UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

EXTRATIVOS VEGETAIS COM PROPRIEDADES SEDATIVAS UTILIZADOS NO TRANSPORTE DE JUNDIÁ (*Rhamdia quelen*)

TESE DE DOUTORADO

Joseânia Salbego

Santa Maria, RS, Brasil

2014

EXTRATIVOS VEGETAIS COM PROPRIEDADES SEDATIVAS UTILIZADOS NO TRANSPORTE DE JUNDIÁ (*Rhamdia quelen*)

Joseânia Salbego

Tese apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito
parcial para a obtenção do grau de
Doutora em Farmacologia

Orientador: Prof. Dr. Bernardo Baldisserotto
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Santa Maria, RS, Brasil

2014

Ficha catalográfica elaborada através do Programa de Geração Automática
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Salbego, Joseânia
EXTRATIVOS VEGETAIS COM PROPRIEDADES SEDATIVAS UTILIZADOS NO TRANSPORTE DE JUNDIÁ (Rhamdia quelen) / Joseânia Salbego.-2014.
119 p.; 30cm

Orientador: Bernardo Baldisserotto
Coorientadora: Vania Lucia Loro
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós-Graduação em Farmacologia, RS, 2014

1. Jundiá 2. Estresse oxidativo 3. Eugenol 4. Lippia alba 5. Condalia buxifolia I. Baldisserotto, Bernardo II. Loro, Vania Lucia III. Título.

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Universidade Federal de Santa Maria
Centro de Ciências da Saúde
Programa de Pós-Graduação em Farmacologia

A Comissão Examinadora, abaixo assinada,
aprova a Tese de Doutorado

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elaborada por
Joseânia Salbego

como requisito parcial para o grau de
Doutora em Farmacologia

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Santa Maria, 27 de junho de 2014.

Athos Giovani Salbego Alves
Filho, tu és assim:
Luz que me conduz,
Amor imensurável,
Parte de mim!

Pai Claro e Mãe Rosa,
Vânia Salbego "Mana Velha, Dinda de todos os sobrinhos..."
Lourenço Salbego, sobrinho-irmão, sentimentos entrelaçados...
Matheus Salbego, sobrinho-confidente...
Todas as filhas da D. Rosa...
Nossas mentes, que conduzem "nossos genes parentais",
compartilham também nossas histórias de buscas, de superações, de acreditar no poder do que semeamos, a exemplo do paciente e bondoso "Seu Claro"!

Artur Alves, a teu modo,
acompanhaste o
desenvolvimento deste
trabalho.

A vocês, com gratidão, dedico!

AGRADECIMENTOS

A Deus e às Forças da Natureza, pela graça da vida, em todas as suas formas.

À Universidade Federal de Santa Maria e aos Programas de Pós Graduação: em Farmacologia, Bioquímica Toxicológica e de Farmácia Industrial pelos espaços concedidos para a elaboração deste trabalho. À CAPES, pela bolsa concedida.

Ao Professor Dr. Bernardo Baldisserotto e à Prof.ª Dr.ª Vania Lucia Loro, orientador e coorientadora, "irmãos científicos", meu terno agradecimento pela oportunidade de fazer parte de seus grupos de pesquisa e pelo respeito as minhas buscas.

Aos professores Jessié M. Gutierres e João Radünz Neto e às professoras Adriana Chippari-Gomes, Ionara I. Dalcol, Roselei Fachinetto e Taís C. Unfer pelo aceite em compor a banca deste trabalho.

Ao grupo da Professora Vania, do Laboratório de Bioquímica Toxicológica: Adriana Santi, Bárbara Clasen, Cândida Toni, Camila Murussi, Charlene Menezes, Eduardo Severo, Jossiele Leitemperger, Luciana Guerra, Maiara Dorneles, Roberta Cattaneo Horn, Thais Lópes – pessoas queridas que fizeram parte desta trajetória: umas, porque receberam-me, lá no início deste processo (depois seguiram novos rumos). Outras, porque foram atuantes nas técnicas. Outras integraram-se ao grupo depois. Todas, porém, deixaram traços de si!

Ao grupo do Prof. Bernardo, do Laboratório de Fisiologia de Peixes: Alessandra J. Becker, Alexssandro G. Becker, Ana Paula Gottlieb, Cândida Toni, Carine F. Souza, Carine Fogliarine, Carla Zeppenfeld, Fernando Sutili, Jaqueline Golombieski, Luciane Gressler, Prof. Mauro Cunha, Sheron Martins, Thaylise Parodi – costumo dizer que a convivência é um contínuo aprendizado e vocês contribuíram para meu aprimoramento!

Aos primeiros 'Colegas de Aula de Doutorado' Alexssandro Becker, Lenise de Lima Silva e Thaylise Parodi: obrigada pela amizade e troca de conhecimentos. À Carine Souza pela oportunidade de ser uma 'colega-tutora'. Às amigas e parceiras de estudos, de longa data, Alexandra Pretto, Jaqueline Ineu Golombieski, Neiva Braun e Vera Maria Machado da Silva: o Tempo e a Ciência nos aproximam cada vez mais. Continuemos então!

Às Prof.as Berta Maria Heinzmann e Maria Amália Pavanato, e à Secretária da Farmacologia, Zeli de Maria Carvalho, pela dedicação que põem, em tudo que fazem.

Ao Ademir Gentil Rossi, Vânia Rossi e seus filhos Cristiane, Cristian e Thaís Rossi (que veio depois), família amável que acolheu-me, lá em Alegrete, em meus primeiros passos à formação de Professora. À Vanessa Bertotti da Silva e Tcharllie Ribeiro Alves, filhota e sobrinho do coração: eu os considero muito!

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria

EXTRATIVOS VEGETAIS COM PROPRIEDADES SEDATIVAS UTILIZADOS NO TRANSPORTE DE JUNDIÁ (*Rhamdia quelen*)

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Data e Local da Defesa: Santa Maria, 27 de junho de 2014.

As práticas de manejo utilizadas na aquicultura estão entre os principais fatores estressantes para peixes e podem causar reações fisiológicas adversas, afetando as suas funções essenciais. Em decorrência da crescente utilização de anestésicos e sedativos no transporte de peixes, é importante a análise do efeito destes agentes para peixes nativos, bem como a padronização de métodos alternativos, como a utilização de extratos de plantas. Com este propósito, o presente trabalho avaliou a eficácia da utilização do eugenol (EUG), do óleo essencial de *Lippia alba* (OEL) e do extrato metanólico de *Condalia buxifolia* (EMC) no transporte de jundiá (*Rhamdia quelen*). No experimento I, peixes (301,2 ± 21,4 g, 28,9 ± 1,30 cm, densidade de carga 169,2 g L^{-1}) foram transportados por 4 h, em sacos plásticos, divididos em cinco tratamentos: controle, EUG 1,5 ou 3,0 μL L^{-1}; OEL 10 ou 20 μL L^{-1}. Os parâmetros analisados foram a atividade da acetilcolinesterase (AChE) (encéfalo e músculo); marcadores oxidativos não enzimáticos tais como o conteúdo de proteína carbonil (PC) e formação de espécies reativas ao ácido tiobarbitúrico (TBARS) e atividade dos antioxidantes glutationa reduzida (NPSH) e glutationa -S- transferase (GST), no fígado. Além disso, foram analisados parâmetros gerais relacionados ao metabolismo: glicogênio, lactato e proteína (fígado e músculo). No experimento II, os peixes (420,1 ± 8,8 g, 21,2 ± 2,3 cm, densidade de carga 275,1 g L^{-1}), foram transportados por 6 h em sacos plásticos, divididos em cinco tratamentos: controle, OEL 30 ou 40 μL L^{-1} e EMC 5 ou 10 μL L^{-1}. Avaliou-se o balanço ionorregulatório, parâmetros da água e do sangue, atividade das enzimas AChE, NTPDase e 5'-nucleotidase (em encéfalo) e parâmetros bioquímicos tais como: níveis de NPSH, CAT, GST, GPx, SOD, ácido ascórbico, TBARS e PC em fígado, brânquias, músculo e rim. Nestes órgãos também foram avaliados parâmetros metabólicos. Os resultados obtidos mostraram que, independente do tempo de transporte (4 ou 6 h), as concentrações e os extrativos testados reduziram as atividades bioquímicas na maioria dos tecidos. Ocorreu estresse oxidativo em alguns órgãos avaliados, mas essas variações não indicam que esses extrativos sejam prejudiciais aos peixes durante o transporte. EMC foi eficaz na redução da perda de íons e excreção de amônia. Portanto, com base nestes dados recomenda-se o uso de OEL e EMC no transporte de jundiás.

Palavras-chave: Jundiá. Estresse oxidativo. Eugenol. *Lippia alba*. *Condalia buxifolia*.

ABSTRACT

Doctoral Thesis
Graduate Program in Pharmacology
Universidade Federal de Santa Maria

VEGETAL EXTRACTIVES WITH SEDATIVE PROPERTY USED IN THE TRANSPORT OF SILVER CATFISH (*Rhamdia quelen*)

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Date and Place of Defense: June 27th, 2014, Santa Maria.

Management practices used in aquaculture are among the main stressful factors to fish and can cause adverse physiological reactions, affecting their essential functions. Due to the increasing use of anesthetics and sedatives on fish transport, it is important to analyze the effect of these agents in native fish, as well as standardization of alternative methods such as the use of plant extracts. Consequently this study evaluated the effectiveness of the use of eugenol (EUG), essential oil of *Lippia alba* (EOL) and methanolic extract of *Condalia buxifolia* (MECB) in the transport of silver catfish (*Rhamdia quelen*). In the first experiment fish (301.2 ± 21.4 g, 28.9 ± 1.3 cm, loading density 169.2 g L^{-1}) were transported for 4 h in plastic bags, divided into five treatments: control, EUG 1.5 or 3.0 μL L^{-1} and EOL 10 or 20 μL L^{-1}. The analyzed parameters were acetylcholinesterase (AChE) activity (brain and muscle); non-enzymatic oxidative markers such as protein carbonyl content (PC) and formation of thiobarbituric acid reactive species (TBARS) and antioxidant activity of reduced glutathione (NPSH) and glutathione -S- transferase (GST) in the liver. In addition, general parameters related to metabolism were analyzed: glycogen, lactate and protein (liver and muscle). In the second experiment fish (420.1 ± 8.8 g, 21.2 ± 2.3 cm, loading density 275.1 g L^{-1}) were transported in plastic bags for 6 h, divided into five treatments: control, EOL 30 or 40 μL L^{-1} and MECB 5 or 10 μL L^{-1}. In this experiment the ionorregulatory balance, water and blood parameters, activity of the enzymes AChE, NTPDase and 5'-nucleotidase (in the whole brain) and biochemical parameters such as: levels of NPSH, CAT, GST, GPx, SOD, ascorbic acid, TBARS and PC in liver, gill, muscle and kidney were evaluated. In these tissues, metabolic parameters were also evaluated. The results showed that, regardless of transport time (4 or 6 h), concentrations and extractives tested reduced biochemical activity in most tissues. There was oxidative stress in some tissues, but these variations do not indicate that these extractives are harmful to fish during transport. MECB was effective in reducing the ions loss and ammonia excretion. Therefore, based on these data it is recommended the use of EOL and MECB in the transport of silver catfish.

Keywords: Silver catfish. Oxidative Stress. Eugenol. *Lippia alba*. *Condalia buxifolia*.

SUMÁRIO

INTRODUÇÃO

A aquicultura apresenta-se como uma alternativa promissora na produção de alimentos. O contínuo aumento do consumo de pescados é um dado presente na indústria alimentícia atual e, por conseguinte, o aumento nas atividades de pesca e aquicultura, no Brasil e no mundo, vem sendo registrado (Food and Agriculture Organization of the United Nations - FAO, 2013). A maior produção de peixes, no entanto, requer uma série de práticas de manejo (biometria, análises patológicas, implante hormonal, manejo, captura e transporte) as quais provocam injúrias aos animais e estão entre os principais fatores estressantes para peixes criados em cativeiro (BARTON; IWAMA, 1991; BARTON, 2000; COOKE et al., 2004).

O transporte de peixes pode ser feito através do sistema aberto, em caixas d'água adaptadas e galões de plástico, entre outros, ou fechado, em sacos plásticos, com adição de oxigênio comercial (GOMES, L.C. et al., 2003, 2006), sendo que na presente tese utilizou-se o sistema de transporte fechado- sacos plásticos suplementados com oxigênio puro (Figura 1).

Figura 1 – Sistema fechado de transporte de peixes: saco de plástico com jundiás (*Rhamdia quelen*), alocados para o transporte.
Fonte: arquivo pessoal.

Estudos com várias espécies de peixes têm mostrado que durante o transporte eles são expostos a diferentes estímulos de estresse destacando-se manejo, captura, altas densidades de carga, qualidade da água inadequada e baixos níveis de oxigênio (SCHRECK et al., 1995; ERIKSON et al., 1997; CUNHA et al., 2010a; GOMES, D.P. et al., 2011; BECKER et al., 2012). O estresse, por sua vez, resulta em variações em parâmetros metabólicos e fisiológicos, como por exemplo, alterações na homeostase (TORT, 2011) e funções vitais (CHANDROO et al., 2004; ZAHL et al., 2009; ZAHL; SAMUELSEN; KIESSLINH, 2012) o que pode, inclusive, levar os peixes ao óbito, resultando em prejuízos aos produtores (BARTON; IWAMA, 1991; BARTON, 2000). O uso de sedativos durante o transporte pode diminuir esse estresse (SNEDDON, 2012; ZAHL; SAMUELSEN; KIESSLINH, 2012), melhorando o bem estar animal (CHANDROO et al., 2004).

O jundiá, *Rhamdia quelen* (Quoy & Gaimard,1824, Heptapteridae) (Figura 2), é um teleósteo de água doce, nativo da América do Sul e Central (BARCELLOS et al., 2004). É a espécie nativa mais importante para a piscicultura do sul do Brasil, pois além de apresentar boa produtividade e aceitação para o consumo, tem boa reprodução e pronunciado crescimento em cativeiro. Sobrevive às divergências climáticas típicas dessa região, podendo alimentar-se de diferentes fontes de nutrientes (LAZZARI et al., 2006; BALDISSEROTTO; RADÜNZ NETO; BARCELLOS, 2013).

Figura 2 – Exemplares de jundiá (*Rhamdia quelen*).
Fonte: arquivo pessoal.

Com esta espécie, trabalhos demonstram a eficácia do eugenol (obtido de *Eugenia caryophillata*) e do óleo essencial de *Lippia alba* como sedativos e anestésicos, associados ou não ao transporte (CUNHA et al., 2010a,b; AZAMBUJA et al., 2011; GOMES, D.P. et al., 2011; BECKER et al., 2012; HELDWEIN et al., 2012). Atualmente, o extrato metanólico da *Condalia buxifolia* é um novo sedativo que vem sendo estudado, visando facilitar as práticas de transporte na piscicultura e que mostrou eficácia para o transporte do jundiá (BECKER et al., 2013).

O cravo, *E. caryophillata* (*Eugenia aromaticum*, *Syzygium aromaticum*) popularmente conhecido como cravo-da-índia, fornece um óleo aromático (SCHULTZ, 1984), conhecido como óleo de cravo (Figuras 3, A e B), o qual tem como constituinte majoritário (70-90%) o fenilpropeno eugenol [2-metoxi-4-(2-propenil) fenol] (União Internacional de Química Pura e Aplicada- IUPAC), consolidado como anestésico e sedativo eficaz para peixes (INOUE et al., 2003, 2004, 2011; ROUBACH et al., 2005; BARBOSA L.G. et al., 2007; GONÇALVES, A.F.N. et al., 2008; HONCZARYK; INOUE, 2009; CUNHA et al., 2010b; GOMES, D.P. et al., 2011).

Figuras 3 A e B – Respectivamente – Cravo-da índia (botões florais secos) e eugenol, adquirido comercialmente e utilizado para o experimento.
Fonte: arquivo pessoal.

O gênero *Lippia* (Verbenaceae) inclui aproximadamente 200 espécies de pequenos arbustos. A *Lippia alba* (Mill.) N.E. Brown é um arbusto aromático (Figura 4), conhecido no Brasil por erva cidreira, capim-limão, falsa melissa, entre outros, e é utilizada popularmente

para diversos fins, em especial por suas propriedades calmantes e analgésicas (Di STASI et al., 2002; BARBOSA, F.G. et al., 2006; HEINZMANN; BARROS, 2007; BARROS et al., 2009). É uma espécie cosmopolita, encontrada em todos os países da América do Sul e Central (PASCUAL et al., 2001; HENNEBELLE et al., 2008), territórios tropicais da África (STASHENKO; JARAMILLO; MARTINEZ, 2004), India (SHUKLA et al., 2009), e Austrália (DAY; McANDREW, 2003).

Figura 4 – *Lippia alba*, aspecto geral da planta.
Fonte: Berta Maria Heinzmann.

Esta espécie mostra uma notável variação na morfologia da planta e composição do óleo essencial, o qual varia dependendo da origem geográfica e, por isso, é classificada em diferentes quimiotipos, de acordo com a constituição do óleo essencial (PASCUAL et al., 2001; TELES et al., 2012). Três quimiotipos já foram estabelecidos no Nordeste do Brasil: limoneno e carvona, limoneno e citral, mirceno e citral (BARBOSA, F. G. et al., 2006)

enquanto que em São Paulo o componente majoritário encontrado foi o linalol (STASHENKO; JARAMILLO; MARTINEZ, 2004), quimiotipo também predominante nas plantas utilizadas para o presente trabalho (linalol-3,7-dimetil-octa-1,6-dien-3-ol, monoterpeno - IUPAC), as quais foram cultivadas em São Luiz Gonzaga e Santa Maria, RS.

A *Condalia buxifolia* Reissek (Rhamnaceae) é uma árvore de aproximadamente quatro metros de altura encontrada no Sul do Brasil, Uruguai e Argentina, com características espinescentes (Figura 5). É usada na medicina popular como febrífuga, anti-inflamatória, contra disenterias e também por suas propriedades calmantes (MOREL et al., 2002, 2005). Plantas da família Rhamnaceae são amplamente utilizadas na medicina popular (GIACOMELLI et al., 2004; MALDANER et al., 2011) e são ricas em peptídeos alcaloides com diversas atividades biológicas, incluindo propriedades sedativas (vide MOREL et al., 2002). Maldaner (2005) descreve quatro metabólitos secundários isolados da *C. buxifolia*: condalina-A (alcaloide ciclopeptídico), β-sitosterol (fitoesteroide), lupeol e taraxerol (triterpenos).

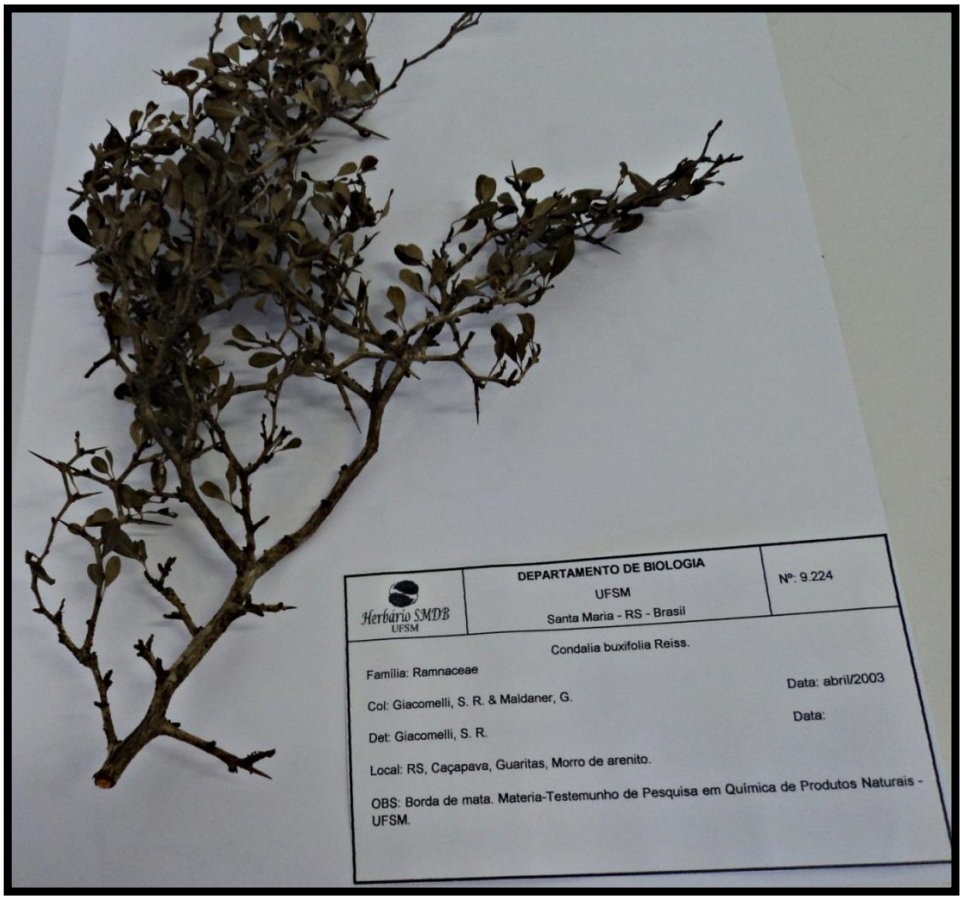

Figura 5 – *Condalia buxifolia*.
Fonte: arquivo pessoal.

