventer cervicis preparation (BC)

Chicks were killed by halothane inhalation, and biventer cervicis muscles were removed [24] and mounted under a tension of 1 g per 0.5 cm in a 5 mL organ bath (Panlab® Four Chamber Organ Bath) maintained at 37°, aerated (95% O_2/5% CO_2), and kept in a Krebs solution with the following composition (mM, pH 7.5): NaCl, 118.1; KCl, 4.8; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 11.1. A bipolar platinum ring electrode was inserted around the tendon within which the nerve trunk supplying the muscle runs. Field stimulation was done using a Pulse Generator & Mainframe for up to 4 units (LE12406TC, Panlab®) stimulator (0.1 Hz, 0.2 ms, 5 - 12 V). Muscle contractions delivered from "intrinsic" receptors, which respond to the neurotransmitter liberated from the nerve terminal, and contractures (depolarizing activity) delivered from "extrinsic" receptors, which respond to acetylcholine added exogenously [24, 25] were recorded isometrically via a force-displacement transducer (MLT0201, ADInstruments®) coupled to a group of 4 software-controlled,

DC bridge transducer amplifiers (FE224, ADInstruments®). The data acquision was made using a PowerLab 4/35 system including LabChart and LabChart Pro Modules software (PL3504/P, ADInstruments®) connected to a Power Unit (LE124060M, ADInstruments®). The BC preparations were allowed to stabilize for at least 20 min before addition of 110 µM acetylcholine (ACh) for 60s or 20 mM potassium chloride (KCl) for 180s. Contractures to exogenous ACh or KCl were recorded in the absence of field stimulation prior to or after the end of the experiment as a test for evaluating pre- or post-synaptic action and, at the same time, the integrity of the sarcolemma. This preparation enables prejunctional effects to be distinguished from postjunctional effects [26]. Control preparations were incubated with Krebs solution.

Creatine kinase (CK) activity

Since phrenic nerve-hemidiaphragm preparations have a spontaneous release of CK resulting in elevated basal levels, this activity was measured only in BC preparations [27]. For the quantification of CK activity, samples (100 µL) of the BC bathing solution were withdrawn from the organ bath at 0 (control) and 60 min after the treatment with facilitatory fraction. The withdrawn volume was replaced with an equal volume of Krebs solution. The samples collected were stored for 2 h at 4°C until CK activity (expressed in units/L) was measured at 340 nm (Shimadzu®, model multispec-1501) , 37°C, using a commercial kit (CK-NAC REF 11.002.00, Biotécnica®, Varginha, MG, Brazil).

Experimental design

Figure 1 shows the potential presynaptic (nerve terminal), synaptic cleft and postsynaptic (muscle fiber) targets, where drugs can act, and the protocols carried out confronting against MeOH fraction in all protocols.

Statistical analysis

Each experimental protocol from pharmacological assays was repeated at least four times and the results are shown as the mean ± SEM. The number of experiments (n) is indicated in the legend of the figure. All results (pharmacological assays, histological analysis, and CK or Cholinesterase determination) were used for statistical comparison of the data using Student's *t*-test and the confidence level was set as 5% (alpha=0.05).

Results and Discussion

The search for substances that can facilitate the neurotransmission is desirable since it open innumerous possibilities of clinical application in the neuromuscular diseases. When these substances are from natural source, as plants, it means that many obstacles were transposed (as chemical synthesis), because a chemical skeleton is already expressed by plant. Most exciting is whether these substances present some pharmacological effects, which justifies their investigation by enormous potential as medicine. In this study, using the plant *Casearia sylvestris* Sw. which exhibits a great medicinal potential including an unexplained facilitatory effect [12], the hexane, dicholoromethane, ethyl acetate and methanol fractions were obtained aiming to exploit the chemical nature of fractions responsible by facilitatory effect as well as an address to future phytochemical study.

Figure 2 shows the pharmacological effects of the obtained fractions (A, 200 µg/mL each), where the facilitatory effect was clearly expressed by ethyl acetate and methanol fractions (both *$p<0.05$ compared to Tyrode control, but not one each other). However, in these concentrations, neither ethyl acetate (C, MI=11.4 ± 0.69) nor methanol (D, MI=7 ± 1.34) fraction caused any damage in cells when compared to control (B, 11.4 ± 0.79), a complementary technique for ensuring the medicinal potential to any drug candidate. It is known that tissue damage caused by disease, trauma, poisons and other factors can lead to formation of increased amounts of putative injury mediators such as prostaglandins, leukotrienes, interleukins, interferons, and tumor necrosis factors [28]. Polyethylene glycol 400 is an example of a safe molecule at histological level [29] justifying its use as a drug carrier.

MeOH fraction of *C. sylvestris* was chosen for clearing the mechanisms by which the fraction induces the facilitatory effect. Figure 3 shows a classic experimental protocol using d-Tc, a competitive antagonist of acetylcholine (ACh), which causes a neuromuscular blockade for occupying nicotinic receptors (A). Using neostigmine, an anticholinesterasic agent, curarized preparations are immediately reversed (B), since the ACh accumulated at the synaptic cleft is enough to replace d-Tc from nicotinic receptors. However, when MeOH fraction was added to the bath containing curarized preparations two different patterns were obtained: 1) recovery of twitch-tension as neostigmine does (C), and 2) no recovery (D). In all experiments the muscular response was restored after washing (W).

In order to clear the dual response of MeOH fraction in curarized preparations, 3,4 DAP, a potassium channel blocker in membranes was used. At 10 µg/mL 3,4 DAP augments indirectly evoked twitches in the mouse hemidiaphragm (Figure 4A, 4C). As consequence, the augment of ACh at the synaptic cleft is able to

replace d-Tc from nicotinic receptors (Figure 4B). When MeOH fraction was added to pretreated preparation with 3,4 DAP there was a reduction of 41 ± 4.7% (n=4) in the muscular response (Figure 4C).

MeOH fraction also decreases the twitches amplitude when it is added to pretreated preparation with neostigmine (1 μg/mL, n=4, not shown). An explanation for the twitches depression appears to reflect a partial postjunctional muscle-type nicotinic receptor desensitization [30, 31], by accumulation of ACh at the synaptic cleft, since all of them (3,4 DAP, neostigmine and MeOH fraction) did cause a facilitatory effect alone. Since 50'decades researchers have showed that maintained exposure of nicotinic ACh receptors to an appropriate agonist results in a reduction in the response to the agonist [31-33]. Alternatively, the ACh can also act on prejunctional autoreceptors to either enhance or depress its own evoked released, at the mammalian skeletal muscle neuromuscular junction [34-36]. Here, there was a clear autoinhibition induced by MeOH fraction. Another possibility would be a depletion of synaptic vesicles, but, the prompt facilitatory recovery of twitches after washing of preparation fragileness this hypothesis.

Aiming to distinguish antichonesterasic action (Figure 3) from presynaptic action (Figure 4) of MeOH fraction, a set of experiments using 40 Hz frequency was carried out in mouse PND, since it is known that skeletal muscles respond with sustained contractions when the nerve is stimulated at physiological frequencies between 30-80 Hz [37].