Na tradicional medicina indiana, algumas espécies de plantas que possuem alcaloides, β-sitosterol e taraxerol são utilizadas por suas propriedades ansiolíticas e antioxidantes (SETHIYA et al., 2009). Também em outros países orientais, é bastante conhecido o uso da semente de *Zizyphus jujuba* Mill var. *spinosa* (Rhamnaceae), rica em peptídeos alcaloides, por suas propriedades tranquilizantes e analgésicas (MA et al., 2008).

Cabe destacar também o estudo de Boligon et al. (2009), com extrativos de folhas e cascas do caule de *Scutia buxifolia* (Rhamnaceae), os quais mostraram propriedades antioxidantes em encéfalo de ratos e, recentemente, o estudo de Becker et al. (2013) o qual comprovou a atividade sedativa do extrato metanólico da *C. buxifolia* em jundiá.

A sedação é uma redução na sensibilidade que resulta em tranquilidade e calma, descrita por alguns autores como anestesia leve (ZAHL; SAMUELSEN; KIESSLING, 2012). Esta ocorre devido à interação do fármaco sedativo com o receptor do ácido gama-aminobutírico (GABA), o qual é um canal de cloreto, facilitando assim a hiperpolarização da célula e depressão do sistema nervoso central (SNC) (HELDWEIN et al., 2012; MA et al., 2008). A anestesia (do grego, sem sensação), por sua vez, compreende diversos componentes, incluindo sedação, relaxamento muscular, imobilização, inconsciência (narcose), amnésia (perda de memória) e analgesia (alívio da dor). O processo de anestesia pode ser geral ou local. Entende-se por anestesia geral um estado de inconsciência, relaxamento do músculo esquelético e inibição dos reflexos sensoriais, causado por um agente depressor do SNC. Embora não haja um mecanismo definido, acredita-se haver o envolvimento da via gabaérgica, entre outras, as quais interagem. A anestesia local resulta do bloqueio da transmissão sensorial de uma determinada região do corpo para o SNC, através do bloqueio dos canais de sódio das membranas excitatórias neuronais (JAVAHERY et al., 2012; SNEDDON, 2012; ZAHL; SAMUELSEN; KIESSLING, 2012).

A utilização de anestésicos em peixes não é recente (McFARLAND, 1959; SCHOETTGER; JULIN, 1967; SEIGNEUR, 1984) e vem sendo investigada com o propósito de facilitar as práticas de manejo e procedimentos veterinários, tornando-os menos invasivos, proporcionando bem-estar, menos estresse e consequentes danos fisiológicos aos animais (CHANDROO et al., 2004; COOKE et al., 2004; SEGNER et al., 2012). Em baixas concentrações os anestésicos são utilizados com a finalidade de sedação para reduzir a atividade e a taxa metabólica, enquanto que as concentrações mais elevadas são rotineiramente especificadas durante procedimentos que são considerados estressantes ou dolorosos para os peixes (KIESSLING et al., 2009).

O processo de sedação em peixes tem sido usado principalmente para as práticas de transporte. Entre os produtos comumente reportados até o momento estão a benzocaína (etil aminobenzoato) (GOMES, L.C. et al., 2006) e o eugenol, os quais são considerados baratos e seguros para os animais, produtor e ambiente (INOUE et al., 2005; GUÉNETTE et al., 2007; CUNHA et al., 2010b; GOMES, D.P. et al., 2011), o metanosulfonato de tricaína (metacaína, MS-222), o hidrocloreto de metomidato, a quinaldina, o sulfato de quinaldina, o isoeugenol (Aqui-S®) e o 2-fenoxietanol, ambos utilizados para diminuir o estresse causado pelo transporte (IVERSEN et al., 2003; SMALL, 2003, 2004; COOKE et al., 2004; SINGH et al., 2004; SNEDDON, 2012; ZAHL; SAMUELSEN; KIESSLING, 2012; GHANAWI; MONZER; SAOUD, 2013).

No entanto, alguns destes produtos apresentam efeitos tóxicos que podem comprometer a saúde dos animais, bem como dos produtores, além de não haver protocolos específicos em relação às concentrações adequadas para as diferentes espécies de peixes cultivadas (GUÉNETTE et al., 2007; SNEDDON, 2012; ZAHL; SAMUELSEN; KIESSLING, 2012). Outras são difíceis de serem obtidas e também têm custo elevado, dificultando o acesso por parte do produtor (PAVLIDIS et al., 2003; SINGH et al., 2004; GOMES, L.C. et al., 2006; ZAHL; SAMUELSEN; KIESSLING, 2012). Além disso, somente o MS-222 é liberado pelo United States Department of Health and Human Services Food and Drug Administration (U.S. FDA) (SMALL, 2003) e o consumo do pescado só é permitido 21 dias após a aplicação (IVERSEN et al., 2003). O Aqui-S® tem aprovação para a produção de peixes destinada à alimentação na Austrália, Chile, Nova Zelândia (SMALL, 2004), Costa Rica e República da Coreia. Na Noruega, é usado em reprodutores e na pesquisa (ZAHL; SAMUELSEN; KIESSLING, 2012) e nos Estados Unidos da América aguarda a aprovação (KIESSLING et al., 2009). Adicionalmente, embora sendo aceito para as práticas de aquicultura na Austrália e Nova Zelândia, entre outros países da Oceania (INOUE et al., 2005; ROSS; ROSS, 2008), o eugenol ou qualquer ingrediente ativo derivado do óleo de cravo, não são liberados pelo U.S. FDA (2007), visto que, estudos comprovam que estas moléculas são carcinogênicas para roedores.

Em vista dessas informações, é evidente a importância do estudo de novos extrativos naturais que minimizem o estresse em peixes. O estresse pode ser descrito como uma alteração na condição biológica basal, em condições de repouso, que resulta em desordens à homeostase e compromete o bem-estar do animal. Bem-estar é um tema complexo e não há um consenso quanto a sua definição. Contudo, a maioria das definições levam em conta três categorias: percepção, natureza e função (CHANDROO et al., 2004; SEGNER et al., 2012).

Estressores podem ser classificados de acordo com diferentes critérios, conforme a duração ou percepção da fonte. A resposta imediata ao estresse resulta na ativação simpática do eixo hipotalâmico-pituitária-interrenal (eixo HPI) levando ao aumento dos níveis de catecolaminas e cortisol no sangue. Dependendo da duração ou intensidade do agente agressor, podem resultar situações muito diferentes, embora em todos os casos leve à ativação de uma sequência de mecanismos para restabelecer a homeostase (BARTON; IWAMA, 1991; GERWICK et al., 1999; TORT, 2011). A ativação do eixo HPI, por sua vez, desencadeia uma série de respostas, classificadas conforme a seguir: 1) resposta primária ou neuroendócrina, a qual é caracterizada por um significativo aumento dos hormônios corticosteróides e catecolaminas; 2) resposta secundária, que corresponde ao aumento das atividades metabólicas, da permeabilidade celular e dos batimentos cardíacos e 3) terciária, decorrente de um maior tempo de exposição, a qual diminui os leucócitos circulantes, a imunidade e o crescimento (MAZEAUD et al.,1977; BARTON; IWAMA, 1991; BARTON et al., 1998; GERWICK et al., 1999).

A resposta primária pode levar a alterações na atividade das enzimas acetilcolinesterase (AChE; E.C. 3.1.1.7), enzima presente no sistema colinérgico, e ectonucleotidases, destacando-se neste trabalho as enzimas NTPDase-1 (ATP difosfohidrolase, Apirase, Ecto/CD39, do grupo das ecto-nucleotídeo trifosfato difosfoidrolases, EC 3.6.1.5) e a ecto- 5′-nucleotidase (CD73; E.C. 3.1.3.5), enzimas presentes no sistema purinérgico, uma vez que esta resposta envolve mecanismos neuronais. Essas enzimas, por sua vez, desempanham mecanismos fundamentais envolvidos na neurotransmissão (SCHETINGER et al., 2000; ABBRACCHIO et al., 2008; GUTIERRES et al., 2012a,b, 2014).

O sistema colinérgico está relacionado com diversas funções vitais, entre elas a cognição. Entre os neutransmissores envolvidos nesse sistema, destaca-se a acetilcolina (ACh), a qual age como moduladora de respostas neuronais desencadeadas por estímulos sensoriais. A AChE é uma enzima que degrada a ACh em colina e ácido acético. Essa reação é necessária para que o neurônio colinérgico retorne a seu estado de repouso após a ativação. Portanto, essa enzima controla a concentração de ACh nas fendas sinápticas e, consequentemente, a transmissão colinérgica (SCHMATZ et al., 2009; BLENAU; RADEMACHER; BAUMANN, 2012; GUTIERRES et al., 2012a, 2014). A diminuição na atividade dessa enzima faz com que a acetilcolina não degrade e acumule-se nessas fendas, alterando as funções cognitivas. Em peixes, esta alteração pode levar à diminuição do movimento e ao nado errático (TIWARI; SINGH, 2004; SALBEGO et al., 2010).

O sistema purinérgico é constituído por uma série de moléculas da membrana plasmática, envolvidas em várias funções celulares, entre elas as relacionadas à neurotransmissão e, portanto, alvo de promissores estudos nesta área (SCHETINGER et al., 2000; ABBRACCHIO et al., 2008; BURNSTOK et al., 2012). A organização desse sistema pode ser assim definida: 1) nucleotídeos e nucleosídeos extracelulares; 2) receptores destes nucleotídeos e nucleosídeos e 3) ectoenzimas responsáveis pelos níveis extracelulares destas moléculas (YEGUTKIN, 2008; SCHMATZ et al., 2009).

A sinalização purinérgica, faz parte de uma cascata de eventos, através dos quais ocorre a comunicação entre as células, sendo o ATP (um nucleotídeo trifosfato) a molécula sinalizadora fundamental, desse sistema. Em adição, o ATP participa de outros processos celulares, atuando em vias metabólicas e participando do transporte e conservação de energia. A exemplo de todos os nucleotídeos, está entre as substâncias mais amplamente distribuídas nas células dos vertebrados (ZIMMERMANN et al., 2007; ABBRACCHIO et al., 2008).

Identificado na década de 70 pela neurotransmissão não adrenérgica e não colinérgica, é atualmente reconhecido como um cotransmissor, atuando nas inervações do sistema nervoso periférico e central. Em conjunto com outros nucleotídeos tais como a adenosina difosfato (ADP) e adenosina monofosfato (AMP) atua como sinalizador purinérgico intercelular em diferentes situações fisiológicas, podendo indicar o estado de saúde de um organismo (SCHETINGER et al., 2000; BURNSTOK, 2006, 2008, 2012; YEGUTKIN, 2008; SCHMATZ et al., 2009), visto que os níveis extracelulares desses nucleotídeos estão diretamente relacionados com o funcionamento celular, manutenção da homeostase e respostas ao estresse oxidativo. Por sua vez, as ectonucleotidases NTPDase e 5′-nucleotidase, entre outras, de forma sincronizada, desencadeiam uma série de ações as quais controlam os níveis extracelulares desses nucleotídeos (entre outras moléculas) (ZIMMERMANN, 2007; 2011; SCHMATZ et al., 2009; GUTIERRES et al., 2012b; GONÇALVES, J.F. et al., 2013).

Estresse oxidativo é definido como um desequilíbrio entre os níveis de pró-oxidantes e antioxidantes, no qual os primeiros se sobressaem causando danos potenciais aos componentes celulares (proteínas, lipídeos, DNA), sendo a membrana celular um dos primeiros alvos (SIES, 1991; HALLIWELL; GUTTERIDGE, 1999). O oxigênio, apesar de essencial aos organismos aeróbicos, naturalmente durante o processo de combustão, utilizado para converter os nutrientes dos alimentos absorvidos, em energia, produz espécies reativas de oxigênio (EROs) como resultado do metabolismo celular. Entre elas, pode-se incluir o peróxido de hidrogênio (H_2O_2), o radical hidroxila ($^{\bullet}OH$), o ânion superóxido ($O_2^{\bullet-}$), entre outros oxidantes (MATÉS et al., 2000; WU et al., 2011), os quais estão diretamente

relacionados com o estresse oxidativo (SIES, 1991; HALLIWELL; GUTTERIDGE, 1999; HERMES-LIMA; ZENTENO-SAVÍN, 2002). Uma das fontes de H_2O_2 é a β-oxidação de ácidos graxos. Alterações nos níveis de oxigênio e ações decorrentes do manejo também podem levar à formação de EROs (AZAMBUJA et al., 2011; LUSHCHAK, 2011; PARODI et al., 2012), as quais levam ao dano oxidativo se houver um desequilíbrio entre a formação e remoção das mesmas, decorrente da diminuição dos antioxidantes endógenos (produzidos pelo organismo) e exógenos (provenientes da dieta) caracterizando um estado pró-oxidante (SIES, 1991; HERMES-LIMA; ZENTENO-SAVÍN, 2002; WILHELM FILHO, 2007).

Um estado pró-oxidante tem como consequência o dano aos componentes celulares, sendo a membrana celular o principal alvo, devido a sua constituição lipoproteica. O dano às proteínas, denominado oxidação ou carbonilação proteica, resulta na formação de grupos carbonil ou proteína carbonil (PC) devido à oxidação das cadeias laterais das proteínas (SIES, 1991; PAVANATTO; LLESUY, 2008; AZAMBUJA et al., 2011; LUSHCSAK, 2011; HELDWEIN et al., 2012; PARODI et al., 2012). O dano aos lipídios, denominado lipoperoxidação (LPO), induz à produção de aldeídos endógenos, destacando-se o malondialdeído (MDA), considerado um importante desencadeador de distúrbios metabólicos. O MDA é um dialdeído formado como um produto secundário durante a oxidação de ácidos graxos poli-insaturados, reativo ao ácido tiobarbitúrico (TBARS), sendo, portanto, principal indicador de dano lipídico (MATÉS et al., 2000; DMITRIEV; TITOV, 2010; SCHMATZ et al., 2012).

O não reparo aos danos oxidativos pode comprometer a funcionalidade das células e consequentemente a homeostase de um organismo (SCHMATZ et al., 2012). Para protegerem-se, as células contam com um complexo sistema de defesa antioxidante enzimático (de alto peso molecular) e não enzimático (de baixo peso molecular), que neutraliza a formação de EROs e seus efeitos negativos sobre os componentes celulares. Entre as enzimas antioxidantes mais importantes dos organismos (constituem a primeira linha de defesa contra as EROs) estão a superóxido dismutase (SOD), a qual catalisa a dismutação do $O_2^{\bullet-}$, em H_2O, a catalase (CAT), que reduz o H_2O_2, e a glutationa peroxidase (GPx), que reduz tanto o H_2O_2 como peróxidos orgânicos por uma reação dependente de glutationa (MATÉS et al.,1999; HALLIWELL; GUTTERIDGE, 1999; WU et al., 2011; SCHMATZ et al., 2012). Associada a essas enzimas, está a glutationa-S-transferase (GST), a qual é considerada uma enzima detoxificadora, uma vez que participa da detoxificação de metabólitos prejudiciais à célula, utilizando mecanismos de conjugação à glutationa reduzida (GSH), reduzindo a

toxicidade desses metabólitos, além de torná-los mais hidrofílicos (MATÉS et al., 2000; WU et al., 2011).

O sistema de defesa antioxidante não enzimático dispõe da GSH, que é um tiol não proteico (por isso também denominada NPSH, conforme será tratada no decorrer desta tese), reconhecido como o tiol mais abundante dos seres vivos, e o ácido ascórbico (vitamina C, único antioxidante exógeno aqui reportado), considerado um dos antioxidantes mais importantes nas células animais (ANDERSON; MEISTER, 1989; MEISTER, 1992; KOCHHANN et al., 2009; SCHMATZ et al., 2012), entre outros, não reportados no presente trabalho. Esse sistema pode atuar de duas formas: 1) inativando radicais livres (SOD, CAT, GST e GPx) e 2) reparando os danos já causados por esses (NPSH e ácido ascórbico).

A resposta secundária leva a alterações no metabolismo proteico e dos carboidratos. As proteínas são os maiores constituintes do metabolismo, enquanto que os carboidratos são os responsáveis por suprir a demanda energética dos animais. Em condições desfavoráveis, as moléculas de proteína sofrem alterações, podendo haver tanto a estimulação da síntese quanto a quebra das mesmas. Do metabolismo das proteínas, resultam proteína total, aminoácido e amônia, enquanto que do metabolismo dos carboidratos resultam glicose, glicogênio e lactato (MAZEAUD, M.M.; MAZEUD, F.; DONALDSON, 1977; BARTON; IWAMA, 1991; BARTON et al., 1998).

Adicionalmente, o controle dos parâmetos sanguíneos e de qualidade da água, juntamente com o balanço ionorregulatório, são vitais para a sobrevivência e bem estar dos animais, durante o transporte (BECKER et al., 2012).

Considerando esses parâmetros e os extrativos propostos neste trabalho, não há estudos que os relacionem ao transporte de peixes. Sendo assim, para uma melhor análise da condição dos peixes após o transporte com os sedativos propostos adicionados à água de transporte, é preciso verificar parâmetros enzimáticos e bioquímicos, os quais, em conjunto, compõem uma série de eventos essenciais para a manutenção da homeostase corporal desses animais.

1 OBJETIVOS

1.1 Objetivo geral

Avaliar o efeito de um fitofármaco (eugenol) e de fitoterápicos (óleo essencial de *Lippia alba* e extrato metanólico de *Condalia buxifolia*) sobre parâmetros bioquímicos relacionados ao metabolismo e indicadores de estresse oxidativo em jundiás submetidos ao transporte.

1.2 Objetivos específicos

- Verificar a eficácia do eugenol (EUG), do óleo essencial de *L. alba* (OEL) e do extrato metanólico de *C. buxifolia* (EMC) para o transporte de jundiá.
- Avaliar os efeitos do eugenol, do OEL e do EMC sobre a atividade da AChE (cerebral e muscular) e da NTPDase e 5′-nucleotidase (cerebral).
- Investigar as respostas do metabolismo energético e proteico, parâmetros da água, do sangue e balanço ionorregulatório.
- Determinar parâmetros pró-oxidantes (PC e TBARS) e a atividade de antioxidantes (NPSH, GST, SOD, CAT, GPX e ácido ascórbico) em diferentes tecidos.

2 DESENVOLVIMENTO

Neste item serão apresentados três manuscritos, um publicado e dois submetidos, resultantes do desenvolvimento desta tese. Os manuscritos estão redigidos de acordo com as normas dos respectivos periódicos aos quais foram submetidos.

2.1 MANUSCRITO 1 – Biochemical parameters of silver catfish (*Rhamdia quelen*) after transport with eugenol or essencial oil of *Lippia alba* added to the water - submetido ao periódico **Anais da Academia Brasileira de Ciências.**

2.2 MANUSCRITO 2 – Essential oil of *Lippia alba* induces oxidative stress in silver catfish, *Rhamdia quelen*, after transportation - publicado no periódico **Neotropical Ichthyology.**

2.3 MANUSCRITO 3 – Methanolic extract of *Condalia buxifolia* added to the water of transport alters biochemical parameters of silver catfish, *Rhamdia quelen* - submetido ao periódico **Aquaculture.**

2.1 MANUSCRITO 1 – Biochemical parameters of silver catfish, *Rhamdia quelen*, after transport with eugenol or essencial oil of *Lippia alba* added to the water

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Running title: Catfish transported with eugenol or *L. alba* oil

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ABSTRACT

Sedatives can be an alternative to prevent or mitigate the deleterious effects of transport stress. This study investigated the effects of eugenol (1.5 or 3.0 µL L^{-1}) and essential oil of *Lippia alba* (10 or 20 µL L^{-1}) in the water for transport on some metabolic and oxidative parameters in the liver and muscle and acetylcholinesterase (AChE) activity in the brain and muscle of silver catfish, *Rhamdia quelen*, submitted to transportation for four hours. The studied concentrations of eugenol and *Lippia alba* would be advisable for transportation of silver catfish, since both extractives improved some oxidative (glutathione - GSH and thiobarbituric acid reactive substances levels - TBARS) and some metabolic (total protein levels) parameters. However, as eugenol is not recommended by United States Department of Health and Human Services Food and Drug Administration (U.S. FDA) as anesthetic for fish, only *Lippia alba* seems to be a promising anesthetic/sedative recommended for fish transport.