Figure 5A shows the augment of contractions amplitude caused by MeOH fraction under a stimulation of 40 Hz (tetanic tension), calculated after the ratio (R) obtention (in %), derived from a modified purpose of Ambiel and Alves-do-Prado [37] which applied 100-120 Hz. The explanation is found in the Figure 5B legend. Moreover, it is noted that neostigmine was unable to sustain contractions during high frequency stimulation (Figure 5C), an effect already described by elsewhere, since the inhibition of acetylcholinesterase (acetylcholine cylhydrolase, E.C. 3.1.1.7) prolongs the postsynaptic action of ACh [38]; as consequence, striated muscle becomes unable to maintain a contraction in response to nerve stimulation at higher frequencies (tetanic fade, Wedensky inhibition). Thus, MeOH fraction, in turn, seems not act as a typical anticholinesterasic, also reinforced by cholinesterase determination, as shown in Table 2.

Aiming to exclude an anticholinesterasic action of MeOH fraction, a set of experiments was carried out using neostigmine and Qualitrol®, as positive and negative controls, respectively. In opposite to Lamiaceae species that are a rich source of various natural AChE inhibitors and antioxidants [15], MeOH fraction did not act by this mechanism.

The facilitatory effect was also studied in chick biventer cervicis, a multiply innervated preparation, useful to study the mechanism by which drugs act at the neuromuscular junction, since the muscle remain in contracture for as long as the depolarizing agent remains activating the receptors [39]. It is known that chick biventer cervicis muscle contains a mixture of focally innervated fibers (by which presynaptic drugs act by changing the release of ACh from the nerve terminals either reducing or increasing responses to nerve stimulation), whereas addition of agonists results in contracture responses of the multiply innervated fibers (by which the responses to added agonists remain unchanged, in case of presynaptic drugs) [25, 39]. Here, MeOH fraction changes the release of ACh from the nerve terminals increasing the response to nerve stimulation, but the responses to added agonists in absence of stimulation remained unchanged ($p>0.05$, $n=4$), as does a good presynaptic drug (Figure 6). The MeOH fraction addition in absence of stimulation did not causes contracture. Indeed, the responsive contracture to exogenous ACh addition at the end of experiment showed that nicotinic receptor is not the target of MeOH fraction.

Even histological analysis from resulting diaphragm muscles exposed to MeOH fraction showed no cell damage, and also confirmed by a biochemical evaluation via CK determination, two pharmacological protocols were performed using PND preparation to evaluate a possible post synaptic action of MeOH fraction (Figure 7): 1) using dantrolene (6 µg/mL), under indirect stimuli (3V), followed by MeOH fraction (A, n=4), and 2) using d-Tc (4 µg/mL), followed by direct stimulation (30V) and MeOH fraction addition (B, n=5).

Ryanodine, an alkaloid from South American plant *Ryania speciosa* [40], affects the calcium release mechanism of sarcoplasmic reticulum [41] and facilitates calcium-dependent release of transmitter at mouse neuromuscular junctions [42]. Dantrolene sodium, an antagonist of ryanodine channel, decreased the force of contraction of mouse skeletal muscle *in vitro* up to 75% [43], by which MeOH fraction did not able to replace. No changes were observed using these pharmacological protocols, absenting MeOH fraction of acting post synaptically.

Thus, in front of these results we focused other targets at presynaptic level as prejunctional muscarinic and nicotinic receptors, which modulate the transmitter release at motor nerve terminals [44, 45]. Abbs and Joseph [46] suggested the existence of presynaptic inhibitory muscarinic receptors that modulate the release of ACh in the phrenic nerves of the rat. These authors used atropine (10 – 5M), an antagonist muscarinic, and they observed an enhance of the release of [3H]-acetylcholine from rat isolated hemidiaphragms, previously incubated with [3H-methyl]-choline, stimulated via their phrenic nerves.

Here, atropine 2 µg/mL augmented the contractile response, corroborating the findings by elsewhere [46]. The MeOH fraction addition had no influence on muscular response (Figure 8), but atropine inhibited the facilitatory effect of fraction.

Could act MeOH fraction as a muscarinic antagonist? Vizi and Somogyi [47] showed a link between positive (nicotinic)- and negative (muscarinic)-feedback modulation. These authors concluded that quantally-released ACh from motor endplates is subject to prejunctional automodulation: (a) ACh facilitates its own release via an effect on prejunctional nicotinic receptors (positive feedback), (b) ACh release is reduced by an action on muscarinic receptors. When the nicotinic receptor-mediated facilitation is fully operative, the muscarinic receptor-mediated negative feedback is much less effective, explaining the link between the two feedback mechanisms possibly at the level of the second messenger system(s).

Fann et al. [48] showed that preincubation with atropine did not facilitate or prevent the increase of miniature end plate potentials (m.e.p.p.) frequency induced by Phenthonium. Authors concluded that antimuscarinic activity does not account for the enhanced spontaneous ACh release. On the other hand, Basu et al. [49] showed that the failure of atropine to antagonize the facilitatory effect of theaflavins (Tfs, *Camellia sinensis*) at a concentration which has earlier been reported to inhibit the release of ACh from the phrenic nerve terminals [50], showed that the effect of Tfs was not mediated by altering ACh release from the nerve ending. Herein atropine-pretreated preparations at a concentration which increase the ACh release from the phrenic nerve terminals, MeOH fraction did not facilitate, which shows that such effect was not mediated by prejunctional nicotinic receptor. At the presence of atropine the modulation by prejunctional receptor nicotinic could be expected an augment of the contractile response. As this twitch increase did not occur a question might arise: MeOH fraction would act as an antagonist of prejunctional nicotinic receptor (nAChR)? According to [51] an antagonist must have no intrinsic activity (no agonist efficacy at the target nAChR) and also must inhibit the response elicited by an appropriate nAChR. Hence, this role should be considered to MeOH fraction that has no agonist efficacy at the postjunctional target nAChR, as showed using biventer cervicis preparation.

Facilitatory effect can also be related to an increase of Ca^{2+} influx and an enhance of neurotransmitter release. Thus, it was used nifedipine (Figure 9), a calcium channel blocker [41], at low concentration (0.346 µg/mL) which allowed the contractile response [49], and the facilitatory effect of MeOH fraction was inhibited (n=5).

When calcium (1.8 mM) from nutritive solution was replaced by strontium chloride (3.6 mM), since Sr^{2+} is able to substitute Ca^{2+} in the neurotransmitter release process [52], the facilitatory effect of MeOH fraction was also concealed (not shown) giving support for an involvement of calcium for this effect.

Tetrodotoxin, a potent neuronal sodium channel blocker at 0.5 µM did not avoid the facilitatory effects of 3,4 DAP, that acts as a potassium channel blocker, nor from neostigmine, an anticholinesterasic agent, but did avoid the MeOH fraction one. In opposite, the prejunctional (facilitatory) effect of Phentonium persisted in the presence of tetrodotoxin, excluding an activation of the voltage sensitive Na^{+} channels at the nerve terminal membrane as a mechanism of action of the drug [48].

The results of this study showed that MeOH fraction of *C. sylvestris* facilitates the axonal propagation via the Na^{+} channels activation can be valuable in medicine, since it causes no cell damage (as confirmed by light microscopy and CK determination) as PEG 400 [29]. From the bio- prospection point of view, we suggested the phytochemical studies in order to identificate the bioactive molecule responsible by this action.