Key words: acetylcholinesterase, fish transportation, metabolism, sedatives, stress.

INTRODUCTION

In a stressful situation, such as transport, fish demand greater energy, which can be obtained from glycogenolysis, gluconeogenesis and increases in protein turnover (Mommsen et al. 1999, Pankhurst 2011). The addition of anesthetics to the water at a sedative concentration during fish transport could be an important tool to prevent harm. Sedation is a component of anesthesia and can lead to reduction in sensitivity, which results in tranquility and calmness (Ross and Ross 2008, Zahl et al. 2012).

Metabolic and antioxidant response in fish submitted to anesthetics has been scarcely investigated (Inoue et al. 2005, Barbosa et al. 2007, Azambuja et al. 2011, Becker et al. 2012). Fish transportation in closed systems lead to changes on the dissolved oxygen levels (Golombieski et al. 2003 Gomes et al. 2006) and this condition can lead to elevated intracellular reactive oxygen species levels (Azambuja et al. 2011). Reactive oxygen species (ROS) are generated in aerobic organisms as a result of oxidative metabolism. These ROS can be harmful, leading to an imbalance between antioxidants and pro-oxidants. This state is called oxidative stress (Halliwell and Gutteridge 1999, Hermes-Lima and Zenteno-Savín 2002).

The increase of ROS increases oxidation of lipid and protein structures and leads to damage of both carbohydrates and nucleic acids (Halliwell and Gutteridge 1999, Wilhelm Filho 2007). Among the markers of oxidative damage are oxidized proteins that form carbonyl groups (PC) and oxidized lipids that result in the formation of malondialdehyde (MDA) whose reaction with 2-thiobarbituric acid (TBA) can be estimated by the thiobarbituric acid-reactive substances (TBARS). In order to protect against oxidative stress, organisms developed antioxidant enzymes such as glutathione -S-transferase (GST) and

nonenzymatic antioxidants like glutathione (GSH) (Winston and Di Giulio 1991, Halliwell and Gutteridge 1999).

Another important parameter to verify possible adverse effects of anesthetics is acetylcholinesterase (AChE). This enzyme, present in the cholinergic synapses and motor end-plates is responsible for degrading acetylcholine (ACh) at the synaptic level, being extremely important for many physiological functions in fish (Blenau et al. 2012). The effect of anesthetics on AChE activity is poorly documented (Mazzanti et al. 1986, Lintern et al. 2000). Although relationship between ACh and anesthesia have emerged (Hudetz et al. 2003, Leung et al. 2011), to our knowledge the effects of anesthetic products on the cholinergic system of fish is still lacking.

Eugenol (EUG) (4-allyl-2-metoxy-phenol), the main component of clove oil, is a well-studied anesthetic for fish (Roubach et al. 2005, Barbosa et al. 2007, Gonçalves et al. 2008, Cunha et al. 2010b). Recently some studies demonstrated that the essential oil of *Lippia alba* (EOL) (Mill.) N.E. Brown (linalool chemotype), is also a suitable anesthetic and/or sedative for aquatic animals (Cunha et al. 2010a, 2011, Parodi et al. 2012).

The silver catfish (*Rhamdia quelen*) was chosen because it is the main native species raised in fish cultures of southern Brazil (Baldisserotto 2009). Thus, the aim of this study was to investigate the effects of EUG and EOL (at concentrations able to induce only sedation in fish) on metabolic response and AChE activity, as well as to analyze the oxidative parameters (PC and TBARS) and enzymatic (GST) and nonenzymatic (GSH) antioxidants in silver catfish after four hours of transport. Both extracts were effective in minimizing the effects of transport stress in some fish species when added to the water during transportation (Cooke et al. 2004, Inoue et al. 2005, Azambuja et al. 2011, Becker et al. 2012). A previous study regarding the effect of these extracts on silver catfish transport analyzed ventilatory

frequency, whole body ion fluxes, water quality parameters, blood pH, PvO_2, $PvCO_2$ and hematocrit (Becker et al. 2012), but not the parameters proposed in this study.

MATERIAL AND METHODS

EXPERIMENTAL PROCEDURE

Fish (301.2 ± 21.4 g; 28.9 ± 1.3 cm) were transported in closed plastic bags with 7 L of water and 8 L of pure oxygen, at a loading density of 169.2 g L^{-1} for four hours, divided in five treatments (in triplicate, n = 12 per group): control, 1.5 or 3.0 µL L^{-1} EUG (99% purity, Odontofarma®, Porto Alegre, Brazil, equivalent to 1.5 or 3.0 mg L^{-1}, respectively, because the density of this extractive is about 1.06), and 10 or 20 µL L^{-1} EOL (equivalent to 8 or 16 mg L^{-1}, respectively, because the density of this EO is about 0.80). This oil was extracted from fresh leaves by hydrodistillation according to Cunha et al. (2010a). These extractive were firstly diluted in ethanol (1:10). The concentrations used in this study cause only sedation in silver catfish (Cunha et al. 2010a, b). The water parameters were measured before and after transportation. Dissolved oxygen and temperature were measured with a YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA). The pH was verified with a DMPH-2 pH meter (Digimed, São Paulo, SP, Brazil). Nesslerization verified total ammonia nitrogen levels according to the method of Eaton et al. (2005). Un-ionized ammonia levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd and Tucker (1992). Carbon dioxide (CO_2) was calculated by the method of Wurts and Durborow (1992).

After transport, fish were euthanized by spinal cord section and tissues (brain, muscle and liver) were carefully removed, placed on ice and then stored at -20 °C for one week until

analysis of the biochemical parameters. The methodology of this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process nº 046/2010).

METABOLIC PARAMETERS

Liver and muscle were homogenized in a proportion of 50 mg mL^{-1} TCA (trichloroacetic acid) 10%, using a Potter-Elvejhem homogenizer type. Afterwards, the homogenates were centrifuged at 1,000 *x g* for 10 minutes and the supernatants were used for the determination of the lactate levels according to Harrower and Brown (1972). Levels of glycogen were estimated according to Dubois et al. (1956), after KOH and ethanol addition for precipitation of glycogen of the tissues. Homogenate (0.08 mL) was used to estimate the total protein level according to the method described by Lowry et al. (1951).

ACETYLCHOLINESTERASE ASSAY

AChE (E.C. 3.1.1.7) activity was measured using the method described by Ellman et al. (1961). Brain and muscle tissues (30 mg) were weighted and homogenized in a Potter Elvejhem glass/Teflon homogenizer with sodium phosphate buffer 50 mM pH 7.2 and Triton X-100 1%. The homogenate was then centrifuged for 10 min at 3,000 *x g* at 5 °C and the supernatant was used as enzyme source. Aliquots of supernatant of brain (50 µL) and muscle (100 µL) were incubated at 30 °C for 2 min with a solution containing 0.1 M sodium phosphate buffer pH 7.5 and 1 mM DTNB. After the incubation period, the reaction was initiated by the addition of acetylthiocoline (0.5 mM). The final volume was 2.0 mL.

Absorbance was measured by spectrophotometry (Femto Scan spectrophotometer 800 XI, São Paulo, Brazil) at 412 nm during 2 min.

OXIDATIVE PARAMETERS

The protein carbonyl (PC) levels in the liver were determined by the method described by Yan et al. (1995), with some modifications. Hepatic tissue was homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer, pH 7.4, using a glass homogenizer. Soluble protein (1 mL) was reacted with 10 mM DNPH in 2N hydrochloric acid (0.2 mL). After incubation at room temperature for 1 h in the dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3.0%), 2.0 mL heptane (99.5%), and 2.0 mL of ethanol (99.8%) were added sequentially, vortexed for 40 s, and centrifuged at 10,000 *x g* for 15 min. Then, the protein isolated from the interface was washed two times by resuspension in ethanol/ethyl acetate (1:1) and suspended in 1 mL of denaturing buffer. Each DNPH sample was measured at 370 nm in a spectrophotometer against the corresponding sample (blank), and total carbonylation was calculated using a molar extinction coefficient of 22,000 M cm^{-1}.

The oxidative damage to lipids can be estimated by a TBARS assay, performed by an MDA reaction with 2-thiobarbituric acid (TBA), which was optically measured according to Buege and Aust (1978). Aliquots of supernatants (0.25 mL) from liver were mixed with 10% trichloroacetic acid (TCA) (0.25 mL) and 0.67% thiobarbituric acid (0.5 mL) to adjust to a final volume of 1.0 mL. The reaction mixture was placed in a microcentrifuge tube and incubated for 15 min at 95 °C. After cooling, it was centrifuged at 5,000 *x g* for 15 min, and optical density was measured by spectrophotometer at 532 nm.

PROTEIN DETERMINATION

The protein levels were spectrophotometrically estimated by the method of Bradford (1976) using bovine serum albumin as standard.

ENZYMATIC AND NONENZYMATIC PARAMETERS

Glutathione -*S*- transferase (GST) activity in the liver was measured according to Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (0.15 mL) as a substrate, which was added to a mixture containing potassium phosphate buffer (20 mM, pH 6.5) (2.5 mL), reduced glutathione (10 mM) (0.3 mL) and homogenate (0.05 mL). The formation of S-2,4-dinitrophenyl glutathione was monitored by the increase in absorbance at 340 nm against blank (buffer and other reagents used to measure the enzyme activity in the absence of sample). The extinction coefficient used for CDNB was 9.6 mM cm^{-1}.

Reduced glutathione (GSH) content in the liver was indirectly determined by the colorimetric method of Ellman (1959). An aliquot of the hepatic supernatant (1.0 mL) was mixed with 1.0 mL 10% trichloroacetic acid followed by centrifugation. Supernatants (0.25 mL) were used for determination with 5,5′-dithio-bis(2-nitrobenzoic acid) 10 mM (DTNB) (0.05 mL) and phosphate buffer 0.5 mM (pH 6.8) (0.7 mL). The optical density of the reaction product was read at 412 nm on a spectrophotometer.

STATISTICAL ANALYSIS

All data are represented as mean ± SEM. Homogeneity of variances was tested with Levene test. Data that presented homogeneous variances were compared by one-way ANOVA

and Tukey test. Nonparametric data were analyzed by Kruskal-Wallis and comparisons of mean ranks. Analyses were performed using the software Statistica version 7.0 (StatSoft, Tulsa, OK), and the minimum significance level was set at $P<0.05$.

RESULTS

No mortality was observed in in this experiment after four hours of transport. After transport, the highest dissolved oxygen levels (7.63 ± 0.46 and 7.77 ± 0.52 mg L^{-1}) and lowest CO_2 levels (40.51 ± 1.09 and 49.63 ± 1.06 mg L^{-1}) were found in the control and in the 10 µL L^{-1} of EO *L. alba* treatment, respectively. Total alkalinity (26.5 ± 1.0 mg $CaCO_3$ L^{-1}), pH (5.8 ± 0.1), hardness (30.3 ± 1.8 mg $CaCO_3$ L^{-1}) and NH_3 (0.0013 ± 0.0002 mg N L^{-1}) levels in the water did not exhibit any significant differences among the treatments at the end of transport. In addition, the total ammonia nitrogen levels were significantly highest in the control (5.3 ± 0.2 mg N L^{-1}) compared with the other groups (4.4 ± 0.2 mg N L^{-1}).

Glycogen and lactate levels in the liver and muscle were significantly lower in all groups transported with EUG and EOL at the end of transport. The total protein values were significantly higher in both tissues of fish transported with EUG when compared to the control group. Furthermore, with EOL, a significant increase of this parameter was observed only in the muscle of fish transported with 20 µL L^{-1} EOL (Table 1). Brain AChE activity was significantly lower in fish transported with all tested concentrations of EUG and EOL. This activity was also significantly lower in the muscle of fish transported with 1.5 µL L^{-1} EUG than in control fish (Figure 1).

In the liver, PC (Figure 2 A) and TBARS (Figure 2 B) levels were significantly higher and lower, respectively, in fish transported with EUG and EOL than in control fish. Hepatic GSH levels were significantly higher in silver catfish transported with both anesthetics, but

the strongest effect was observed in those transported with 20 µL L^{-1} EOL (Figure 3 A). Fish transported with both EUG concentrations presented lower GST activity when compared to the control group (Figure 3 B).

DISCUSSION

In aquaculture practices there are many instances where light sedation is sufficient and in fact desirable over deeper sedation, especially for the transport of fish. For example, largemouth bass, *Micropterus salmoides*, lightly sedated with clove oil exhibited reduced activity and interaction with conspecifics during transportation, but were able to maintain equilibrium, swimming capacity, and avoid physical damage resulting from collision with the tank walls (Cooke et al. 2004). In fact, concentrations of EUG and EOL that sedate or calm silver catfish (Cunha et al. 2010a, b) are sufficient to improve some physiological indicators during transportation (Azambuja et al. 2011, Becker et al. 2012).

In response to stressors, disturbance in metabolic processes can occur (Iwama et al. 2004, Pankhurst 2011). Silver catfish transported with EUG and EOL have shown lower glycogen levels in both liver and muscle. Decreased glycogen reserves are usually associated with exhaustive activities (Inoue et al. 2005). In silver catfish transported with EUG and EOL, presumably a higher amount of glycogen was broken down by glycogenolysis and was responsible for supplying energy up to transport.

Lactate is the end product of glucose anaerobic metabolism and increased levels are observed in hypoxia conditions (Omlin and Weber 2010). In the current study, the addition of EUG and EOL to the water avoided the increase of lactate levels in silver catfish tissues, suggesting the reduction of anaerobic metabolism, common in exhaust physiological conditions or when no oxygen is available for the aerobic metabolism (Iversen et al. 2003).

Matrinxã, *Brycon amazonicus*, also presented lower lactate levels after handling when anesthetized with benzocaine or 2-phenoxyethanol (Inoue et al. 2004), or transported with clove oil added to water (Inoue et al. 2005). The results suggest that the addition of EUG and EOL improve the availability of oxygen to tissues, contributing to ameliorate the physiological condition of fish during transport.

Stress negatively affects the protein metabolism, inhibiting the synthesis and stimulating protein catabolism (Mommsen et al. 1999). The addition of EUG and EOL to water transportation of silver catfish had a protective role against protein catabolism. Higher levels of total protein indicate that the carbohydrate metabolism was sufficient to meet the demand of energy, favoring the use of proteins for other vital functions.

The addition of EUG and EOL in the water of transport decreased AChE activity in brain of silver catfish. This effect of EOL was expected because of the main compound of this chemotype is linalool (Vale et al. 1999, 2002), and this compound inhibits AChE (see Blenau et al. 2012). The activity of this enzyme was lower in rat brain anesthetized with ketamine (Mazzanti et al. 1986) and in hippocampus and midbrain of guinea pigs after anesthesia with halothane (Lintern et al. 2000).

Protein carbonyl content is actually the most commonly used marker of protein oxidation. The carbonyl groups can be introduced in proteins by different pathways, including secondary mechanisms as a result of reactions of ROS with lipids, carbohydrates and nucleic acids (Almroth et al. 2005). In this study, silver catfish transported with EUG and EOL showed higher PC content than control fish. These results are unexpected because another indicator of oxidative damage (TBARS) remained lower, indicating the absence of lipid peroxidation. So, the addition of EUG and EOL to the transport water protected against oxidative damage of lipids, since TBARS levels were lower in the liver of silver catfish transported with both compounds than those transported without any substance added to

water. In a similar study, TBARS levels were lower in the liver of silver catfish transported for 6 h with EOL added to water when compared to those transported without EOL (Azambuja et al. 2011). The attenuation of oxidative damage may also have been favored by the efficiency of the antioxidant defense system.

In a stress condition, components of an antioxidant defense system can be increased, inhibited, or unaltered depending on the intensity and the duration of the stress applied, as well as the susceptibility of the exposed species (Winston and Di Giulio 1991, Ballesteros et al. 2009, Kavitha and Rao 2009). Activity of GST was lower in the liver of silver catfish transported with EUG, while the addition of EOL to the water of transport did not change enzymatic activity. In both cases, the higher GSH content in the liver of fish after transport with EUG and EOL added to water seems to have contributed to prevent damage to lipids. Other authors suggest that the increase of GSH levels attenuates the oxidative stress (Ungvari et al. 2009, Yonar 2012).

CONCLUSION

The addition of EUG and EOL to the transport water of silver catfish improved the oxidative status, evidenced mainly by lower TBARS and higher GSH levels. Aerobic metabolism of carbohydrates was sufficient to meet the energy demand, indicating that (1) the tissues received a sufficient supply of oxygen; (2) anaerobic metabolism was reduced; (3) there was no protein catabolism. The addition of the tested concentrations of EUG and EOL in the water would be advisable for transportation of this species. However, neither clove oil nor any individual active ingredient of clove oil (including eugenol) should be used as an anesthetic in fish according to the United States Department of Health and Human Services Food and Drug Administration (U.S. FDA) because the National Toxicology Program studies

determined that EUG is an equivocal carcinogen to rodents (U.S. FDA, 2007). On the other hand, *L. alba* is a popular plant that is considered useful against several illness, and no significant toxicity was reported (Hennebelle et al. 2008). Therefore, only EOL seems to be a promising anesthetic/sedative recommended for fish transport.

ACKNOWLEDGMENTS

This study was supported by research funds from the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS/PRONEX, process 10/0016-8) and the Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq, process 470964/2009-0). J. Salbego, C. Toni, C. C. Zeppenfeld and C.C. Menezes received PhD fellowships from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). B. Baldisserotto, V. L. Loro and B. M. Heinzmann received research fellowships, and A.G. Becker received a PhD fellowship from CNPq. The authors are grateful to Dr. Gilberto Dolejal Zanetti for the identification of *Lippia alba*.

RESUMO

Anestésicos podem ser uma alternativa para evitar ou atenuar os efeitos deletérios do estresse decorrente do transporte. Este estudo investigou os efeitos do eugenol (1,5 ou 3,0 µL L^{-1}) e do óleo essencial de *Lippia alba* (10 ou 20 µL L^{-1}) adicionados à água para o transporte, sobre alguns parâmetros metabólicos e oxidativos no fígado e no músculo, e acetilcolinesterase (AChE) no cérebro e músculo do jundiá, submetido ao transporte, durante quatro horas. As concentrações de eugenol e *Lippia alba* estudadas seriam aconselháveis para o transporte de jundiá, já que ambos os extrativos melhoraram alguns parâmetros oxidativos (glutationa -

GSH e níveis de substâncias reativas ao ácido tiobarbitúrico - TBARS) e alguns parâmetros metabólicos (níveis de proteína total). No entanto, como o eugenol não é recomendado pelo United States Department of Health and Human Services Food and Drug Administration (U.S. FDA) como anestésico para peixes, apenas a *Lippia alba* parece ser um anestésico / sedativo promissor recomendado para o transporte de peixe.

Palavras-chave: acetilcolinesterase, estresse, metabolismo, sedativos, transporte de peixes.

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TABLE 1

Metabolites in tissues of silver catfish, *Rhamdia quelen,* after transport (four hours) with eugenol (EUG) or essential oil of *Lippia alba* (EOL) added to the water. Values are expressed as mean ± SEM (n = 12 per group). Different letters in the rows indicate significant difference between treatments (P < 0.05). Glycogen and lactate were expressed in µmol g tissue^{-1}. Protein was expressed in mg protein g tissue^{-1}.

Tissues		Treatments				
	Metabolites	Control	EUG (1.5 µL L^{-1})	EUG (3.0 µL L^{-1})	EOL (10 µL L^{-1})	EOL (20 µL L^{-1})
Liver						
	Glycogen	30.61 ± 1.59^{a}	16.76 ± 0.92^{b}	14.87 ± 0.79^{b}	15.22 ± 0.30^{b}	14.80 ± 0.58^{b}
	Lactate	17.84 ± 0.30^{a}	12.24 ± 0.36^{b}	7.27 ± 0.18^{c}	6.51 ± 0.22^{c}	6.64 ± 0.16^{c}
	Protein	186.25 ± 7.07^{c}	252.05 ± 9.07^{b}	319.00 ± 4.22^{a}	195.12 ± 8.50^{c}	199.25 ± 8.11^{c}
Muscle						
	Glycogen	7.24 ± 0.13^{a}	4.27 ± 0.15^{b}	3.86 ± 0.08^{bc}	3.54 ± 0.12^{c}	3.61 ± 0.04^{c}
	Lactate	21.50 ± 0.31^{a}	17.35 ± 0.23^{b}	15.74 ± 0.20^{c}	17.31 ± 0.25^{b}	15.86 ± 0.36^{c}
	Protein	85.37 ± 4.22^{c}	139.50 ± 3.85^{b}	151.00 ± 1.86^{ab}	90.44 ± 4.01^{c}	153.62 ± 2.76 ^{a}

FIGURE CAPTIONS

FIGURE 1**.** Brain and muscle AChE activity of silver catfish, *Rhamdia quelen*, after four hours of transportation using eugenol (EUG) and essential oil of *Lippia alba* (EOL) added to the water. Values are represented as mean ± SEM (n = 12 per group). Different letters indicate significant differences between groups for the same tissue ($P < 0.05$).