Conclusion

This study provided evidences in order to clear on the facilitatory effect firstly described by Oshima-Franco et al. [12]. The selected MeOH fraction acts presynaptically, activating neuronal sodium channels, which facilitatory effect can be modulated by extracellular calcium.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgement

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Legends

Figure 1. Figure shows the potential targets where drugs can act: nerve terminal (presynaptic agents), synaptic cleft (cholinesterase inhibitors) and muscle fiber (post synaptic agents) and the rationale drugs used as pharmacological tools.

Figure 2. **A**: Mouse phrenic nerve-diaphragm preparation, indirect stimuli. Among the assayed hexane, dichloromethane, ethyl acetate and methanol fractions obtained from liquid-liquid partition of *Casearia sylvestris leaves* (lyophilized hydroalcoholic extract), only the two latter fractions showed the facilitatory effect (an increase of twitches amplitude). The number of experiments is shown in the legend of figure. Each point represents the mean ± SEM. * = $p<0.05$ in comparison with the control. **B**, **C** and **D**: Qualitative images are shown of cross-sections (5 μm thick) of diaphragm muscle exposed to following treatments (200 μg/mL and n=3, each): control (B); Ethyl acetate fraction (C), Methanol fraction (D). Quantitave data are showed in the text. Note that C and D showed a normal appearance, like control, with polygonal aspect, peripheral nuclei, although sometimes the edema is visible; bar = 50 μm.

Figure 3. Representative myographical records of mice phrenic nerve-diaphragm preparations, indirect stimuli. **A**, curarized preparation (d-Tubocurarine, d-Tc, 4μg/mL, n=5). **B**, Reversal effect of neostigmine (1 μg/mL, n=4) in pre curarized preparations. **C**, Reversal effect of methanol fraction (MeOH, n=4) in pre curarized preparations. D, No reversal effect of MeOH fraction (n=5) in pre curarized preparations.

Figure 4. Mouse phrenic nerve-diaphragm preparation (PND). **A**, myographical register showing facilitatory effect of 3,4-diaminopyridine (3,4 DAP, n=4), a potassium neuroblocker. **B,** myographical record showing the replace of d-Tubocurarine (d-Tc) from nicotinic receptors, by 3,4 DAP, as a consequence of an ACh augment in the synaptic cleft (n=3). **C**, Mouse PND, indirect stimuli. Note the increase of contractile response induced by 3,4 DAP alone. The MeOH fraction addition (arrow) reduces the amplitude of twitches-induced by 3,4 DAP (n=4). Each point represents the mean ± SEM. * = $p<0.05$ in comparison with the control. ACh, acetylcholine.

Figure 5. Mouse phrenic nerve-diaphragm preparation, indirect stimuli. High electrical frequency of stimulation (40 Hz) was given at motor nerve during 5 seconds every 15 min interval. The values of curve were obtained according to the modified purpose of Ambiel and Alves-do-Prado [37]. MeOH fraction exhibited an increase of tetanic tension. The number of experiments is shown in the legend of figure. Each point represents the mean ± SEM. * = $p<0.05$ in comparison with the control. **B**, The tetanic fade is calculated as the ratio (R) between the tension at the end (B) and the tension at the beginning (A) of the tetanic response (R = B/A). C and D correspond to pre-tetanic and post-tetanic twitches, respectively. F represents the rate of stimulation required to obtain R =

0.75, at 100-120 Hz [37]. **C**, A standard curve using neostigmine at 40 Hz of frequency was appropriate and it was corroborated with data from the literature showing its tension decay [38].

Figure 6. Chick biventer cervicis preparation, indirect stimuli and creatine kinase (CK) evaluation, U/L. **A**, myographical register of Krebs control and responses to added KCl and ACh (before and after the experiment). **B**, myographical register of 240 µg/mL MeOH fraction (arrow: addition time) showing a slight but significant increase of twitch tension amplitude. Note that the contracture caused by agonist on the nicotinic receptor after the experiment remained unchanged. At the end of experiment, the contracture response to added KCl was slightly changed, in both (A and B), but not statistically different from control. **C**, under CK parameter MeOH fraction did not significantly different from control. Green lines inserted in graphic means the normal range of CK in human serum according to the manufacturer.

Figure 7. Mouse phrenic nerve-diaphragm preparation (PND). **A**, myographical register showing dantrolene effect (6 µg/mL), a Ca^{2+} antagonist of ryanodyne receptor, followed by MeOH fraction addition (200 µg/mL, n=4). No significant change was observed. **B,** myographical record showing no effect of MeOH fraction under direct stimuli (DS), in curarized preparation (d-Tc, 4 µg/mL). IS, indirect stimuli. W, washing.

Figure 8. Mouse phrenic nerve diphragm preparation, indirect stimuli. Atropine (2 µg/mL) induces an increase of ACh resulting in a slight augment of twitches amplitude. The addition of MeOH fraction (200 µg/mL, n=4) did not change the amplitude pattern of atropine, but avoid the facilitatory exhibition. Tension: 5 g/cm. W, washing.

Figure 9. **A**, Mouse phrenic nerve-diaphragm preparation (PND). Experiments using nifedipine (0.346 µg/mL) + MeOH fraction (200 µg/mL) showed that facilitatory effect of fraction depends on the calcium from extracellular medium. The points are the means ± SEM of 5-10 experiments. * $p<0.05$ vs. MeOH fraction. **B**, Myographic register representative of 5 experiments using nifedipine + MeOH fraction mixture. Arrow, mixture addition. W, washing.

Figure 10. Myographical records of mouse phrenic nerve-diaphragm preparation (PND), indirect stimuli, showing the influence of **A**, tetrodotoxin (TTX, 0.5 µM) on the facilitatory effect of MeOH fraction (**E**, 200 µg/mL, n=3**)** compared to MeOH fraction alone (**B**); **C**, 3,4 diaminopyridine (3,4 DAP, 10 µg/mL); and **D**, neostigmine (1 µg/mL). Note the facilitatory effect in C and D even in presence of TTX. Arrow, addition time. W, washing.