FIGURE 2. Protein carbonyl (PC) (A) and TBARS levels (B) in the liver of silver catfish, *Rhamdia quelen*, after four hours of transportation using eugenol (EUG) and essential oil of *Lippia alba* (EOL) added to the water. Values are represented as mean ± SEM (n = 12 per group). Different letters indicate significant differences between groups for the same tissue ($P < 0.05$).

FIGURE 3. Reduced glutathione (GSH) (A) levels and glutathione-S-transferase (GST) (B) activity in the liver of silver catfish, *Rhamdia quelen*, after four hours of transportation using eugenol (EUG) and essential oil of *Lippia alba* (EOL) added to the water. Values are represented as mean ± SEM (n = 12 per group). Different letters indicate significant differences between groups for the same tissue ($P < 0.05$).

FIGURE 1.

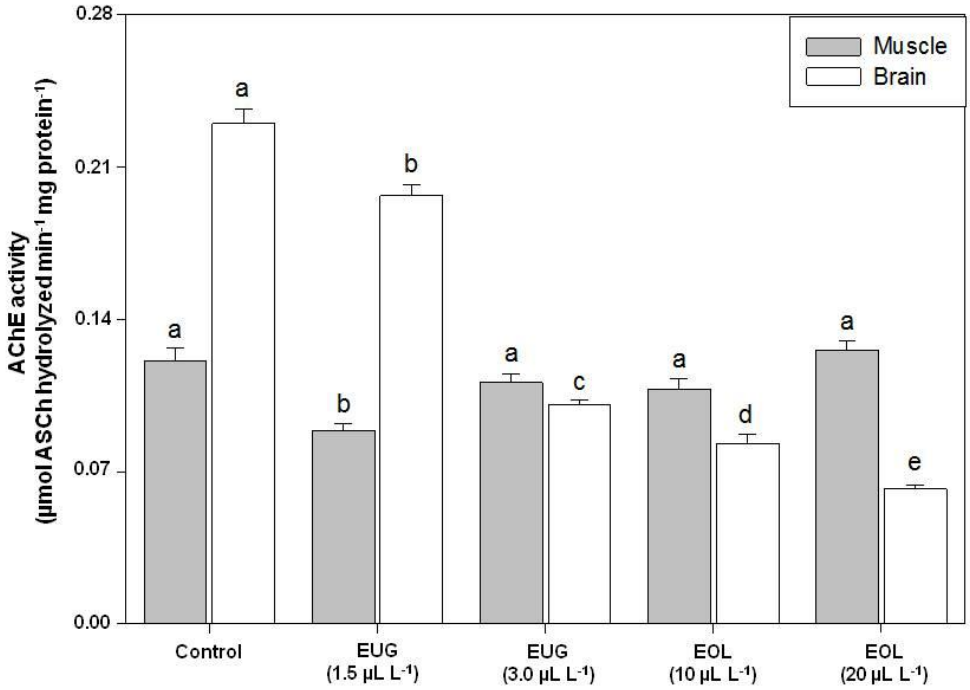

AChE activity
(µmol ASCh hydrolyzed min-1 mg protein-1)
0.28
0.21
0.14
0.07
0.00
Muscle
Brain
a
a
b
b
a
c
a
d
a
e
Control
EUG
(1.5 µL L-1)
EUG
(3.0 µL L-1)
EOL
(10 µL L-1)
EOL
(20 µL L-1)

FIGURE 2.

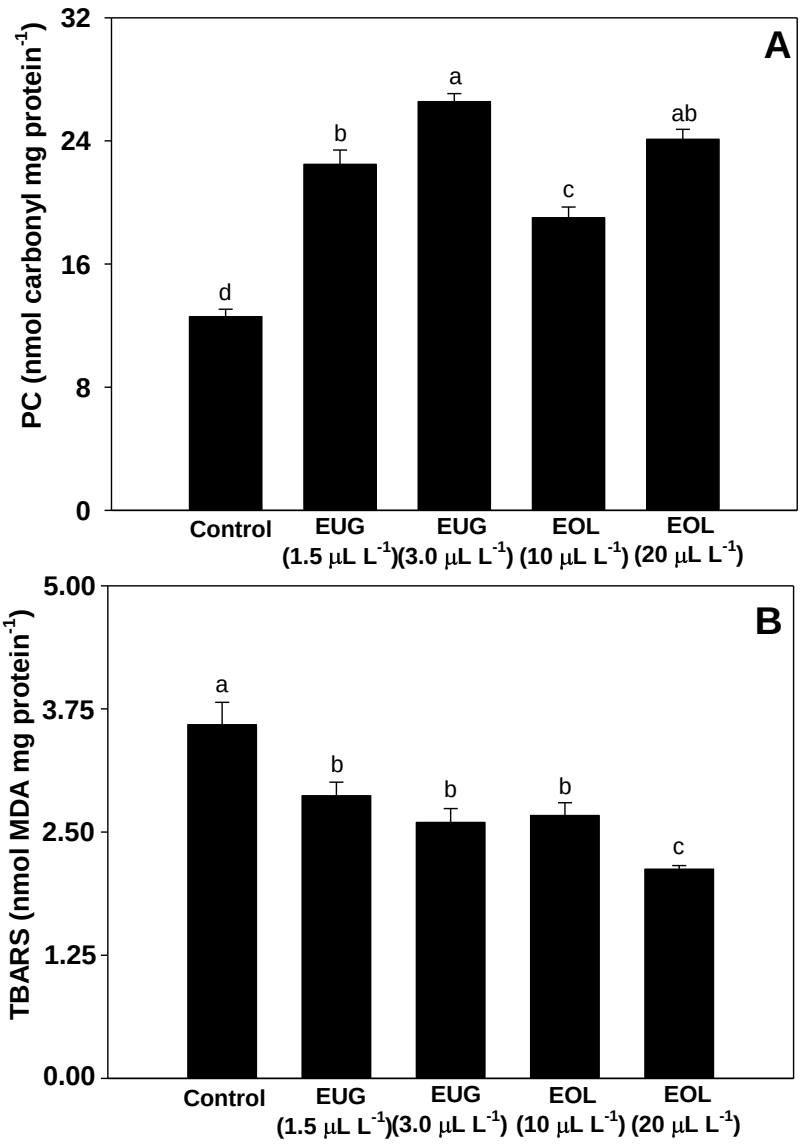
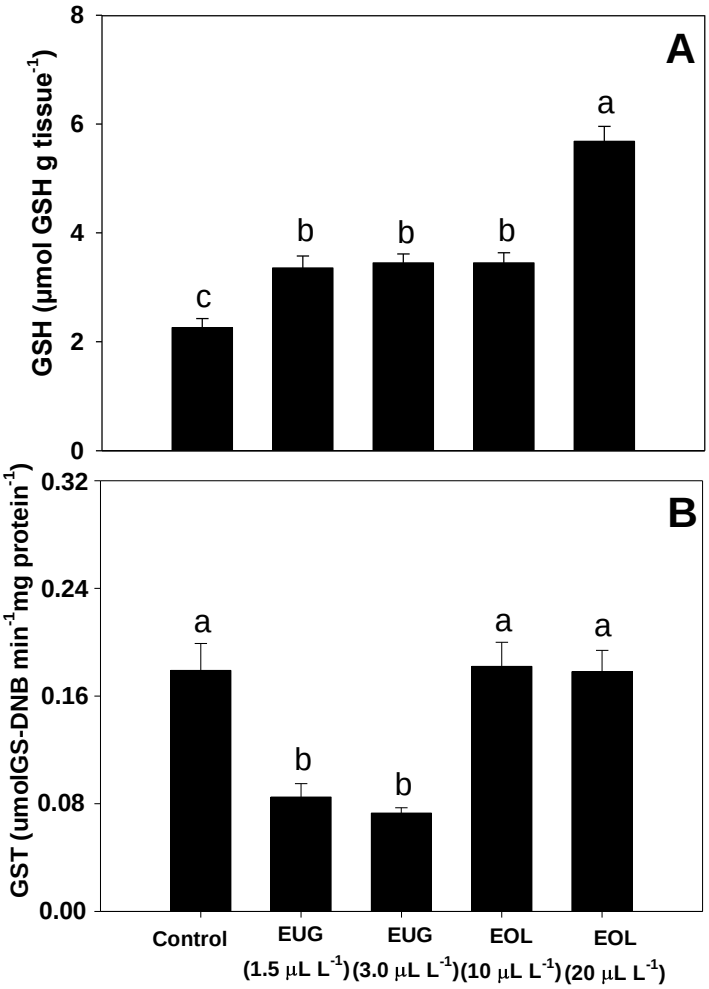

A
PC (nmol carbonyl mg protein-1)
32
24
16
8
0
a
b
c
d
ab
Control
EUG (1.5 μL L-1)
EUG (3.0 μL L-1)
EOL (10 μL L-1)
EOL (20 μL L-1)
B
TBARS (nmol MDA mg protein-1)
5.00
3.75
2.50
1.25
0.00
a
b
b
b
c
Control
EUG (1.5 μL L-1)
EUG (3.0 μL L-1)
EOL (10 μL L-1)
EOL (20 μL L-1)

FIGURE 3.


A
B
GSH (µmol GSH g tissue-1)
GST (umolGS-DNB min-1mg protein-1)
0
2
4
6
8
0.00
0.08
0.16
0.24
0.32
a
b
c
Control
EUG (1.5 µL L-1)
EUG (3.0 µL L-1)
EOL (10 µL L-1)
EOL (20 µL L-1)

2.2 MANUSCRITO 2 – The essential oil from *Lippia alba* induces oxidative stress in the silver catfish (*Rhamdia quelen*) after transportation

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This study investigated the effects of the essential oil (EO) from *Lippia alba* on whole brain enzymes and biochemical parameters related to oxidative stress in the liver of silver catfish (*Rhamdia quelen*) after six hours of transport. Fish were transported in plastic bags and divided into three treatments groups: control, 30 μL L^{-1} EO from *L.alba* and 40 μL L^{-1} EO from *L. alba*. Prior to transport, the fish were treated with the EO from *L. alba* (200 μL L^{-1} for three minutes), except for the control group. Fish transported in bags containing the EO did not have any alterations in acetylcholinesterase, ecto-nucleoside triphosphate diphosphohydrolase and 5'-nucleotidase activity in the brain or superoxide dismutase activity in the liver. The hepatic catalase (CAT), glutathione-S-transferase GST, glutathione peroxidase (GPx), nonprotein thiol and ascorbic acid levels were significantly lower compared to the control group. However, the hepatic thiobarbituric acid-reactive substances, protein oxidation levels and the lipid peroxidation/catalase+glutathione peroxidase (LPO/CAT+GPx) ratio were significantly higher in fish transported with both concentrations of the EO, indicating oxidative stress in the liver. In conclusion, considering the hepatic oxidative stress parameters analyzed in the present experiment, the transport of previously sedated silver catfish in water containing 30 or 40 μL L^{-1} of EO from *L. alba* is less effective than the use of lower concentrations.

Este estudo investigou os efeitos do óleo essencial (OE) de *Lippia alba* sobre enzimas do encéfalo e parâmetros bioquímicos relacionados ao estresse oxidativo em fígado de jundiá (*Rhamdia quelen*), após seis horas de transporte. Os peixes foram transportados em sacos plásticos e divididos em três tratamentos: controle, 30 μL L^{-1} ou 40 mL L^{-1} de OE de *L.alba*. Antes do transporte, os peixes foram tratados com o OE de *L. alba* (200 μL L^{-1} por três minutos), exceto para o grupo controle. Os peixes transportados em sacos contendo o OE não tiveram alterações na atividade da acetilcolinesterase (AChE), ecto-nucleosídeo trifosfato

difosfohidrolase (NTPDase) e 5'-nucleotidase, em cérebro ou superóxido dismutase (SOD) no fígado. O tiol não proteico (NPSH), os níveis de ácido ascórbico, catalase (CAT), glutationa-S-transferase GST e glutationa-peroxidase (GPx) hepáticos, foram significativamente mais baixos em comparação com o grupo controle. No entanto, as substâncias reativas ao ácido tiobarbitúrico (TBARS), os níveis de oxidação proteica e a taxa de peroxidação lipídica/catalase+glutationa peroxidase (LPO/CAT+GPx) foram significativamente maiores nos peixes transportados com ambas as concentrações de OE, indicando estresse oxidativo no fígado. Em conclusão, considerando os parâmetros de estresse oxidativo do fígado analisados no presente experimento, o transporte de jundiás previamente sedados em água contendo 30 ou 40 μL L^{-1} de OE de *L.alba* é menos efetivo que utilizando concentrações menores.
Keywords: Anesthetics; Antioxidant defenses; Fish transport; Biochemical parameters; Oxidative stress.

Introduction

The transportation of fish in Brazil involves the use of plastic bags. This system has limitations, such as a finite oxygen supply and the build-up of ammonia and carbon dioxide levels (Golombieski *et al.*, 2003; Carneiro *et al.*, 2009; Becker *et al.*, 2012). Generally, fish farmers add pure oxygen to the plastic bags prior to transport, which can cause variations in the dissolved oxygen levels, depress the metabolic rate and provoke blood flow rearrangement and effective methods of energy production (Nilsson and Renshaw, 2004).

The neurotransmitter acetylcholinesterase (AChE) is an important regulatory enzyme, which hydrolyses acetylcholine, a neurotransmitter with important role in the regulation of cognitive functions, mainly found in the brain, muscles, erythrocytes and cholinergic neurons. AChE besides cholinergic transmission is related to several non-cholinergic actions such as

responses to stress situations. Stress can increase due to free radicals which impair enzyme function (Gutierres *et al*., 2012, 2014) and lead to disorders in locomotion (Blenau *et al*., 2012) and fish erratic swimming (Salbego *et al*., 2010).

Adenosine triphosphate (ATP), is the primary intracellular energy source is which is also one of the most important neurotransmitters in the purinergic system and is responsible for modulating signaling and biosynthetic processes, including vascular homeostasis, cell size maintenance, neuronal signaling, immune function, and protein and lipid modifications (Marcus *et al.*, 2003; Tort, 2011). The enzyme ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) hydrolyzes ATP and adenosine diphosphate (ADP) to adenosine monophosphate (AMP). The resulting AMP is subsequently hydrolyzed to adenosine by 5'-nucleotidase (Stefan *et al.*, 2005; Colgan *et al.*, 2006; Schmatz *et al.*, 2009).

Exposure to hyperoxic, anoxic or hypoxic environments may result in oxidative changes because oxygen consumption contributes to the levels of reactive oxygen species (ROS) generated and the antioxidant status of the cells (Wilhelm-Filho *et al.*, 2001, 2002; Lushchak *et al.*, 2001, 2005; Azambuja *et al.*, 2011). The oxidative metabolism of cells is a continuous source of ROS (resulting from the univalent reduction of O_2) that can damage most cellular components such as carbohydrates, lipids and proteins as well as promoting cell death (Ahmad *et al.*, 2000; Morales *et al.*, 2004). To protect themselves from these highly reactive intermediates, living organisms possess a biochemical defense system consisting of enzymatic and non-enzymatic antioxidants that scavenge any reactive species. However, in several situations, the rate of ROS generation exceeds that of their removal, resulting in oxidative stress (Halliwell and Gutteridge, 2000; Livingstone, 2001). The most important antioxidant enzymes are superoxide dismutase (SOD), which detoxifies $O_2^{\bullet-}$; catalase (CAT), which reduces H_2O_2; glutathione peroxidase (GPx), which reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction and glutathione reductase (GR),which

catalyzes the NADPH-dependent regeneration of glutathione (a nonprotein thiol [NPSH]) from the oxidized form (GSSG) generated by GPx (Halliwell and Gutteridge, 2000).

Variations in the water parameters, such as ammonia and carbon dioxide levels, can provoke stress that could be minimized, at least in some species, through the addition of either sedatives or anesthetics in the transport water (Azambuja *et al.*, 2011; Cunha *et al.*, 2011; Becker *et al.*, 2012). The essential oil (EO) from *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) is a novel anesthetic whose action has been established for the silver catfish *Rhamdia quelen* (Cunha *et al.*, 2010; Heldwein *et al.*, 2012), and the slender seahorse *Hippocampus reidi* (Cunha *et al.*, 2011).When this oil was added to the transport water, the redox state (Azambuja *et al.*, 2011) and ionoregulation (Becker *et al.*, 2012) were improved, and lipid oxidation was delayed in the fillets of silver catfish (Veeck *et al.*, 2013). However, an increased ventilation rate was observed during the 30 min of transport (Becker *et al.*, 2012).

Increases in the plasma cortisol and glucose levels are classics indicators of stress responses (Iwama *et al.*, 2004; Urbinati and Carneiro, 2004; Tort, 2011). However, other biochemical parameters, such as enzymatic activities, are also important to understand stress at the cellular level (Lushchak *et al.*, 2001, 2005). In this context, the purpose of this study was to investigate if a rapid pre-transport sedation and transport with the EO from *L. alba* could change enzymes related to purinergic neurotransmitters in the whole brain (AChE, NTPDase and 5'-nucleotidase activities) and improve enzymatic parameters (SOD, CAT, GST and GPx) that act by inactivating free radicals, in the liver. Furthermore, this study also measured non-enzymatic parameters that can repair damage caused by oxidative stress (NPSH and ascorbic acid), changes in pro-oxidant parameters, such as thiobarbituric acid-reactive substance (TBARS) levels and protein oxidation resulting from damage to lipids and proteins respectively, in the liver of *Rhamdia quelen* after transport.

Material and Methods

Essential oil extraction. *L. alba* was cultivated in São Luiz Gonzaga, Rio Grande do Sul State, Brazil. The plant material was identified by botanist Dr. Gilberto Dolejal Zanetti, Department of Industrial Pharmacy, Universidade Federal de Santa Maria (UFSM), and a voucher specimen (SMDB No. 10050) was deposited in the herbarium of the Department of Biology, UFSM.

Essential oil (EO) was obtained from the fresh leaves of the plant by steam distillation for 2 h using a Clevenger-type apparatus. In this method, the distillate is collected and the aqueous phase is automatically reused by returning it to the distillation flask (European Pharmacopoeia, 2007). The EO samples were stored at -4°C in amber glass bottles.

Experimental procedure. Silver catfish (mean weight ± SEM 420.1 ± 8.8 g; mean length ± SEM 21.2 ± 2.3 cm) were captured from a cage net at a fish farm. Fish did not go through a depuration period because this procedure, despite its recommendation (Amend *et al.*, 1982), is not implemented by most fish producers in southern Brazil (Golombieski *et al.*, 2003). Fish were transported at a loading density of 275.1 g L^{-1} for six hours in nine plastic bags with 7 L of water and 8 L of pure oxygen, and they were divided into three treatment groups (three replicates each, n= 12 per group) that were treated with different concentrations of EO from *L. alba*: control, 30 µL L^{-1} and 40 µL L^{-1} of EO from *L. alba* (equivalent to 24 or 32 mg L^{-1}, respectively, because the density of this EO is about 0.80 g mL^{-1}) diluted 1:10 in ethanol. Fish that were transported with either of the EO treatments were rapidly sedated with a higher concentration of the same EO (200 µL L^{-1}) for three minutes before placing them into the plastic bags. This concentration induces sedation within the time proposed (Cunha *et al.*, 2010). Control fish were placed directly into the plastic bags. The transport time and

concentrations of EO from *L. alba* were chosen according to Becker *et al.* (2012) and were within a sedative safe range for silver catfish (Cunha *et al.*, 2010). The loading density was chosen according to Carneiro *et al.* (2009).

Water parameters were monitored before and after transport, with the values (mean ± SEM) at the end of transport as follows: dissolved oxygen (8.29 ± 0.98 mg L^{-1}), carbon dioxide (58.13 ± 2.51 mg L^{-1}), alkalinity (30.89 ± 2.09 mg $CaCO_3$ L^{-1}), water hardness (22.78 ± 1.64 mg $CaCO_3$ L^{-1}), pH (6.07± 0.07), temperature (26.33 ± 0.81 ºC), total ammonia nitrogen (3.21 ± 0.16 mg L^{-1}) and un-ionized ammonia (0.0023 ± 0.0002 mg L^{-1}). Dissolved oxygen and temperature were measured with an YSI oxygen meter. The pH was verified with a DMPH-2 pH meter. Nesslerization was used to verify the total ammonia nitrogen levels using the method of Eaton *et al.* (2005). Un-ionized ammonia levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd and Tucker (1992). Carbon dioxide was calculated by the method of Wurts and Durborow (1992).

After transport, all of the fish were euthanized by spinal cord section, and whole brain and liver tissues were carefully removed and frozen to posterior analysis of biochemical parameters.

Enzymatic assays in the brain

Acetylcholinesterase (AChE; E.C. 3.1.1.7). Brain was homogenized with 150 mM NaCl. The homogenate was centrifuged for 15 min at 3000 *g* at 5 ºC, and the supernatant was used as the enzyme source. AChE activity was measured as described by Ellman *et al.* (1961).

NTPDase (ecto-apyrase, ecto/CD39; E.C. 3.6.1.5). Enzymatic assay of the whole brain was carried out in a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10

mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer (pH 8.0) in a final volume of 200 μL, as described by Schetinger *et al.* (2000).

5′-nucleotidase (CD73; E.C. 3.1.3.5). Activity was determined by the method of Heymann *et al.* (1984) in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris–HCl buffer (pH 7.5) in a final volume of 200 μL. The tubes were then chilled on ice for 10 min, and the released inorganic phosphates (Pi) were assayed by the method of Chan *et al.* (1986).

Enzymatic antioxidant activity in the liver

Superoxide dismutase (SOD; E.C. 1.15.1.1). Activity was determined as the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine (0.017 mL) and 50 mM glycine–NaOH (pH 10.5) (1 mL). A unit of SOD is defined as the amount of enzyme that inhibits the speed of detector (epinephrine) reduction by 50%. Enzyme activity was expressed in units mg protein^{-1} using the method described by Misra and Fridovich (1972).

Catalase (CAT; E.C. 1.11.1.6). Activity was assayed by ultraviolet spectrophotometry. Change of H_2O_2 absorbance after 60 s was measured at 240 nm. Catalase activity was calculated and expressed in μmol min^{-1} mg protein^{-1} using the method described by Nelson and Kiesow (1972).

Glutathione -S-transferase (GST). Activity was measured based on the method described by Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (0.15 mL) as a substrate. The extinction coefficient used for CDNB was 9.6 mM cm^{-1}, and the activity was expressed as μmol GS-DNB min^{-1} mg protein^{-1}.

Glutathione peroxidase (GPx; EC 1.11.1.9). The enzyme activity was measured according to Paglia and Valentine (1967). The assay solution contained 100 mM potassium phosphate buffer (pH 7.0) 1 mM GSH, 0.15 mM NADPH, 0.1 U mL^{-1} glutathione reductase, 100 mM

azide and a suitable sample of enzyme solution. Enzyme activity was determined at 37 °C by measuring the depletion of NADPH at 340 nm and expressed as μmol NADPH min^{-1} mg $protein^{-1}$**.**

Nonenzymatic antioxidants in the liver

Non-protein thiol groups (NPSH). NPSH levels were determined by the method of Ellman (1959) with 0.05 mL of 10 mM 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 0.7 mL of 0.5 mM phosphate buffer (pH 6.8) added to 0.25 mL of supernatant. The results were expressed as μmol non-protein thiols g $tissue^{-1}$.

Ascorbic acid content (AsA). It was determined by the method of Roe (1954). To measure the ascorbic acid levels, an aliquot of the supernatant was mixed with 2,4-dinitrophenylhydrazine (4.5 mg mL^{-1}), 0.6 mg mL^{-1} thiourea, $CuSO_4$ (0.075 mg mL^{-1}), and 13.3% trichloroacetic acid followed by incubation for 3 h at 37 ºC. Afterwards, H_2SO_4 65% (v/v) was added to the medium. The results were expressed as μmol AsA g $tissue^{-1}$.

Prooxidants in the liver

Lipid peroxidation estimation. The lipid peroxidation was estimated by a thiobarbituric acid-reactive substances (TBARS) assay and performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured according to Buege and Aust (1978). TBARS levels were expressed as nmol MDA mg $protein^{-1}$.

Protein carbonyl assay. The protein carbonyl content was assayed by the method described by Yan *et al.* (1995). The assay was performed in duplicate, and two blank tubes treated with 2N HCl (0.2 mL) without DNPH were included for each sample. The total carbonylation was calculated using a molar extinction coefficient of 22000 M cm^{-1}. The protein carbonyl content was expressed as nmol carbonyl mg $protein^{-1}$.

Protein total determination. The protein concentration was determined by the Coomassie Blue method following the Bradford method (1976) using bovine serum albumin as a standard, and the absorbance of the samples was measured at 595 nm.

Statistical analyses. All data are expressed as the mean ± SEM. The homogeneity of variances between treatments was tested with Levene's test. The data presented homogeneous variances, so comparisons between the different treatments were made using a one way ANOVA and Tukey's test. Analysis was performed using the Statistica ver. 7.0 software (Stat Soft, Tulsa, OK), and the minimum significance level was set at $P < 0.05$.

Results

No mortality was recorded in any treatment following transport. The AChE, NTPDase and 5'-nucleotidase activities in the whole brain of silver catfish showed no significant differences between the treatments (Table 1).

The SOD activity in the liver did not present significant difference between the treatments (Fig. 1A), and CAT activity was significantly lower in fish transported with both EO concentrations compared to the control fish (Fig. 1B).The GST activity in the liver was significantly lower in silver catfish transported with 30 µL L^{-1} of EO compared to the control fish (Fig. 2A). The GPx activity in the liver was significantly lower in all EO treatments compared to the control (Fig. 2B). Moreover, the lowest activity was observed in fish transported with 40 µL L^{-1} of EO (Fig. 2B).The levels of NPSH and ascorbic acid in the liver were significantly lower in fish transported with both concentrations of EO compared to the control (Fig. 2C and D).

The TBARS and protein carbonyl levels in the liver were higher in the fish transported in water treated with 30 μL L^{-1} of EO from *L. alba* compared to the control group, but the fish transported in water treated with 40 μL L^{-1} of EO showed similar results to the control (Fig. 3A and 3B). The LPO/CAT + GPx ratio indicated that the balance between lipoperoxidation and total antioxidant enzyme activities was significantly higher in treatments with either 30 or 40 μL L^{-1} of EO compared to the control fish (Fig. 4).

Discussion

Generally, anesthetics cause a depression of the central nervous system by interrupting the action potential of axons, release of neurotransmitters, excitability of the membrane or a combination of all of these actions (Ross and Ross, 2008). However, in the present study, the EO from *L. alba* had no effect on the activities of the enzymes NTPDase and 5'-nucleotidase, which constitute an enzymatic complex able to regulate the extracellular concentrations of adenine nucleotides and nucleosides (Stefan *et al.*, 2005; Colgan *et al.*, 2006; Schmatz *et al.*, 2009; Gutierres *et al*. 2012, 2014).

Studies by Heldwein *et al.* (2012) suggest the involvement of the gamma-aminobutyric acid -A ($GABA_A$) benzodiazepine receptor in the anesthetic effect of the EO from *L. alba*. Silver catfish transported in water containing the EO from *L. alba* were less agitated (Becker *et al.*, 2012). The results of the AChE activity assay corroborate with the aforementioned findings because the enzyme activity was unaltered and the change in the locomotor behavior was not due to alterations in its function. Furthermore, consolidates that EO from *L. alba* is able to reduce or maintain the basal levels of the cholinergic neurotransmitters.

This study did not detect any significant differences in the SOD activity between the treatments after transport of the catfish. The lower CAT and GST activities in the liver of silver catfish transported with 30 µL L^{-1} of EO in the water could be attributed to the increase of the oxidant levels, as observed by the highest levels of TBARS that were generated by this treatment. It is possible that in silver catfish transported with 40 µL L^{-1} of EO in the water the decrease of CAT was enough to avoid the increase of TBARS. Azambuja *et al.* (2011) reported that silver catfish transported (fish density 140 – 200 g L^{-1}) for 6 h in normoxic conditions with 10 µL L^{-1} of EO of *L. alba* exhibited no significant alterations in hepatic GST, SOD and CAT activities, but TBARS levels also did not change.

Alterations in GPx activity are generally accompanied by changes in NPSH levels because the NPSH is a co-substrate for H_2O_2 breakdown by GPx (Sies, 1999). The major cellular thiol that participates in cellular redox reactions – NPSH – displayed an important role in the detoxification of electrophilic metabolites catalyzed by glutathione-S-transferase (GST) (Sies, 1999; Latha and Pari, 2004).

High NPSH levels may protect cellular proteins against oxidation either via the NPSH redox cycle or by directly detoxifying the ROS generated by exposure to stressor agents (Ruas *et al.*, 2008), but the low NPSH content could modulate the activity of GPx and GST enzymes (Brouwer and Brouwer, 1998) as suggested in the present study in silver catfish transported in water containing the EO from *L. alba*. The formation of peroxides and TBARS was lower in the frozen fillets of silver catfish transported with either 30 or 40 µL L^{-1} of EO (Veeck *et al.*, 2013). In the present study, lower levels of ascorbic acid were also observed in the liver of silver catfish transported in water containing the EO from *L. alba*. Antioxidants such as ascorbic acid are active ROS scavengers involved in the lipid peroxidation process (Halliwell and Gutteridge, 2000; Trenzado *et al.*, 2006; Kochhann *et al.*, 2009). This study showed that the TBARS and protein carbonyl levels in the liver of silver catfish were higher when

transported in water containing 30 µL L^{-1} of EO, indicating higher lipid peroxidation and protein oxidation activity and demonstrating that the antioxidant defenses were not completely able to effectively scavenge the ROS produced. The increased LPO/CAT+GPx ratio also suggests that hydrogen peroxide were produced, overcoming the capacity of CAT and GPx in neutralizing ROS production (Ruas *et al.*, 2008) and resulting in a LPO in the liver.

The use of 10-20 µL L^{-1} of EO from *L. alba* increased ventilation rate of silver catfish during the first 30 min of transport, indicating a higher agitation of the fish (Becker *et al.*, 2012). Therefore, it was hypothesized that the use of a pre-transport sedation with 200 µL L^{-1} of EO from *L. alba* would reduce this initial agitation and the transport with 30 or 40 µL L^{-1} of EO from *L. alba* in the water would be more effective. However, this hypothesis was not confirmed considering the hepatic oxidative stress parameters analyzed in the present experiment and the lower concentrations (10-20 µL L^{-1} of EO from *L. alba*) used by Azambuja *et al.* (2011) and Becker *et al.* (2012) are more effective for transporting silver catfish.

Acknowledgments

This study was supported by research funds from the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS/PRONEX, process 10/0016-8), the Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq, process 470964/2009-0) and the Ministério da Pesca e Aquicultura/FINEP (process 01.12.0130.00). B. Baldisserotto, V. L. Loro, M. R. C. Schetinger, V. M. Morsch, B. M. Heinzmann and R. M. Spanevello received research fellowship. J. Salbego, J. F. Gonçalves and C. G. Heldwein received fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). A.G. Becker and C.C. Menezes received fellowship from CNPq. The authors are grateful to Dr. Gilberto Dolejal Zanetti for the identification of *L. alba*.

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Table 1. Enzymatic activity in the brain. The levels of AChE expressed as µmol ASCh hydrolyzed min^{-1} mg $protein^{-1}$ and the levels of NTPDase and 5'-nucleotidase activity using either ATP or ADP and AMP as substrates, respectively (values expressed as nmol Pi min^{-1} mg $protein^{-1}$).

	L. alba concentration		
	0 µL L^{-1}	30 µL L^{-1}	40 µL L^{-1}
AChE	0.301 ± 0.014	0.296 ± 0.010	0.306 ± 0.019
NTPDase - ATP	201.51 ± 24.51	233.85 ± 28.29	257.85 ± 41.85
NTPDase - ADP	81.60 ± 5.43	101.10 ± 8.82	96.18 ± 4.65
5'-nucleotidase - AMP	64.35 ± 8.16	65.25 ± 5.58	54.12 ± 4.80

Values are expressed as the mean ± SEM (n = 12 per group), $P < 0.05$. No significant differences between groups were observed.

Figures captions

Fig. 1. Superoxide dismutase (SOD) and catalase (CAT) activities (A and B, respectively) in the liver of silver catfish transported in plastic bags containing water treated with the essential oil from *Lippia alba*. The values are expressed as the means ± SEM (n = 12 per group). Different letters indicate levels of significance between the treatments ($P < 0.05$).

Fig. 2. Glutathione-S-transferase (GST) activity (A), glutathione peroxidase (GPx) activity (B), non-protein thiol group (NPSH) content (C) and ascorbic acid (D) content in the liver of silver catfish transported in plastic bags containing water treated with the essential oil from *Lippia alba*. The values are expressed as the means ± SEM (n = 12 per group). Different letters indicate levels of significance between the treatments ($P < 0.05$).

Fig. 3. TBARS (A) and protein oxidation (B) levels in the liver of silver catfish transported in plastic bags containing water treated with the essential oil from *Lippia alba*. The values are expressed as the means ± SEM (n = 12 per group). Different letters indicate difference levels of significance between the treatments ($P < 0.05$).

Fig. 4. LPO/CAT+GPx ratio in the liver of silver catfish transported in plastic bags containing water treated with the essential oil from *Lippia alba*. The values are expressed as the means ± SEM (n = 12 per group). Different letters indicate levels of significance between the treatments ($P < 0.05$).

Fig. 1

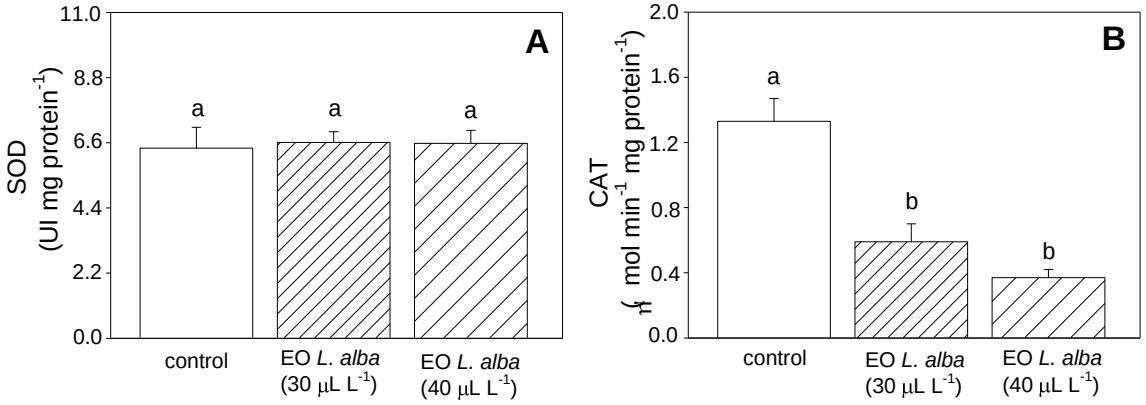

A
SOD
(UI mg protein-1)
11.0
8.8
6.6
4.4
2.2
0.0
a
a
a
control
EO L. alba
(30 μL L-1)
EO L. alba
(40 μL L-1)
B
CAT
(μ mol min-1 mg protein-1)
2.0
1.6
1.2
0.8
0.4
0.0
a
b
b
control
EO L. alba
(30 μL L-1)
EO L. alba
(40 μL L-1)

Fig. 2

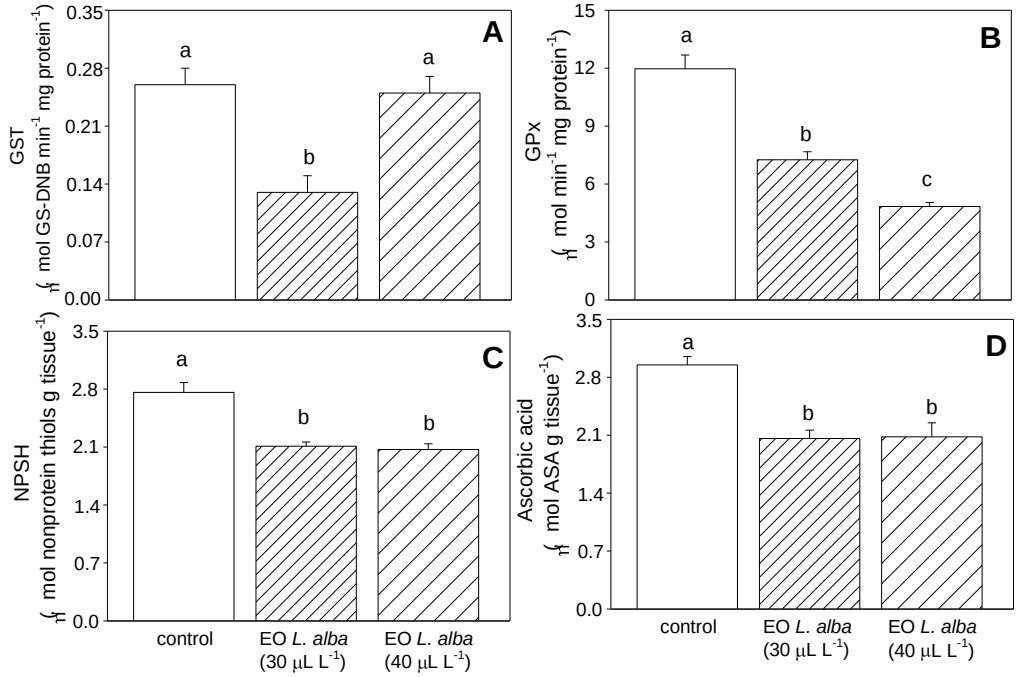

A
GST
(μ mol GS-DNB min-1 mg protein-1)
0.35
0.28
0.21
0.14
0.07
0.00
a
b
a
B
GPx
(μ mol min-1 mg protein-1)
15
12
9
6
3
0
a
b
c
C
NPSH
(μ mol nonprotein thiols g tissue-1)
3.5
2.8
2.1
1.4
0.7
0.0
a
b
b
control
EO L. alba
(30 μL L-1)
EO L. alba
(40 μL L-1)
D
Ascorbic acid
(μ mol ASA g tissue-1)
3.5
2.8
2.1
1.4
0.7
0.0
a
b
b
control
EO L. alba
(30 μL L-1)
EO L. alba
(40 μL L-1)

Fig. 3

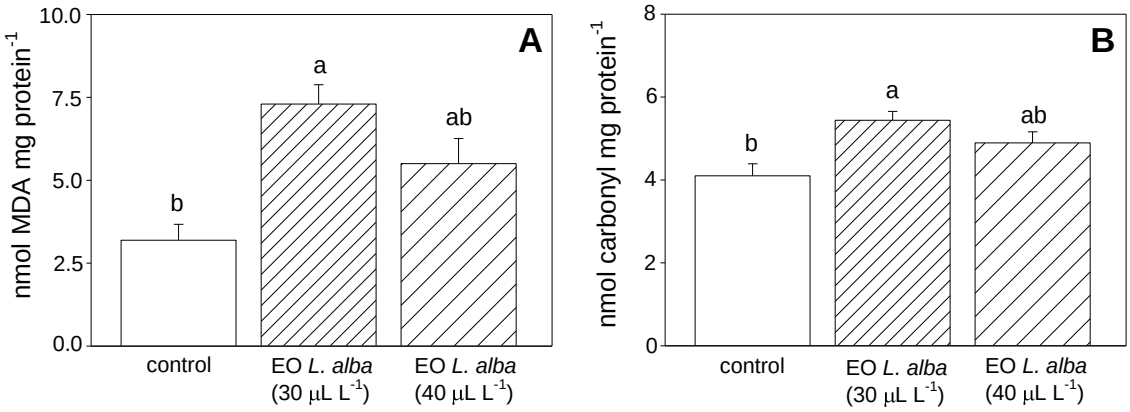
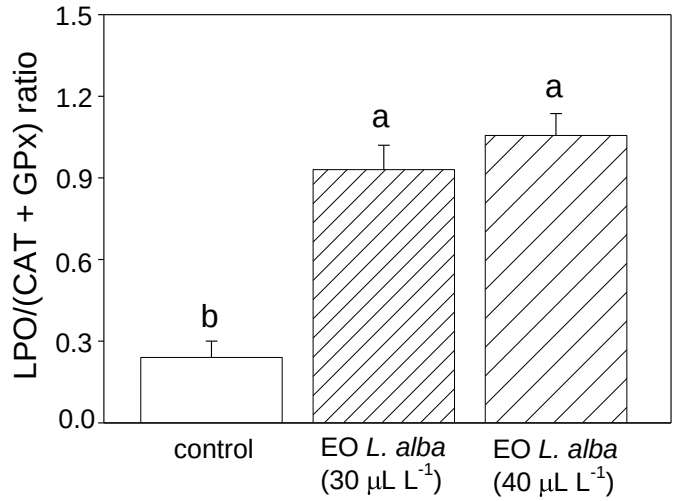

A
nmol MDA mg protein-1
10.0
7.5
5.0
2.5
0.0
b
a
ab
control
EO L. alba (30 μL L-1)
EO L. alba (40 μL L-1)
B
nmol carbonyl mg protein-1
8
6
4
2
0
b
a
ab
control
EO L. alba (30 μL L-1)
EO L. alba (40 μL L-1)

Fig. 4


LPO/(CAT + GPx) ratio
1.5
1.2
0.9
0.6
0.3
0.0
b
a
a
control
EO L. alba (30 μL L-1)
EO L. alba (40 μL L-1)

2.3 MANUSCRITO 3 – Methanolic extract of *Condalia buxifolia* added to transport water alters biochemical parameters of the silver catfish *Rhamdia quelen*

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Abstract

The effects of the methanolic extract of *Condalia buxifolia* (MECB) were investigated in silver catfish (*Rhamdia quelen*) transported for 6 h in plastic bags at 0, 5 or 10 µL L^{-1} MECB. Prior to transport, the fish were sedated with 10 µL L^{-1} MECB for 5 min, except the control group. At the end of transport, dissolved oxygen, alkalinity, pH, temperature, and un-ionized ammonia levels in the transport water were not different between treatments, but the control group presented the highest total ammonia levels. Net Na^+, Cl^- and K^+ effluxes were highest in fish from the control group compared to those transported with MECB. $Pv O_2$, $Pv CO_2$ and HCO_3^- were higher after transport in fish transported with 5 µL L^{-1} MECB, but no significant difference between treatments was found regarding blood pH and plasma cortisol levels. The metabolic parameters (glycogen, lactate, total amino acid, total ammonia and total protein) were lower or no significant difference was found in fish transported with MECB. There was no difference between treatments on the ecto-nucleoside triphosphate diphosphohydrolase, 5'-nucleotidase and acetylcholinesterase activities in the whole brain. The ascorbic acid content and nonprotein thiol levels in the gills were higher and catalase and glutathione peroxidase in the liver were lower in fish transported with MECB. Glutathione-S-transferase values were lower in the kidney and liver at 5 µL L^{-1} and higher in the muscle at 10 µL L^{-1} MECB. The thiobarbituric acid-reactive substances levels in the kidney and muscle were lower at both MECB concentrations but higher in the liver of fish transported with 10 µL L^{-1} MECB. The protein carbonyl content in the kidney was higher in fish transported with 10 µL L^{-1} MECB and in the muscle at both concentrations of this extract. In conclusion, the use of MECB for the transport of silver catfish is advisable because MECB improves antioxidant defenses.