Figures

Figure 1

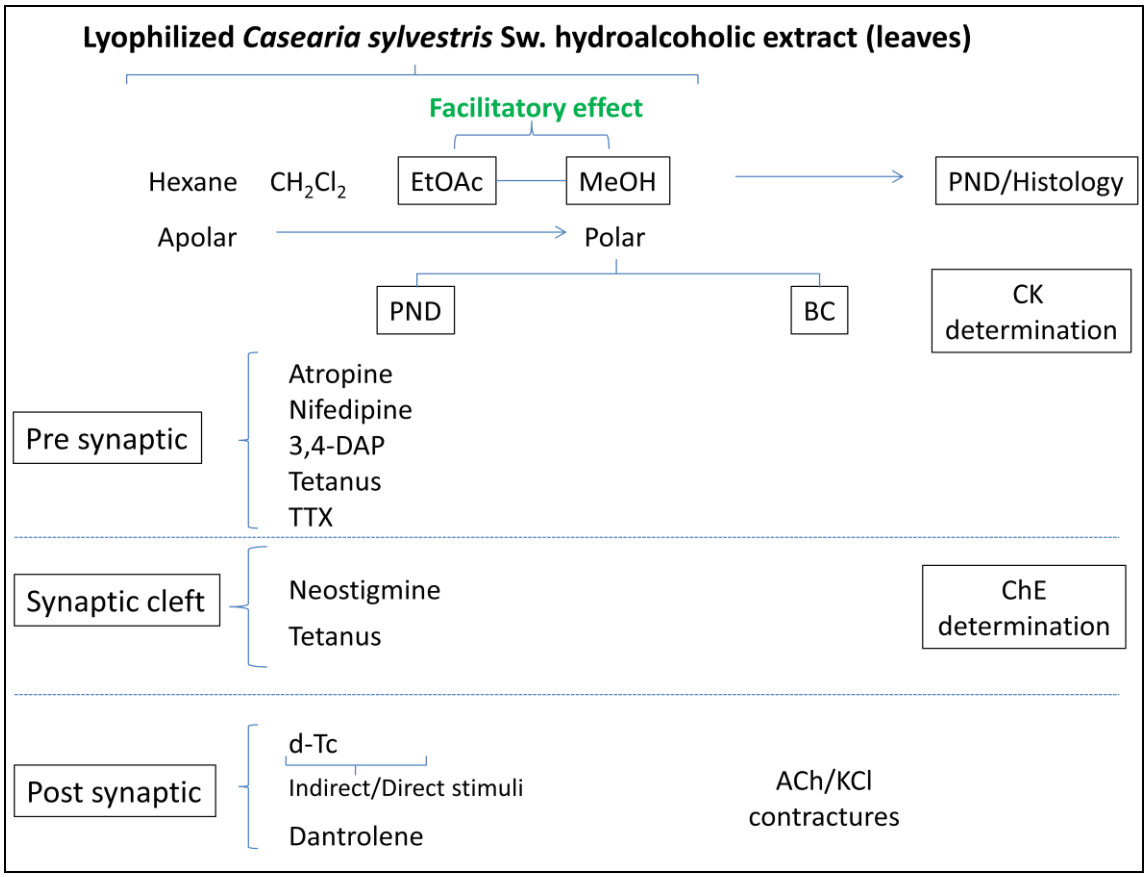


Figure 2

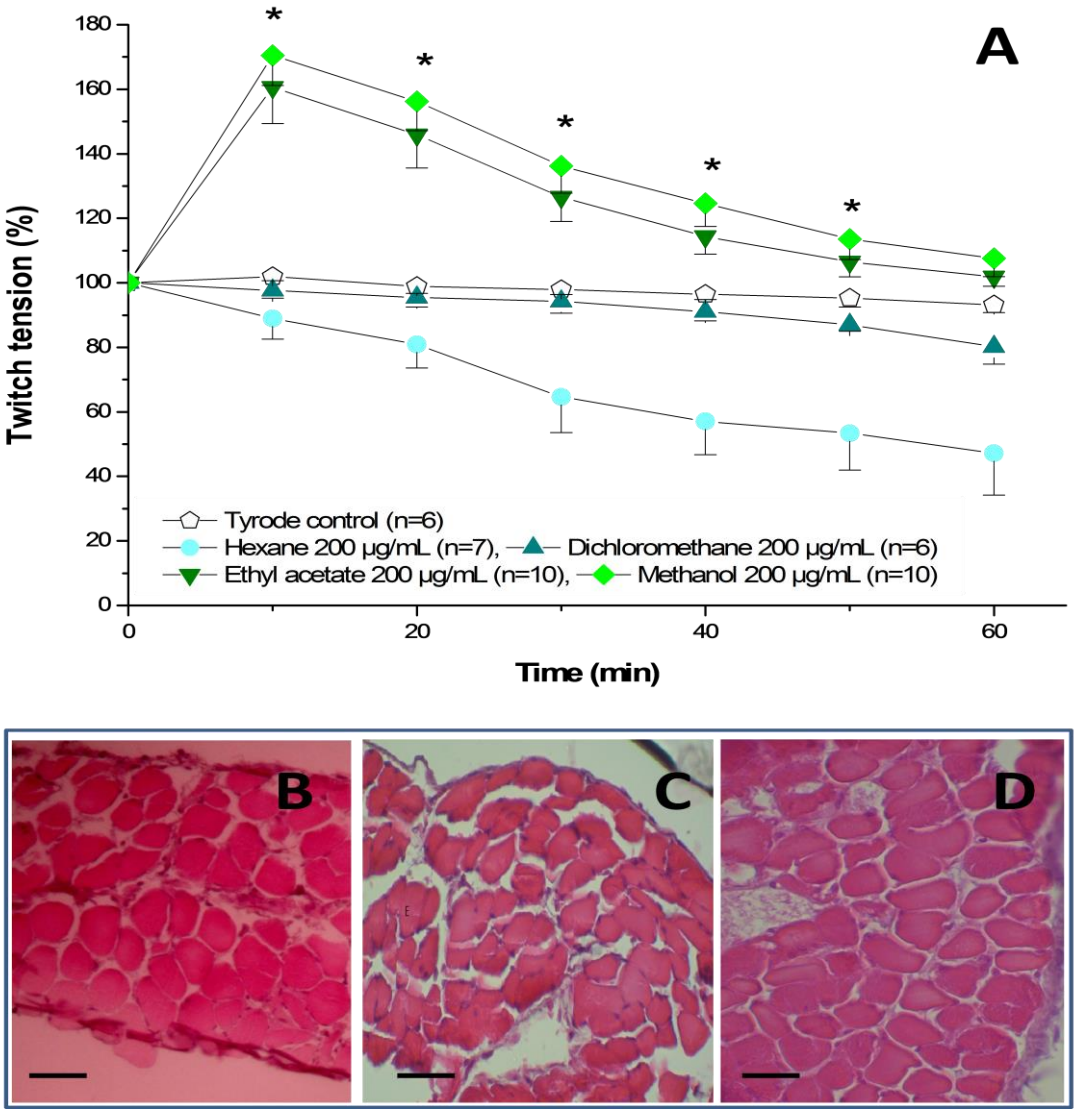

A
Twitch tension (%)
Time (min)
Tyrode control (n=6)
Hexane 200 µg/mL (n=7),
Dichloromethane 200 µg/mL (n=6)
Ethyl acetate 200 µg/mL (n=10),
Methanol 200 µg/mL (n=10)
B
C
D