Keywords: sedative; antioxidant defense; oxidative stress; fish transport

1. Introduction

Fish in culture systems are susceptible to stress caused by capture, handling and transportation, among other stressors (Barton and Iwama, 1991; Tort, 2011). The transportation of fish in plastic bags has limitations, such as the supply of oxygen and the build-up of ammonia and carbon dioxide levels (Golombieski et al., 2003; Carneiro et al., 2009; Becker et al., 2012, 2013). These variations in water parameters provoke stress (Tort, 2011) that could be minimized, at least in some species, through the addition of natural sedatives in the transport water (Azambuja et al., 2011; Becker et al., 2012, 2013; Parodi et al., 2014; Zeppenfeld et al., 2014).

Condalia buxifolia Reissek (Rhamnaceae) is a tree rich in peptide alkaloids. Several secondary metabolites were isolated from *C. buxifolia*: condaline A (alkaloid), β-sitosterol (steroid), lupeol and taraxerol (terpenes) (Maldaner, 2005). The methanolic extract of *C. buxifolia* (MECB) induced only slight sedation in silver catfish (*Rhamdia quelen*), and the best concentration range of MECB is 0.5-10 μL L^{-1} because higher concentration levels increase the time to induce sedation. The addition of this extract to the transport water within the above mentioned concentration range is advisable because it reduces fish mortality and ionoregulatory imbalance (Becker et al., 2013). However, no analysis of the effect of MECB on biochemical parameters was performed. Enzymatic activities are important in understanding stress at a cellular level, how fish respond to the transport and if additions to the water could be beneficial or harmful (Salbego et al., in press). The silver catfish is a native freshwater teleost from Central and South America, and the main native species is raised in southern Brazil (Baldisserotto, 2009).

The purpose of this study was to investigate the effects of the addition of MECB to the transport water with regards to water parameters, survival, ionoregulatory balance, blood parameters (pH, $Pv\mathrm{O_2}$, $Pv\mathrm{CO_2}$, hematocrit and HCO_3^-), cortisol and several biochemical

parameters (glycogen, lactate, total amino acids, total ammonia and total protein); brain enzymes (acetylcholinesterase-AChE, ecto-nucleoside triphosphate phosphohydrolase-NTPDase, and 5′-nucleotidase activities) and oxidative stress parameters (superoxide dismutase-SOD, catalase-CAT, glutathione peroxidase -GPx, ascorbic acid, nonprotein thiol-NPSH, glutathione-S-transferase-GST, thiobarbituric acid-reactive substance-TBARS and protein carbonylation levels) in several tissues of silver catfish after 6 h of transport.

2. Material and Methods

2.1. Experimental procedure

The silver catfish (420.1 ± 8.8 g and 21.2 ± 2.3 cm) were captured from a cage net in a fish farm and then transported at a loading density of 275.1 g L^{-1} for 6 h in nine plastic bags (7 L of water and 8 L of pure oxygen), divided into three groups (three replicates each, n = 12 per group): 0 (control), 5 and 10 µL L^{-1} MECB. This extract was obtained according to Becker et al. (2013) and diluted in ethanol (1:10) before use. Fish that were transported with 5 or 10 µL L^{-1} MECB were rapidly sedated with 10 µL L^{-1} (the concentration that induced sedation within the time proposed) for five minutes, as reported by Becker et al. (2013), before placement in the plastic bags. Control fish were placed directly in the plastic bags. The loading density was chosen according to Carneiro et al. (2009), and the MECB concentrations used have been shown to keep silver catfish sedated and do not induce anesthesia (Becker et al. 2013), avoiding any mortality during transport. After transport, fish were euthanized by spinal cord section, and tissues (brain, gills, kidney, liver and muscle) were carefully removed, placed on ice and stored at -20 ºC for one week until the biochemical parameters were analyzed. The methodology of this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process nº 046/2010).

2.2. Water parameters: sampling and determinations

Water parameters were measured before and after transportation. Dissolved oxygen and temperature were measured with a YSI oxygen meter (YSI Inc., Yellow Springs, OH, USA). The pH was verified with a DMPH-2 pH meter. The total ammonia nitrogen (TAN) levels were verified according to Eaton et al. (2005), and un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd and Tucker (1992), and carbon dioxide (CO_2) was calculated by the method of Wurts and Durborow (1992).

Net ion fluxes were calculated according to the following equation:

$$Jnet = V([ion]_1 - [ion]_2).(M.t)^{-1}$$

where $[ion]_1$ and $[ion]_2$ are the ion concentrations in the water of transport at the beginning and the end of the transport period, respectively, V is the water volume (in L), M is the mass of the fish (in kg) and t is the duration of the transport (in h). The Cl^- levels were determined according to Zall et al. (1956), and Na^+, K^+ and Ca^{2+} levels were determined with a B262 flame spectrophotometer. Standard solutions were made with analytical-grade reagents dissolved in deionized water, and standard curves of each ion to be tested were made for five different concentrations.

2.3. Blood parameters: sampling and determinations

Blood samples (1–1.5 mL) were collected from the caudal vein of each fish using heparinized 3-mL syringes before and after the transport procedure and subsequently were kept in ice to determine, using a clinical analyzer, the following variables: pH, PvO_2, $PvCO_2$, hematocrit (Hct) and HCO_3^-. In addition, the temperature of the clinical analyzer was corrected to water temperature (27 °C), with the assumption that ambient water temperature and individual fish body temperatures were equivalent (Hanley et al., 2010). The plasma

cortisol levels were measured using a commercially available immunoluminometry kit (Immulite, Diagnostic Products Corporation, Los Angeles CA, USA), and the specificity of the test for silver catfish has been previously evaluated by Cunha et al. (2010).

2.4. Biochemical parameters: sampling and determinations

2.4.1. Metabolic parameters

The tissues (gills, kidney, liver and muscle) were homogenized in a proportion of 50 mg mL^{-1} TCA (trichloroacetic acid) 10% using a Potter-Elvejhem homogenizer. Afterwards, the homogenates were centrifuged at 1000 *g* for 10 min, and the supernatants were used for the determination of metabolic parameters. The glycogen was determined by Dubois et al. (1956), lactate by Harrower and Brown (1972), total amino acid by Spies (1957), total ammonia according to Verdouw et al. (1978) and total protein according to Lowry et al. (1951).

2.4.2. Enzymatic assays in the brain

2.4.2.1. Acetylcholinesterase (AChE; E.C. 3.1.1.7)

Brain was homogenized with 150 mM NaCl. The homogenate was centrifuged for 15 min at 3000 *g* at 5 ºC, and the supernatant was used as the enzyme source. AChE activity was measured as described by Ellman et al. (1961).

2.4.2.2. NTPDase (ecto-apyrase, ecto/CD39; E.C. 3.6.1.5)

An enzymatic assay of the entire brain was carried out in a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer (pH 8.0) in a final volume of 200 μL, as described by Schetinger et al. (2000).

2.4.2.3. 5′-nucleotidase (CD73; E.C. 3.1.3.5)

Activity was determined by the method of Heymann et al. (1984) in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris–HCl buffer (pH 7.5) in a final volume of 200 μL. The tubes were then chilled on ice for 10 min, and the released inorganic phosphates (Pi) were assayed by the method of Chan et al. (1986).

2.4.3. Nonenzymatic antioxidants in the gill, liver and muscle

2.4.3.1. Ascorbic acid content (AsA)

Ascorbic acid content was determined by the method of Roe (1954). To measure the ascorbic acid levels, an aliquot of the supernatant was mixed with 2,4-dinitrophenylhydrazine (4.5 mg mL^{-1}), 0.6 mg mL^{-1} thiourea, $CuSO_4$ (0.075 mg mL^{-1}), and 13.3% trichloroacetic acid, followed by incubation for 3 h at 37 ºC. Afterwards, H_2SO_4 65% (v/v) was added to the medium. The results are expressed as μmol AsA g $tissue^{-1}$.

2.4.3.2. Non-protein thiol groups (NPSH) in the gill, liver and muscle

NPSH levels were determined by the method of Ellman (1959), with 0.05 mL of 10 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 0.7 mL of 0.5 mM phosphate buffer (pH 6.8) added to 0.25 mL of supernatant. The results are expressed as μmol non-protein thiols g $tissue^{-1}$.

2.4.4. Enzymatic antioxidant activity in the liver

2.4.4.1. Superoxide dismutase (SOD; E.C. 1.15.1.1)

Activity was determined as the inhibition rate of autocatalytic adrenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine (0.017 mL) and 50 mM glycine–NaOH (pH 10.5) (1 mL). A unit of SOD is defined as the amount of enzyme that

inhibits the speed of detector (epinephrine) reduction by 50%. Enzyme activity was expressed in units mg protein^{-1} using the method described by Misra and Fridovich (1972).

2.4.4.2. Catalase (CAT; E.C. 1.11.1.6)

Activity was assayed by ultraviolet spectrophotometry. Changes in H_2O_2 absorbance after 60 s was measured at 240 nm. Catalase activity was calculated and expressed in μmol min^{-1} mg protein^{-1} using the method described by Nelson and Kiesow (1972).

2.4.2.3. Glutathione peroxidase (GPx; EC 1.11.1.9)

The enzyme activity was measured according to Paglia and Valentine (1967). The assay solution contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM GSH, 0.15 mM NADPH, 0.1 U mL^{-1} glutathione reductase, 100 mM azide and a suitable aliquot of enzyme solution. Enzyme activity was determined at 37 °C by measuring the depletion of NADPH at 340 nm and expressed as μmol NADPH min^{-1} mg protein^{-1}**.**

2.4.4.4. Glutathione-S-transferase (GST; EC 2.5.1.18) (also in the kidney and muscle)

Activity was measured based on the method described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (0.15 mL) as a substrate. The extinction coefficient used for CDNB was 9.6 mM cm^{-1}, and the activity was expressed as μmol GS-DNB min^{-1} mg protein^{-1}.

2.4.5. Prooxidants in the kidney, liver and muscle

2.4.5.1. Lipid peroxidation estimation

Lipid peroxidation was estimated by a thiobarbituric acid-reactive substances (TBARS) assay and performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric

acid (TBA), which was optically measured according to Buege and Aust (1978). TBARS levels are expressed as nmol MDA mg protein^{-1}.

2.4.5.2. Protein carbonyl assay

The protein carbonyl content was assayed by the method described by Yan et al. (1995). The assay was performed in duplicate, and two blank tubes treated with 2 N HCl (0.2 mL) without DNPH were included for each sample. The total carbonylation was calculated using a molar extinction coefficient of 22000 M cm^{-1}. The protein carbonyl content was expressed as nmol carbonyl mg protein^{-1}.

2.4.6. Protein total determination

The protein concentration was determined by the Coomassie blue method following the Bradford method (1976) using bovine serum albumin as a standard, and the absorbance of the samples was measured at 595 nm.

2.4.7. Statistical analyses

All data are expressed as the mean ± SEM. The homogeneity of variances between groups was tested with Levene's test. Data presented homogeneous variances; therefore, comparisons between different groups were made by one-way ANOVA and Tukey's test. Analysis was performed using the software Statistica ver. 7.0 (Stat Soft, Tulsa, OK), and the minimum significance level was set at $P < 0.05$.

3. Results

3.1. Water quality and blood parameters

No mortality was recorded in any treatment following transport. In all water parameters, no significant difference between groups after transport was observed, except TAN levels, where the lowest levels were reported in fish transported with MECB (Table 1). The net Na^+, Cl^- and K^+ effluxes were also significantly lower in fish transported with MECB added to the water. However, the net Ca^{2+} fluxes did not show any significant difference between groups (Figure 1).

No significant difference was found in the blood pH between the groups. However, $Pv O_2$, $Pv CO_2$ and HCO_3^- values after transport were higher in fish transported with 5 µL L^{-1} MECB added to the water compared to those in the other groups. In addition, Htc values decreased after transport compared to those before transport but without significant differences between the groups (Table 2). Plasma cortisol levels were not affected by the addition of MECB to the transport water (Figure 2).

3.2. Biochemical parameters

3.2.1. Metabolic parameters

Glycogen levels were significantly lower in the gills and higher in the liver of fish transported with 5 µL L^{-1} MECB added to the water compared to those in the control fish. Groups transported with MECB also presented significantly lower muscle glycogen compared to that in the control fish. Fish transported with MECB added to the water showed significantly higher lactate levels in the kidney and lower levels in the muscle, and those transported with 10 µL L^{-1} MECB presented lower lactate levels in the liver (Table 3). Total amino acid levels in the gills and muscle were significantly lower and higher in the kidney at

10 μL L^{-1} MECB compared to those in the other groups. Total amino acid levels in the liver were not altered by adding MECB to the water. Total ammonia levels in the liver were significantly higher at 5 μL L^{-1} and lower at 10 μL L^{-1} MECB compared to those from fish from the control group. Total ammonia levels in the gills, kidney and muscle were not affected by MECB addition to the water of transport. The total protein in the gills did not show significant difference between groups, but in the kidney, lower values were found at 10 μL L^{-1} MECB, and in the liver and muscle, the highest and lowest values, respectively, were reported at 5 μL L^{-1} MECB (Table 3).

3.2.2. Enzymatic assays in the brain

The AChE, NTPDase and 5′-nucleotidase activities in the whole silver catfish brains transported with MECB did not show significant differences compared to the control (Table 4).

3.2.3. Antioxidants and prooxidants

The ascorbic acid contents were significantly higher and lower in the gills and liver, respectively, at both concentrations of MECB compared to those in the control fish. The lowest ascorbic acid content of the muscle was observed in the fish transported with 5 μL L^{-1} MECB. The levels of NPSH were significantly higher and lower in the gills and muscle, respectively, in fish transported with MECB, but in the liver, there was no significant difference between groups (Table 5).

The SOD activity in the liver did not present any significant difference between groups (Fig. 3A), but CAT and GPx activities were significantly lower in fish transported with MECB compared to those of the control (Figs. 3B and 3C). The GST activity in the kidney and liver were significantly lower in silver catfish transported with 5 μL L^{-1} of MECB and

higher in the muscle of fish transported with 10 μL L^{-1} of MECB compared to those in the control fish (Fig. 4).

The TBARS levels in the kidney and muscle were significantly lower in fish transported with both concentrations of MECB (Figs. 5A and 5E). However, in the liver, the highest TBARS levels were observed in fish transported with the highest concentration of MECB (Fig. 5C). Protein carbonyl did not change significantly in the liver; the highest levels were observed in the kidney of fish transported at the highest MECB concentration and in the muscle at both concentrations of this extract (Figs. 5B, 5D and 5F).

The LPO/CAT + GPx ratio indicated that the balance between lipoperoxidation and total antioxidant enzymes activities in the liver was significantly higher at 10 μL L^{-1} MECB compared to that in the control fish (Fig. 6).

4. Discussion

The dissolved oxygen levels were higher after 6 h of transport because pure oxygen was added to the plastic bags at the beginning of transport. The increase in CO_2 levels observed in all treatments most likely was responsible for the decrease in water pH at the end of transport, as observed by Golombieski et al. (2003) and Becker et al. (2012, 2013). Alkalinity and water hardness levels, regardless of the treatment, increased after transport, and this increase could be explained by the presence of regurgitated food, as observed by Golombieski et al. (2003) and Becker et al. (2012, 2013) with the same species.

Total ammonia nitrogen levels in the water were lower at the end of the transport in the groups with MECB added to the water. As transport with MECB did not reduce total ammonia levels in the tissues (except in the liver of those transported with 10 μL L^{-1}) compared to the control fish, it is most likely that this lower ammonia excretion is related to

slower metabolism. The lower Na^+, Cl^- and K^+ loss by the fish transported with MECB is also an indication supporting this hypothesis. The use of eugenol, MECB, and essential oils of *Lippia alba* (Becker et al., 2012, 2013) and *Aloysia triphylla* (Parodi et at., 2014; Zeppenfeld et al., 2014) in the transport water also reduced ion loss in silver catfish. In the present study, plasma cortisol levels were not affected significantly by the addition of MECB, indicating that the reduced ion loss was not due to reduced stress through transport, as observed with the use of the essential oil of *A. triphylla* in the transport water (Zeppenfeld et al., 2014). However, before transportation, fish were rapidly sedated with 10 μL L^{-1} MECB. Consequently, increased cardiovascular responses and gill blood flow, which are stress responses as side effects of anesthetics (Sneddon, 2012), were most likely minimized before transport because fish were less agitated. In agreement with this hypothesis, β-sitosterol and taraxerol, compounds found in MECB (Maldaner, 2005), caused drowsiness and lower mobility in mice (Sethiya et al., 2009).

Blood parameters values before transport were similar to those reported for the same species by Becker et al. (2012). Blood gases and HCO_3^- values increased and Hct decreased in silver catfish after transport. Most likely, these alterations are related to hyperoxia exposure through transport because Atlantic salmon (*Salmo salar*) exposed to hyperoxia have presented the same changes (Hoslfeld et al., 2010; Kristensen et al., 2010). However, as even long-term alterations of these blood parameters did not impair the growth of Atlantic salmon (Hoslfeld et al., 2010), it is most likely that no significant physiological perturbation in silver catfish resulted from these changes. Pv_{O_2} and Pv_{CO_2} values after transport were higher in fish transported with 5 μL L^{-1} MECB added to the water compared to the other groups. Most likely, the blood HCO_3^- values in this group after transport were higher to compensate for the higher Pv_{CO_2} values and to maintain blood pH similar to the other groups.

Sedatives can reduce or block the activation of the hypothalamus–pituitary–interrenal (HPI) axis (Tort, 2011) and render the fish unable to respond to additional stressors, resulting in a cascade of physiological and metabolic changes (Trenzado et al., 2003) to maintain homeostasis (Barton and Iwama 1991; Vijayan et al., 1994; Iversen et al., 2003; Small, 2003; Segner et al., 2012). Lactate levels were significantly lower in the liver of silver catfish transported with 10 μL L^{-1} MECB and in the muscle at both MECB concentrations. Most likely, these lower lactate values are related to the lower muscular activity caused by MECB sedation. Furthermore, in general, the metabolic responses in the tissues of silver catfish transported with MECB are similar to those observed by Inoue et al. (2005) in the plasma metabolites of matrinxã (*Brycon amazonicus*) subjected to transport with clove oil added to water: stress responses were prevented, attenuated or not altered, keeping a balance in body homeostasis. A depression of the central nervous system is the main response to the use of anesthetics, which interrupts the action potential of neurons, the release of neurotransmitters, the excitability of the membrane or a combination of all these actions (Ross and Ross, 2008).

However, AChE, a well-known enzyme that degrades the neurotransmitter acetylcholine (Blenau et al., 2012; Gutierres et al., 2014; Salbego et al., in press), and the ecto-enzymes NTPDase and 5′-nucleotidase, which among others, regulate the extracellular concentrations of nucleoside adenosine and adenine nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), and play an important role in the maintenance of normal cellular status (Fields and Burnstock, 2006; Yegutkin, 2008; Schmatz et al., 2009; Gutierres et al., 2012), did not change with the addition of MECB to the transport water.

The imbalance of the pro and antioxidants equilibrium leads to ROS production that can induce potential damage to cellular constituents (Halliwell and Gutteridge, 2000; Trenzado et al., 2006; Lushchak, 2011; Tort, 2011). Antioxidant enzymes, such as SOD,

detoxifies superoxide anion ($O_2^{\bullet-}$), CAT reduces hydrogen peroxide (H_2O_2), and GPx reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction (Halliwell and Gutteridge, 2000). Recent studies have shown that plant extracts are able to improve the antioxidant capacity in silver catfish (Azambuja et al., 2011; Zeppenfeld et al., 2014; Salbego et al., in press).

The activity of antioxidants increased in the gill but decreased in the liver and muscle of silver catfish transported with MECB. The increase in GST activity in the muscle (at 10 μL L^{-1}) is justified by the increase in protein carbonyls in the same treatment, most likely due to the scavenger role of this enzyme. In the kidney, GST (at 5 μL L^{-1}) and TBARS levels decreased, and there was carbonylation of protein at 10 μL L^{-1}, most likely because this is one of the key organs for organizing a response after stressful situations in fish (Tort, 2011; Segner et al., 2012). The increased LPO/CAT+GPx ratio in the liver at 10 μL L^{-1} MECB showed that the amount of H_2O_2 produced overcame the capacity of CAT and GPx enzymes to neutralize ROS production, resulting in more LPO in this organ. However, taken together, the results of all studied organs indicated that MECB addition to the transport water improved the oxidative status of silver catfish. In agreement with the present study, the methanolic extract of Indian plants that contained β-sitosterol and taraxerol (compounds found in the MECB) showed antioxidant properties in mouse brain homogenate (Sethiya et al., 2009). β-sitosterol also induced apoptosis in human colon cancer cell lines by scavenging ROS (Baskar et al., 2010), and lupeol (another compound of MECB) ameliorated aflatoxin B_1-induced toxicity due to its combined antioxidant potential as well as hepatoprotective action in a rat model (Preetha et al., 2006).

5. Conclusion

The use of MECB in the transport water at both concentrations tested in the present study is recommended for silver catfish transport because MECB improved the antioxidant capacity in some tissues and was effective in reducing waterborne total ammonia levels and ion loss.

Acknowledgements

This study was supported by research funds from the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS/PRONEX, process 10/0016-8) and Ministério da Pesca e Aquicultura/ Ministério da Ciência e Tecnologia/FINEP/INCT ADAPTA – CNPQ-FAPEAM. B. Baldisserotto, A. G. Becker, G. Maldaner, V. L. Loro, V. M. Morsch, M. R. C. Schetinger and A. F. Morel received research fellowships from CNPq. J. Salbego, T. V. Parodi, C. C. Zeppenfeld and J. F. Gonçalves received fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Table 1. Water parameters before and after transport (6 h) of silver catfish, *Rhamdia quelen*, in plastic bags with methanolic extract of *Condalia buxifolia* (MECB) added to the water.

Water parameters	Before transport	After transport (groups)		
		Control	MECB (5 µL L^{-1})	MECB (10 µL L^{-1})
Dissolved oxygen	5.35 ± 0.68	8.68 ± 1.09*[a]	8.74 ± 1.64*[a]	8.02 ± 0.52*[a]
Carbon dioxide	6.35 ± 0.47	54.84 ± 2.47*[a]	60.69 ± 3.21*[a]	57.49 ± 2.79*[a]
Alkalinity	20.30 ± 1.77	29.14 ± 2.20*[a]	32.25 ± 1.85*[a]	30.55 ± 2.15*[a]
Water hardness	15.73 ± 1.72	21.00 ± 1.80*[a]	24.12 ± 1.90*[a]	23.32 ± 1.30*[a]
pH	6.74 ± 0.04	6.01 ± 0.05*[a]	6.11 ± 0.05*[a]	6.13 ± 0.07*[a]
Temperature (ºC)	27.53 ± 0.56	27.30 ± 0.62[a]	26.10 ± 0.91[a]	25.85 ± 1.07[a]
Total ammonia nitrogen	1.36 ± 0.12	3.89 ± 0.19*[a]	2.74 ± 0.15*[b]	2.72 ± 0.13*[b]
Un-ionized ammonia	0.005 ± 0.0001	0.0026 ± 0.0002*[a]	0.0021 ± 0.0002*[a]	0.0022 ± 0.0002*[a]

Values are presented as the mean ± SEM (n = 12 per group). Asterisks indicate significant differences compared to values before transport ($P < 0.05$). Different letters in the rows indicate significant differences between groups after transport ($P < 0.05$). Dissolved oxygen and carbon dioxide are expressed as mg L^{-1}, and total ammonia nitrogen and un-ionized ammonia are expressed as mg N L^{-1}. Alkalinity and water hardness are expressed as mg $CaCO_3$ L^{-1}.

Table 2. Blood parameters before and after transport (6 h) of silver catfish, *Rhamdia quelen*, in plastic bags with methanolic extract of *Condalia buxifolia* (MECB) added to the water.

Blood parameters	Before transport	After transport (groups)		
		Control	MECB (5 µL L^{-1})	MECB (10 µL L^{-1})
pH	7.32 ± 0.07	7.26 ± 0.04[a]	7.25 ± 0.04[a]	7.27 ± 0.05[a]
PvO_2 (mm Hg)	10.21 ± 1.88	16.08 ± 1.69*[b]	28.23 ± 1.91*[a]	16.11 ± 1.14*[b]
$PvCO_2$ (mm Hg)	13.79 ± 0.71	21.57 ± 0.93*[b]	32.28 ± 0.99*[a]	23.21 ± 1.86*[b]
Hct (%)	30.46 ± 0.48	24.39 ± 0.85*[a]	25.32 ± 0.94*[a]	25.15 ± 0.89*[a]
HCO_3 (mmol L^{-1})	6.27 ± 0.16	12.45 ± 0.47*[b]	15.58 ± 0.29*[a]	12.44 ± 0.36*[b]

Values are represented as the mean ± SEM (n = 12 per group). Asterisks indicate significant differences compared to values before transport ($P < 0.05$). Different letters in the rows indicate significant differences between groups after transport ($P < 0.05$).

Table 3. Metabolites in tissues of silver catfish, *Rhamdia quelen*, after transport (6 h) in plastic bags with methanolic extract of *Condalia buxifolia* (MECB) added to the water.

Metabolites	Tissues	Groups		
		Control	MECB (5 µL L^{-1})	MECB (10 µL L^{-1})
Glycogen				
	Gills	22.22 ± 0.61[a]	17.40 ± 0.75[b]	20.33 ± 1.05[a]
	Kidney	21.74 ± 0.95[a]	22.55 ± 0.77[a]	22.72 ± 0.60[a]
	Liver	518.25 ± 16.15[b]	605.20 ± 13.82[a]	450.88 ± 21.75[b]
	Muscle	10.58 ± 0.43[a]	8.41 ± 0.37[b]	6.64 ± 0.43[b]
Lactate				
	Gills	8.86 ± 0.39[a]	6.94 ± 0.33[a]	8.30 ± 0.68[a]
	Kidney	3.23 ± 0.29[b]	4.45 ± 0.24[a]	4.46 ± 0.19[a]
	Liver	9.99 ± 0.37[a]	11.81 ± 0.43[a]	7.23 ± 0.28[b]
	Muscle	23.78 ± 0.68[a]	20.15 ± 0.67[b]	18.91 ± 0.66[b]
Total amino acid				
	Gills	52.01 ± 2.78[a]	51.05 ± 1.96[a]	42.50 ± 1.21[b]
	Kidney	61.90 ± 2.51[b]	55.67 ± 2.03[b]	68.09 ± 2.39[a]
	Liver	62.27 ± 5.37[a]	72.96 ± 5.20[a]	72.56 ± 6.06[a]
	Muscle	21.97 ± 0.39[a]	24.75 ± 0.59[a]	17.55 ± 0.54[b]
Total ammonia				
	Gills	4.66 ± 0.21[a]	4.82 ± 0.26[a]	4.68 ± 0.14[a]
	Kidney	2.47 ± 0.21[a]	2.83 ± 0.28[a]	3.38 ± 0.34[a]
	Liver	5.63 ± 0.41[b]	7.10 ± 0.37[a]	4.31 ± 0.37[c]
	Muscle	4.46 ± 0.15[a]	4.50 ± 0.29[a]	4.43 ± 0.23[a]
Total protein				
	Gills	25.19 ± 1.03[a]	23.62 ± 1.50[a]	23.06 ± 0.74[a]
	Kidney	59.32 ± 2.44[a]	59.32 ± 2.78[a]	45.04 ± 1.86[b]
	Liver	47.78 ± 4.05[b]	82.35 ± 4.54[a]	46.05 ± 4.81[b]
	Muscle	38.95 ± 0.44[a]	36.15 ± 0.54[b]	41.49 ± 0.67[a]

Values are expressed as the mean ± SEM (n = 12 per group). Different letters in the rows indicate significant differences compared to the control ($P < 0.05$). Glycogen, lactate, total amino acid and total ammonia are expressed in µmol g $tissue^{-1}$. Protein is expressed in mg protein g $tissue^{-1}$.

Table 4. Enzymatic activities in whole brain of silver catfish, *Rhamdia quelen*, after transport (6 h) in plastic bags with methanolic extract of *Condalia buxifolia* (MECB) added to the water. The AChE is expressed as µmol ASCh hydrolyzed min^{-1} mg $protein^{-1}$. NTPDase (using ATP or ADP as the substrate) and 5'-nucleotidase (using AMP as the substrate) activities are expressed as nmol Pi min^{-1} mg $protein^{-1}$.

	Groups		
	Control	MECB ($5\ \mu L\ L^{-1}$)	MECB ($10\ \mu L\ L^{-1}$)
AChE	0.301 ± 0.014	0.293 ± 0.011	0.325 ± 0.012
NTPDase - ATP	201.51 ± 24.51	206.04 ± 20.79	223.65 ± 30.69
NTPDase - ADP	81.60 ± 5.43	85.71 ± 7.77	103.98 ± 8.85
5'-nucleotidase - AMP	64.35 ± 8.16	69.30 ± 8.85	62.52 ± 8.37

Values are expressed as the mean ± SEM (n = 12 per group), $P < 0.05$. No significant differences between groups were observed.

Table 5. Antioxidant parameters in tissues of silver catfish, *Rhamdia quelen*, after transport (6 h) in plastic bags with methanolic extract of *Condalia buxifolia* (MECB) added to the water.

Antioxidant parameters	Tissues	Groups		
		Control	MECB (5 µL L^{-1})	MECB (10 µL L^{-1})
Ascorbic acid (µmol AsA g $tissue^{-1}$)				
	Gills	0.40 ± 0.06^{b}	1.01 ± 0.04^{a}	0.90 ± 0.07^{a}
	Liver	2.95 ± 0.10^{a}	2.40 ± 0.22^{b}	2.56 ± 0.16^{b}
	Muscle	1.82 ± 0.15^{a}	1.27 ± 0.07^{b}	1.76 ± 0.09^{a}
Nonprotein thiols groups (µmol NPSH g $tissue^{-1}$)				
	Gills	5.41 ± 0.73^{b}	12.16 ± 0.81^{a}	14.44 ± 0.55^{a}
	Liver	2.76 ± 0.12^{a}	2.52 ± 0.23^{a}	2.71 ± 0.14^{a}
	Muscle	2.29 ± 0.25^{a}	0.94 ± 0.13^{b}	1.60 ± 0.11^{b}

Values are expressed as the mean ± SEM (n = 12 per group). Different lowercase letters indicate significant differences between groups in the same antioxidant parameter ($P < 0.05$).

Figure captions

Fig. 1 Net ion (Na^+, Cl^-, K^+ and Ca^{2+}) fluxes measured in the water after transport (6 h) of silver catfish, *Rhamdia quelen*, with methanolic extract of *Condalia buxifolia* added to the water. Values are expressed as the mean ± SEM. Different lowercase letters indicate significant differences between groups for the same ion ($P < 0.05$; n = 12 per group).

Fig. 2 Effect of methanolic extract of *Condalia buxifolia* added to the water on plasma cortisol levels after transport (6 h) of silver catfish, *Rhamdia quelen*. Values are expressed as the mean ± SEM. Different lowercase letters indicate significant differences between groups for the same ion ($P < 0.05$; n = 12 per group).

Fig. 3 Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities (A, B and C, respectively) in the liver of silver catfish, *Rhamdia quelen*, after transport (6 h) with methanolic extract of *Condalia buxifolia* added to the water. Values are expressed as the mean ± SEM. Different lowercase letters indicate significant difference between the groups ($P < 0.05$; n = 12 per group).

Fig. 4 Glutathione-S-transferase (GST) activity in the kidney (A), liver (B) and muscle (C) of silver catfish, *Rhamdia quelen*, after transport (6 h) with methanolic extract of *Condalia buxifolia* added to the water. Values are expressed as the mean ± SEM. Different lowercase letters indicate significant difference between the groups ($P < 0.05$; n = 12 per group).

Fig. 5 TBARS levels and protein oxidation in the kidney (A and B), liver (C and D) and muscle (E and F, respectively) of silver catfish, *Rhamdia quelen*, after transport (6 h) with methanolic extract of *Condalia buxifolia* added to the water. Values are expressed as the mean ± SEM. Different lowercase letters indicate significant difference between the groups ($P < 0.05$; n = 12 per group).

Fig. 6 LPO/CAT+GPx ratio in the liver of silver catfish, *Rhamdia quelen*, after transport (6 h) with methanolic extract of *Condalia buxifolia* added to the water. Values are expressed as the mean ± SEM. Different lowercase letters indicate significant difference between the groups ($P < 0.05$; n = 12 per group).

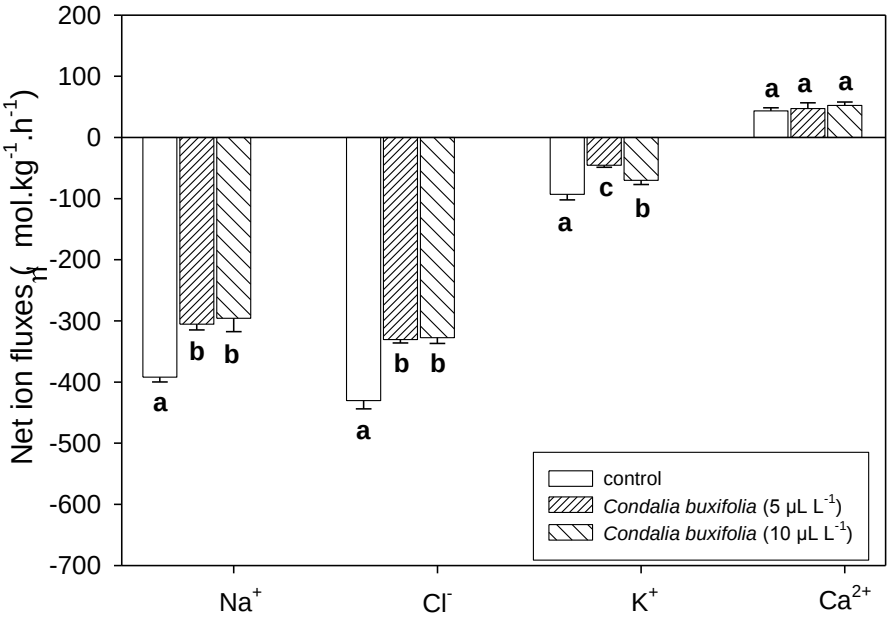
Net ion fluxes (mol.kg-1.h-1)
200
100
0
-100
-200
-300
-400
-500
-600
-700
a
b
b
a
b
b
a
c
b
a
a
a
control
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)
Na+
Cl-
K+
Ca2+

Fig. 1

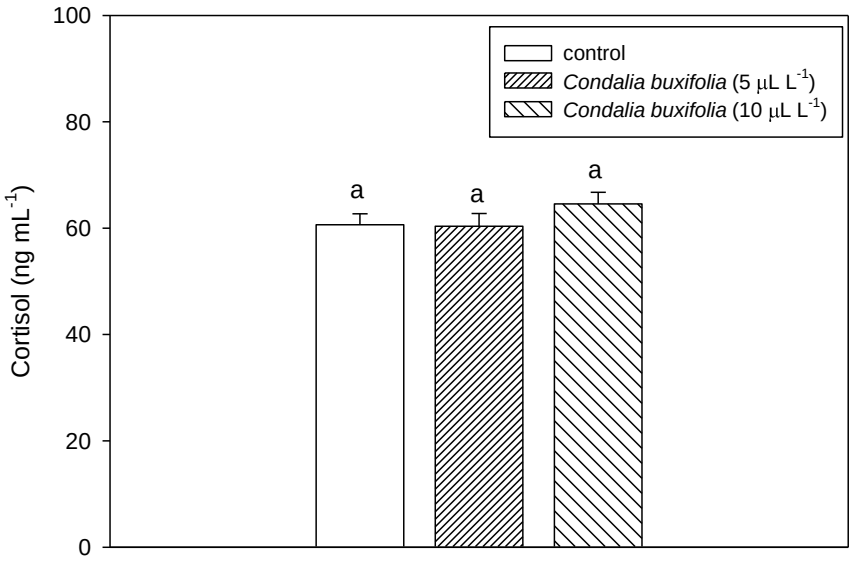
Cortisol (ng mL-1)
100
80
60
40
20
0
a
a
a
control
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Fig. 2

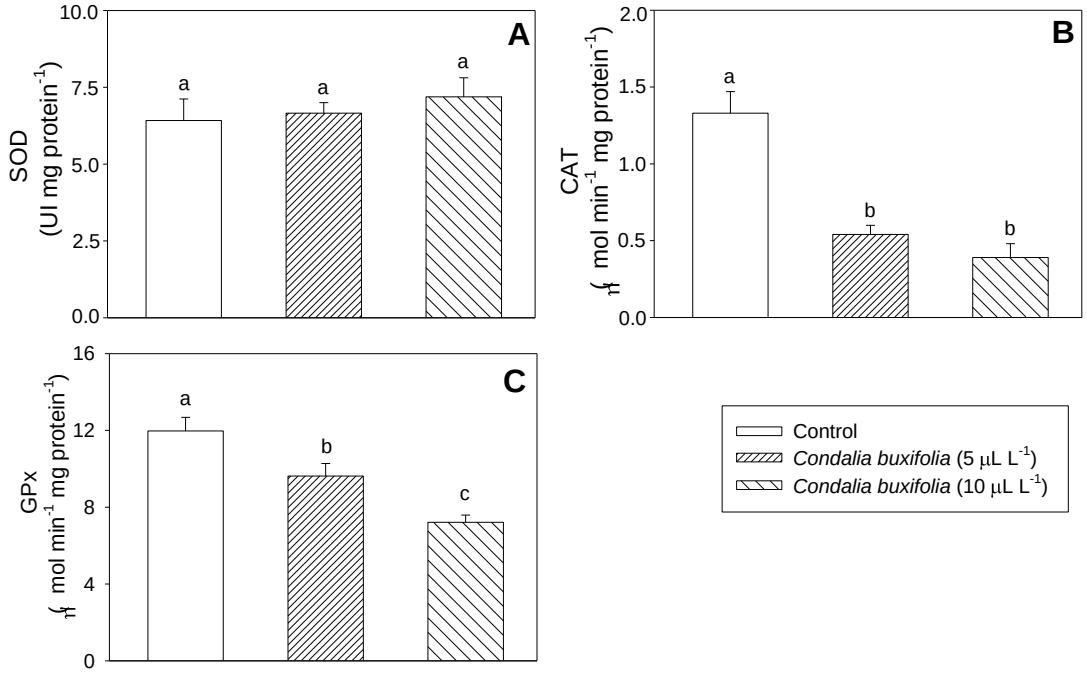
A
SOD
(UI mg protein-1)
10.0
7.5
5.0
2.5
0.0
a
a
a
B
CAT
(μ mol min-1 mg protein-1)
2.0
1.5
1.0
0.5
0.0
a
b
b
C
GPx
(μ mol min-1 mg protein-1)
16
12
8
4
0
a
b
c
Control
Condalia buxifolia (5 μL L-1)
Condalia buxifolia (10 μL L-1)