Figure 3

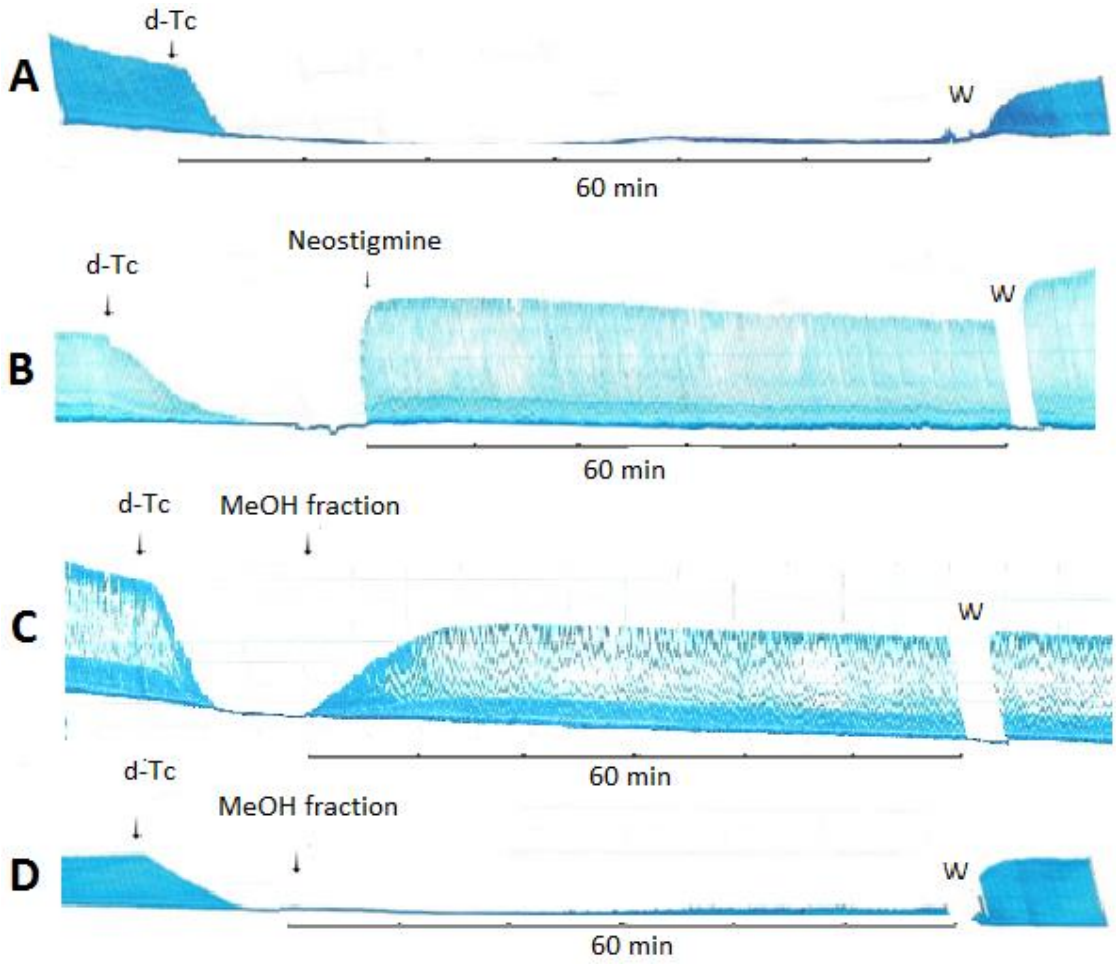

A
d-Tc
W
60 min
B
d-Tc
Neostigmine
W
60 min
C
d-Tc
MeOH fraction
W
60 min
D
d-Tc
MeOH fraction
W
60 min

Figure 4

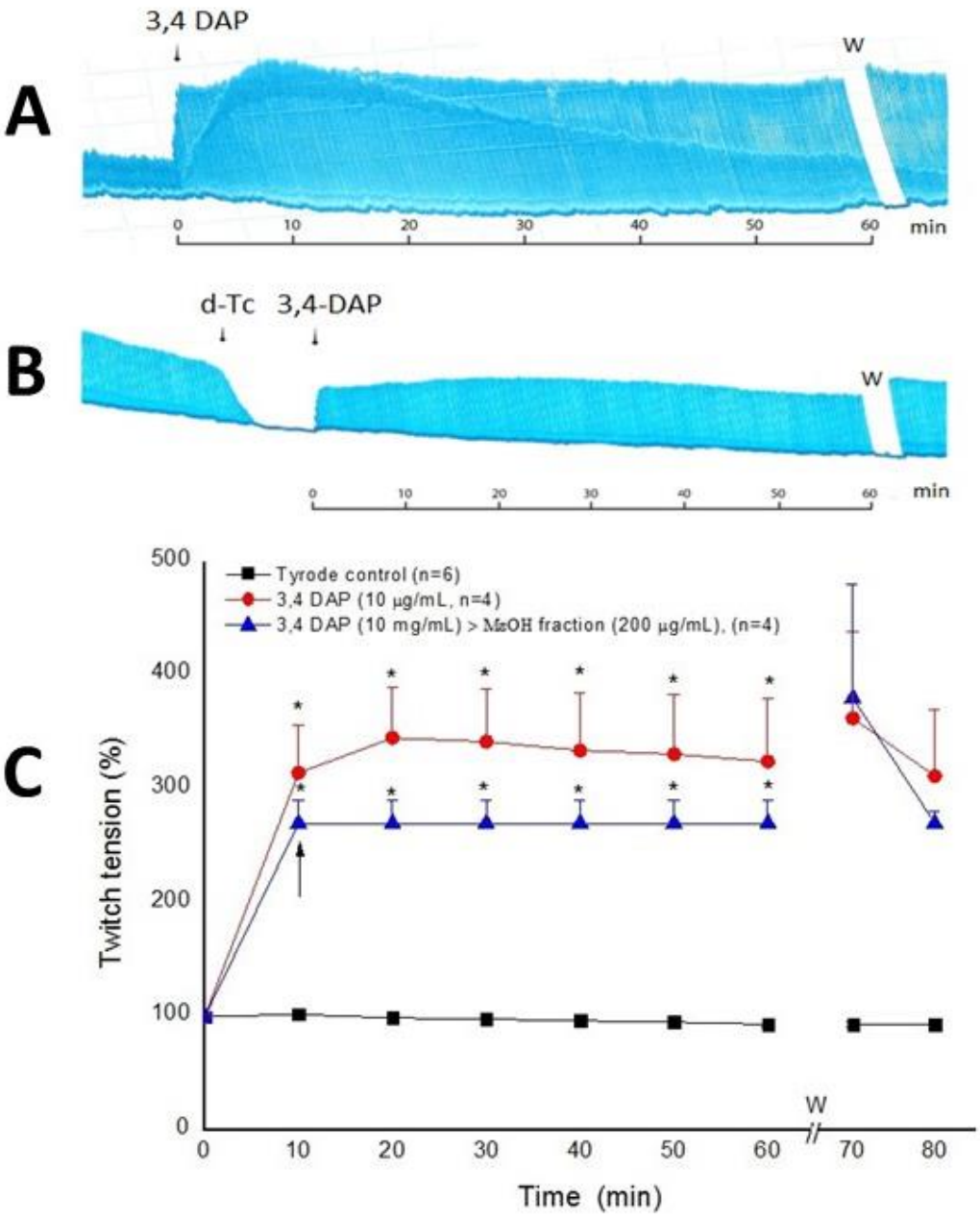

A
3,4 DAP
W
0
10
20
30
40
50
60
min
B
d-Tc
3,4-DAP
W
0
10
20
30
40
50
60
min
C
Tyrode control (n=6)
3,4 DAP (10 μg/mL, n=4)
3,4 DAP (10 mg/mL) > MeOH fraction (200 μg/mL), (n=4)
Twitch tension (%)
500
400
300
200
100
0
W
0
10
20
30
40
50
60
70
80
Time (min)

Figure 5

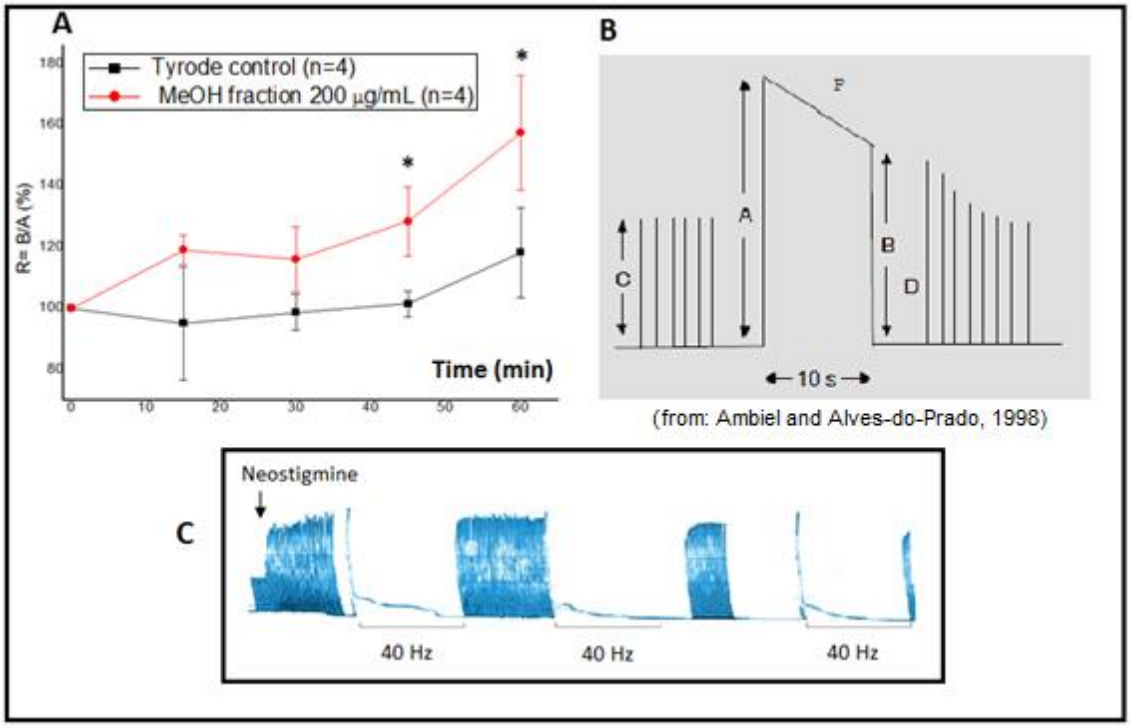

A
Tyrode control (n=4)
MeOH fraction 200 μg/mL (n=4)
R= B/A (%)
Time (min)
B
A
B
C
D
10 s
(from: Ambiel and Alves-do-Prado, 1998)
C
Neostigmine
40 Hz
40 Hz
40 Hz

Figure 6

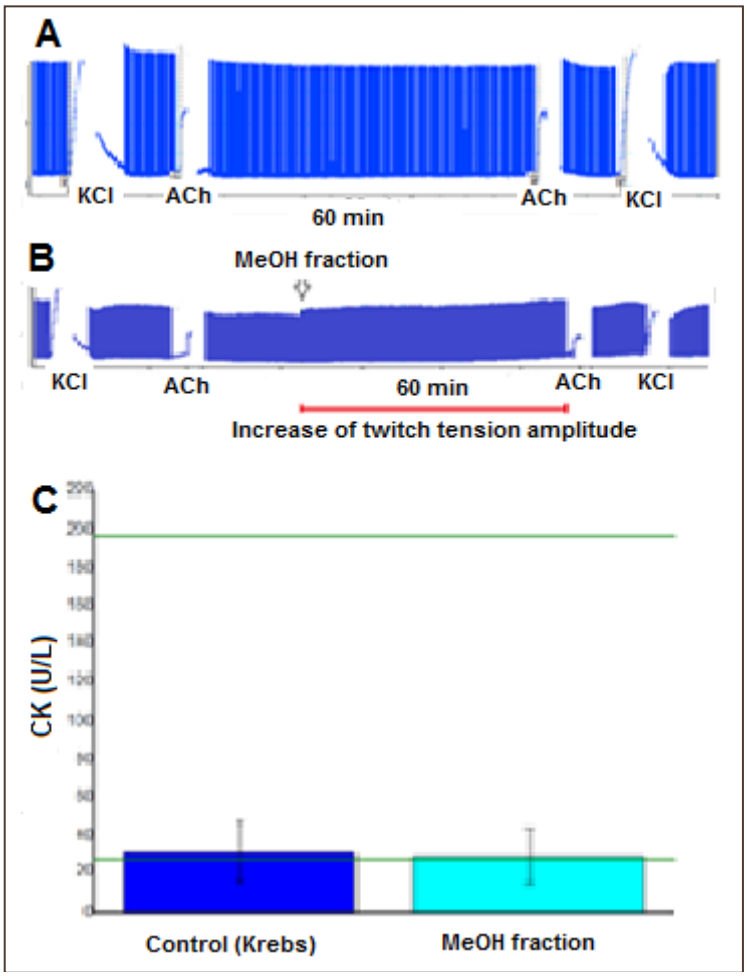

A
KCl
ACh
60 min
ACh
KCl
B
MeOH fraction
KCl
ACh
60 min
ACh
KCl
Increase of twitch tension amplitude
C
CK (U/L)
Control (Krebs)
MeOH fraction