Fig. 3

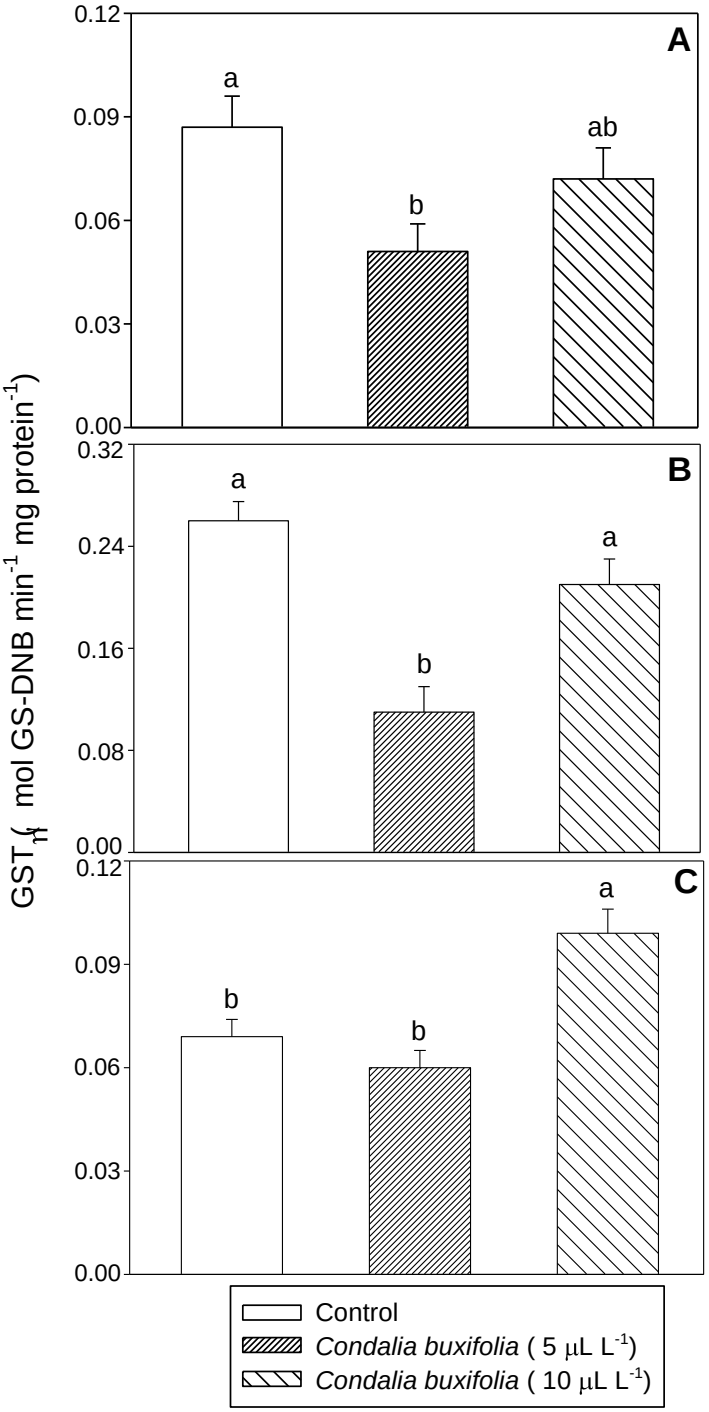
A
B
C
a
b
ab
a
b
a
b
b
a
0.12
0.09
0.06
0.03
0.00
0.32
0.24
0.16
0.08
0.00
GST (μ mol GS-DNB min-1 mg protein-1)
Control
Condalia buxifolia (5 μL L-1)
Condalia buxifolia (10 μL L-1)

Fig. 4

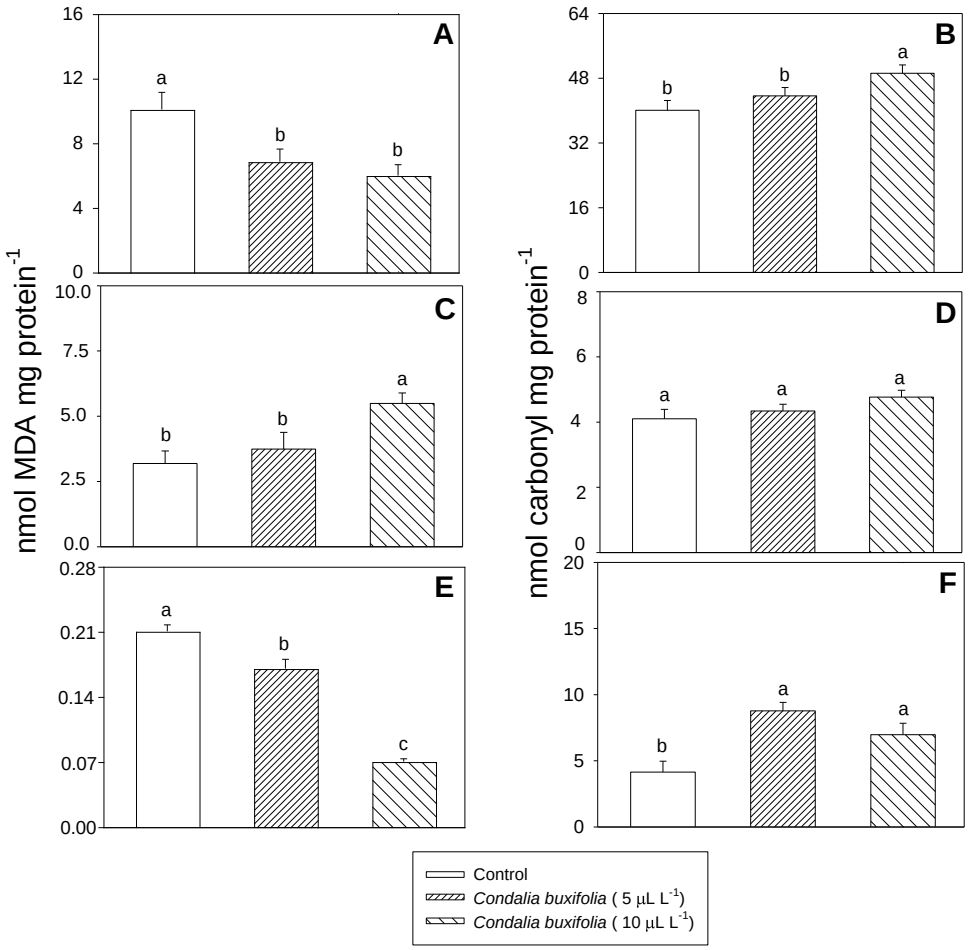
A
B
C
D
E
F
nmol MDA mg protein-1
nmol carbonyl mg protein-1
Control
Condalia buxifolia (5 μL L-1)
Condalia buxifolia (10 μL L-1)

Fig. 5

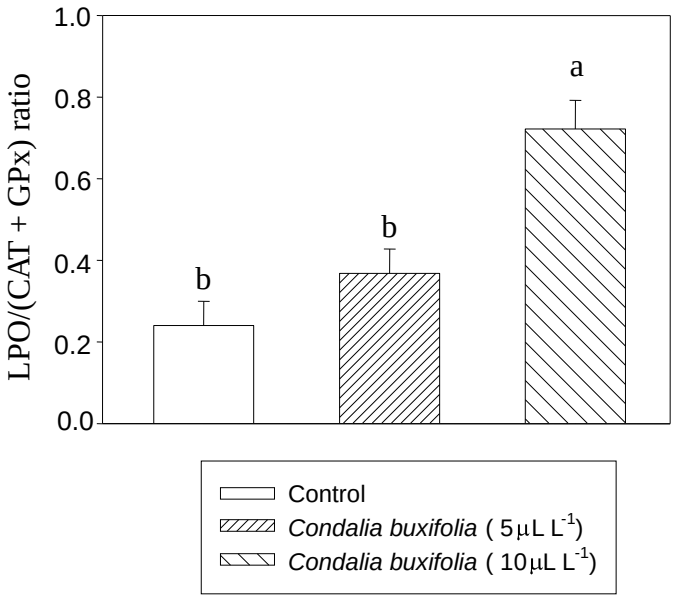
LPO/(CAT + GPx) ratio
Control
Condalia buxifolia (5μL L-1)
Condalia buxifolia (10μL L-1)

Fig. 6

3 DISCUSSÃO GERAL

Reportando aos resultados do experimento I, no qual foi utilizado EUG (1,5 ou 3,0 μL L^{-1}) e OEL (10 ou 20 μL L^{-1}), houve uma menor atividade da AChE no encéfalo, para ambas as concentrações testadas, enquanto que em músculo, somente na concentração 1,5 μL L^{-1}. Essas respostas foram acompanhadas pela menor quantidade de glicogênio e lactato no fígado e músculo e pelo aumento da proteína nestes tecidos (exceto em algumas concentrações, em que não houve alterações).

Entre os neurotransmissores clássicos estão a acetilcolina (CROSSLAND; MERRICK, 1953; TIWARI; SINGH, 2004; BLENAU; RADEMACHER; BAUMANN, 2012) e as catecolaminas (VIJAYAN; PEREIRA; MOON, 1994; GERWICK; DEMERS; BAYNE, 1999). Ambos podem se ligar a receptores específicos na membrana pós-sináptica e influenciar uma atividade em particular. A inibição da AChE leva ao aumento da acetilcolina e consequentemente, sua acumulação nas fendas neurais, comprometendo a transmissão do impulso motor para o músculo, resultando na desorientação ou redução do movimento e alterações metabólicas (SALBEGO et al., 2010; BLENAU; RADEMACHER; BAUMANN, 2012; GUTIERRES et al., 2014). Em adição, Schetinger et al (2000) e Müller et al. (2002), respectivamente, reportaram a inibição da AChE em córtex cerebral de ratos expostos a benzodiazepínicos (BDZ) e em sangue e eritrócitos de humanos tratados com diferentes antidepressivos.

Nesse contexto, pode-se inferir que a inibição da AChE no encéfalo, para ambos os extrativos, está relacionada à interação destes, com a via gabaérgica, uma vez que, os receptores de GABA mais abundantes no SNC são os receptores ionotrópicos do tipo ‘A’ ($GABA_A$), que constituem canais iônicos mediados por neurotransmissores rápidos, incluindo receptores neuronais de acetilcolina. A maioria dos agentes neurotransmisores via gabaérgica atua sobre os receptores $GABA_A$ ionotrópicos (YOUNG; CHU, 1990; HELDWEIN et al., 2012). Além disso, estudos de Heldwein et al. (2012) sugerem o envolvimento do receptor $GABA_A$ no efeito anestésico do OEL, uma vez que este óleo mostrou sinergismo com BDZ comprovando assim sua ação sedativa sobre o SNC. O mesmo pode-se afirmar para o efeito do eugenol, visto que estudos relatam que este extrativo, além de atuar como anestésico local (JAVAHERY et al., 2012) tem seus efeitos mediados pela via gabaérgica (GUÉNETTE et al., 2007).

Alterações no eixo HPI e maior liberação de catecolaminas, levando ao aumento nos níveis de lactato e redução no conteúdo de proteína total, estão diretamente relacionadas com situações de estresse (MAZEAUD, M.M.; MAZEUD, F.; DONALDSON, 1977; BARTON; IWAMA, 1991; VIJAYAN; PEREIRA; MOON, 1994; GERWICK; DEMERS; BAYNE, 1999). Pode-se inferir então, que no experimento aqui reportado, os extrativos testados foram eficazes, impedindo a cadeia de eventos acima citados, uma vez que os níveis de lactato e proteína foram menores e maiores, respectivamente, em fígado e músculo, enquanto que o glicogênio foi degradado para suprir a demanda energética, sem comprometer o metabolismo proteico.

Além disso, algumas das concentrações utilizadas mostraram ter eficácia no que se refere à redução de estresse oxidativo uma vez que, para proteger-se dos produtos do estresse, o organismo conta com a atividade conjugada do sistema antioxidante. Esta relação pode ser observada pela menor atividade (nas concentrações de EUG) ou ausência de resposta (nas concentrações de OEL) da GST no fígado e uma maior atividade de NPSH, em todas as concentrações. Nesse mesmo tecido, também houve um acréscimo no conteúdo de PC e redução de TBARS. Pode-se afirmar que esses resultados estão de acordo com os encontrados por Azambuja et al. (2011), relacionando OEL e estresse oxidativo em jundiá. Complementando essas afirmações, estudos de Veeck et al. (2013) também comprovaram a eficiência do OEL (30 ou 40 $\mu L\ L^{-1}$) para filés congelados, provenientes de jundiás tratados com as referidas concentrações, nos quais os níveis de TBARS foram menores, o que garante a melhor qualidade da carne estocada.

No experimento II, no qual os peixes foram previamente tratados com OEL (200 $\mu L\ L^{-1}$, por 3 minutos) ou EMC (10 $\mu L\ L^{-1}$, por 5 minutos) e imediatamente transportados em água contendo OEL (30 ou 40 $\mu L\ L^{-1}$) ou EMC (5 ou 10 $\mu L\ L^{-1}$), não houve alteração na atividade das enzimas AChE, NTPDase e 5' -nucleotidase no encéfalo. Os níveis de ATP presentes nas fendas sinápticas são controlados por estas enzimas e podem ser coarmazenados e colançados com outros neurotransmissores, entre eles o GABA (ABBRACCHIO et al., 2008). A partir dessa constatação, é possível relacionar a ação desse óleo, nessas concentrações, bem como do EMC, à manutenção dos níveis basais dessas enzimas, isto é, os extrativos não diminuiram as atividades neurotransmissoras, no entanto, contribuíram com a homeostase das mesmas.

Essa afirmação, em relação ao EMC, pode ser embasada nas descrições de Ma et al. (2008) para as propriedades farmacológicas da semente de planta *Zizyphus jujuba,* da família Rhamnaceae, comumente utilizada em países orientais para o tratamento da insônia, ansiedade e, também, como analgésico. Dessa semente, diversos componentes já foram

estudados, entre eles triterpenos, flavonas, β-sitosterol e ciclopeptídeos alcaloides sendo que este último, em experimentos realizados com camundongos, evidenciou o envolvimento da via gabaégica em seu mecanismo de ação. Reforçando essas afirmações, estudos de Sethiya et al. (2009), descrevem os benefícios de diferentes extrativos de plantas da India (cujos componentes também são encontrados na *C. buxifolia*), os quais são amplamente utilizados no tratamento da ansiedade e insônia, nesse país.

Pode-se inferir também, que o tratamento recebido antes do transporte, no segundo experimento, e que não ocorreu no primeiro, justifique a alteração (redução) dos níveis encefálicos da AChE neste e a manutenção dos níveis basais daquele. A presença de estresse oxidativo em fígado, decorrente do transporte com adição de OEL, conforme descrito no segundo manuscrito, sugere que concentrações menores sejam mais eficazes, em relação a esse tecido (AZAMBUJA et al., 2011).

Estudos de Becker et al. (2012, 2013) e Salbego et al. (in press) sugerem que anestésicos e sedativos diminuem as taxas metabólicas. Contudo, os resultados do artigo 3, mostram que em rim e fígado, houve uma tendência ao aumento nos níveis de alguns metabólitos. Nos peixes, o rim é um dos principais órgãos para organizar as respostas ao estresse, além do cérebro e da hipófise, uma vez que nesse órgão as catecolaminas são liberadas através da ativação dos tecidos cromafins, ocorrendo além de resposta ao estresse, a interação entre os sistemas nervoso, endócrino e imunitário (TORT, 2011). Quanto ao fígado, é o primeiro órgão envolvido nas atividades de detoxificação do organismo (SALBEGO et al., in press), justificando-se assim essas respostas tecido-específicas.

Observa-se também, nesse trabalho, que os níveis dos antioxidantes ácido ascórbico e NPSH foram elevados nas brânquias, enquanto que no músculo e fígado foram menores em relação ao grupo controle. O aumento na capacidade antioxidante nas brânquias sugere que o extrativo utilizado foi eficaz, neutralizando efeitos tóxicos gerados direta ou indiretamente pelos anestésicos (PARODI et al., 2012). Contudo, pode-se afirmar que a adição de EMC à água de transporte de jundiá, foi eficaz em manter o equilíbrio entre as respostas pró-oxidantes e antioxidantes, entre os diferentes tecidos e concentrações avaliadas. Também auxiliou no controle dos parâmetros da água, mantendo-os dentro dos padrões aceitáveis para a espécie, similarmente aos encontrados por Becker et al. (2012, 2013), impediu o aumento dos níveis de cortisol plasmático e pH sanguíneo, e reduziu a perda de íons na água, sugerindo que os movimentos operculares e o fluxo sanguíneo foram menores nas brânquias.

Em adição, dados reportados por estes autores mostram que jundiás transportados com OEL, EUG e EMC tiveram uma menor perda de íons, provavelmente associada ao menor

fluxo sanguíneo em brânquias, uma vez que os peixes estavam com as atividades metabólicas reduzidas. Agregando-se a este achado, Toni et al. (2014) e Zeppenfeld et al. (2014), comprovaram menor perda de íons em plasma de jundiás anestesiados com óleo essencial de *Hesperozygis ringens* e sedados com *Aloysia triphylla*, respectivamente.

Por se tratar de um trabalho pioneiro envolvendo extrativos naturais derivados de plantas nativas (OEL e EMC) e eugenol, e seus efeitos sobre diversificados parâmetros enzimáticos, bioquímicos e oxidativos, trabalhos relacionados que possam servir de referência são escassos. Assim, consolida-se a importância e a necessidade de maior exploração nessas áreas, com a finalidade de encontrar concentrações sedativas e/ou anestésicas eficazes para o transporte de jundiá, entre outros organismos aquáticos, com o propósito de melhorar a produção e bem estar animal e, em adição, propor novos extrativos que possam substituir a utilização do eugenol e produtos sintéticos.

4 CONCLUSÕES

- O eugenol, o óleo essencial de *L. alba* (OEL) e o extrato metanólico de *C. buxifolia* (EMC) melhoram as condições de transporte de jundiá.
- A adição de eugenol (1,5 ou 3,0 μL L^{-1}) e OEL (10 ou 20 μL L^{-1}) à água de transporte de jundiá, reduziu a atividade da AChE em cérebro enquanto que em músculo a redução ocorreu apenas na concentração 1,5 μL L^{-1} de eugenol. Contudo, em músculo, contribuiu para que o metabolismo aeróbico dos carboidratos suprisse a demanda energética, em pról das reservas proteicas e melhorou o estado oxidativo, evidenciado principalmente por menores níveis de TBARS e maiores níveis de GSH.
- O OEL (30 ou 40 μL L^{-1}) e o EMC (5 ou 10 μL L^{-1}), com pré-sedação, não levaram à alterações na atividade das enzimas AChE, NTPDase e 5' -nucleotidase.
- O EMC foi eficaz quanto ao aumento dos antioxidantes ácido ascórbico e NPSH em brânquias e redução nos níveis de TBARS (pró-oxidante) em rim e músculo, além da manutenção dos níveis basais de cortisol, redução dos níveis de amônia na água e perda de íons.
- Para todos os extrativos testados, o metabolismo aeróbico dos carboidratos foi suficiente para atender à demanda de energia, o que indica que: 1) os tecidos receberam uma oferta suficiente de oxigênio, 2) o metabolismo anaeróbico não foi necessário, comprovado com a estabilidade ou menores níveis de lactato na maioria dos tecidos e 3) houve uma redução no catabolismo proteico.
- Os resultados, tomados em conjunto, demonstram que jundiá transportado com os extrativos e concentrações propostas no presente trabalho, apresentaram um equilíbrio quanto às atividades das enzimas AChE, NTPDase e 5' -nucleotidase, metabólicas, pró-oxidantes e antioxidantes.

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