Figure 7

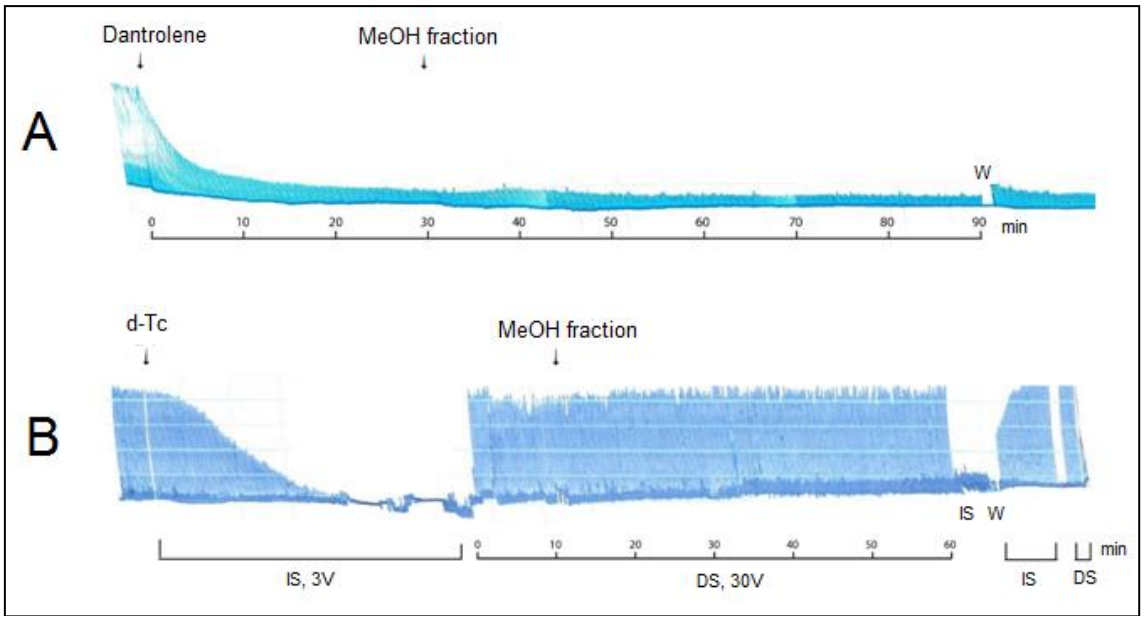


Figure 8

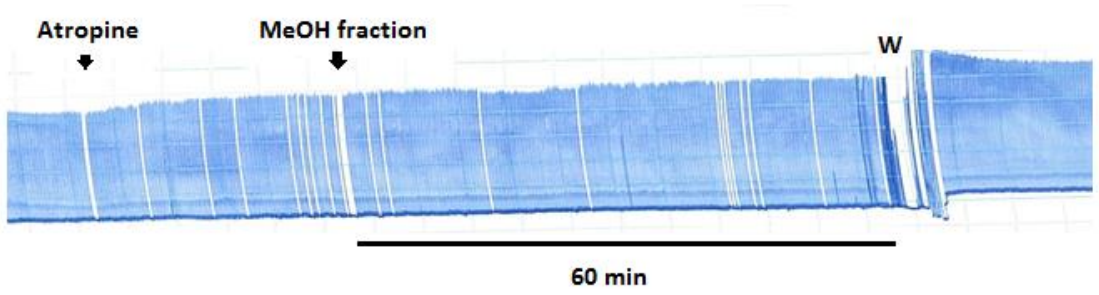


Figure 9

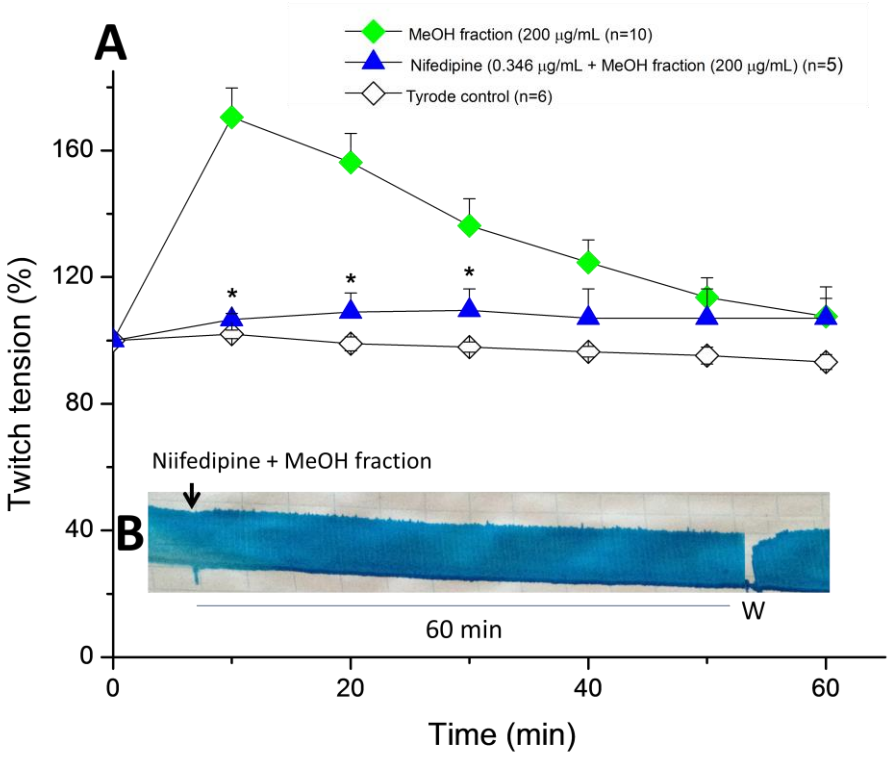
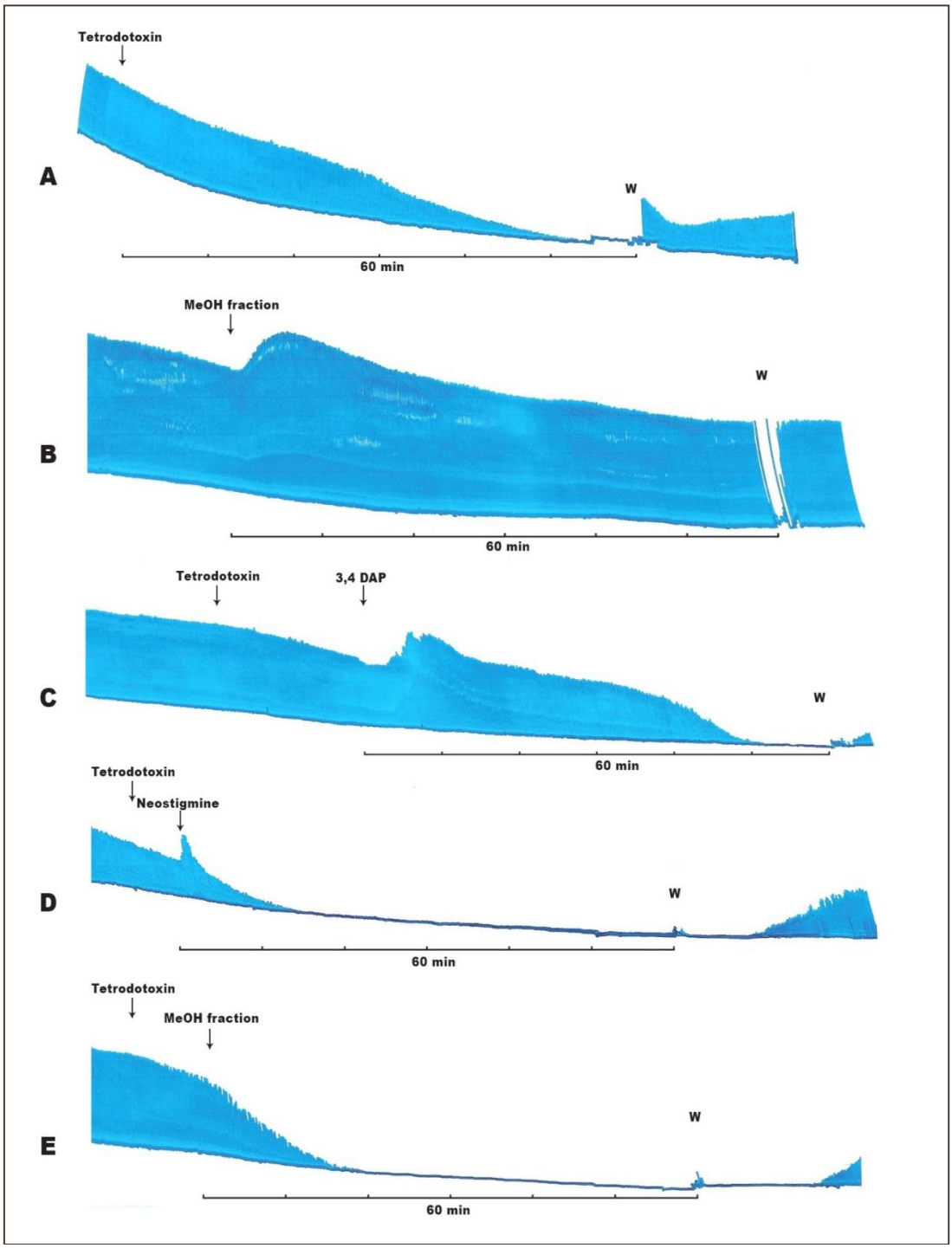

A
MeOH fraction (200 μg/mL (n=10)
Nifedipine (0.346 μg/mL + MeOH fraction (200 μg/mL) (n=5)
Tyrode control (n=6)
Twitch tension (%)
160
120
80
40
0
*
*
*
Niifedipine + MeOH fraction
B
60 min
W
0
20
40
60
Time (min)

Figure 10


A
Tetrodotoxin
W
60 min
B
MeOH fraction
W
60 min
C
Tetrodotoxin
3,4 DAP
W
60 min
D
Tetrodotoxin
Neostigmine
W
60 min
E
Tetrodotoxin
MeOH fraction
W
60 min

Tables

Table 1

Table 1. Active agents on the neuromuscular junction

Agent	Pharmacological action [22]	(Concentration) PND	Uses
Atropine	Parasympatholytic Muscarinic antagonist	2 µg/mL	Adjuvant in anesthesia, Anti-arrhythmia, Bronchodilator, Mydriatic
Dantrolene	Skeletal muscle relaxant that acts by interfering with excitation-contraction coupling in the muscle fiber	6 µg/mL	Spasticity and other neuromuscular abnormalities
3,4-Diaminopyridine (3,4 DAP)	Potassium channel blocker in membranes	10 µg/mL	Neuromuscular diseases
d-Tubocurarine (d-Tc)	Neuromuscular nondepolarizing Nicotinic antagonist	4 µg/mL	Muscle relaxant
Neostigmine	Parasympathomimetic Cholinesterase inhibitor	1 µg/mL	Anticholinesterasic
Nifedipine	Calcium antagonist	0.346 µg/mL	Vasodilator agent, useful as anti-anginal agent that also lowers blood pressure
Strontium chloride	A substitute of calcium chloride in Tyrode solution	3.6 mM	Experimental use
Tetrodotoxin (TTX)	A neuronal sodium channel blocker	5 µL of a 0.5 µM TTX solution	Pharmacological tool

Table 2

Table 2. Comparison of the cholinesterase inhibitory activity

Samples (TV= 40 µL)	**Cholinesterase determination (U/L)**			
	Serum	1 mg/mL	0.5 mg/mL	0.25 mg/mL
Qualitrol ®	3080.3 ± 66 (2652 — 3978)	-	-	-
Neostigmine	-	105.5 ± 28 *	230.9 ± 74.4 *	641.6 ± 84.8 *
MeOH fraction	-	3542.0 ± 256	3167.2 ± 64	3255.5 ± 163.5

*$p<0.05$ compared to Qualitrol ®. TV, Total volume.

5 CONCLUSÃO

A tetrodotoxina antagonizou o efeito facilitador da FM, sendo que o mesmo não ocorreu com 3,4 DAP e neostigmina. Assim, a FM age ativando os canais de sódio neuronais e a neurofacilitação é também modulada pelo cálcio extracelular.

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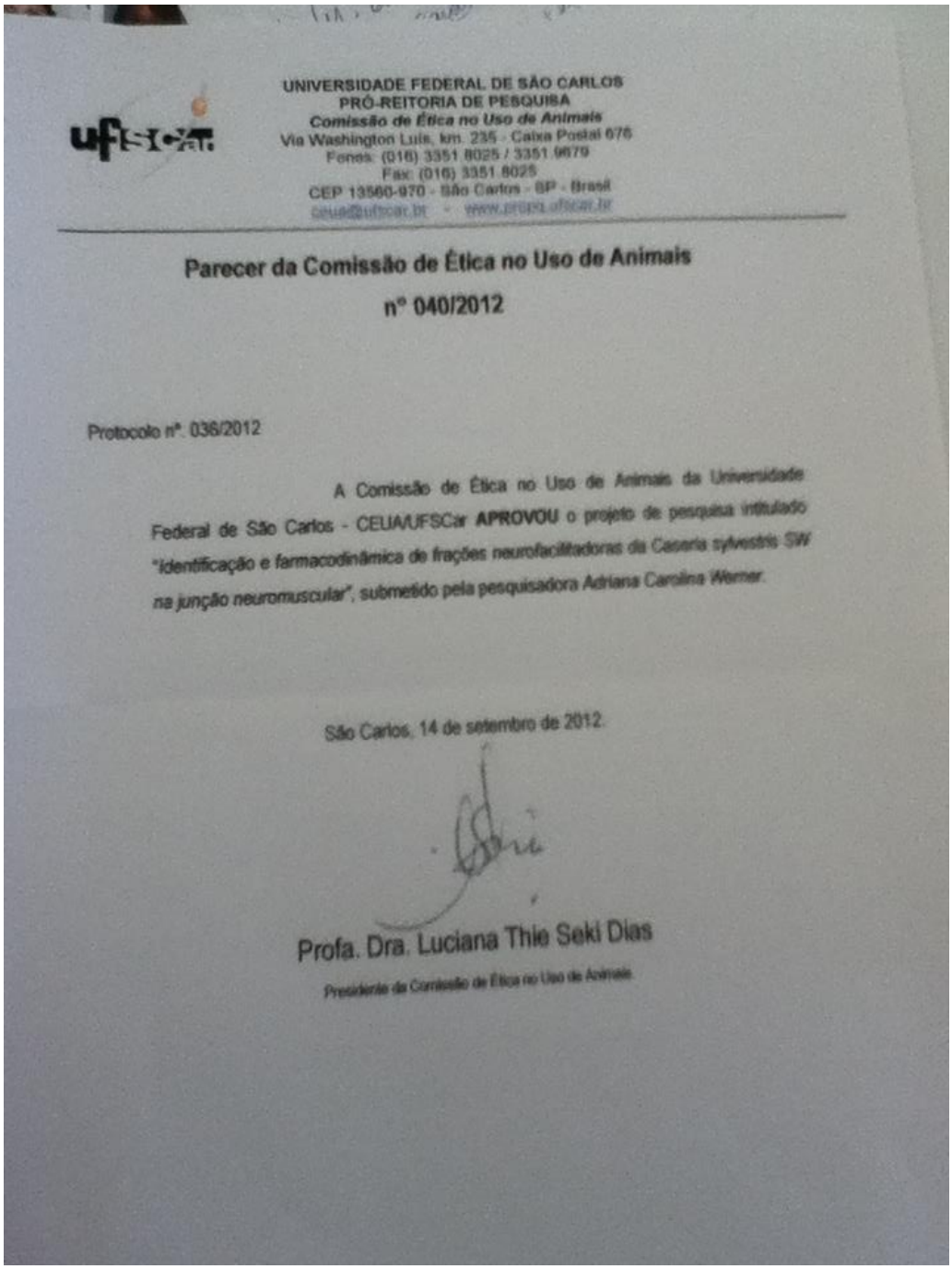
ANEXO A – Comissão de Ética no Uso de Animais (CEUA).



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Parecer da Comissão de Ética no Uso de Animais

nº 040/2012

Protocolo nº. 036/2012

A Comissão de Ética no Uso de Animais da Universidade Federal de São Carlos - CEUA/UFSCar **APROVOU** o projeto de pesquisa intitulado "Identificação e farmacodinâmica de frações neurofacilitadoras da Casearia sylvestris SW *na junção neuromuscular*", submetido pela pesquisadora Adriana Carolina Werner.

São Carlos, 14 de setembro de 2012.

Profa. Dra. Luciana Thie Seki Dias

Presidente da Comissão de Ética no Uso de Animais